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Hypotensive hypovolemia and hypoglycemia activate different hindbrain catecholamine neurons with projections to the hypothalamus

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Drug treatment with the antiglycolytic glucose analog, 2-deoxy-D-glucose, have been reported to stimulate AVP secretion (1, 4, 5, 13–16, 21, 22) and to induce Fos expression in magnocellular AVP neurons (5, 10, 11). Hypoglycemia also alters AVP gene expression in the rat paraventricular hypothalamus (38). Indeed, AVP has been proposed to contribute significantly to ACTH secretion (21) and to the inhibition of luteinizing hormone (30) under hypoglycemic conditions. Although alpha adrenergic blockade has been reported to reduce hypoglycemic stimulation of AVP secretion (24), a role for hindbrain catecholamine neurons in the AVP response to hypoglycemia has not been established (19). However, in view of the range of glucoregulatory responses that require hindbrain catecholamine neurons for their elicitation and the demonstration that hypovolemic stimulation of AVP secretion is catecholamine-dependent, it is reasonable to hypothesize that glucoprivation-induced AVP secretion is also mediated by hindbrain catecholamine neurons. If so, this would raise the possibility that responses to glucoprivation and hypovolemia are mediated by the same catecholamine neurons.

To examine these issues, we lesioned catecholamine neurons with projections to the medial hypothalamus using the selective

HINDBRAIN CATECHOLAMINE NEURONS are responsive to a variety of physiological stimuli. For example, noradrenergic neurons of the A1 cell group provide monosynaptic innervation of magnocellular vasopressinergic neurons (47, 48). These A1 neurons are activated by hypovolemia, as indicated by induction of Fos expression and electrophysiological results (12, 18, 19, 41, 51), and their stimulation results in secretion of AVP into the posterior pituitary blood (8). Lesions in the caudal ventrolateral medulla causing destruction of A1 neurons, or suppression of A1 neuron activity with agonists of the inhibitory neurotransmitter, GABA, impairs the AVP response to hypovolemia (26, 29). Compelling data indicate that hindbrain catecholamine neurons are also strongly activated by glucoprivation, as indicated by induction of Fos expression in both cell bodies and hypothalamic target sites (43) and by increases in turnover and release of catecholamines in hypothalamic terminal fields (6, 7, 45). Studies using selective immunotoxin lesions have shown that hindbrain catecholamine neurons are essential for elicitation of a number of glucoregulatory responses, including feeding (31, 42), corticosterone secretion (44), and suppression of estrous cycling (35). As demonstrated by these studies, immunotoxin lesions causing deficits in the latter responses, profoundly reduce numbers of A1 neurons.

In addition to stimulation of glucoregulatory responses, glucoprivation induced by hypoglycemic doses of insulin or by the antiglycolytic glucose analog, 2-deoxy-D-glucose, have been reported to stimulate AVP secretion (1, 4, 5, 13–16, 21, 22) and to induce Fos expression in magnocellular AVP neurons (5, 10, 11). Hypoglycemia also alters AVP gene expression in the rat paraventricular hypothalamus (38). Indeed, AVP has been proposed to contribute significantly to ACTH secretion (21) and to the inhibition of luteinizing hormone (30) under hypoglycemic conditions. Although alpha adrenergic blockade has been reported to reduce hypoglycemic stimulation of AVP secretion (24), a role for hindbrain catecholamine neurons in the AVP response to hypoglycemia has not been established (19). However, in view of the range of glucoregulatory responses that require hindbrain catecholamine neurons for their elicitation and the demonstration that hypovolemic stimulation of AVP secretion is catecholamine-dependent, it is reasonable to hypothesize that glucoprivation-induced AVP secretion is also mediated by hindbrain catecholamine neurons. If so, this would raise the possibility that responses to glucoprivation and hypovolemia are mediated by the same catecholamine neurons.

To examine these issues, we lesioned catecholamine neurons with projections to the medial hypothalamus using the selective
and retrogradely-transported immunotoxin, anti-dopamine β-hydroxylase conjugated to saporin (DSAP), which lesions norepinephrine (NE) and epinephrine (Epi) neurons (56, 57). When injected into NE and Epi terminal areas, DSAP is internalized selectively by the binding of the monoclonal antibody to its target, dopamine β-hydroxylase (dβh), which is uniquely expressed by NE and Epi neurons. The conjugate is then retrogradely transported to the soma, where saporin (SAP) causes cell death by ribosomal disruption. We demonstrated previously that microinjections of DSAP into the hypothalamic paraventricular nucleus (PVH) will lesion only those catecholamine neurons that innervate the medial hypothalamus and do not lesion other catecholamine neurons, such as those projecting spinally (42). Moreover, microinjections of DSAP produce minimal nonspecific damage at the injection site. For example, we showed that PVH DSAP injections profoundly reduced glucoprivic stimulation of corticosterone secretion without apparent damage to CRH neurons and without impairing their response to a nonglucoprivic stimulus (forced swimming) (44).

In the present experiment, we microinjected DSAP into the PVH and measured AVP responses to hypovolemic and glucoprivic (hypoglycemic) stimulation. Our hypothesis was that both stimuli would increase AVP secretion and that the DSAP lesion would impair responses to both stimuli. As a control for the functional integrity of the AVP neurons in the DSAP-lesioned rats, we also measured the AVP response to osmotic challenge, which has been shown to be catecholamine-independent (2, 32, 49). We reasoned that the catecholamine-independent AVP response to osmotic challenge would not be impaired by DSAP.

MATERIALS AND METHODS

Animals. Adult female and male Sprague-Dawley rats were obtained from Simonsen Laboratories. They were housed individually in suspended wire mesh cages under standard American Association for Accreditation of Laboratory Animal Care-approved conditions in a temperature-controlled room (21 ± 1°C) illuminated between 0700 and 1900. Rats had ad libitum access to pelleted rat food and tap water, except as noted. Tests were conducted between 0900 and 1300 and 1900. Rats had ad libitum access to water, except as noted. Tests were conducted between 0900 and 1300.

Intravenous catheters. One week before the first experiment, catheters constructed from Silastic tubing (ID 0.64 mm, OD 1.19 mm, Dow Corning, Midland, MI) were implanted intra-atrially through the right jugular vein. When not in use, catheters were filled with polyvinylpyrrolidone solution (40,000 mol wt, Sigma Aldrich, St. Louis, MO), 11 g polyvinylpyrrolidone in 20 ml 0.9% saline containing 1,000 U/ml of heparin (Elkins-Sinn, Cherry Hill, NJ) and 2 mg/ml gentamicin (Schering-Plough Animal Health, Kenilworth, NJ). Before experiments requiring blood withdrawal, rats were habituated to opaque 4" × 12" Plexiglas testing chambers designed for remote blood sampling. On test days, they were placed in the chambers without food and connected to the sampling lines 1 h before collection of the first blood sample. Blood samples were collected remotely.

Hypovolemia. Hypovolemia was induced by blood withdrawal (1 ml/min for 4.5 min). Blood was also withdrawn (2.5 ml/sample) 20 and 50 min later for analysis of AVP levels. The total volume initially withdrawn was 4.5 ml, or ~28% of total blood volume (54.3 ml/kg body wt) for rats of the weight used in this experiment (280–300 g). This amount of blood loss decreases mean arterial pressure (MAP) to ~60 mmHg (36). Adding the volume of the 20-min sample to the volume of blood initially withdrawn, the total blood withdrawn (7 ml) was ~43% of total blood volume. After the 50-min sample was withdrawn in the latter experiment, blood volume was replaced by slow infusion of an equal volume of washed and resuspended erythrocytes, which were obtained from heparinized donor blood, washed and recentrifuged 3 times, and resuspended in a volume of Nutricil (Medseq, Covina, CA) equal to the original plasma volume.

Osmotic and hypoglycemic challenges. Osmotic challenge was induced by subcutaneous administration of hypertonic saline (1 M NaCl, 15 ml/kg). When assessed in a separate group of rats, this challenge increased plasma osmolality from the basal value of 294 mOsmol at time 0 to 316 mOsmol at 50 min after the 1 M saline injection. Response to a control solution (0.15 M NaCl, 15 ml/kg) was tested separately in the same rats with at least 1 wk intervening between tests. Hypoglycemic challenge was induced by administration of a hypoglycemic dose of regular insulin (R. Humulin, Eli Lilly, Indianapolis, IN; 1.5 U/kg). Control injections were an equal volume of 0.15 M saline. For these tests, a single blood sample (2.5 ml) was drawn 50 min after the insulin and hypertonic or isotonic saline injections to minimize the contribution of hemodynamic changes to the AVP response. Blood samples from each treatment were assayed for glucose and corticosterone as independent measures of the efficacy of insulin-induced hypoglycemia.

We examined more fully the time course of the AVP response to osmotic and hypoglycemic challenge using male rats (350–400 g). Rats were prepared by injection of SAP or DSAP into PVH and by implantation of chronic catheters for blood withdrawal, as described above for female rats, except that both femoral vein and intra-atrial catheters were inserted. On the test days, rats were injected with hypertonic saline (1 M, 15 ml/kg), insulin (1.5 U/kg), or isotonic saline control at 0 min. Blood was sampled for AVP analysis 30 min before injection and at 20, 60, and 120 min after the injection. For this experiment, blood replacement (via a femoral vein catheter) and blood sample withdrawal (via the intra-atrial catheter) were done simultaneously to minimize hemodynamic changes. Erythrocytes for replacement were obtained by centrifugation of heparinized donor blood, washed and recentrifuged 3 times, and then resuspended in a volume of Nutricil (Medseq, Covina, CA) equal to the original plasma volume. Blood volume replaced was equal to the volume withdrawn at each sampling time.

Glucoprivic feeding. Previous experiments have shown that glucoprivic feeding is abolished by effective PVH injections of DSAP. Therefore, glucoprivic feeding was used as an independent measure of the effectiveness of the DSAP lesion, as well as to verify the effectiveness of the hypoglycemic challenge in the SAP controls. All DSAP and SAP rats used in the above experiments were tested. For these tests, rats were given a weighed quantity of pelleted rat chow in...
their home cages and injected subcutaneously with a hypoglycemic dose of regular insulin (R. Humulin, Eli Lilly; 1.5 U/kg). Baseline intakes were measured after injection of sterile saline (0.9%, 1 ml/kg). Remaining pellets and spillage were measured in the 4-h period immediately after injections.

**Assay of AVP and corticosterone.** Plasma was separated from blood samples by centrifugation, transferred to fresh vials, frozen and stored at −80°C until assayed. Plasma AVP was extracted using 1% tri-fluoro acetic acid buffer and C-18 column technique. Each sample was analyzed in triplicate. The recovery from the extraction protocol is routinely >70%. Plasma AVP concentrations were determined using a commercially available ELISA (Assay Designs, Ann Arbor, MI; sensitivity for AVP ~2 pg/ml-1,000 pg/ml) (28). Corticosterone, measured during hypoglycemia, was assayed using a radioimmunoassay kit obtained from Diagnostic Products, (Los Angeles, CA, Cat. No. TKRC-1). The lower limit of sensitivity for corticosterone was 20 ng/ml. Samples from the insulin test were assayed for glucose concentration using the glucose oxidase protocol (46).

**Immunohistochemistry.** After testing was completed, rats were killed by lethal dose of pentobarbital sodium (Abbott Laboratories, North Chicago, IL; 300 mg/kg) and perfused transcardially with PBS followed by 4% formalin (both pH 7.4). Brains were removed, postfixed at room temperature for 2 h, and cryoprotected overnight in 25% sucrose. Coronal sections (40 μm) of the brain stem were immunoreacted for detection of dβH. Hypothalamic sections were processed to detect dβH-immunoreactive (ir) or AVP-ir. The dβH-ir was used to verify the DSAP-induced lesion, and AVP-ir was used to evaluate the effect of the PVH DSAP injection on AVP neurons, but the latter were not quantified.

Immunohistochemical staining was done using standard avidin-biotin-peroxidase techniques described previously (43). Briefly, sections were treated with 50% ethanol for 30 min and then washed (3 × 5 min) in 0.1 M PB 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 incubated for 48 h in mouse monoclonal anti-dβH (Chemicon 1:100,000) or rabbit polyclonal anti-AVP (Chemicon 1:15,000) made in 10% normal horse serum-TPBS. The primary antibody was removed, and the sections were washed and incubated in biotintylated donkey anti-mouse or anti-rabbit IgG (both 1:500 in 1% normal horse serum-TPBS, Jackson ImmunoResearch Laboratories). After 24 h, the tissue was washed (3 × 10 min), incubated with Extravidin-peroxidase (Sigma, 1:1,500 in TPBS) overnight, washed again (3 × 10 min), and reacted for visualization of dβH- or CRF-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 Zeiss Axioplan 2 photomicroscope equipped with a Zeiss AxioCam digital camera and linked to a computer running Zeiss AxioVision imaging software. Plates of multiple sections were assembled using Adobe Photoshop (Adobe Systems). Brightness only was altered digitally in some cases to achieve uniformity among photomicrographs grouped in composite figures.

**Assessment of the DSAP lesion.** In referring to catecholamine cell groups, we use the conventional terminology: NE cell groups are designated A1–A6 and Epi cell groups as C1–C3 (39). To verify the effectiveness of PVHI DSAP injections in lesioning NE/Epi neurons, dβH-ir cell bodies were quantified in cell groups A1, A2, A5, C1, C2, and C3. For each cell group, three nonconsecutive 30-μm sections anatomically matched across animals were quantified by counting immunoreactive cell profiles, regardless of the presence of a nucleus. Cell fragments were not counted. No correction factor for double-counting was applied because of the use of relatively thick nonconsecutive sections for the quantification. Because of the overlap of A1 and C1, cells in the area of overlap were counted as a single group, extending from the most caudal extent of the area postrema to the rostral pole of the area postrema and are referred to as group A1/C1. Cell group A5 was quantified at the level of the locus coeruleus, just rostral to the exit of cranial nerve 7 from the ventral brain stem. Hypothalamic sections were examined for the presence of dβH-ir terminals and AVP-ir cell bodies, but these were not quantified.

**Statistical analysis.** AVP, corticosterone, glucose determinations, and food intake were analyzed using two-factor ANOVA, with repeated measures, as appropriate, using post hoc tests to isolate significant differences. Catecholamine cell bodies in each area of quantification were compared between DSAP and SAP using a Student’s t-test. A probability level of 0.05 was used as the level for significance.

**RESULTS**

**Immunohistochemistry.** Figures 1 and 2 show the most caudal and most rostral of the three levels chosen for quantification of dβH-ir cell bodies. Cell bodies were analyzed separately in male and female rats, but results did not differ between sexes, so results were pooled for the final statistical analysis and for presentation in Fig. 3, which provides cell counts for each quantified catecholamine cell group. Cell numbers were decreased significantly (P < 0.002) by DSAP in all quantified areas. Results are similar to those reported previously using the same DSAP injection parameters (31, 35, 42, 44). PVH DSAP injections caused profound decrease in cell numbers in cell groups containing the largest proportions of cell bodies with projections to the medial hypothalamus; namely, A1 and A1/C1 (Fig. 4, A and D). Other cell groups are of more mixed composition, including a significant number of cell bodies that project to other sites but not to the PVH (A2, C1, A5). As expected, these groups sustained correspondingly less damage from PVH DSAP injections. In the PVH, dβH-ir terminals were nearly eliminated in the DSAP-injected rats, compared with SAP controls (Fig. 4, B and E). The DSAP injections also produced an almost complete loss of dβH-ir terminals in the supraoptic nuclei of female rats (Fig. 4, C and F). However, we noted that the supraoptic nuclei appeared to be less completely denervated in the male than in the female DSAP-injected rats, although the pattern and extent of cell loss in the hindbrain were equivalent in both sexes. To determine whether this observation reflects an actual sex difference in catecholamine innervation of the supraoptic nucleus would require additional experimentation. AVP cell bodies in the PVH did not appear to be damaged by the DSAP injection (Fig. 5).

**Hypovolemia.** The effect of hypovolemia on AVP levels is shown in Fig. 6. The mean baseline AVP level of SAP controls at 2 min was 2.9 (SE 0.3) pg/ml, and this response was not significantly increased at 5 min (6.02 pg/ml, SE 1.4, P < 0.71). However, hypovolemia significantly increased AVP levels at 20 min to 46.9 (SE 13.2) pg/ml (P < 0.001 vs. baseline) and at 50 min to 50.7 (SE 12.1) pg/ml (P < 0.001 vs. baseline) in the SAP group. Baseline AVP level of DSAP-treated rats at 2 min 2.6 (SE 0.3) pg/ml and at 5 min was 4.1 (pg/ml, SE 0.8, P = 0.92). These values did not differ from those of SAP-treated rats (P = 0.83 in both cases). In DSAP-lesioned rats, however, AVP levels were not elevated significantly above...
baseline levels at either 20 (12.7 pg/ml, SE 3.8, P = 0.35) or 50 min (16.2 pg/ml, SE 6.3, P = 0.11). These levels were significantly attenuated compared with SAP controls at both 20 min (P < 0.001) and 50 min (P < 0.001).

**Osmotic challenge.** The AVP response to injection of 1 M and 0.15 M NaCl (15 ml/kg) 50 min after injection is shown in Fig. 7. Osmotic challenge significantly increased AVP responses (F = 43.9, P = 0.001), but there was no interaction between the DSAP lesion and the AVP response (F = 0.48, P = 0.51). In the SAP group, AVP levels after isotonic and hypertonic saline were 8.9 (SE 0.53) pg/ml and 20.6 (SE 1.72) pg/ml, respectively (P < 0.004). In the DSAP groups, responses were 8.9 (SE 0.92) pg/ml and 18.5 (SE 1.9) pg/ml, respectively (P < 0.003).

AVP levels did not differ between DSAP and SAP groups after either isotonic (P = 0.73) or hypertonic (P = 0.21) saline.

**Insulin-induced hypoglycemia.** The effect of insulin-induced hypoglycemia on AVP levels 50 min after insulin injection is shown in Fig. 8 (top). There was a significant effect of insulin-induced hypoglycemia (F = 0.15, P = 0.03). However, in both SAP and DSAP rats, levels of AVP were significantly lower after insulin than after saline injection. This change was opposite in direction from the expected change, and the absolute difference between saline and insulin treatments was very small. In the SAP group, AVP levels after saline and insulin injections were 8.5 (SE 0.5) pg/ml and 7.2 (SE 0.7) pg/ml, respectively. In the DSAP group, AVP levels were 8.9 (SE 0.9) pg/ml and 7.9 (SE 0.4) pg/ml, respectively. The mean AVP levels did not differ between groups either after saline or insulin. Corticosterone secretion (Fig. 8, bottom) was significantly increased by hypoglycemia in both SAP and DSAP rats, but there was a significant difference between groups. In SAP rats, hypoglycemia increased corticosterone significantly above the saline control values (265.69, SE 38.4 vs. 38.33, SE 3.6, pg/ml, respectively; P < 0.001). In DSAP rats, corticosterone levels were also significantly elevated above baseline (91.2, SE 17.6 pg/ml vs. 24.8, SE 2.3, pg/ml respectively; P = 0.04). The between-group difference in the corticosterone response was highly significant (P < 0.001), but the basal corticosterone levels did not differ between the two groups (P = 0.66).

**Osmotic challenge, time course.** The AVP response to injection of 1 M and 0.15 M NaCl (15 ml/kg), as measured at four sampling times, is shown in Fig. 9. Although this protocol is a more powerful one, with multiple samples and both within-day and across-day baseline values, the results are similar to those obtained at the single, 50-min sampling time. There was a significant effect of osmotic challenge on AVP levels across sampling times, but there were no differences between groups at any time point. Basal values (at −30 min) for SAP and DSAP groups were 1.84 and 2.72 and pg/ml, respectively; (P = 0.83). For SAP rats, AVP levels were elevated significantly above baseline at 20 (12.0 pg/ml, SE 0.2 P = 0.017), 60 (22.7 pg/ml, SE 4.2, P < 0.001) and 120 min (23.81 pg/ml; SE 2.0, P < 0.001). For DSAP rats, AVP levels were elevated significantly above baseline at 20 (10.6 pg/ml, SE 0.2, P = 0.003), 60 min (18.32 pg/ml, SE 3.9, P < 0.001), and 120 min (17.41 pg/ml, SE 2.1, P < 0.001). There were no significant differences between groups at −30 (P = 0.83), 20 (P = 0.74), 60 (P = 0.29), or 120 min (P = 0.13). After isotonic saline, AVP values in the DSAP group ranged from 1.4 to 1.8 pg/ml and in the SAP group from 1.3 to 3.6 pg/ml. Values did not differ between or within groups and did...
not differ significantly from the −30-min levels during the hypertonic saline test.

Insulin-induced hypoglycemia, time course. The effect of insulin-induced hypoglycemic challenge, as measured at four sampling times, is shown in Fig. 10. Insulin produced a significant reduction of blood glucose at 20, 60, and 120 min (P < 0.001). Glucose values for SAP rats at 0, 20, 60, and 120 min were 93, 39, 39, and 25 mg/dl, and for the DSAP group, the means were 87, 42, 37, and 29 mg/dl, and these values did not differ between SAP- and DSAP-treated rats (P = 0.6, 0.8, and 0.5, respectively) (Fig. 10, top). There was no evidence for stimulation of AVP secretion by insulin-induced hypoglycemia (Fig. 10, bottom). All of the AVP values fell within the expected unstimulated range for freely moving, unanesthetized rats. The baseline SAP/saline control values ranged across the four sampling times from 1.3 pg/ml, SE 0.2, to 3.6 pg/ml, SE 2.4. There were no effects of insulin at any time point for DSAP or SAP groups that fell outside this range.

Fig. 2. Drawings of the ventral medulla of rat brain, adapted from Paxinos and Watson (39), indicating the most caudal and most rostral of the three levels used for quantification of δβh-ir cell bodies in cell groups A1, A1/C1, C1, and A5. Ovals indicate the approximate anatomical regions containing the cell bodies from each group.

Fig. 3. Numbers of δβh-ir cell bodies present in catecholamine cell groups in rats microinjected with anti-δβh-saporin (DSAP) or unconjugated saporin control (SAP) into the paraventricular nucleus of the hypothalamus (PVH).
Glucoprivic feeding. All PVH DSAP rats used in these studies had significant deficits in glucoprivic feeding compared with SAP controls (P < 0.001), as reported previously (31, 35, 42, 44). Effects of insulin-induced hypoglycemia on food intake in male rats are shown in Fig. 11. Insulin-induced hypoglycemia produced a significant stimulation of food intake above baseline in SAP rats (3.78 vs. 0.79, respectively, P < 0.001), but not in DSAP rats (0.91 vs. 0.63 g, respectively, P = 0.61). The effect of hypoglycemia on intake was significantly different between groups (P < 0.001).

DISCUSSION

Hypovolemia, brought about by remote blood withdrawal in SAP controls, resulted in a robust AVP response that was 17-fold above basal values 50 min after initiation of blood withdrawal. In contrast, the peak AVP response in PVH DSAP-injected rats did not differ significantly from basal values, demonstrating the crucial role of hindbrain catecholamine neurons in eliciting this response. The PVH DSAP lesion is more selective for catecholamine neurons than lesions previously used to examine catecholamine involvement in hypovolemia-induced AVP secretion. Previous experiments have used excitotoxins or GABA agonists to eliminate the contribution of A1 neurons (26, 29), but these treatments affect noncatecholamine, as well as catecholamine neurons at the injection sites, and bilateral excitotoxin lesions extensive enough to destroy the majority of the A1 neurons can be lethal (50). In contrast, PVH DSAP injections selectively lesioned nearly all hindbrain catecholamine neurons with projections to or through the injection site, and animals remained healthy. Nevertheless, PVH DSAP produced a severe deficit in the AVP response to hypotensive hypovolemia. Therefore, the present results support the essential role of catecholamine neurons in hypotensive hypovolemia-induced AVP secretion.

Previous work has specifically implicated neurons in the A1 cell group as being the key mediators of hypovolemia-induced AVP secretion (17, 26, 29). Although more selective for catecholamine neurons, PVH DSAP injection is less selective for particular catecholamine cell groups than techniques used in some previous studies in which A1 neurons were destroyed or suppressed by injections made directly into the A1 cell group. All catecholamine neurons projecting to the medial hypothalamus were lesioned by PVH DSAP injection, including subsets of A6, A2, and C1–C3 cell groups, as well as A1 neurons. However, like direct hindbrain injections, the PVH DSAP lesion profoundly reduced A1 cell numbers, while producing a deficit similar to that resulting from direct lesions restricted to the A1 region. Therefore, our results strongly support the hypothesis that neurons that specifically mediate hypovolemia-induced AVP secretion are present in the A1 cell group.

Other investigators have suggested, on the basis of Fos expression and electrophysiological recording from AVP neurons, that AVP secretion in response to moderate hypotensive hemorrhage is dependent on hindbrain catecholamine neurons, but that secretion in response to more severe hemorrhage, resulting in a fall in MAP to 30 mmHg, involves additional vasopressin-stimulating pathways (51). Although we did not measure blood pressure, we estimate the total amount of blood withdrawn during the initial 4.5 min to be ~28% of total blood volume and after withdrawal of the 20-min sample to be ~43% of total blood volume (3). This amount of blood loss would be considered to be a hypotensive hypovolemic stimulus (12). On the basis of previous experiments in which MAP was measured, our initial blood loss would be expected to decrease MAP to ~60 mmHg (36), with an additional decrease resulting from the 20-min blood sample. It is clear that, at our level of hypovolemic stimulation, the pathway proposed to bypass the
A1 region was not activated or was not sufficient to overcome the severe deficit in AVP secretion in the DSAP lesioned rats. However, given the substantial reduction in blood volume in our study, our results suggest that hindbrain catecholamines are the main stimulatory control of hypotensive hypovolemia-induced AVP secretion, even during severe hemorrhage.

Osmotic challenge produced a robust AVP response that, in contrast to the AVP response to hypovolemic challenge, was not impaired by the DSAP lesion. This finding confirms previous work, suggesting that the AVP response to osmotic challenge is catecholamine independent (2, 32, 49). Osmotic control of AVP secretion is attributed primarily to the innervation of AVP neurons by terminals from neurons of the lamina terminalis (37, 53, 54) and from the intrinsic osmosensitivity of AVP neurons (9). The presence of a normal AVP response to osmotic challenge in DSAP rats provides functional confirmation that PVH DSAP injection did not destroy the PVH AVP neurons themselves or impair their secretory capacity. Although not quantified, the normal immunohistochemical appearance of AVP neurons in the vicinity of the DSAP injections also suggests that they were not significantly damaged by the injections. These results strengthen our interpretation of the hypovolemia experiment by showing that the impairment of AVP secretion in response to hypovolemia is not due to damage of AVP neurons but can be attributed to loss of a specific catecholaminergic control of secretion.

In contrast to previous reports, glucoprivation in our experiments did not elevate plasma AVP levels in either SAP controls or DSAP-lesioned rats. The failure to elicit an AVP response cannot be attributed to an ineffective glucoprivic stimulus. The rats were severely hypoglycemic during the test. Furthermore, corticosterone levels, measured in the same samples assayed for AVP, were robustly elevated by hypoglycemia in SAP rats. As reported previously, this effect was impaired by DSAP. Finally, in a separate test, the same dose of insulin used in the AVP test strongly stimulated appetite, leading to a 4.6-fold increase in food intake that was completely abolished.
by DSAP. In addition, the failure to stimulate AVP secretion cannot be due to the choice of an inappropriate sampling time. In one experiment, we sampled blood at 50 min only, but in the second experiment, we sampled blood over a longer time course. AVP was not elevated at any sampling time. Finally, the failure to stimulate AVP secretion is not due to damage of AVP neurons by the PVH microinjections because, as noted above, osmotic stimulation caused AVP secretion in both SAP and DSAP rats.

Fig. 8. Plasma AVP (top) and corticosterone (bottom) levels in rats previously microinjected into the PVH with DSAP (n = 8) or SAP (n = 7) and subsequently subjected to hypoglycemic challenge induced by administration of a hypoglycemic dose of regular insulin (1.5 U/kg). Baseline responses to injection of 0.9% saline were tested separately in the same rats with at least 1 wk intervening between tests. Blood samples were drawn 50 min after the insulin or saline injections. Hypoglycemia did not elevate AVP levels in either group. Corticosterone secretion, however, was significantly increased by hypoglycemia in both SAP (*P < 0.001) and DSAP (*P < 0.04) groups, compared with saline control, but the response was severely attenuated in the DSAP group (#P < 0.001, DSAP vs. SAP).

Fig. 9. Plasma AVP levels in male rats in response to osmotic challenge induced by injection of hypertonic saline (1 M, 15 ml/kg) or to isotonic saline control injection at 0 min (arrow). Washed and resuspended erythrocytes from donor blood were transfused via a femoral vein catheter simultaneously with blood sample withdrawal via an intra-atrial catheter to minimize hemodynamic changes due to sampling. Rats were previously microinjected into the PVH with DSAP (n = 8) or SAP (n = 3). AVP levels were significantly increased above baseline in response to hypertonic saline in both SAP and DSAP groups at 20 (*P < 0.01), 60 (*P < 0.001), and 120 min (*P < 0.001), compared with time 0 and isotonic saline control, but the levels did not differ significantly between SAP and DSAP groups at any time point.

Fig. 10. Plasma glucose (top) and AVP (bottom) levels in PVH DSAP (n = 8) and SAP (n = 6)-injected male rats after insulin administration (1.5 U/kg) at time 0 (arrow). Insulin produced hypoglycemia but did not stimulate AVP secretion.

Fig. 11. Intake of pelleted rat food in a 4-h test after injection of a hypoglycemic dose of insulin (1.5 U/kg). Rats were injected previously into the PVH with DSAP or SAP. The DSAP lesion abolished the feeding response to hypoglycemia, indicating its dependence on hindbrain catecholamine neurons. Data shown are for male rats: DSAP (n = 8) and SAP (n = 6). *P ≤ 0.001 SAP saline vs. SAP insulin; *P ≤ 0.001 SAP insulin vs. DSAP insulin.
In previous studies, both 2-deoxy-d-glucose induced glucoprivation and insulin-induced hypoglycemia have been reported to increase AVP secretion or Fos expression in magnocellular neurons of a number of species (1, 4, 5, 10, 13–16, 21, 22). Nevertheless, not all experiments have observed such changes (34) and when present, the changes in AVP are often small (40). Although AVP secretion may be attributable to osmotic stimulation in some experiments using 2-deoxy-d-glucose, this possible source of osmotic stimulation cannot be the mechanism for all reported cases of glucoprivation-induced AVP secretion, since hyperosmolality is not a factor with insulin-induced hypoglycemia. In our experiments, precaution was taken to avoid blood sampling-induced or handling-induced hemodynamic stimulation or handling stress. Possibly, these measures reduced the incidence of inadvertent stimuli for AVP secretion that may have contributed to positive findings in some other studies. We conclude from our results that hypoglycemia is not a direct stimulus for AVP secretion and that AVP release or increased Fos expression in AVP neurons observed in some cases during glucoprivation are attributable to other, unidentified factors.

The present findings confirm that the AVP response to hypotensive hypovolemia is dependent on hindbrain catecholamine neurons that project to the medial hypothalamus. Previous studies using the same DSAP injection protocol and the same injection site used in this study have shown that the feeding and corticosterone responses to glucoprivation are dependent on catecholamine neurons contributing axons to this same projection (30, 35, 44). However, the fact that hypoglycemia, but not glucoprivation, increased AVP secretion strongly suggests that glucoprivation and hypoglycemia stimulate different catecholamine neurons. This conclusion can be drawn because levels of glucoprivation adequate to elicit feeding and corticosterone responses in SAP controls were used in this study, showing that catecholamine neurons, which are required for elicitation of these responses, were activated by our hypoglycemic stimulus. Yet, an AVP response was not elicited. Thus hindbrain catecholamine neurons that are differentially sensitive to glucoprivation and hypoglycemia must exist in the hindbrain. Although evidence discussed above suggests that those neurons that increase AVP secretion in response to hypoglycemia are located in A1, those responsible for elicitation of glucoregulatory responses have not been precisely localized.

Hypoxia is another stimulus for AVP secretion (25, 27, 55). Hypoxia has also been shown to activate some hindbrain catecholamine neurons (20, 23, 33, 50, 52), suggesting that catecholamine neurons are involved in the AVP response to that stimulus. We assume that our DSAP lesion, which eliminated nearly all catecholamine neurons with projections to the magnocellular areas of the hypothalamus, would have destroyed these hypoxia-responsive catecholamine neurons. Therefore, if our hypovolemic stimulus was hypoxic, the loss of hypoxia-responsive catecholamine neurons may have contributed to the deficits in AVP secretion observed in our study. This possibility was not specifically investigated.

In summary, hindbrain catecholamine neurons contribute to a variety of homeostatic responses. However, it is not yet clear which responses are mediated by a common set of neurons and which are mediated by phenotypically or anatomically distinguishable subgroups. The present study demonstrates that hindbrain catecholamine neurons mediate and are essential for the AVP response to hypotensive hypovolemia but are not required for the AVP response to osmotic stimulation. They also clearly indicate for the first time that hypotensive hypovolemia and glucoprivation activate distinct subsets of hypothalamically projecting catecholamine neurons.

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