Repeated 2-Deoxy-D-Glucose–Induced Glucoprivation Attenuates Fos Expression and Glucoregulatory Responses During Subsequent Glucoprivation

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A condition of reduced responsiveness to hypoglycemia, known as hypoglycemia-associated autonomic failure (HAAF), occurs in diabetic patients in the wake of a prior hypoglycemic episode. This condition suggests that hypoglycemia alters central glucose-sensing mechanisms. This experiment examined the effects of repeated 2-deoxy-D-glucose (2DG)-induced glucoprivation on subsequent 2DG-induced feeding and hyperglycemic responses in rats. Fos immunoreactivity (ir) in adrenal medulla and brain sites involved in these responses was also examined. Rats were injected daily for 10 days with 2DG (200 mg/kg) or saline (0.9%) or were handled. On day 11, rats were injected with 2DG (200 mg/kg). After injection, food intake was measured in one group. In another group, food was withheld, and multiple blood samples were collected for glucose determination. In a third group, food was withheld, and rats were killed after 2 h for evaluation of Fos-ir. Prior repeated glucoprivation reduced subsequent feeding and hyperglycemia responses to 2DG to baseline levels. Double-label immunohistochemistry showed that Fos-ir was reduced or abolished in catecholamine cell groups A1, A1/C1, C1, C3, and A6 and in the paraventricular nucleus of the hypothalamus and adrenal medulla. In other brain sites, 2DG-induced Fos-ir was diminished or unaffected by prior glucoprivation. Sites in which Fos-ir was abolished have been implicated previously in glucoprivic control of feeding and adrenal medullary secretion. Therefore, the present findings may identify crucial neuroanatomical sites that are altered by prior glucoprivation and that mediate some of the physiological deficits observed in HAAF. *Diabetes* 49:1865–1874, 2000

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2DG, 2-deoxy-D-glucose; ANOVA, analysis of variance; CeM, central nucleus of the amygdala; HAAF, hypoglycemia-associated autonomic failure; ir, immunoreactivity; LPBN, lateral parabrachial nucleus; NE, norepinephrine; NPY, neuropeptide Y; NTS, nucleus of the solitary tract; PNMT, phenethanolamine-*N*-methyl-transferase; PVH, paraventricular nucleus of the hypothalamus; PVT, paraventricular nucleus of the thalamus; R2DG, recurrent 2DG; RS, recurrent saline; TH, tyrosine hydroxylase; TPBS, Tris sodium phosphate-buffered saline.

Glucoprivation is a metabolic event that elicits multiple glucoregulatory responses, including adrenal medullary secretion of epinephrine, secretion of glucocorticoids, and stimulation of feeding. These responses serve to prevent and correct glucoprivic conditions by mobilizing glucose stores and promoting glucose delivery to the brain. The integrity of glucoregulatory mechanisms is essential for survival, since the brain requires an uninterrupted supply of glucose, the primary metabolic fuel for the brain.

Recently, a clinical syndrome known as hypoglycemia-associated autonomic failure (HAAF) has drawn attention to the fact that glucoregulatory mechanisms are impaired by prior exposure to glucoprivation (1–3). HAAF occurs in diabetic patients as a consequence of prior inadvertent hypoglycemic bouts associated with intensive insulin therapy. In HAAF, plasma epinephrine, norepinephrine (NE), and glucagon responses to a hypoglycemic challenge are severely diminished. In addition, increased appetite and autonomic signs, such as sweating and cardiac palpitations, that normally occur during hypoglycemia are reduced (1,2,4). Consequently, affected individuals are not able to detect an ongoing or developing hypoglycemic emergency. Although HAAF occurs in diabetic patients, diabetes is not a necessary condition for HAAF. HAAF has also been produced experimentally in nondiabetic humans (5) and in rats (6). The reduced glucoregulatory responsiveness caused by prior hypoglycemia is not permanent. After chronic recurrent insulin-induced hypoglycemia, glucoregulatory responses had returned to control values when rats were tested 3–4 weeks after restoration of normoglycemia (6). However, the time course of the recovery has not been examined parametrically.

Neither the mechanisms responsible for the impairment of glucoregulatory responses by antecedent hypoglycemia nor the neural substrates involved have been identified. However, the gravity of the HAAF syndrome for diabetic patients clearly poses a challenge in understanding the mechanisms underlying this disruption of glucoregulation. Furthermore, it seems possible that HAAF may be an exaggerated expression of processes that operate normally to adjust the sensitivity of glucoregulatory mechanisms to chronic metabolic demands associated with differing levels of glucose availability. Thus, the paradigm for experimental production of

HAAF potentially provides an opportunity to more fully understand how glucoregulatory systems operate in normal animals. The present experiments examine several aspects of this phenomenon. First, one goal was to determine whether prior exposure to glucoprivation, rather than hypoglycemia or insulin per se, is the causative factor leading to reduction of glucoregulatory responses. Therefore, we used the antimetabolic glucose analog, 2-deoxy-D-glucose (2DG), rather than insulin as the glucoprivic agent in these experiments. Second, the effects of the antecedent glucoprivic bouts on feeding and hyperglycemic responses to subsequent glucoprivic challenge were assessed. Although loss of the normal appetite stimulatory effects of hypoglycemia is often mentioned as a component of HAAF, the effect of antecedent glucoprivation on glucoprivic feeding has not been specifically studied.

The primary goal of this experiment was to identify central neural pathways in which the response to acute glucoprivation is altered by prior repeated glucoprivic bouts and which might therefore be involved in the production of HAAF. Neurons activated by an acute glucoprivic episode have been identified previously using Fos immunoreactivity (ir) (7,8). Fos is the protein product of c-fos, an immediate early gene that is induced by neural activation (9). The normal pattern of Fos expression induced in the brain by glucoprivation is highly reproducible. In the present study, we used Fos-ir to determine whether repeated antecedent glucoprivation alters the normal pattern of neural activation in response to a glucoprivic challenge.

RESEARCH DESIGN AND METHODS

Animals and procedures. Adult male Sprague-Dawley rats weighing 340–380 g were obtained from Simonson Laboratories (Gilroy, CA). They were individually housed in suspended wire mesh cages in a temperature-controlled room (21 ± 1°C) illuminated between 0630 and 1830. Food and tap water were available ad libitum throughout the study, except 2 h during daily drug treatment and during the final drug challenge, when food was withheld during the Fos immunohistochemical experiment and blood glucose determination. Before and during the experiment, rats were handled and habituated to the testing environment and procedures. All treatment injections were performed at 0900, and food was returned to the cages at 1100.

Feeding tests. Feeding tests were conducted between 0900 and 1200. On each of 10 treatment days, rats received one subcutaneous injection of NaCl (recurrent saline [RS]; $n = 6$), one subcutaneous injection of 2DG (200 mg/kg) (Sigma, St. Louis, MO) (recurrent 2DG [R2DG]; $n = 6$), or were handled (control; $n = 6$). On day 11 (the test day), all animals where challenged with 200 mg/kg 2DG. In addition, another group of animals received one subcutaneous injection of 2DG (200 mg/kg) daily for 10 days and were challenged on day 11 with saline (R2DG + saline; $n = 6$). At the beginning of the test period on day 11, food was removed from the cages and weighed. The rats were then injected subcutaneously with 2DG (200 mg/kg) or saline. Food was returned immediately after injection. Food consumption was measured by weighing the remaining food in the cages and the spilled food collected on aluminum trays placed under the cages. Cumulative food intakes, measured to the nearest 0.1 g, were recorded for 3 h after injection. Two or more saline tests were conducted in each treatment group to establish baseline levels of food intake before the treatments began. These baseline saline tests were performed between 0900 and 1200.

Blood glucose analysis. Animals undergoing blood glucose tests received the same treatments on days 1–10 as described above (control, $n = 4$; RS, $n = 6$; R2DG, $n = 6$; and R2DG + saline, $n = 6$). On day 11 at 0900, blood glucose was measured in response to 2DG (200 mg/kg) or saline. Food was removed ~1 h before collection of the first blood sample and was not returned until the end of the test. Blood (50 µl) was collected from the tail 15 min before and 30, 60, 90, and 120 min after 2DG injection. Glucose was analyzed using the glucose oxidase method (10). Body weight was recorded each day before treatment injections over the course of the 10-day period in all four treatment groups. Two or more saline tests were conducted in each treatment group to establish baseline blood glucose levels before the beginning of the 10-day treatment period. These baseline saline tests were also conducted at 0900.

Immunohistochemical detection of c-fos and tyrosine hydroxylase. In the last experiment, the effects of repeated 2DG or control solution on subsequent 2DG-induced Fos expression were investigated. Rats received either 10 daily injections of control solution (0.9% NaCl) (RS; $n = 4$), 2DG (200 mg/kg) (R2DG; $n = 4$), or were handled daily (control; $n = 4$), as described above. A fourth group of rats received 10 daily injections of 2DG (200 mg/kg) and were challenged on day 11 with saline (R2DG + saline; $n = 3$). Food was removed from the cages for a 2-h period after each injection. On day 11, rats were challenged with 2DG (200 mg/kg) or saline and placed back into their cages without food. Two hours after the injection, when 2DG-induced Fos-ir is optimal (8), rats were anesthetized lightly by inhalation of methoxyflurane (Metofane; Pitman Moore) followed by a lethal injection of sodium pentobarbital. They were perfused transcardially, first with phosphate-buffered saline (pH 7.4) and then with a 4% paraformaldehyde solution prepared with phosphate buffer. Brains and adrenals were removed, postfixed at room temperature for 3 h in paraformaldehyde solution, cryoprotected in 25% sucrose solution, and sectioned in the coronal plane (40-µm thick) on a cryostat in preparation for Fos and tyrosine hydroxylase (TH) immunohistochemistry. Sections were placed into 0.1 mol/l phosphate buffer (pH 7.4) and processed using previously described immunohistochemical techniques (11). Briefly, after pretreatment with 50% ethanol/distilled water for 20 min, sections were washed (3 × 10 min) in 0.1 mol/l phosphate buffer and preincubated for 1 h in 10% normal horse serum (made in Tris sodium phosphate-buffered saline [TPBS] containing 0.05% thimerosol). Sections were then incubated for 48 h in rabbit anti-Fos (1:50,000; Arnel) and mouse monoclonal anti-TH (1:1,000; Boehringer Mannheim) in 10% normal horse serum TPBS. Sections were washed (3 × 10 min) in TPBS, incubated overnight in biotinylated donkey anti-rabbit IgG (1:500 in 1% normal horse serum TPBS; Jackson ImmunoResearch Laboratories), washed again (3 × 10 min), incubated 4 h in Extravidin-peroxidase (1:1,500 in TPBS; Sigma), washed again (3 × 10 min), and reacted to reveal Fos protein. Nickel-intensified diaminobenzidine was used as the chromogen in the peroxidase reaction. After Fos-ir, sections were subsequently incubated overnight in biotinylated donkey anti-mouse for TH-ir (1:500 in 1% normal horse serum TPBS; Jackson ImmunoResearch Laboratories) and then in Extravidin-peroxidase. They were then reacted to reveal TH using imidazole-intensified diaminobenzidine as the chromogen in the peroxidase reaction. Controls included omission of the primary antibody rabbit anti-Fos and preabsorption of the peptide with the appropriate commercially available peptide. None of the immunoreactivities described below were observed in the controls.

Sections were mounted on slides for microscopic examination. Quantification of Fos-positive nuclei and cell bodies immunoreactive for both cytoplasmic TH and nuclear Fos was performed bilaterally in two sections from each animal. Catecholamine cell groups were identified from published descriptions (12–15) and designated using the conventional terminology (14). C1 through C3 designate epinephrine cell groups containing both TH-ir and phenethanolamine-*N*-methyl-transferase (PNMT)-ir. The C1 cell group consists of all of the PNMT-ir neurons in the ventrolateral medulla. These form a column ventral to the nucleus ambiguous beginning at the level of the calamus scriptorius (the most caudal extent of the area postrema) and extending rostrally to the caudal aspect of the nucleus of the facial nerve. The rostral C1 (rC1) cell group is concentrated between the levels defined by the rostral pole of the inferior olivary nucleus and the caudal border of the facial nucleus (i.e., the retrofacial area). The C3 group is defined as the cells in the medial longitudinal fasciculus and in the floor of the fourth ventricle at the level of the prepositus hypoglossal nucleus (13). A1 and A6 designate NE cell groups containing TH-ir but not PNMT-ir. The A1 cell group is in the ventrolateral medulla and extends from the upper cervical spinal cord approximately to the level of the obex, overlapping the C1 group between the calamus scriptorius and obex. The area of overlap is referred to here as the A1/C1 area. Means and SEs of the mean for each quantified area were calculated for all rats in a given treatment group.

Statistical analyses. Data are expressed as means ± SE. Data were analyzed separately using analysis of variance (ANOVA) with repeated measures. Significant individual differences were identified using Bonferroni's t test. The critical level for significance was set at $P < 0.05$ for all comparisons.

RESULTS

2DG-induced hyperglycemia. Changes in body weight over the course of the 10-day treatment period are shown in Fig. 1. There was no difference in body weight among the animals in each of the four treatment groups. Blood glucose results are presented in Fig. 2. Injection of 2DG elicited a significant

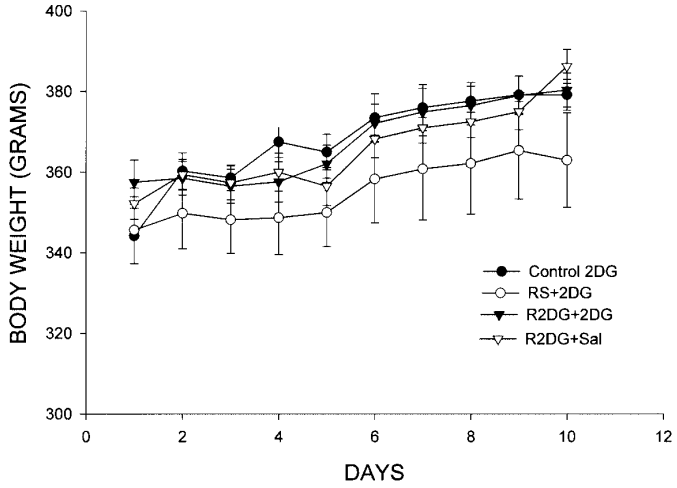

FIG. 1. Changes in body weight during the 10-day treatment period in control (n = 6), RS challenged with 2DG (RS + 2DG) (n = 6), R2DG challenged with 2DG (R2DG + 2DG) (n = 6), and R2DG challenged with saline (R2DG + Sal) (n = 6) animals undergoing blood glucose assessment. Body weights were recorded daily before treatment injections or handling. There was no difference in body weights among the four groups over the course of the 10-day period.

increase in blood glucose concentration in both control (Fig. 2*A*) and RS (Fig. 2*B*) animals from baseline levels of 74.0 ± 1.1 and 66.0 ± 1.3 mg/dl at –15 min to a maximum of 181 ± 5.9 and 158 ± 6.6 mg/dl in control and RS animals, respectively. The increase in blood glucose was significant at all time points after injection of 2DG ($P < 0.001$). There was no significant difference in peak 2DG-induced hyperglycemia in control and RS animals. Animals exposed to prior repeated episodes of glucoprivation did not exhibit hyperglycemia in the 2DG challenge test (Fig. 2*C*). After injection of 2DG, blood glucose levels rose from baseline levels of 66.3 ± 1.7 mg/dl and reached a maximum of 101 ± 1.5 at 120 min. Blood glucose concentration after 2DG injection was statistically different from baseline saline values only at 90 and 120 min ($P < 0.05$). Blood glucose concentration in response to saline injection after 10 prior daily 2DG injections was not different from saline blood glucose concentrations recorded before the onset of repeated 2DG treatment (Fig. 2*D*).

2DG-induced feeding. Results from the feeding tests are presented in Fig. 3. One-way repeated-measures ANOVA indicated a significant difference among treatment groups (F [df 5,47] = 37.2, $P < 0.001$). Bonferroni's test revealed that food intake was significantly greater after 2DG injection in control (5.3 ± 0.3 g) and RS (5.6 ± 0.5 g) animals compared with R2DG animals (2.3 ± 0.3 g). In both control and RS animals, 2DG significantly increased food intake above baseline saline levels ($P < 0.001$). However, in R2DG animals, 2DG-induced food intake was not significantly different from food intake after saline injection (2.3 ± 0.3 g vs. 1.9 ± 0.1 g, $P > 0.05$).

2DG-induced Fos expression. The photomicrographs in Figs. 5–7 correspond to the drawings of the brain sites shown in Fig. 4. In control and RS animals, glucoprivation induced Fos expression in several discrete brain sites. In the hindbrain, Fos-positive nuclei were located in the dorsal medial nucleus of the solitary tract (NTS), medial NTS, dorsal motor nucleus of the vagus, lateral borders of the area postrema, catecholaminergic cell groups in the ventral lateral and dorsal medial medulla, and the lateral parabrachial nucleus (LPBN). Forebrain sites included the paraventricular nucleus of the thalamus (PVT), supraoptic nucleus, paraventricular nucleus of the hypothalamus (PVH) parvocellular division, and lateral part of the central nucleus of the amygdala (CeM) as well as the adrenal medulla. RS rats did not differ from control rats with respect to the number of Fos-positive nuclei induced in these brain areas by 2DG treatment (Table 1). However, the number of Fos-positive nuclei was significantly different in R2DG animals. The number of Fos-positive nuclei at every brain site analyzed, except the CeM, was significantly different from the number of Fos-positive nuclei in control and RS animals ($P < 0.001$) (Table 1). Some sites, such as catecholaminergic cell groups A1, A1/C1, and rC1, the PVH, and adrenal medulla, exhibited a complete loss of 2DG-induced Fos expression (Figs. 5 and 6), whereas Fos expression was present, although reduced or unchanged, in the NTS, LPBN, PVT, and CeM (Fig. 7). Rats that received daily 2DG and were then challenged with saline on day 11 (R2DG + saline) did not express Fos in any of the brain sites analyzed (Figs. 5–7).

DISCUSSION

Experimental findings in humans have shown that antecedent insulin-induced hypoglycemia reduces peripheral epinephrine, NE, and glucagon secretion as well as appetite and autonomic signs in response to a hypoglycemic challenge (1,2,4). The present findings demonstrate that antecedent glucoprivation induced by 2DG also impairs glucoregulatory responses in the rat, suggesting that the effect of antecedent insulin-induced hypoglycemia on glucoregulatory responses is not dependent on insulin or hypoglycemia per se but rather is a consequence of decreased intracellular glucose metabolism. Previous work in rats has shown that repeated insulin-induced hypoglycemia blocks adrenal medullary and glucagon responses to subsequent central 2DG administration, supporting the assumption that the reduced glucoregulatory responses are due to central effects of the prior glucoprivic bouts (16).

Increased appetite is a well-recognized and crucial response to glucoprivation, occurring simultaneously with adrenal medullary secretion. Food intake potentially provides a rapidly available source of glucose and is the sole mechanism by which caloric deficits can be repaired. Until now, food intake has not been measured either in human or animal models of HAAF. Results of the present study demonstrate that food intake, as well as plasma indicators of glucoprivation, are reduced or eliminated by prior repeated glucoprivation. The fact that both food intake and adrenal medullary secretion are subject to inhibitory modulation by prior glucoprivation and the recent finding that feeding and hyperglycemia can be elicited from the same hindbrain cannula sites are consistent with the possibility that both responses are initiated by activation of the same glucoreceptor cells (17).

Catecholamine neurons in the ventrolateral medulla are distributed within a caudal medullary area known as the cardiovascular depressor zone (18,19) and a rostral medullary area known as the cardiovascular pressor zone (20,21). Glucoprivation is a potent stimulus for adrenal medullary secretion, which could potentially alter cardiovascular function and lead to changes in Fos expression in these cardiovascular areas. However, expression of Fos in TH-ir neurons after

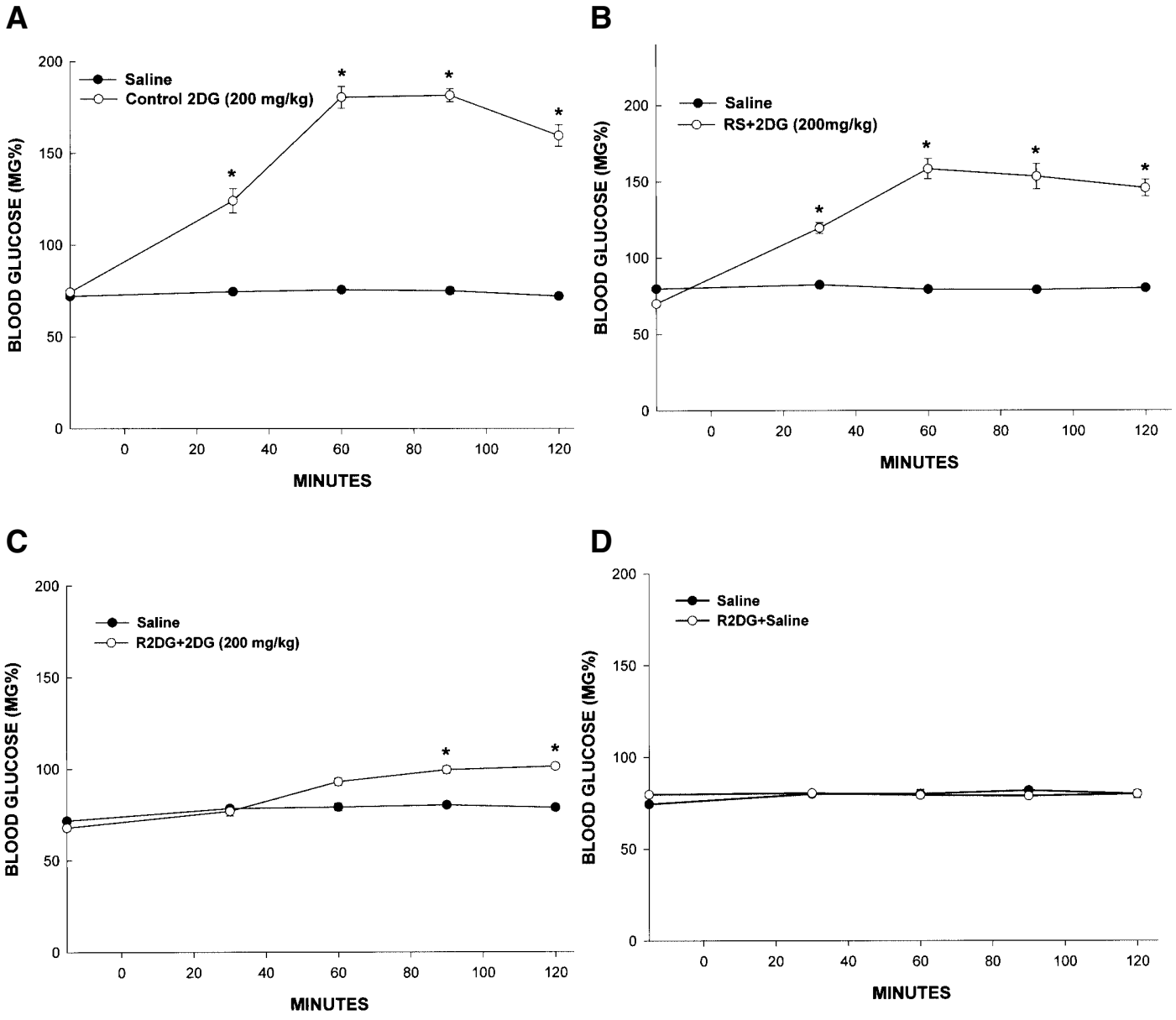

FIG. 2. Blood glucose responses after saline (1 ml/kg) or 2DG (200 mg/kg) in control (*A*), RS (*B*), R2DG (*C*), and R2DG challenged with saline (R2DG + Saline) (*D*) animals. Venous blood samples for glucose determination were collected from the tail 15 min before subcutaneous injection and at 30-min intervals thereafter for 2 h. Food was removed from the animals' cages 1 h before the injection and was not returned until the last blood sample was collected. Saline baseline tests were conducted before the beginning of the 10-day treatment period, and the 2DG or saline challenge tests were conducted on day 11. Data are expressed as mean blood glucose concentration ± SE. *$P < 0.001$.

2DG administration is not secondary to changes in cardiovascular parameters induced by glucoprivation, since 2DG does not alter blood pressure (22). In addition, 2DG-induced expression of Fos in TH-ir neurons is not altered by adrenal denervation, which completely blocks adrenal medullary secretion (7). Finally, catecholamine neurons activated by 2DG appear to be anatomically distinct from the neurons with cardiovascular function. In the caudal ventrolateral medulla, the majority of the baroreceptive neurons in the cardiovascular depressor zone express the GABAergic, not the catecholaminergic, phenotype (18,19). Catecholamine neurons in the rostral ventrolateral medulla in the cardiovascular pressor zone express Fos in response to sustained hypertension and may be involved in cardiovascular regulation. However, the latter cells are described as being located in the rostral third of the C1 cell group in a region extending ~450 μm caudally from the facial nucleus. In this most rostral region of C1, very few TH-ir neurons express Fos in response to 2DG. The C1 neurons activated by 2DG are located primarily in the caudal two-thirds of the cell group (7). These previous findings indicate that hindbrain catecholamine neurons are anatomically and functionally heterogeneous. The current data further implicate a specific subpopulation of catecholamine neurons in glucoregulatory responses.

Prior repeated exposure to glucoprivation not only reduced 2DG-induced feeding and hyperglycemia to control

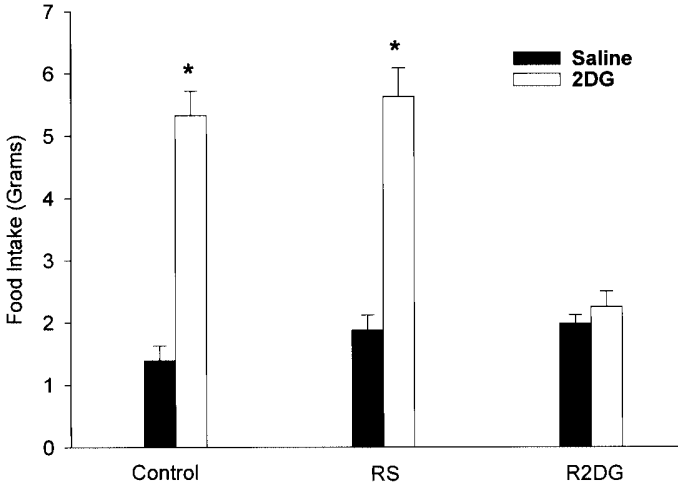

FIG. 3. Cumulative intake of pelleted rat food during the 3 h immediately after subcutaneous injection of saline (1 ml/kg) or 2DG (200 mg/kg) in control, RS, and R2DG animals. In R2DG animals challenged with saline on day 11, food intake in response to saline was not different from baseline saline food intake recorded before the onset of repeated 2DG treatment (data not shown). Data are expressed as mean 3-h food intake ± SE. *P < 0.001.

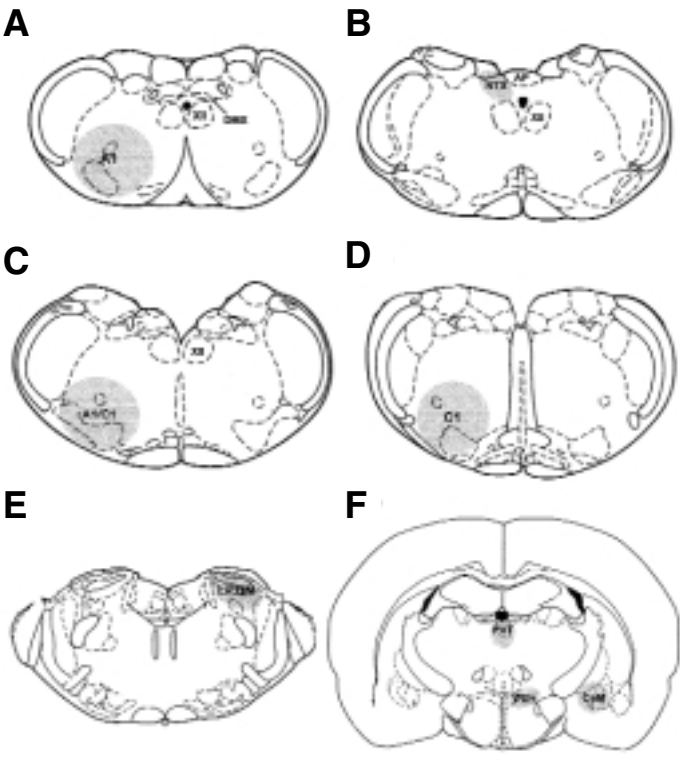

FIG. 4. Representative brain atlas drawings of the photomicrographs shown in Figs. 5–7. *A*: A1 norepinephrine cell group; *B*: dorsomedial nucleus of the solitary tract; *C*: A1/C1 overlap; *D*: rostral C1 epinephrine cell group; *E*: LPBN; *F*: PVT, PVH (parvocellular division), and the CeM. AP, area postrema; DMX, dorsal motor nucleus of the vagus; XII, hypoglossal nerve.

TABLE 1
Number of neurons in hindbrain and forebrain regions expressing Fos-ir in response to 2DG challenge in control, RS, and R2DG treatment

Cell Group	Control	RS	R2DG
A1	9.4 ± 1.1	9.5 ± 1.5	0.0 ± 0.0*†
A1/C1	24.6 ± 2.2	24.0 ± 1.5	1.7 ± 0.5*†
C1r	25.0 ± 2.1	20.0 ± 1.5	2.3 ± 0.8*†
C3	5.0 ± 0.8	5.4 ± 0.5	0.0 ± 0.0*†
DMNTS	46.4 ± 4.8	48.3 ± 3.4	34.3 ± 2.2*†
LPBN	70.6 ± 3.3	67.5 ± 4.1	44.3 ± 4.2*†
PVT	53.0 ± 5.2	67.8 ± 4.6	29.8 ± 2.2*†
PVH	109 ± 5.7	111 ± 6.1	0.0 ± 0.0*†
ArcM	22.4 ± 1.5	21.9 ± 1.9	3.1 ± 1.9*†
CeM	107 ± 4.0	122 ± 4.9	119 ± 5.6

Data are means ± SE. ArcM, medial arcuate nucleus; DMNTS, dorsal medial nucleus of the solitary tract. *P < 0.05 vs. control; †P < 0.05 vs. RS.

levels, but it also blocked expression of Fos-ir in some, but not all, sites normally responsive to 2DG. Fos-ir was abolished in the adrenal medulla, in the PVH, and in catecholaminergic cell groups A1, A1/C1, rC1, C3, and A6. Anatomical studies have shown that the PVH, A6, and adrenal medullary preganglionic neurons are all heavily innervated by catecholamine neurons in C1 through C3. Spinal and hypothalamic projections arise from separate populations of epinephrine neurons in these cell groups (23–29). Based on these anatomical connections, it is possible that the loss of 2DG-induced Fos expression could be due to a selective effect of the antecedent glucoprivation on these epinephrine neurons and the resulting inability of these neurons to activate their target sites in the PVH and intermediolateral column.

This possibility would also be consistent with results showing that hindbrain glucoreceptive sites, where localized glucoprivation elicits feeding and hyperglycemic responses, are codistributed with cell groups C1 through C3 (17) and the extensive literature showing the involvement of epinephrine and NE in feeding and glycemic control. Destruction of central epinephrine and NE neurons (30) and blockade of PVH α-noradrenergic receptors (31) significantly impairs glucoprivic feeding, whereas injection of epinephrine or NE into the PVH stimulates feeding (32). Selective destruction of epinephrine and NE neurons projecting to the PVH permanently abolishes 2DG-induced feeding but not 2DG-induced hyperglycemia. Selective destruction of NE and epinephrine neurons projecting to the spinal cord permanently abolishes 2DG-induced hyperglycemia, whereas the feeding response is preserved (33). In addition, neuropeptide Y (NPY) is colocalized with catecholaminergic neurons that project to the PVH (34). NPY stimulates feeding when injected into the PVH (32), whereas injection of NPY antibodies into the PVH impairs glucoprivic feeding (35). In addition, 2DG-induced glucoprivation increases NPY mRNA in the arcuate nucleus (36).

Prior glucoprivation did not uniformly reduce 2DG-induced Fos-ir at all brain sites. Fos-ir was reduced but not abolished in the NTS, LPBN, and PVT. In the CeM, no attenuation of Fos-ir was detected. Therefore, the areas where Fos-ir was abolished by prior glucoprivation appear to be different in some way from other brain sites activated by glucoprivation.

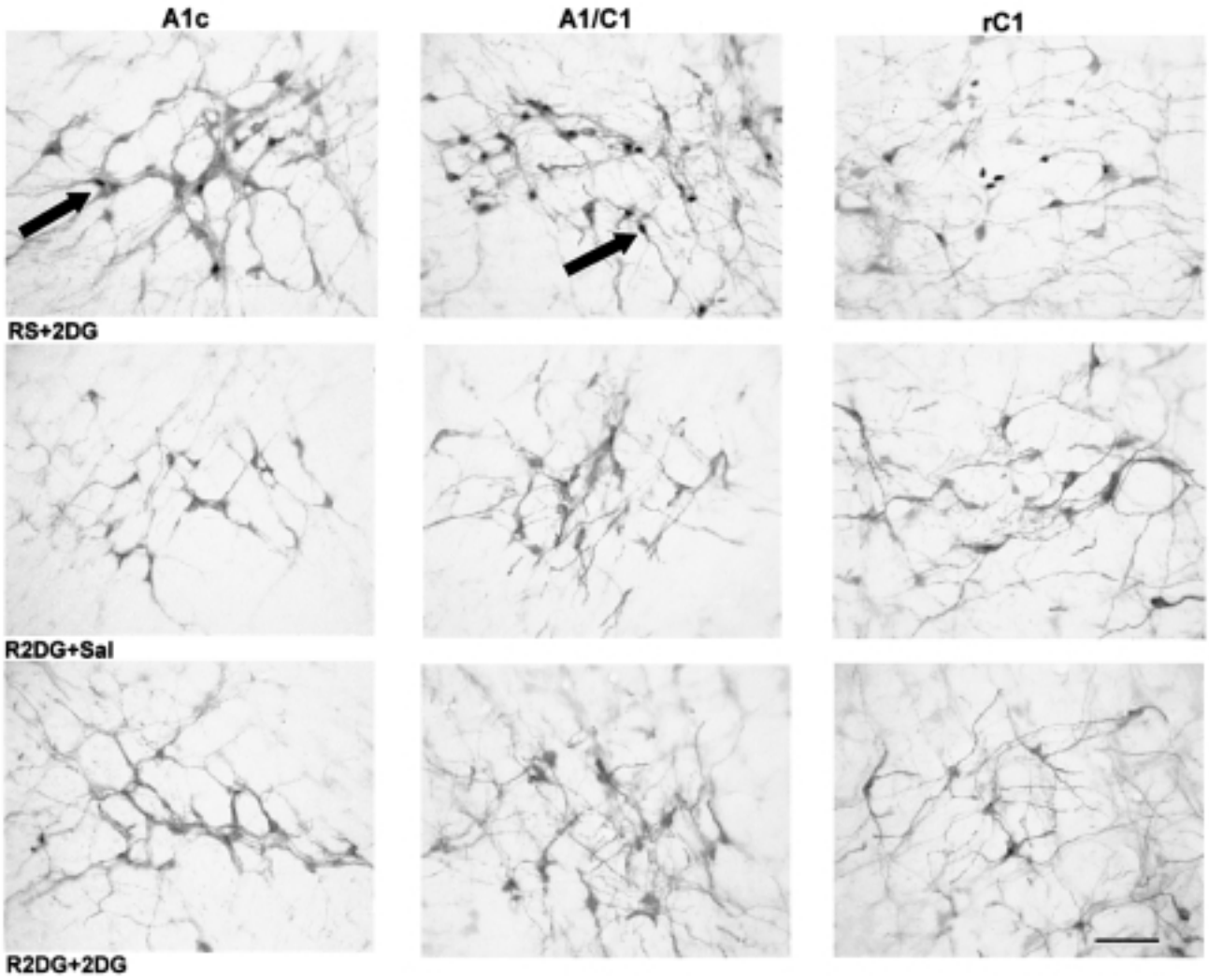

FIG. 5. Photomicrographs showing Fos-ir and TH-ir in neurons of the ventral hindbrain catecholamine cell groups: A1, A1/C1, and rC1 in RS animals receiving 2DG for the first time (RS + 2DG), in R2DG animals challenged with saline on day 11 (R2DG + Sal), and in R2DG animals challenged with 2DG after 10 prior glucoprivic episodes (R2DG + 2DG). Fos-ir appears as the black nuclear staining, whereas TH-ir appears as the gray cytoplasmic staining (arrow indicates doubly labeled cells). Fos was induced 2 h before perfusion with 2DG (200 mg/kg) in the absence of food. In the ventral hindbrain, doubly labeled neurons were concentrated in the area of A1/C1 in control and RS animals. Calibration bar = 200 µm.

In this regard, it is of interest that the majority of epinephrine neurons projecting to the PVH and the spinal cord express glucocorticoid type II receptors (37). In addition, it has been shown that chronic reduction of glucocorticoid levels increases basal and stress-induced activity of hindbrain catecholaminergic neurons (38,39) and increases catecholamine release in the PVH (40,41). Chronic elevation of glucocorticoid levels decreases basal catecholamine levels and reduces stress-induced catecholamine release, metabolism, turnover, and synthesis in the PVH (42). Glucocorticoids, which are released by each glucoprivic bout (43), have been implicated in the pathophysiology of impaired glucoregulation in HAAF (44,45). Our results reveal C1 through C3 to be potential sites in which glucocorticoids could act to produce a loss of sensitivity to a subsequent glucoprivic challenge.

Stress exposure, like glucoprivation, is associated with both catecholamine neuron activation and elevated glucocorticoids. Impaired neuronal activation of hindbrain catecholaminergic cells after repeated glucoprivation may be similar to effects of chronic stress on these neurons. For example, daily restraint stress significantly decreases A1/C1 catecholaminergic activity, as shown by reduced 3,4-dihydroxyphenylacetic acid (DOPAQ) levels in the cell bodies during the subsequent restraint session (46). Similarly, acute restraint stress induces c-fos mRNA in hindbrain catecholaminergic cells, but after 4 days of repeated exposure to restraint stress, c-fos mRNA expression is significantly reduced and is nonexistent after 9 days (47).

Clinical and experimental results in humans indicate that the glucoregulatory deficits associated with HAAF are reversible. Preliminary indications are that these deficits are also reversible in the chronically glucodeprived rat, but this question has not been subjected to parametric analysis. In addition, the number of glucoprivic episodes required to produce impairment of glucoregulatory responses is not known in the rat, but in humans, deficits are observed even after a single glu-

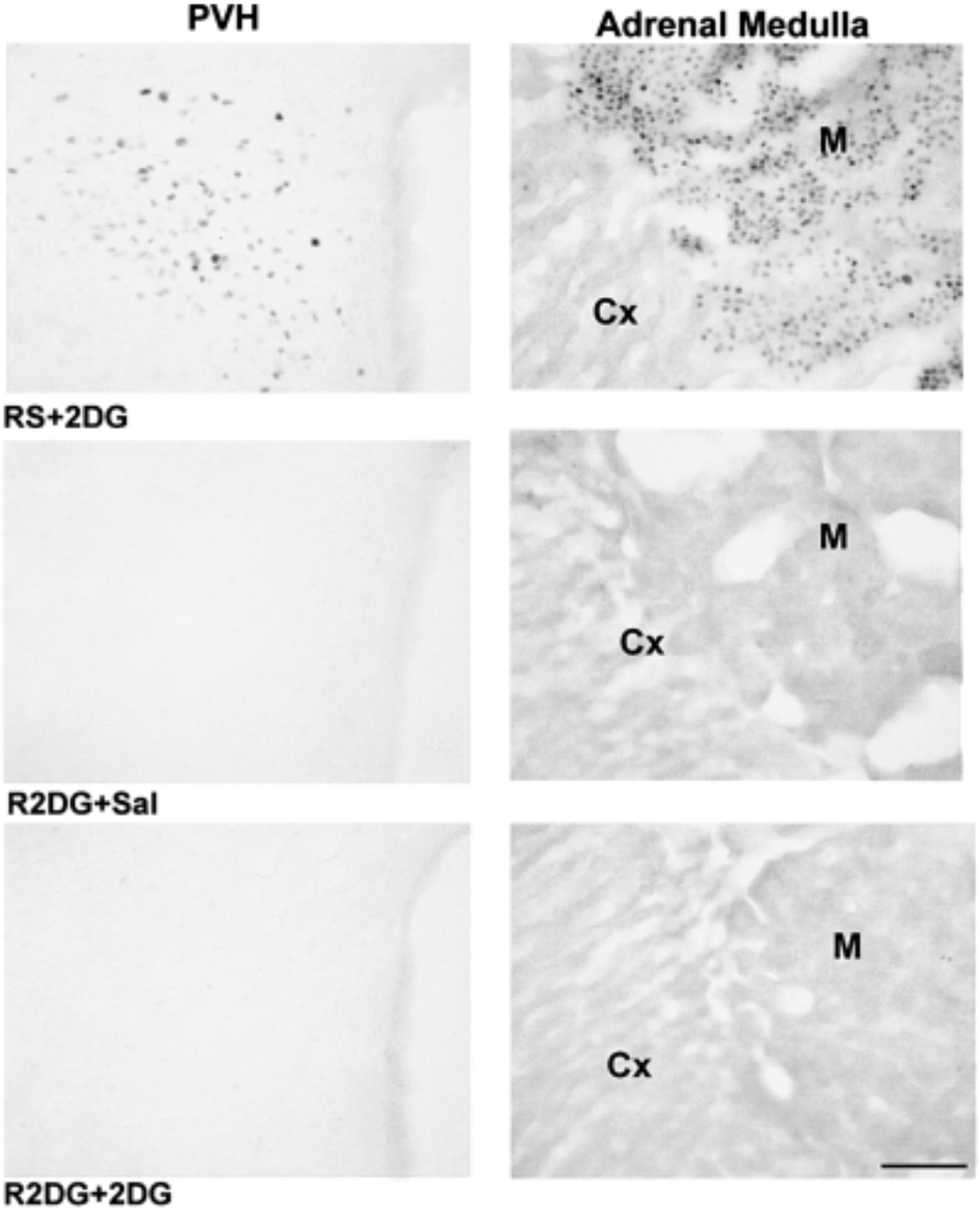

FIG. 6. Photomicrographs showing 2DG-induced Fos-ir in neurons of the PVH, primarily in the medial parvocellular division (left), and the adrenal medulla (right) in RS animals receiving 2DG for the first time (RS + 2DG), in R2DG animals challenged with saline on day 11 (R2DG + Sal), and in R2DG animals challenged with 2DG on day 11 (R2DG + 2DG). Fos-ir appears as the black nuclear staining. Fos-ir was induced by 2DG (200 mg/kg) 2 h before perfusion in the absence of food. Cx, adrenal cortex; M, adrenal medulla. Calibration bar = 200 µm.

coprivic episode (48). These issues have a practical importance for experiments using repeated-measures designs to study glucoprivic responses. The time course of effects of prior glucoprivation obviously must be taken into account to avoid testing responses during periods of reduced sensitivity unless it is the purpose of the experiment to do so.

HAAF is a life-threatening pathological consequence of inadvertent hypoglycemic bouts occurring in the course of intensive insulin therapy. However, the occurrence of HAAF prompts us to consider the possibility that under more natural conditions, reduced responsiveness may be a physiologically adaptive response to chronic reductions in glucose availability. In such cases, the glycemic threshold for elicitation of glucoregulatory responses may be shifted toward a lower set point. A lowered set point may facilitate other metabolic adaptations to reduced availability and, in addition, would avoid unnecessary elicitation of the metabolically costly emergency response.

The present data indicate that 2DG-induced stimulation of c-fos expression, food intake, and adrenal medullary secretion are reduced by repeated prior exposure to glucoprivation in the rat. Catecholaminergic cell groups A1/C1, rC1, and C3

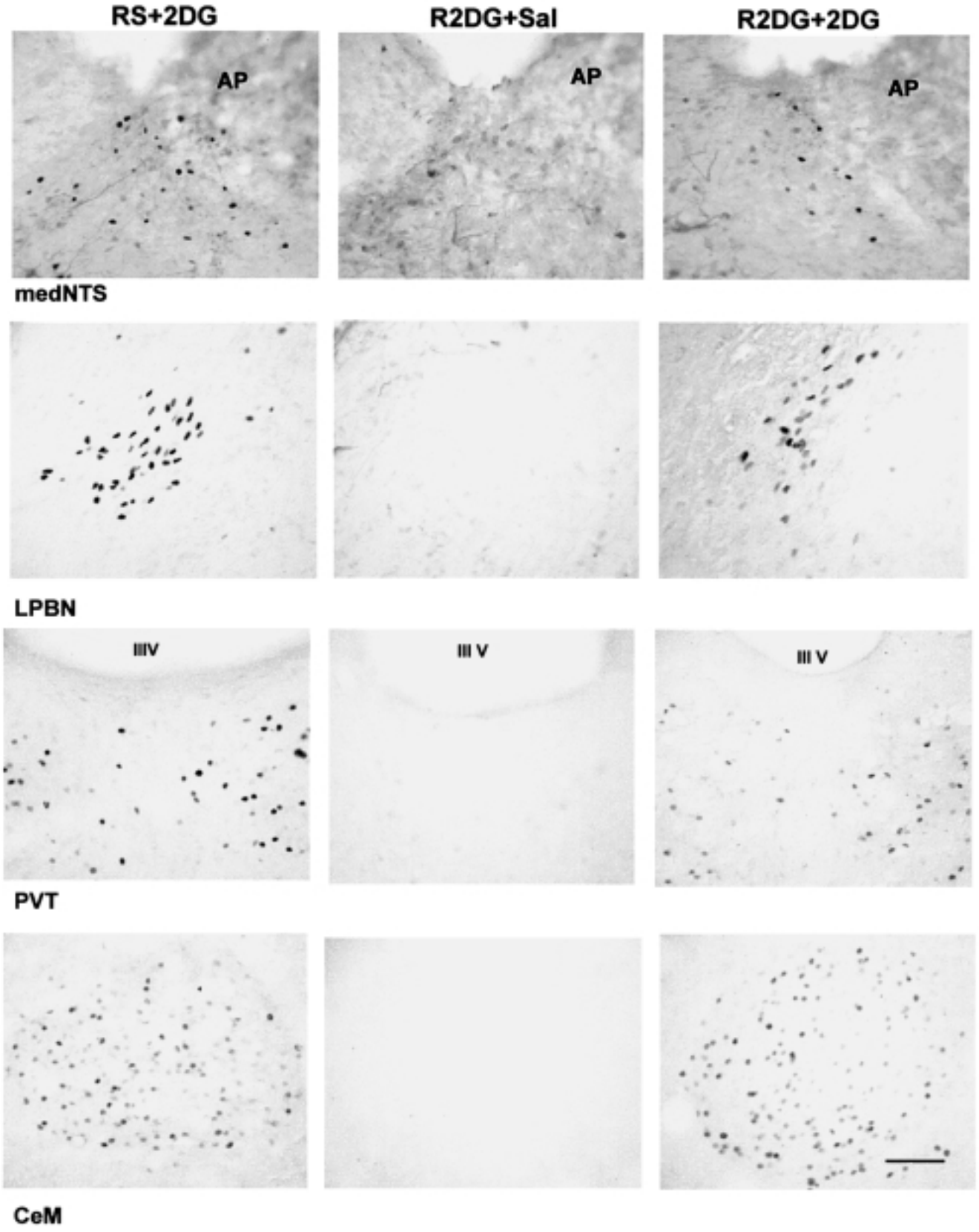

FIG. 7. Photomicrographs showing Fos-ir in response to 2DG-induced glucoprivation in hindbrain and forebrain sites in RS animals receiving 2DG for the first time (RS + 2DG), in R2DG animals challenged with saline (R2DG + Sal), and in R2DG challenged with 2DG on day 11 (R2DG + 2DG). 2DG (200 mg/kg) was administered in the absence of food 2 h before perfusion. Fos-ir appears as the black nuclear staining. AP, area postrema; III V, third ventricle; LPBN, lateral parabrachial nucleus (external subnucleus); MedNTS, dorsomedial nucleus of the solitary nucleus. Calibration bar = 200 µm.

and their rostral and caudal projection targets, the PVH and adrenal medullary preganglionic neurons, appear to be important neural substrates through which this reduced responsiveness is effected. These results reveal an anatomical focal point for study of the pathogenic mechanisms underlying HAAF as well as potentially adaptive mechanisms for normal modulation of brain glucoregulatory responses.

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