

Immunotoxin Lesion of Hypothalamically Projecting Norepinephrine and Epinephrine Neurons Differentially Affects Circadian and Stressor-Stimulated Corticosterone Secretion

SUE RITTER, ALAN G. WATTS, THU T. DINH, GRACIELA SANCHEZ-WATTS, AND CHRISTI PEDROW

Programs in Neuroscience (S.R., T.T.D., C.P.), Washington State University, Pullman, Washington 99164-6520; and Neuroscience Program (A.G.W., G.S.-W.), University of Southern California, Los Angeles, California 90089-2520

Hindbrain norepinephrine (NE) and epinephrine (E) neurons play a pivotal role in the central distribution of sensory signals derived from the internal environment. Their projections influence the various secretory patterns of the hypothalamo-pituitary-adrenal axis and are essential for feeding and adrenal medullary responses to glucoprivation. NE and E terminals in the paraventricular nucleus of the hypothalamus (PVH) and associated hindbrain cell bodies can be virtually eliminated by PVH microinjection of a retrogradely transported conjugate of saporin (SAP, a ribosomal toxin) and a monoclonal antibody against dopamine β -hydroxylase (d β h), *i.e.* d β h mouse monoclonal antibody conjugated to SAP (DSAP). To examine the effects of selective elimination of NE/E afferents on hypothalamo-pituitary-adrenal activation,

we injected DSAP into the PVH and measured corticosterone secretion under basal circadian conditions and in response to two distinct challenges: glucoprivation and forced swim. DSAP lesions profoundly impaired glucoprivation-induced corticosterone secretion and induction of CRH heteronuclear RNA and Fos mRNA in the PVH, without impairing basal CRH mRNA expression, circadian corticosterone release, or the corticosterone response to swim stress. Thus, NE/E projections influence corticosterone secretion only in certain circumstances. They are required for the response to glucoprivation, but are dispensable for circadian activation and for the response to swim stress. (*Endocrinology* 144: 1357-1367, 2003)

CRH NEURONS IN the medial parvicellular (mp) part of the hypothalamic paraventricular nucleus (PVH) are innervated by a variety of afferents that control the different activation patterns of the hypothalamo-pituitary-adrenal (HPA) axis. A prominent afferent input originates from hindbrain catecholaminergic neurons (1-5). A variety of evidence indicates that both pre- and post-synaptic adrenergic receptors control CRH neuronal function and thus shape HPA activation patterns (3, 6).

Despite the recognized importance of hindbrain catecholamines for HPA function, major questions still exist regarding the precise nature of their involvement in the reactions to stress (7, 8). For example, it is not clear whether NE and E neurons encode only specific modalities of stress-related sensory information or whether they are broadly activated by many and diverse stress-related stimuli, allowing them to contribute in a more general way to many or all forms of HPA activation. The functional complexity of the hindbrain cell groups and the physiological complexity of many stressors have impeded the resolution of these questions. However, a major obstacle has been the lack of sufficiently selective tools for lesioning these neurons, which has

made it difficult to attribute disruption of HPA function specifically to loss of PVH catecholamine terminals.

We have approached this problem using a novel immunolesioning technique that selectively destroys norepinephrine (NE) and epinephrine (E) neurons. The lesioning agent is a conjugate of saporin (SAP, a ribosomal toxin), and a monoclonal antibody against dopamine β -hydroxylase (d β h, a biosynthetic enzyme present exclusively in NE/E neurons). This conjugate, referred to here as DSAP, is internalized by targeted neurons (9-12) and transported retrogradely to their cell bodies (13-15), which are then destroyed (16). Previously, we demonstrated that injection of DSAP into the PVH selectively destroys hypothalamically directed NE/E neurons, producing a nearly complete loss of d β h-immunoreactive (ir) terminals in the medial hypothalamus without significant nonspecific damage at the injection site or to spinally projecting NE/E neurons (14).

Using this technique, we here examined the contribution of catecholaminergic afferents to the PVH on corticosterone secretion under basal circadian conditions and in response to two distinct challenges: metabolic perturbation (glucoprivation) and external aversive stress (forced swim). Additionally, we have determined the effects of DSAP lesions on CRH and *c-fos* gene transcriptional activity in the PVH after 2-deoxy-D-glucose (2DG) injections. 2DG interferes specifically with glucose use (17) and is thus a selective metabolic stimulus. Our previous work with DSAP demonstrated that feeding and adrenal medullary responses to glucoprivation are completely dependent on NE or E neurons (14). Corticoste-

Abbreviations: d β h, Dopamine β -hydroxylase; 2DG, 2-deoxy-D-glucose; DSAP, d β h mouse monoclonal antibody conjugated to SAP; E, epinephrine; hn, heteronuclear; HPA, hypothalamo-pituitary-adrenal; ir, immunoreactive; mp, medial parvicellular; NE, norepinephrine; PVH, paraventricular nucleus of the hypothalamus; SAP, saporin; TPBS, Tris sodium phosphate buffer; UTP, uridine triphosphate.

rone secretion is also potentially stimulated by glucoprivation (18, 19), but the degree to which this response depends on catecholamine afferents is not known. Forced swim is a complex aversive stress that may involve some degree of telencephalic processing (19, 20) and, therefore, may activate the HPA axis by a completely different afferent pathway(s). Whether catecholamine afferents are an essential component of this pathway is unclear. The highly selective DSAP lesioning technique provides the opportunity to clearly establish the contribution of NE/E neurons to stimulation of the HPA axis by qualitatively distinct stressors.

Materials and Methods

Preparation of animals

Adult male Sprague-Dawley rats weighing 320–340 g at the start of the experiment were obtained from Simonsen Laboratories, Inc. (Gilroy, CA). Rats were housed individually in suspended wire mesh cages under standard Association for Assessment and Accreditation of Laboratory Animal Care-approved conditions in a temperature-controlled room (21 ± 1 C) illuminated between 0600 and 1800 h. Rats had *ad libitum* access to pelleted rat food and tap water, except as noted. Tests were conducted between 0900 and 1300 h. All experimental animal protocols were approved by the Washington State University Institutional Animal Care and Use Committee, which conforms to National Institutes of Health guidelines.

For intracranial injections, the rats were anesthetized using chloroform anesthesia (3 ml/kg ip). Chloroform anesthesia was made by combining 21.25 g chloral hydrate, 10.6 g magnesium sulfate, 4.43 g pentobarbital sodium, 75.26 ml ethyl alcohol, and 169.00 ml propylene glycol, brought to 500 ml with sterile double-distilled H₂O and filtered. Injections of d β h mouse monoclonal antibody conjugated to SAP [DSAP; Chemicon, Temecula, CA; 42 ng/200 nl in phosphate buffer (pH 7.4), n = 7] or unconjugated SAP control solution [Advanced Targeting Systems, San Diego, CA; 8.82 ng/200 nl in phosphate buffer (pH 7.4), n = 7] were directed bilaterally into the PVH, using the following stereotaxic coordinates: 7.3 mm ventral to dura mater, 1.8 mm caudal to bregma, ± 0.4 mm lateral from midline. The amount of unconjugated SAP in the control solution approximated the amount of SAP present in the DSAP conjugate (21%), as indicated in the manufacturer's product information. Injections were made through a stereotaxically positioned drawn glass capillary micropipette (tip diameter, 30 μ m) connected to a microinjector (Picospritzer, General Valve Corp., Fairfield, NJ) with polyethylene tubing. The delivery of solution was monitored microscopically. The dose and volume of DSAP and SAP injected at each site were determined from previous experiments using similar protocols (14). Previous immunohistochemical studies indicate that 2 wk is adequate for transport of the immunotoxin and degeneration of the affected neurons (12, 15). Therefore, responses to glucoprivation were assessed beginning approximately 3 wk after DSAP injections.

Glucoprivic feeding

Injections of DSAP into the PVH have been shown previously to abolish 2DG-induced feeding (14). Therefore, glucoprivic feeding tests were conducted in all SAP- and DSAP-injected rats before blood collection experiments to obtain an independent *in vivo* assessment of the effectiveness of the DSAP injections. Feeding in response to both insulin-induced hypoglycemia and 2DG were tested, because both methods of producing glucoprivation were used to assess the effect of DSAP lesions on corticosterone responses, and the effect of DSAP on feeding induced by hypoglycemia has not been examined previously. For these tests, rats were given a weighed quantity of pelleted rat chow in their home cages and injected sc with a hypoglycemic dose of regular insulin (Humulin R, Eli Lilly and Co., Indianapolis, IN; 1.5 U/kg) or 2DG (Sigma-Aldrich Corp., St. Louis, MO; 250 mg/kg, 1 ml/kg), a nonmetabolizable glucose analog that competitively reduces glucose use (17). Baseline intakes were measured after injection of sterile saline (0.9%, 1 ml/kg). Remaining pellets and spillage were measured over the 4-h period immediately following the injection. Rats were tested with each drug and saline. Tests

for 2DG- and hypoglycemia-induced feeding were separated by at least a 1-wk interval. Saline tests were conducted on the days before drug tests. DSAP rats that ate more than 1 g above their own saline baseline intake in response to 2DG or insulin were considered to have incomplete lesions and were excluded from further testing.

Corticosterone responses to insulin-induced hypoglycemia and 2DG

For these studies, rats were extensively habituated to opaque 4 \times 12-inch Plexiglass testing chambers designed for remote blood sampling. One week before the first experiment, catheters constructed from Silastic tubing (inside diameter, 0.64 mm; outside diameter, 1.19 mm; Dow Corning Corp., Midland, MI) were implanted intraatrially through the right jugular vein. When not in use, catheters were filled with polyvinylpyrrolidone solution (40,000 molecular weight, Sigma-Aldrich Corp.), 11 g polyvinylpyrrolidone in 20 ml 0.9% saline containing 1000 U/ml heparin (Elkins-Sinn, Inc., Cherry Hill, NJ), and 2 mg/ml Gentamicin (Schering-Plough Animal Health Corp., Kenilworth, NJ). On test days, which were at least 1 wk apart, rats were placed in the chambers without food 1 h before collection of the first (time 0) blood sample, which was followed immediately by injection of 2DG (250 mg/kg, 1 ml/kg), insulin (1.5 U/kg), or saline (0.9%, 1 ml/kg). Additional blood samples (800 μ l) were collected remotely 30, 60, 90, 120, 180, and 240 min after the 2DG or saline injection. Plasma was separated by centrifugation. After removing an aliquot for determination of glucose concentration, the samples were stored at -80 C for later assay of corticosterone. Glucose was analyzed using the glucose oxidase method (21). Corticosterone concentrations were determined in duplicate aliquots using RIA kits obtained from Diagnostic Products Corp. (Los Angeles, CA; catalog no. TKRC-1). The lower limit of sensitivity for corticosterone was 20 ng/ml. At each sampling time, blood volume withdrawn was replaced with an equal volume of resuspended erythrocytes, which were obtained from heparinized donor blood, washed, and centrifuged three times and resuspended in a volume of Nutricil (Medsep Corp., Covina, CA) equal to the original plasma volume. Donor blood was prepared the afternoon before the experiment and refrigerated overnight.

Corticosterone responses to 5-min forced swim

The corticosterone and glucose responses to a 5-min swim were examined in the same rats used for 2DG and insulin tests. On the test day, rats were attached to the blood collection lines and placed in the test chambers for 1 h before collection of the preswim baseline sample. Rats were placed individually for 5 min in a bucket of water maintained at 37 C. They were then removed from the water, towel-dried, and returned to the test chambers for the remainder of the test. Blood samples were collected at 30, 60, 90, 120, and 240 min after the start of the swim.

Basal circadian rhythm of corticosterone secretion

Seven DSAP-injected and six SAP-injected rats were used in this experiment. At 0800 h, they were given fresh food and water, and their jugular catheters were connected to the blood sampling lines, which remained connected for the duration of the experiment. Blood (800 μ l) was collected remotely at 4-h intervals for 24 h, beginning at 0900 h, and replaced with an equal volume of donor blood, as described above. An aliquot of blood was removed for blood glucose determination. The remaining blood was centrifuged, and plasma was stored for RIA of corticosterone.

Immunohistochemistry

At the conclusion of testing, rats were killed rapidly by injection of a lethal dose of pentobarbital sodium (Abbott Laboratories, Irving, TX; 300 mg/kg) through the jugular catheter. They were perfused using a pH shift procedure to optimize visualization of CRH-ir without use of colchicine (22). Successive transcardial perfusion of 0.1 M potassium PBS, 4% paraformaldehyde in 0.1 M acetate buffer (pH 6.5), and 0.4% paraformaldehyde plus 0.05% glutaraldehyde in 0.1 M borate buffer (pH 9.5) was followed by 5–7 h post fixation and 0.4% paraformaldehyde in 0.1 M borate buffer (pH 9.5). Brains were then cryoprotected overnight in 0.1 M potassium PBS in 25% sucrose and sectioned on a cryostat.

Coronal sections of the brain stem (40 μ m) and hypothalamus (14 μ m) were cut in multiple sets. Hindbrain sections were processed for immunocytochemical detection of d β h-ir. Hypothalamic sections were processed for d β h-ir and CRH-ir. The d β h-ir was used to verify the DSAP-induced lesion, and CRH-ir was used to evaluate the effect of the PVH DSAP injection on CRH neurons controlling ACTH secretion.

Immunohistochemical staining was performed using standard avidin-biotin-peroxidase techniques described previously (23, 24). Briefly, sections were treated with 50% ethanol for 30 min, then washed (3×5 min) in 0.1 M phosphate buffer, and incubated for 45 min in 10% normal horse serum made in Tris sodium phosphate buffer [TPBS (pH 7.4)] with 0.05% thimerosal. The blocking solution was removed from the tissue, and the sections were coincubated for 48 h in mouse monoclonal anti-d β h (Chemicon, 1:100,000) or rabbit anti-CRH (a gift from Dr. Wylie Vale, The Salk Institute, La Jolla, CA; 1:50,000) made in 10% normal horse serum-TPBS. The primary antibody was removed, and the sections were washed and incubated in biotinylated donkey antimouse or antirabbit IgG (both 1:500 in 1% normal horse serum-TPBS; Jackson Immuno-Research Laboratories, Inc., West Grove, PA). After 24 h, the tissue was washed (3×10 min), incubated with extravidin-peroxidase (Sigma; 1:1500 in TPBS) overnight, washed again (3×10 min), and reacted for visualization of d β h-ir or CRH-ir using nickel-intensified diaminobenzidine in the peroxidase reaction to produce a black reaction product. Sections were then mounted on slides and coverslipped for microscopic evaluation. All antibodies used in the experiment were titrated before use to determine optimal concentrations. Standard controls for specificity of primary antibodies were used, including the incubation of the tissue with normal instead of immune serum and preincubation of the immune serum with the antigen before its application to tissue. Histological sections used in figures were captured using a Nikon photomicroscope equipped with a digital camera (RS Photometrics, Roper Scientific Inc., Tucson, AZ) and linked to a computer running CoolSNAP software (Roper Scientific Inc.). Plates of multiple sections were assembled using Adobe Photoshop (Adobe Systems Inc., San Jose, CA). Brightness only was altered digitally in some cases to achieve uniformity among photomicrographs grouped in composite figures.

In situ hybridization

For *in situ* hybridization experiments, corticosterone responses to 2DG and saline were tested, as described, in separate groups of rats given PVH SAP or DSAP injections ($n = 8$ per group). Hypothalamic tissue from these rats was later processed for *in situ* hybridization of CRH mRNA or heteronuclear RNA (hnRNA). At the time the rats were killed, jugular catheters were connected to infusion lines, and animals were allowed to rest quietly for approximately 1 h. Half of the DSAP and SAP rats were then injected remotely through their infusion lines with 2DG (250 mg/kg) and half with 0.9% saline. Thirty minutes later, they were killed rapidly by remote injection of a lethal dose of pentobarbital. Within 2 min, transcardial perfusion was initiated, as described above, using the pH shift procedure. Brains were then removed from the skull and were post-fixed for 24 h in the pH 9.5 borate buffer containing 12–15% (wt/vol) sucrose. Hypothalamic tissue was sectioned (14 μ m) through the level of the PVH using a cryostat (Leica Corp., Deerfield, IL). Sections used for *in situ* hybridization were mounted on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA) and stored desiccated at -70 C until processed for *in situ* hybridization.

Sections were hybridized with a 35 S-uridine triphosphate (UTP)-labeled cRNA probe transcribed from a 700-bp cDNA sequence encoding for part of the pre-proCRH mRNA (25), a 2.1-kbp sequence encoding for the rat *c-fos* mRNA, or a 35 S-UTP/ 35 S-CTP-labeled cRNA probe transcribed from a 536-bp *PvuII* fragment complementary to the sequence within the single CRH intron. cRNA probes were synthesized using the Promega Gemini kit (Promega Corp., Madison, WI) and the appropriate RNA polymerase, as described previously (25, 26). The characterization of each probe has been reported elsewhere (25–28). Sections were exposed to Cronex Microvision x-ray film (DuPont, Wilmington, DE) for 1 d (CRH and *c-fos* mRNAs) or 42 d (CRH hnRNA), then dipped in nuclear track emulsion (Kodak NTB-2, Eastman Kodak Co., Rochester, NY; diluted 1:1 with distilled water). Slides were exposed for 4 d (*c-fos* mRNA), 7 d (CRH mRNA), or 42 d (CRH hnRNA), developed, and counterstained with thionin.

Quantitation of 35 S-UTP-cRNA hybridization signals

Mean gray levels of the RNA hybridization signals in the PVHmp were measured from images on Microvision C x-ray film using IP-Lab Spectrum imaging software (Signal Analytics Corp., Vienna, VA) as described elsewhere (25). Hybridization values were expressed on a 0–255 grayscale. Parcellation of the hypothalamus was determined using the scheme and nomenclature of Swanson (29). We have previously demonstrated the linearity of the *in situ* hybridization signal response on the x-ray film and our detection system (28). That part of the PVHmp in which CRH hnRNA measurements were taken was defined using the adjacent CRH mRNA hybridized section.

Assessment of the DSAP lesion

To verify the effectiveness of PVH DSAP injections in lesioning NE/E neurons, d β h-ir cell bodies were quantified at representative levels through hindbrain cell groups A1, C1 (caudal part), and A2, where the majority of cell bodies project to the hypothalamus and which provide the major NE/E innervation of the PVH (30, 31). To assess the specificity of the DSAP lesion for PVH-projecting NE/E neurons, groups A5 and A7 were also analyzed. Cell groups A5 and A7 project predominantly to the spinal cord and do not innervate the PVH. One of three sets of hindbrain sections from each rat was used for quantification. Three 40- μ m sections, anatomically matched across rats, were selected from each area of interest for quantification. Cells were counted bilaterally on each section, and the mean number of cells per section was calculated for each cell group. All immunoreactive cells were counted, regardless of the presence of a cell nucleus. No correction factor for double counting was applied due to the use of relatively thick nonconsecutive sections for the quantification. A1 and A2 cells were counted between the pyramidal decussation and the calamus scriptorius (*i.e.* the most caudal extent of the area postrema). Cells in the area of A1/C1 overlap were counted between the calamus scriptorius and obex (*i.e.* the most rostral level of the area postrema). Cell group A5 was quantified at the level of the caudal locus coeruleus, at the exit of cranial nerve 7 from the ventral brain stem, and A7 was assessed at the level of the Kölliker-Fuse nucleus. Hypothalamic sections were examined for the presence of d β h-ir terminals and CRH-ir cell bodies. However, CRH-ir cell bodies and d β h-ir terminals in the PVH were not quantified.

Catecholamine cell groups are referred to using conventional terminology; NE cell groups are designated A1–A6, and E cell groups as C1–C3 (32). The area of overlap of rostral A1 and caudal C1 cell bodies is referred to as A1/C1. The respective distribution of NE and E neurons has been described in detail (4, 33–35).

Statistical analysis

Data from RIA, feeding tests, and glucose determinations were analyzed using two-factor repeated measures ANOVA and appropriate *post hoc* tests to isolate significant differences. A probability level of 0.05 was used as the level for significance.

Results

Rats recovered rapidly from intracranial injections without evidence of illness or neurological impairment. Body weights of DSAP and SAP rats did not differ at the time of PVH injections (374.8 ± 4.6 g and 362.0 ± 8.8 g, respectively), but DSAP rats weighed significantly more than SAP rats 5 months later at the conclusion of testing (537.5 ± 3.9 g and 465.5 ± 4.6 g, respectively; $P < 0.05$), as observed previously for PVH DSAP-injected rats (14).

Effects of DSAP on glucoprivic feeding

Injections of DSAP into the PVH completely eliminated feeding in response to both insulin-induced hypoglycemia and 2DG (Fig. 1). SAP controls ate 1.6 ± 0.4 , 5.9 ± 0.2 , and 6.1 ± 0.3 g of food in response to saline, insulin, and 2DG, respectively ($P < 0.001$ for insulin and 2DG *vs.* saline),

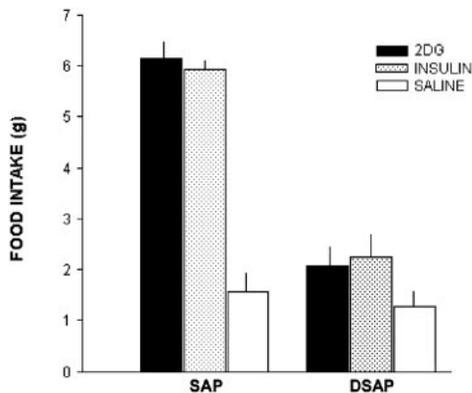


FIG. 1. Total amount of food consumed by SAP- or DSAP-treated rats in a 4-h test immediately after systemic administration of 2DG (250 mg/kg), insulin (1.5 U/kg), or saline (0.9%). DSAP or SAP was microinjected bilaterally into the PVH at least 3 wk before the feeding tests to selectively eliminate the innervation of the PVH by hindbrain NE and E neurons. DSAP rats did not increase their food intake in response to either direct metabolic blockade of glucose use by 2DG or to a hypoglycemic dose of insulin.

whereas DSAP rats ate 1.3 ± 0.3 , 2.2 ± 0.4 , and 2.0 ± 0.4 g of food in response to saline, insulin, and 2DG injection ($P > 0.05$ for insulin and 2DG vs. saline). The amounts consumed in response to insulin-induced hypoglycemia and 2DG were significantly greater in SAP than in DSAP rats ($P < 0.001$). All DSAP-injected rats exhibited severe deficits in glucoprivic feeding, and none were excluded from the data analysis.

Corticosterone and glycemic responses to 2DG, hypoglycemia, and forced swim

Figure 2 shows that 2DG significantly elevated blood glucose concentrations above baseline levels in both SAP and DSAP rats, beginning 30 min after 2DG injection. Glucose responses to saline and 2DG did not differ between SAP and DSAP rats. Corticosterone levels measured after saline injection did not differ between SAP and DSAP groups at any sampling time. In contrast, the corticosterone response to 2DG was significantly reduced in the DSAP-treated rats, compared with SAP controls. For SAP rats, corticosterone levels after 2DG differed significantly from levels after saline injection at all sampling times between 30 and 240 min ($P < 0.001$). For DSAP rats, corticosterone levels after 2DG injection differed significantly from levels at the corresponding time points after saline injection between 30 and 120 min after 2DG ($P \leq 0.05$), but the 2DG response was severely diminished in the DSAP compared with the SAP rats at all post-injection sampling times ($P < 0.001$). The peak corticosterone response, occurring at 60 min after 2DG injection, was 480 ± 23.2 ng/ml for SAP and 268 ± 13.8 ng/ml for DSAP rats. The corticosterone response of DSAP rats to 2DG, as measured by the calculated area under the curve (36), was only 34% of the response to 2DG in SAP rats.

Figure 3 shows that insulin produced significant hypoglycemia between 30 and 240 min after the injection ($P < 0.001$) that did not differ between SAP and DSAP groups ($P > 0.05$). In SAP rats, corticosterone levels were elevated significantly above those present after saline injection at all post-injection sampling times ($P < 0.001$). In DSAP rats,

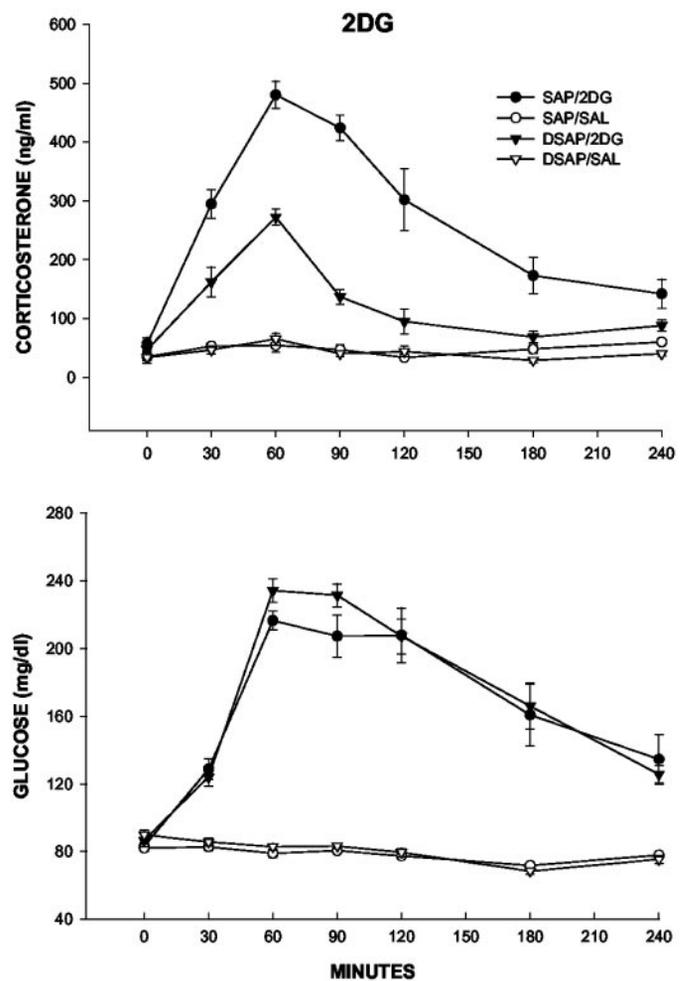


FIG. 2. Plasma corticosterone (top) and glucose (bottom) concentrations in SAP- and DSAP-treated rats after systemic injection 2DG (250 mg/kg) or saline (0.9%). DSAP or SAP was microinjected bilaterally into the PVH at least 3 wk before initiation of testing to selectively eliminate the innervation of the PVH by hindbrain NE and E neurons. Basal levels of corticosterone did not differ between groups, but the plasma corticosterone response to 2DG was significantly attenuated by the DSAP treatment. The corticosterone response of DSAP rats to 2DG, as measured by the calculated area under the curve, was only 34% of the response to 2DG in SAP rats. Glucose concentrations were increased to a similar extent above baseline by 2DG in SAP and DSAP rats, indicating that the glucoprivic stimulus for corticosterone secretion was similar for both groups.

corticosterone levels at 60, 90, and 120 min after insulin injection were elevated significantly above saline baseline levels ($P < 0.001$), but the corticosterone response in DSAP rats was significantly less than the response in SAP rats at all post-injection time points ($P < 0.001$). The peak corticosterone response, occurring at 120 min after insulin injection, was 369.1 ± 18.5 ng/ml in SAP rats and 165.4 ± 14.6 ng/ml in DSAP rats ($P < 0.001$). The corticosterone response to insulin-induced hypoglycemia in the DSAP rats, as measured by the calculated area under the curve, was only 28% of the response in SAP rats.

Forced swim did not significantly alter blood glucose concentrations in either SAP or DSAP rats, but it did significantly elevate corticosterone secretion (Fig. 4). However, the corti-

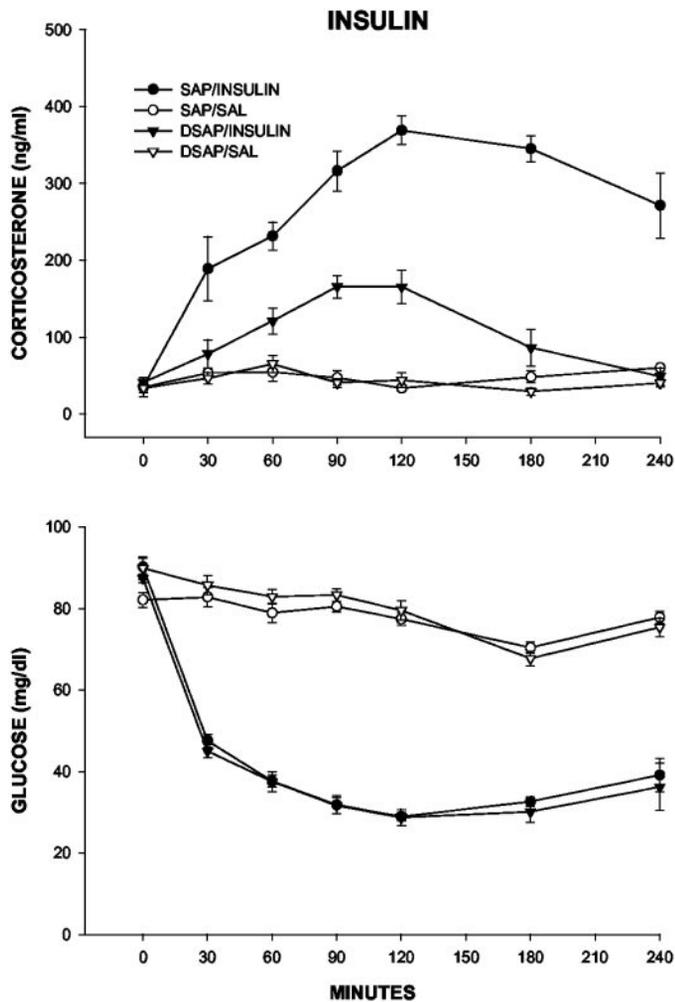


FIG. 3. Plasma corticosterone (*top*) and glucose (*bottom*) concentrations in SAP- and DSAP-treated rats after a hypoglycemic dose of insulin (1.5 U/kg) or saline (0.9%). DSAP or SAP was microinjected bilaterally into the PVH at least 3 wk before testing to selectively eliminate the innervation of the PVH by hindbrain NE and E neurons. Basal corticosterone concentrations did not differ between groups, but the plasma corticosterone response to hypoglycemia was significantly attenuated in DSAP-treated rats. The corticosterone response to insulin-induced hypoglycemia in the DSAP rats, as measured by the calculated area under the curve, was only 28% of the response in SAP rats. Glucose concentrations were reduced from baseline levels by insulin in both SAP and DSAP rats, but did not differ between groups under either condition, demonstrating that the glucoprivic stimulus was similar in both groups.

corticosterone response to forced swim did not differ between SAP and DSAP rats. The peak corticosterone responses of SAP and DSAP rats were 312 ± 12.8 and 316 ± 17.3 ng/ml, respectively. The total corticosterone response of DSAP rats to forced swim, as measured by the calculated area under the curve, was 104% of the response to swim in SAP rats.

Basal secretion of corticosterone did not differ between SAP and DSAP rats across the circadian cycle (Fig. 5). Corticosterone levels were highest just before the onset of the dark phase of the circadian light cycle and lowest just before onset of the light phase, as described in many previous reports (37, 38).

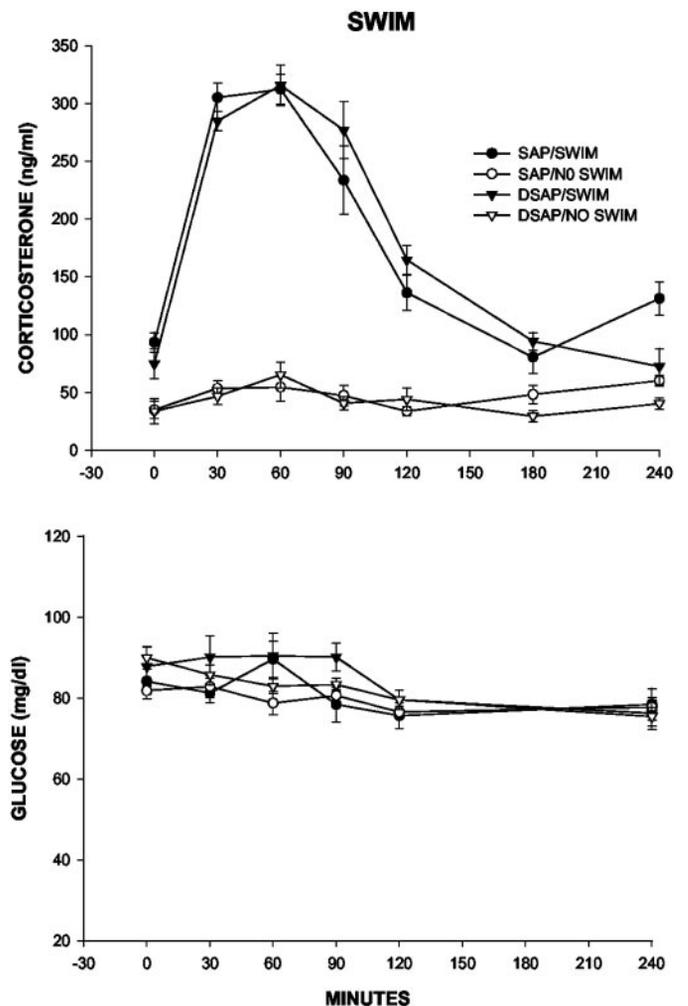


FIG. 4. Plasma corticosterone (*top*) and glucose (*bottom*) concentrations in SAP- and DSAP-treated rats after a 5-min forced swim. DSAP or SAP was microinjected bilaterally into the PVH at least 3 wk before initiation of testing to selectively eliminate the innervation of the PVH by hindbrain NE and E neurons. Basal levels of corticosterone and glucose did not differ between SAP and DSAP groups. In addition, the plasma corticosterone response to forced swim did not differ between groups, indicating that NE and E terminals are not required for the corticosterone response to this particular stressor. Glucose concentrations were not altered by the swim in either group.

DSAP lesions

Deficits in the screening test for glucoprivic feeding were consistent with the histological data in indicating lesion severity. In all DSAP-injected rats, the injections produced a nearly complete loss of d β h-ir terminals in medial hypothalamic structures, including the PVH and arcuate nucleus, and loss of NE/E cell bodies known to innervate these structures (30, 31, 39, 40). In the PVH itself, d β h-ir terminals were almost completely eliminated by the DSAP injection (Fig. 6, *left column*). However, histological sections revealed no evidence of nonspecific damage at the injection site in either DSAP- or SAP-injected rats. Most importantly, CRH-ir cell bodies in the PVH were not altered by DSAP (Fig. 6, *right column*). Similarly, the PVH DSAP lesions did not diminish axonal processes and synaptic terminals of these CRH cell bodies in the median eminence, although the lesion virtually elimi-

FIG. 5. Circadian rhythm of corticosterone secretion in rats given bilateral PVH microinjections of unconjugated SAP (n = 6) control solution or the immunotoxin anti-d β h-SAP (DSAP, n = 7) at least 3 wk before the test to selectively eliminate NE and E innervation to the PVH area. Blood samples for RIA of corticosterone were collected remotely at 4-h intervals across the circadian light/dark cycle. *Black bar* indicates the dark portion of the circadian cycle. The circadian rhythm of corticosterone secretion did not differ in DSAP and SAP rats.

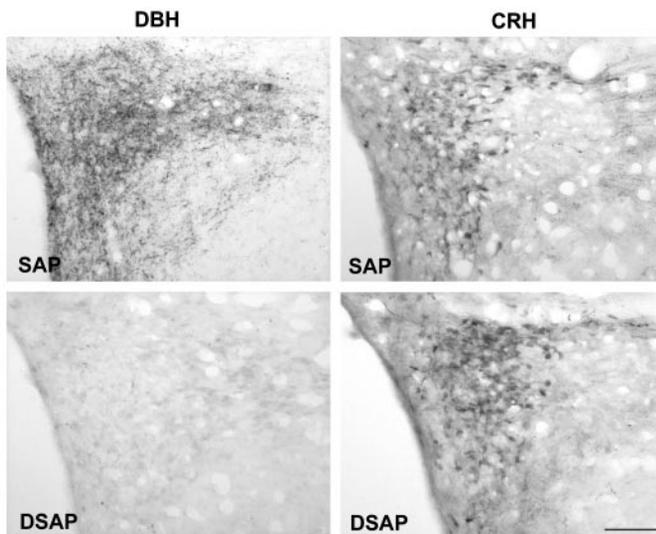
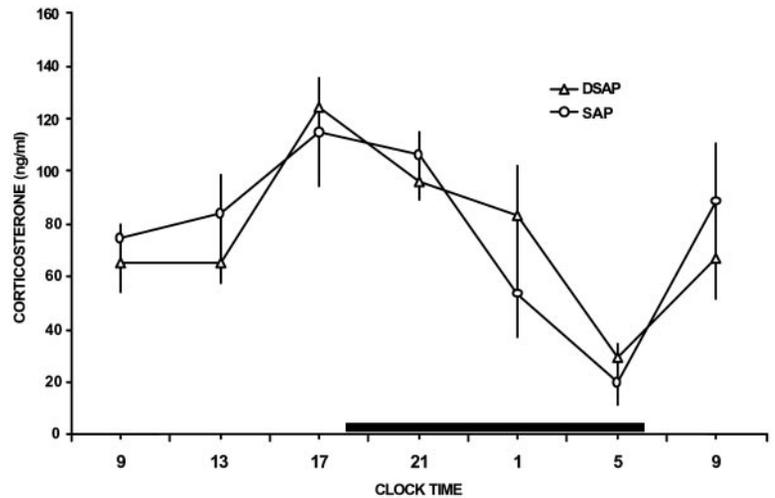


FIG. 6. Histological sections (14 μ m) showing the parvicellular area of the PVH from representative rats given bilateral injections of unconjugated SAP control solution or the immunotoxin anti-d β h-SAP (DSAP) into the PVH approximately 5 months before death. Sections show d β h-ir terminals (*left side*) and CRH-ir cell bodies (*right side*). Rats were perfused using a pH shift procedure to visualize CRH-ir without using colchicine. DSAP injections virtually abolished d β h-ir in the PVH, but did not alter the appearance of CRH-ir cell bodies in the same area. *Calibration bar* (100 μ m) applies to all sections.

nated d β h-ir terminals from this area (Fig. 7). The selectivity of the immunotoxin lesion observed here is consistent with our previous study using the same PVH DSAP microinjection protocol in which lesion selectivity was investigated using a variety of histological and functional tests (14).

Injections of DSAP into the PVH reduced or eliminated d β h-ir terminals in several additional medial diencephalic structures in addition to the PVH and median eminence, as described above. These included the dorsomedial and arcuate nucleus of the hypothalamus and the paraventricular nucleus of the thalamus. Notably, d β h-ir terminals were reduced only slightly in the supraoptic nucleus of the hypothalamus. Loss of terminals throughout the medial hypothalamus may indicate that the same NE/E neurons that innervate the injection site also provide collateral innervation

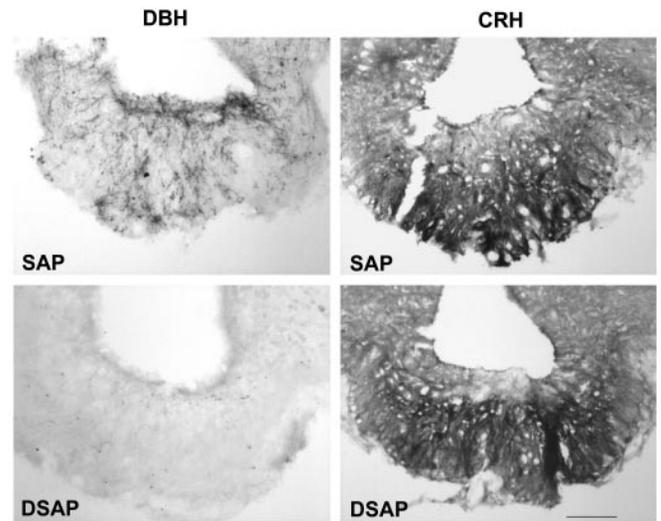


FIG. 7. Histological sections (14 μ m) showing the median eminence region from representative rats given bilateral injections of unconjugated SAP control solution or the immunotoxin anti-d β h-SAP (DSAP) into the PVH approximately 5 months before death. Sections show d β h-ir terminals (*left side*) and CRH-ir terminals (*right side*). DSAP injections virtually abolished d β h-ir terminals in the median eminence, but did not alter the appearance of CRH-ir terminals in the same area. *Calibration bar* (100 μ m) applies to all sections.

of this entire region. Destruction of the cell body would eliminate all of the terminals of that cell, even those distant from the injection site. Alternatively, this pattern of denervation may reflect the diffusion radius of the injected toxin. In the medial forebrain bundle at levels caudal to the PVH, d β h-ir was reduced significantly by PVH DSAP, whereas reductions in d β h-ir in the rostral medial forebrain bundle and in the bed nucleus of the stria terminalis were minimal, indicating that many of the catecholamine cell bodies contributing fibers or terminals to these regions do not extend collaterals into our PVH injection site.

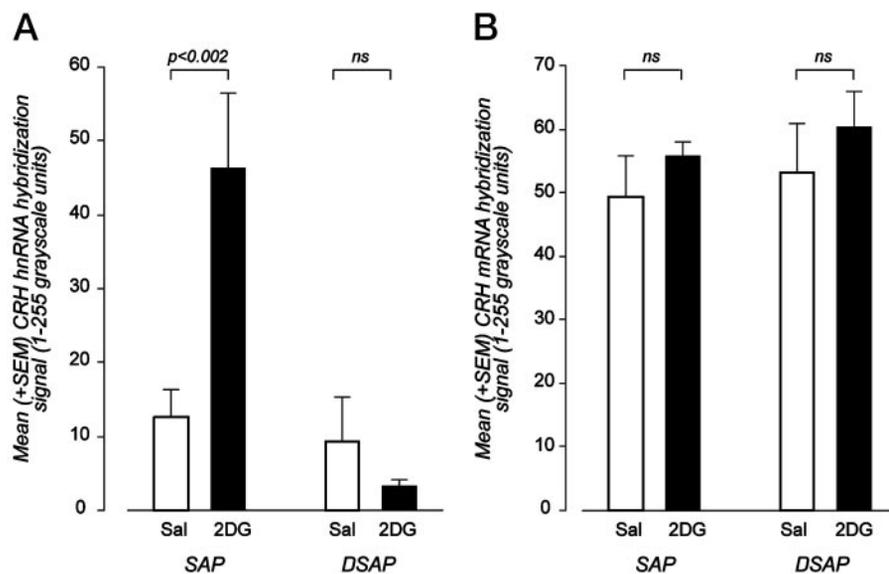
As reported previously (14), microinjection of DSAP into the PVH produced profound reductions in the number of cell bodies in PVH-projecting NE/E cell groups. Table 1 shows numbers of d β h-ir cell bodies (mean number per section \pm SEM) for each of 5 quantified levels from 5 SAP and 14 DSAP

TABLE 1. No. (mean \pm SEM) of d β h-ir cell bodies at representative levels of the hindbrain showing effects of bilateral PVH microinjections of SAP or DSAP on PVH-projecting (A1, A1/C1, and A2) and spinally projecting (A5 and A7) NE and E cell groups

	A1	A1/C1	A2	A5	A7
SAP (n = 5)	39.1 \pm 1.9 ^a	55.9 \pm 1.8 ^a	64.6 \pm 2.9 ^a	37.4 \pm 1.7	47.3 \pm 1.6
DSAP (n = 14)	5.7 \pm 1.0	5.4 \pm 1.1	28.9 \pm 4.0	43.5 \pm 2.9	51.2 \pm 3.1

^a $P < .001$, SAP *vs.* DSAP.

FIG. 8. The effect of 2DG (250 mg/kg) or saline (0.9%) injections on the mean (\pm SEM) CRH hnRNA (A) and CRH mRNA (B) levels from rats previously given bilateral PVH microinjections of unconjugated saporin (SAP) or anti-d β h-SAP (DSAP). The hybridization values are based on a 0–255 U gray-scale.



rats used for corticosterone determinations and *in situ* hybridization experiments. The PVH DSAP injections produced a selective and nearly complete loss of d β h-ir in cell groups A1 and the area of A1/C1 overlap between the levels of the calamus scriptorius and obex and significantly reduced cell number in group A2. In contrast, spinally projecting NE/E neurons in A5, A7, or subcoeruleus (5, 40–42) were not damaged by PVH DSAP injections. Although not quantified, cell numbers in A6, C2, and C3 (all of which contain some cells with projections to the medial hypothalamus) appeared to be reduced by PVH DSAP, as described previously (14), but numbers of subcoeruleus area cells (which project spinally) did not appear to be reduced.

The effects of 2DG on CRH mRNA, CRH hnRNA, and c-fos mRNA levels in the PVHmp

Basal and 2DG-induced transcriptional activity in the PVHmp are shown in Figs. 8 and 9. Neither CRH hnRNA nor CRH mRNA levels differed significantly between SAP and DSAP rats given saline control infusions (Figs. 8 and 9). This result is consistent with the CRH immunohistochemical findings, described above, and provides additional evidence that the DSAP lesion does not damage CRH neurons nonspecifically. There was a robust and significant increase in CRH hnRNA levels in the SAP rats given 2DG, whereas 2DG produced no response in the DSAP rats (Figs. 8A and 9). In contrast, CRH mRNA hybridization signal was not altered by 2DG in either SAP or DSAP rats (Figs. 8B and 9). This is consistent with studies using other stressors in which changes in CRH mRNA levels usually are not detected sooner than 3 h after stress onset (26, 43). The lack of effect

of the DSAP lesion on CRH mRNA indicates that basal CRH mRNA expression is not dependent on catecholamine neurons and also provides another indication that the DSAP lesion did not destroy CRH-expressing cell bodies. Figure 9 also shows that 2DG significantly increased *c-fos* mRNA levels in the PVH of the SAP rats but produced no response in the PVH of DSAP rats, indicating that the activation of CRH neurons, as well as any other cell types in the PVH area, is diminished by the DSAP lesion.

Discussion

Microinjection of DSAP into the PVH produced virtually complete destruction of d β h-ir terminals in the PVH but did not damage CRH-ir cell bodies. This selective lesion profoundly impaired induction of CRH hnRNA, Fos mRNA, and corticosterone secretion by glucoprivation, without altering the corticosterone response to forced swim, the basal circadian pattern of corticosterone secretion, or basal CRH mRNA expression. Thus, hindbrain NE or E neurons are essential for glucoprivic stimulation of neuroendocrine CRH neurons, but are completely dispensable for their stimulation during the daily corticosterone surge or during swim stress.

An extensive literature clearly documents that catecholaminergic afferents release NE/E within the PVH and can activate both CRH gene expression and ACTH release either by direct innervation of CRH neurons or indirectly through glutamatergic interneurons (2–5, 44–53). Despite this wealth of data, the relative importance of catecholamine neurons in the shaping of the HPA axis response to particular stressors has been difficult to establish. This difficulty has been due largely to lack of selective lesioning techniques.

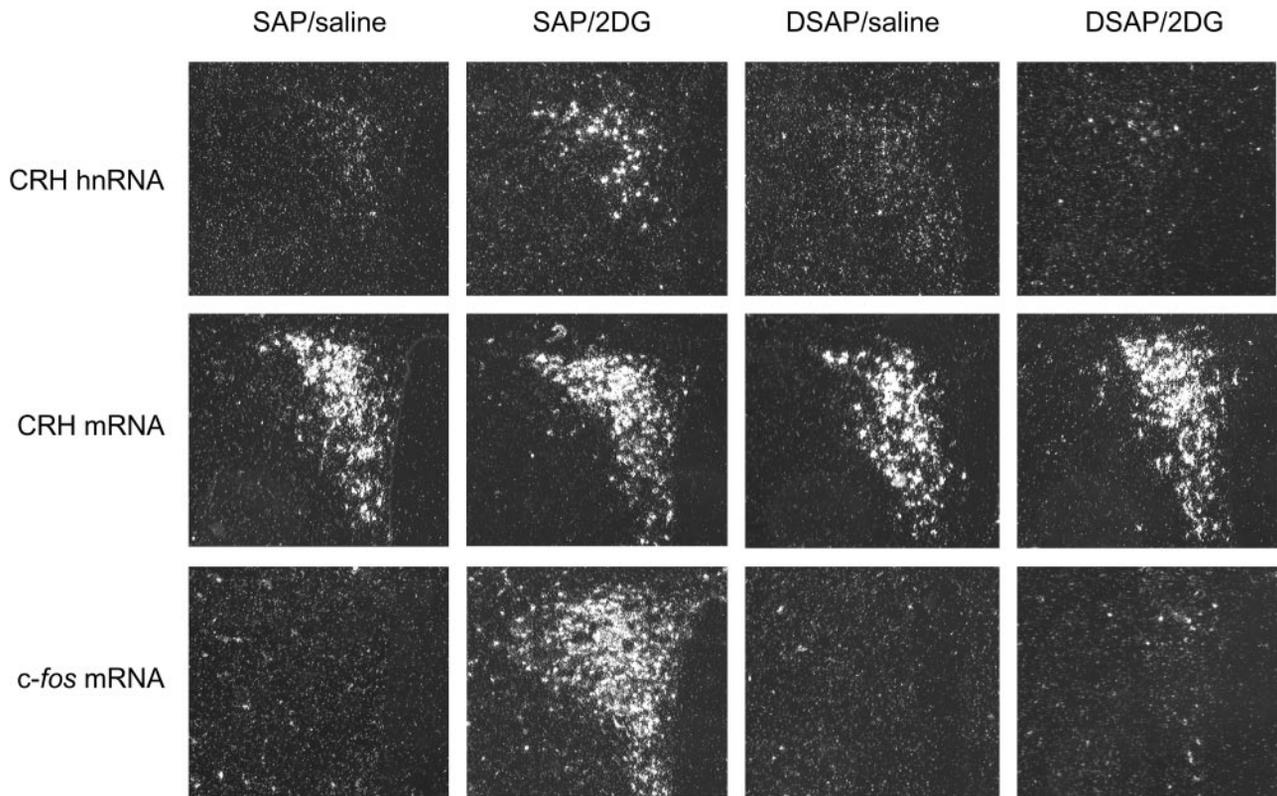


FIG. 9. Darkfield photomicrographs showing CRH hnRNA (*top row*), CRH mRNA (*middle row*), and *c-fos* mRNA (*bottom row*) hybridization signal in the PVH from representative rats previously given bilateral PVH microinjections of unconjugated SAP (*columns 1 and 2*) or anti-d β h-SAP (DSAP, *columns 3 and 4*) to selectively eliminate NE and E terminals in the PVH area. Saline (0.9%) or 2DG (250 mg/kg) was administered by remote iv injection 30 min before death. 2DG-induced glucoprivation did not induce CRH hnRNA or *c-fos* mRNA in DSAP-treated rats. The expression of CRH mRNA, which was not altered at this time point by 2DG, was similar in both SAP and DSAP rats, indicating that the CRH-expressing neurons were not damaged by the DSAP injection.

Even 6-hydroxydopamine, the most selective lesioning agent previously available, causes nonspecific damage at the injection site, is highly variable in its effect on different parts of the catecholamine neuron and on different catecholamine phenotypes, and may produce severe behavioral deficits (54–56). The sensory complexity of many stressors has also been an obstacle. In analyzing neural activation associated with a complex stressor, it is often difficult to distinguish the primary sensory signals driving the CRH response from those generated by secondary physiological reactions to the stressor. Furthermore, hindbrain catecholaminergic neurons are functionally heterogeneous, such that evidence of catecholamine neuron activation by a stressor does not reveal if or how that neuron contributes to the subsequent neuroendocrine response. For these reasons, studies attempting to identify catecholamine pathways controlling CRH neurons by examining the patterns of *c-fos* induction, NE/E release, or electrophysiological changes in response to various stressors have not produced unequivocal results regarding stimulus specificity (51, 57–60). Thus, the ability to eliminate catecholamine afferents selectively using DSAP is an important and novel aspect of the present work. The clear differential effect of the DSAP lesion on the CRH neuroendocrine response to glucoprivation strongly supports the hypothesis that NE/E afferents exert stimulus-specific control of the HPA axis and are not required for all modes of CRH neuronal activation.

Mapping of stressor-induced Fos expression suggests that subpopulations of NE and E neurons may each encode stressor-specific information (23, 59, 61). Certainly the evidence for anatomical and functional heterogeneity among these catecholamine neurons suggests that different subgroups are organized to respond to distinct physiological conditions (14, 39, 62–70). On the other hand, the fact that swim stress has been shown to induce Fos-ir in catecholaminergic neurons (59) would seem to contradict the present findings, which show that these neurons are not required for the CRH response to swim. A possible explanation is that the same catecholamine neurons activated by glucoprivation may also be activated during longer swim bouts because increased physical exertion increases the demand for glucose. Regardless, our findings are unambiguous in showing that any catecholaminergic neurons projecting to the PVH are far more critical for the CRH neuronal response to glucoprivation than to forced swimming. They clearly indicate that catecholaminergic neurons that are Fos-positive after forced swimming either have functions other than the activation of CRH neurons or are not obligatory for a CRH response to swim stress.

Based on a variety of results, it has been hypothesized that stressors can be grouped into two broad categories that control the HPA axis by different neural pathways (7, 59, 71, 72). These categories can be described as interoceptive (or systemic, physiological, or homeostatic) and exteroceptive (or

neurogenic, psychological, or emotional). Glucoprivation would be categorized as an interoceptive stressor, and the importance of catecholamine neurons for the CRH response to this stressor is consistent generally with the reported roles of the hindbrain, and specifically of catecholamine neurons, in a wide variety of homeostatic responses (71–74). The categorization of our swim protocol as an exteroceptive stressor would receive support from recent Fos mapping studies indicating that swim produces an activational footprint that is similar to other proposed exteroceptive stressors and unlike the footprint of proposed interoceptive stressors (59). The neural mediators of the corticosterone response to forced swim are not known. Catecholaminergic neurons clearly are not required, but γ -aminobutyric acid and glutamate are candidates for this role because they both influence CRH secretion (45, 75, 76). Our present results therefore indicate clearly that forced swim and glucoprivation activate CRH neurons by distinct pathways. But whether information conveyed by these different pathways converges at the level of the PVH, or whether it is conveyed by a final common pathway shared with a broader category of stressors remains to be determined (77).

DSAP microinjections produced a nearly complete loss of the corticosterone response to glucoprivation, but a slight residual response remained. This residual response might have been due to incomplete denervation of the PVH or to activation of an indirect catecholaminergic or noncatecholaminergic pathway not damaged by our lesion. Noncatecholaminergic neurons with projections to the PVH have been described in the vicinity of the A1/C1 and A2/C2 cell groups (42). In addition, we reported previously that DSAP abolishes 2DG-induced increases in Fos-immunoreactivity in the PVH, but not in the nucleus of the solitary tract, lateral parabrachial nucleus, or central nucleus of the amygdala (14), supporting the possibility of an alternative pathway from the hindbrain that may influence CRH neurons during glucoprivation. The possibility that the residual response is mediated by a neural or endocrine factor that does not involve CRH neurons should also be considered because DSAP lesions abolished the glucoprivation-induced increase in *c-fos* mRNA in the PVH. This is not due to an inability of PVH neurons to express Fos after DSAP treatment. We showed previously in DSAP-lesioned rats that Fos protein can be increased in PVH neurons by intraventricular injection of E, despite the lack of responsiveness of PVH neurons to 2DG (14). Thus, any stimulation of corticosterone secretion by glucoprivation that survives after the DSAP lesion does not require the immediate-early gene response, does not bring that response to the detection threshold of either immunohistochemical or transcriptional detection methods, or does not require CRH neurons.

Our data extend previous studies demonstrating that catecholaminergic afferents are not required for basal CRH gene expression (78, 79). We now show that both CRH mRNA levels in the PVH and circadian corticosterone secretion are completely unaffected by the DSAP lesions. We recently demonstrated that CRH mRNA (and presumably peptide) levels in the PVH are maintained by a robust circadian rhythm of CRH gene transcription, the amplitude of which is independent of circulating corticosterone (80). Together

with the fact that basal CRH mRNA levels are unaffected by DSAP lesions, at least at the midpoint of the light cycle, these findings show that catecholaminergic afferents are not required for maintaining the basal CRH gene transcriptional activity that accompanies circadian corticosterone secretion. mRNA levels would be expected to fall significantly after the lesion if catecholaminergic afferents were required to sustain basal transcription. However, further experiments are required to establish whether NE/E inputs contribute to the integrity of the basal rhythm of CRH gene transcription.

The present findings add to the developing picture of the neural organization of central systems controlling brain glucose homeostasis. In particular, they define the essential role hindbrain catecholamine neurons play in this regard. Cell groups A1 and C1–C3 are situated in sites where localized glucoprivation stimulates feeding and hyperglycemia and are strongly activated by systemic glucoprivation (24). In other studies using DSAP, we have shown that catecholamine neurons with projections to the medial hypothalamus are required for glucoprivic feeding (14) and for arousal of hypothalamic orexigenic circuitry by glucoprivation (81, 82). A separate, spinally projecting population is required for the adrenal medullary response to glucoprivation (14). The present demonstration of the importance of hindbrain catecholamine neurons in the glucoprivic stimulation of corticosterone secretion confirms their essential role in coordinating multiple regulatory responses that assure glucose delivery to the brain as a whole.

Acknowledgments

We thank Dr. Wylie Vale (The Salk Institute, Peptide Biology Laboratory, La Jolla, CA) for supplying CRH antibody and Robert Bierwirth for help with preliminary studies.

Received October 17, 2002. Accepted January 6, 2003.

Address all correspondence and requests for reprints to: Sue Ritter, Department of Veterinary and Comparative Anatomy, Pharmacology, and Physiology, Washington State University, Pullman, Washington 99164-6520. E-mail: sjr@vetmed.wsu.edu.

This work was supported by the Juvenile Diabetes Research Foundation International and U.S. Public Health Service Grants DK-40498 (to S.R.) and NS-29728 (to A.G.W.).

References

- Swanson LW, Sawchenko PE 1983 Hypothalamic integration: organization of the paraventricular and supraoptic nuclei. *Annu Rev Neurosci* 6:269–324
- Plotsky PM, Otto S, Sutton S 1987 Neurotransmitter modulation of corticotropin releasing factor secretion into the hypophysial-portal circulation. *Life Sci* 41:1311–1317
- Plotsky PM, Cunningham Jr ET, Widmaier EP 1989 Catecholaminergic modulation of corticotropin-releasing factor and adrenocorticotropin secretion. *Endocr Rev* 10:437–458
- Ruggiero DA, Ross CA, Anwar M, Park DH, Joh TH, Reis DJ 1985 Distribution of neurons containing phenylethanolamine *N*-methyltransferase in the medulla and hypothalamus of rat. *J Comp Neurol* 239:127–154
- Tucker DC, Saper CB, Ruggiero DA, Reis DJ 1987 Organization of central adrenergic pathways. I. Relationships of ventrolateral medullary projections to the hypothalamus and spinal cord. *J Comp Neurol* 259:591–603
- Whitnall MH 1993 Regulation of the hypothalamic corticotropin-releasing hormone neurosecretory system. *Prog Neurobiol* 40:573–629
- Herman JP, Cullinan WE 1997 Neurocircuitry of stress: central control of the hypothalamo-pituitary-adrenocortical axis. *Trends Neurosci* 20:78–84
- Van de Kar LD, Blair ML 1999 Forebrain pathways mediating stress-induced hormone secretion. *Front Neuroendocrinol* 20:1–48
- Picklo MJ, Wiley RG, Lappi DA, Robertson D 1994 Noradrenergic lesioning with an anti-dopamine β -hydroxylase immunotoxin. *Brain Res* 666:195–200
- Picklo MJ, Wiley RG, Lonce S, Lappi DA, Robertson D 1995 Anti-dopamine

- β -hydroxylase immunotoxin-induced sympathectomy in adult rats. *J Pharmacol Exp Ther* 275:1003–1010
11. Picklo MJ 1997 Methods of sympathetic degeneration and alteration. *J Auton Nerv Syst* 62:111–125
 12. Wrenn CC, Picklo MJ, Lappi DA, Robertson D, Wiley RG 1996 Central noradrenergic lesioning using anti-DBH-saporin: anatomical findings. *Brain Res* 740:175–184
 13. Studelska DR, Brimjoin S 1989 Partial isolation of two classes of dopamine β -hydroxylase-containing particles undergoing rapid axonal transport in rat sciatic nerve. *J Neurochem* 53:622–631
 14. Ritter S, Bugarith K, Dinh TT 2001 Immunotoxic destruction of distinct catecholamine subgroups produces selective impairment of glucoregulatory responses and neuronal activation. *J Comp Neurol* 432:197–216
 15. Blessing WW, Lappi DA, Wiley RG 1998 Destruction of locus coeruleus neuronal perikarya after injection of anti-dopamine- β -hydroxylase immunotoxin into the olfactory bulb of the rat. *Neurosci Lett* 243:85–88
 16. Barthelemy I, Martineau D, Ong M, Matsunami R, Ling N, Benatti L, Cavallaro U, Soria M, Lappi DA 1993 The expression of saporin, a ribosome-inactivating protein from the plant *Saponaria officinalis*, in *Escherichia coli*. *J Biol Chem* 268:6541–6548
 17. Brown J 1962 Effects of 2-deoxy-D-glucose on carbohydrate metabolism: review of the literature and studies in the rat. *Metabolism* 11:1098–1112
 18. Kartesz M, Dallman MF, Makara GB, Stark E 1982 Regulation of the adrenocortical response to insulin-induced hypoglycemia. *Endocrinology* 111:535–541
 19. Duncan GE, Johnson KB, Breese GR 1993 Topographic patterns of brain activity in response to swim stress: assessment by 2-deoxyglucose uptake and expression of Fos-like immunoreactivity. *J Neurosci* 13:3932–3943
 20. Cullinan WE, Herman JP, Battaglia DF, Akil H, Watson SJ 1995 Pattern and time course of immediate early gene expression in rat brain following acute stress. *Neuroscience* 64:477–505
 21. Saifer A, Gerstenfeld S 1958 The photometric microdetermination of blood glucose with glucose oxidase. *J Lab Clin Med* 51:445–460
 22. Kay-Nishiyama C, Watts AG 1999 Dehydration modifies somal CRH immunoreactivity in the rat hypothalamus: an immunocytochemical study in the absence of colchicine. *Brain Res* 822:251–255
 23. Ritter S, Dinh TT, Bugarith K 1998 2-Deoxy-D-glucose-induced Fos-immunoreactivity in brain and adrenal medulla after injections of saporin-anti- α -SAP. *Soc Neurosci Abstr* 28:389
 24. Ritter S, Llewellyn-Smith I, Dinh TT 1998 Subgroups of hindbrain catecholamine neurons are selectively activated by 2-deoxy-D-glucose induced metabolic challenge. *Brain Res* 805:41–54
 25. Watts AG, Sanchez-Watts G 1995 Physiological regulation of peptide messenger RNA colocalization in rat hypothalamic paraventricular medial parvocellular neurons. *J Comp Neurol* 352:501–514
 26. Kovacs KJ, Sawchenko PE 1996 Regulation of stress-induced transcriptional changes in the hypothalamic neurosecretory neurons. *J Mol Neurosci* 7:125–133
 27. Watts AG 1992 Disturbance of fluid homeostasis leads to temporally and anatomically distinct responses in neuropeptide and tyrosine hydroxylase mRNA levels in the paraventricular and supraoptic nuclei of the rat. *Neuroscience* 46:859–879
 28. Tanimura SM, Sanchez-Watts G, Watts AG 1998 Peptide gene activation, secretion, and steroid feedback during stimulation of rat neuroendocrine corticotropin-releasing hormone neurons. *Endocrinology* 139:3822–3829
 29. Swanson LW 1998 Brain maps: structure of the rat brain. 2nd ed. Amsterdam: Elsevier Publishers
 30. Cunningham Jr ET, Bohn MC, Sawchenko PE 1990 Organization of adrenergic inputs to the paraventricular and supraoptic nuclei of the hypothalamus in the rat. *J Comp Neurol* 292:651–667
 31. Cunningham Jr ET, Sawchenko PE 1988 Anatomical specificity of noradrenergic inputs to the paraventricular and supraoptic nuclei of the rat hypothalamus. *J Comp Neurol* 274:60–76
 32. Paxinos G, Watson C 1997 The rat brain in stereotaxic coordinates. 3rd ed. San Diego: Academic Press
 33. Hokfelt T, Johansson O, Goldstein M 1984 Central catecholamine neurons as revealed by immunohistochemistry with special reference to adrenaline neurons. In: Bjorklund A, Hokfelt T, eds. *Handbook of chemical neuroanatomy*. Amsterdam: Elsevier; 157–276
 34. Kalia M, Fuxe K, Goldstein M 1985 Rat medulla oblongata. II. Dopaminergic, noradrenergic (A1 and A2) and adrenergic neurons, nerve fibers, and presumptive terminal processes. *J Comp Neurol* 233:308–332
 35. Minson J, Llewellyn-Smith I, Neville A, Somogyi P, Chalmers J 1990 Quantitative analysis of spinally projecting adrenaline-synthesising neurons of C1, C2 and C3 groups in rat medulla oblongata. *J Auton Nerv Syst* 30:209–220
 36. Matthews JN, Altman DG, Campbell MJ, Royston P 1990 Analysis of serial measurements in medical research. *BMJ* 300:230–235
 37. Sage D, Maurel D, Bosler O 2001 Involvement of the suprachiasmatic nucleus in diurnal ACTH and corticosterone responsiveness to stress. *Am J Physiol Endocrinol Metab* 280:E260–E269
 38. Rusak B, Zucker I 1979 Neural regulation of circadian rhythms. *Physiol Rev* 59:449–526
 39. Sawchenko PE, Swanson LW, Grzanna R, Howe PR, Bloom SR, Polak JM 1985 Colocalization of neuropeptide Y immunoreactivity in brainstem catecholaminergic neurons that project to the paraventricular nucleus of the hypothalamus. *J Comp Neurol* 241:138–153
 40. Sawchenko PE, Swanson LW 1982 The organization of noradrenergic pathways from the brainstem to the paraventricular and supraoptic nuclei in the rat. *Brain Res* 257:275–325
 41. Westlund KN, Bowker RM, Ziegler MG, Coulter JD 1983 Noradrenergic projections to the spinal cord of the rat. *Brain Res* 263:15–31
 42. Sawchenko PE, Swanson LW 1982 Immunohistochemical identification of neurons in the paraventricular nucleus of the hypothalamus that project to the medulla or to the spinal cord in the rat. *J Comp Neurol* 205:260–272
 43. Watts AG 1991 Ether anesthesia differentially affects the content of prepro-corticotropin-releasing hormone, prepro-neurotensin/neuromedin N and prepro-enkephalin mRNAs in the hypothalamic paraventricular nucleus of the rat. *Brain Res* 544:353–357
 44. Alonso G, Szafarczyk A, Balmefrezol M, Assenmacher I 1986 Immunocytochemical evidence for stimulatory control by the ventral noradrenergic bundle of parvocellular neurons of the paraventricular nucleus secreting corticotropin releasing hormone and vasopressin in rats. *Brain Res* 397:297–307
 45. Cole RL, Sawchenko PE 2002 Neurotransmitter regulation of cellular activation and neuropeptide gene expression in the paraventricular nucleus of the hypothalamus. *J Neurosci* 22:959–969
 46. Daftary SS, Boudaba C, Tasker JG 2000 Noradrenergic regulation of parvocellular neurons in the rat hypothalamic paraventricular nucleus. *Neuroscience* 96:743–751
 47. Day HE, Campeau S, Watson Jr SJ, Akil H 1999 Expression of α (1b) adrenoceptor mRNA in corticotropin-releasing hormone-containing cells of the rat hypothalamus and its regulation by corticosterone. *J Neurosci* 19:10098–10106
 48. Guillaume V, Conte-Devolx B, Szafarczyk A, Malaval F, Pares-Herbutte N, Grino M, Alonso G, Assenmacher I, Oliver C 1987 The corticotropin-releasing factor release in rat hypophysial portal blood is mediated by brain catecholamines. *Neuroendocrinology* 46:143–146
 49. Itoi K, Helmreich DL, Lopez-Figueroa MO, Watson SJ 1999 Differential regulation of corticotropin-releasing hormone and vasopressin gene transcription in the hypothalamus by norepinephrine. *J Neurosci* 19:5464–5472
 50. Leibowitz SF, Diaz S, Tempel D 1989 Norepinephrine in the paraventricular nucleus stimulates corticosterone release. *Brain Res* 496:219–227
 51. Pacak K, Palkovits M 2001 Stressor specificity of central neuroendocrine responses: implications for stress-related disorders. *Endocr Rev* 22:502–548
 52. Spinedi E, Johnston CA, Chisari A, Negro-Vilar A 1988 Role of central epinephrine on the regulation of corticotropin-releasing factor and adrenocorticotropin secretion. *Endocrinology* 122:1977–1983
 53. Szafarczyk A, Alonso G, Ixart G, Malaval F, Assenmacher I 1985 Diurnal-stimulated and stress-induced ACTH release in rats is mediated by ventral noradrenergic bundle. *Am J Physiol* 249:E219–E226
 54. Marshall JF, Richardson JS 1974 Nigrostriatal bundle damage and the lateral hypothalamic syndrome. *J Comp Physiol Psychol* 87:808–830
 55. Stricker EM, Friedman MI, Zigmond MJ 1975 Glucoregulatory feeding by rats after intraventricular 6-hydroxydopamine or lateral hypothalamic lesions. *Science* 189:895–897
 56. Jonsson G 1983 Chemical lesioning techniques: monoamine neurotoxins. In: Bjorklund A, Hokfelt T, eds. *Handbook of chemical neuroanatomy: methods in chemical neuroanatomy*. New York: Elsevier Science Publishers; 463–507
 57. Pacak K, Palkovits M, Kopin IJ, Goldstein DS 1995 Stress-induced norepinephrine release in the hypothalamic paraventricular nucleus and pituitary-adrenocortical and sympathoadrenal activity: in vivo microdialysis studies. *Front Neuroendocrinol* 16:89–150
 58. Beverly JL, De Vries MG, Bouman SD, Arseneau LM 2001 Noradrenergic and GABAergic systems in the medial hypothalamus are activated during hypoglycemia. *Am J Physiol Regul Integr Comp Physiol* 280:R563–R569
 59. Buller K, Xu Y, Dayas C, Day T 2001 Dorsal and ventral medullary catecholamine cell groups contribute differentially to systemic interleukin-1 β -induced hypothalamic pituitary adrenal axis responses. *Neuroendocrinology* 73:129–138
 60. Gaillet S, Lachuer J, Malaval F, Assenmacher I, Szafarczyk A 1991 The involvement of noradrenergic ascending pathways in the stress-induced activation of ACTH and corticosterone secretions is dependent on the nature of stressors. *Exp Brain Res* 87:173–180
 61. Ritter S, Dinh TT 1994 2-Mercaptoacetate and 2-deoxy-D-glucose induce Fos-like immunoreactivity in rat brain. *Brain Res* 641:111–120
 62. Sawchenko PE, Bohn MC 1989 Glucocorticoid receptor-immunoreactivity in C1, C2, and C3 adrenergic neurons that project to the hypothalamus or to the spinal cord in the rat. *J Comp Neurol* 285:107–116
 63. Pau KY, Ma YJ, Yu JH, Yang SP, Airhart N, Spies HG 1997 Topographic comparison of the expression of norepinephrine transporter, tyrosine hydroxylase and neuropeptide Y mRNA in association with dopamine β -hydroxylase neurons in the rabbit brainstem. *Brain Res Mol Brain Res* 48:367–381
 64. Everitt BJ, Hokfelt T, Terenius L, Tatemoto K, Mutt V, Goldstein M 1984 Differential co-existence of neuropeptide Y (NPY)-like immunoreactivity with catecholamines in the central nervous system of the rat. *Neuroscience* 11:443–462

65. Nagatani S, Bucholtz DC, Murahashi K, Estacio MA, Tsukamura H, Foster DL, Maeda KI 1996 Reduction of glucose availability suppresses pulsatile luteinizing hormone release in female and male rats. *Endocrinology* 137:1166–1170
66. Simonian SX, Delaleu B, Caraty A, Herbison AE 1998 Estrogen receptor expression in brainstem noradrenergic neurons of the sheep. *Neuroendocrinology* 67:392–402
67. Sawchenko PE, Swanson LW 1983 The organization and biochemical specificity of afferent projections to the paraventricular and supraoptic nuclei. *Prog Brain Res* 60:19–29
68. Schreihof AM, Guyenet PG 2000 Sympathetic reflexes after depletion of bulbospinal catecholaminergic neurons with anti-D β H-saporin. *Am J Physiol Regul Integr Comp Physiol* 279:R729–R742
69. Yeomans DC, Clark FM, Paice JA, Proudfit HK 1992 Antinociception induced by electrical stimulation of spinally projecting noradrenergic neurons in the A7 catecholamine cell group of the rat. *Pain* 48:449–461
70. Conde GL, Bicknell RJ, Herbison AE 1995 Changing patterns of Fos expression in brainstem catecholaminergic neurons during the rat oestrous cycle. *Brain Res* 672:68–76
71. Behan DP, De Souza EB, Potter E, Sawchenko P, Lowry PJ, Vale WW 1996 Modulatory actions of corticotropin-releasing factor-binding protein. *Ann N Y Acad Sci* 780:81–95
72. Bale TL, Contarino A, Smith GW, Chan R, Gold LH, Sawchenko PE, Koob GF, Vale WW, Lee KF 2000 Mice deficient for corticotropin-releasing hormone receptor-2 display anxiety-like behaviour and are hypersensitive to stress. *Nat Genet* 24:410–414
73. Blessing WW 1997 The lower brainstem and bodily homeostasis. New York: Oxford University Press
74. Akabayashi A, Watanabe Y, Gabriel SM, Chae HJ, Leibowitz SF 1994 Hypothalamic galanin-like immunoreactivity and its gene expression in relation to circulating corticosterone. *Brain Res Mol Brain Res* 25:305–312
75. Brann DW 1995 Glutamate: a major excitatory transmitter in neuroendocrine regulation. *Neuroendocrinology* 61:213–225
76. Boudaba C, Szabo K, Tasker JG 1996 Physiological mapping of local inhibitory inputs to the hypothalamic paraventricular nucleus. *J Neurosci* 16:7151–7160
77. Watts AG, Sanchez-Watts G 2002 Interactions between heterotypic stressors and corticosterone reveal integrative mechanisms for controlling corticotropin-releasing hormone gene expression in the rat paraventricular nucleus. *J Neurosci* 22:6282–6289
78. Harbuz MS, Chowdrey HS, Jessop DS, Biswas S, Lightman SL 1991 Role of catecholamines in mediating messenger RNA and hormonal responses to stress. *Brain Res* 551:52–57
79. Swanson LW, Simmons DM 1989 Differential steroid hormone and neural influences on peptide mRNA levels in CRH cells of the paraventricular nucleus: a hybridization histochemical study in the rat. *J Comp Neurol* 285:413–435
80. Tanimura SM, Watts AG 2001 Corticosterone modulation of ACTH secretagogue gene expression in the paraventricular nucleus. *Peptides* 22:775–783
81. Fraley GS, Dinh TT, Ritter S 2002 Immunotoxic catecholamine lesions attenuate 2DG-induced increase in AGRP mRNA. *Peptides* 23:1093–1099
82. Fraley GS, Ritter S 2003 Immunolesion of norepinephrine and epinephrine afferents to medial hypothalamus alters basal and 2DG-induced NPY and AGRP mRNA expression in the arcuate nucleus. *Endocrinology* 144:75–83