Third Ventricular Alloxan Reversibly Impairs Glucose Counterregulatory Responses

Nicole M. Sanders,[1] Ambrose A. Dunn-Meynell,[1,2] and Barry E. Levin[1,2]

Glucokinase (GK) is hypothesized to be the critical glucosensor of pancreatic β-cells and hypothalamic glucosensing neurons. To understand the role of GK in glucoprivic counterregulatory responses, we injected alloxan, a GK inhibitor and toxin, into the third ventricle (3v) to target nearby GK-expressing neurons. Four and 6 days after 3v, but not 4v, alloxan injection, alloxan-treated rats ate only 30% and their blood glucose area under the curve was only 28% of saline controls' after systemic 2-deoxy-D-glucose. In addition, their hyperglycemic response to hindbrain glucoprivation induced with 5-thio-glucose was impaired, whereas fasting blood glucose levels and food intake after an overnight fast were elevated. These impaired responses were associated with the destruction of 3v tanycytes, reduced glial fibrillary acidic protein-immunoreactivity surrounding the 3v, neuronal swelling, and decreased arcuate nucleus neuropeptide Y (NPY) mRNA. Nevertheless, hypothalamic GK mRNA was significantly elevated. Two weeks after alloxan injection, 3v tanycyte destruction was reversed along with restoration of feeding and hyperglycemic responses to both systemic and hindbrain glucoprivation. At this time there were significant decreases in GK, NPY, and proopiomelanocortin mRNA. Thus, neural substrates near and around the 3v affected by alloxan may be critically involved in the expression of these glucoprivic responses. *Diabetes* 53: 1230–1236, 2004

The brain relies on a continuous supply of glucose as a primary energy source (1) and has evolved mechanisms that detect decreases in blood glucose levels and elicit autonomic, neuroendocrine, and behavioral counterregulatory responses (CRRs) that prevent and correct glucoprivation. One way in which the brain senses alterations in glucose availability is through specialized glucosensing neurons (2–6). These neurons utilize glucose as a signaling molecule to alter their membrane potential and firing rate, whereas the majority of neurons utilize glucose primarily to fuel their metabolic needs (2,3). As extracellular glucose levels decline, glucose-excited neurons decrease and glucose-inhibited neurons increase their action potential frequency (4,7–11). As is the case with pancreatic β-cells, it is hypothesized that the high-K_m glycolytic enzyme, glucokinase (GK), is a regulator of neuronal glucosensing within the physiological range (10,12–14). GK inhibitors alter calcium flux (10,14) and neuronal firing (12) in ventromedial hypothalamus (VMH) glucosensing neurons, and GK mRNA is expressed in glucosensing neurons in the hypothalamic paraventricular nucleus (PVN), dorsomedial nucleus (DMN), ventromedial nucleus (VMN), and arcuate nucleus (ARC) (15,16). Specifically, GK is coexpressed in both ARC neuropeptide Y (NPY) and proopiomelanocortin (POMC) neurons (10). GK immunoreactivity (ir) is also localized in the ependymocytes lining the ventricles (17) and serotonin neurons in the midline medulla (17), where glucoprivic stimuli elicit CRRs (18).

In vitro studies suggest that GK is an important component of brain glucosensing. The current study was initiated to examine the potential regulatory role of GK in glucosensing in vivo. To do this, we used alloxan, which pharmacologically inhibits GK activity at low doses (19,20), but induces cell death at higher concentrations, presumably through the production of reactive oxygen radicals (21,22). Previous studies have reported that pharmacological concentrations of alloxan delivered centrally stimulate feeding, but not sympathoadrenal activation (23). Conversely, toxic concentrations permanently abolish the feeding, but not the sympathoadrenal response to glucoprivation (24–27). When those studies were carried out, it was not known that alloxan's primary site of action was GK or that GK-expressing neurons were localized in both the hindbrain and the hypothalamus. Because there have been conflicting studies implicating the hypothalamus (28–31) or hindbrain (18,32,33) as primary mediators of the CRR to glucose deficit, we also compared the effects of toxic doses of alloxan administered into the third ventricle (3v) versus the fourth ventricle (4v) on glucoprivic CRRs.

RESEARCH DESIGN AND METHODS

All work was approved by the institutional animal care and use committee of the East Orange Veterans Affairs Medical Center. Animals were housed individually on a 12:12-h light:dark schedule (lights on at 0600, off at 1800) at 22–23°C, with food (Purina rat chow #5001) and water available ad libitum, except where otherwise specified. Adult male SD rats (Charles River, Kingston, NY) weighing 325–350 g were handled and habituated to the testing environment and procedures throughout the study. In preliminary experiments, alloxan had differential effects on glucoprivic responses depending on the time after its injection. Therefore, glucoprivic responses were assessed at

From the [1]Department of Neurology and Neurosciences, New Jersey Medical School, Newark, New Jersey; and the [2]Neurology Service, Veterans Affairs Medical Center, Neurology Service, East Orange, New Jersey.

Address correspondence and reprint requests to Barry E. Levin, MD, Neurology Service (127C), VA Medical Center, 385 Tremont Ave., E. Orange, NJ 07018-1095. E-mail: levin@umdnj.edu.

Received for publication 7 November 2003 and accepted in revised form 16 February 2004.

2DG, 2-deoxy-D-glucose; 5TG, 5-thio-glucose; ARC, arcuate nucleus; CCR, counterregulatory response; CSF, cerebrospinal fluid; DMN, dorsomedial nucleus; GFAP, glial fibrillary acidic protein; GK, glucokinase; ir, immunoreactivity; NeuN, neuron-specific nuclear protein; NPY, neuropeptide Y; POMC, proopiomelanocortin; PVN, paraventricular nucleus; VMH, ventromedial hypothalamus; VMN, ventromedial nucleus.

differing time points after alloxan or saline injection. In one group, rats were tested 4 and 6 days after intraventricular injection and killed 4 or 9 days later. In a second group, testing occurred 4, 6, and 8 days after injections; the same rats were then retested at 14–20 days and killed 22 days after injection.

Surgical procedures. Rats were anesthetized with chloropent (0.3 ml/100 g body wt, i.p.) and their skull was exposed and trephined at the injection site. For experiments using only intraventricular injections, sterile saline (pH 3.0, 5 μl) or alloxan (120 μg in 5 μl; 5,6-dioxyuracil monohydrate, lot no. 116H0888; Sigma, St. Louis, MO) was directly injected into the 3v or 4v using a Hamilton syringe equipped with a 26G injector attached to the stereotaxic apparatus. Coordinates for 3v and 4v injections were determined from Paxinos and Watson's stereotaxic atlas of the rat brain (34). Body weight was monitored daily throughout the experiments. Two additional groups of rats (n = 8 per group) were stereotaxically implanted with two permanent 26G cannulas (Plastics One, Roanoke, VA) using dental acrylic (Plastics One) and mounting screws (Small Parts, Miami Lakes, FL). One cannula was placed in the caudal dorsomedial medulla, a site previously identified by Ritter et al. (18) as eliciting both feeding and hyperglycemia in response to 5-thio-glucose (5TG) injection. The second was placed in the 3v and obturators (Plastics One) were placed into the lumen of both cannulas.

Experiment 1. Rats received direct injections of saline (n = 6) or alloxan (n = 6) into the 3v; 3 days later, food intake was measured in response to saline injection (1 ml/kg, s.c.). Four days postinjection, food intake in response to systemic 2-deoxy-D-glucose (2DG; 250 mg/kg, s.c.; grade III; Sigma) was determined. All feeding tests were performed in nonfasted rats at 0800. After receiving injections, rats were placed back into their home cages and their cumulative food intake was measured over 3 h. Six days after injections, 2DG-induced hyperglycemia was determined. Blood (20 μl) was collected from a nick in the tip of the tail 15 min before and 30, 60, 90, and 120 min after 2DG injection. Blood glucose was determined with a hand-held glucometer (Lifescan; Johnson and Johnson). Food was withheld during the blood glucose test. At 4 or 9 days after 3v injections, rats were decapitated and their brains were removed, immediately frozen on dry ice, and stored at −80°C for subsequent in situ hybridization processing (see below).

In a second group of rats, saline (n = 6) or alloxan (n = 6) was injected into the 3v. Three-hour food intake in response to saline was assessed 3 days after injection, and 2DG-induced hyperglycemic and feeding responses were assessed 4 and 6 days after injection, respectively. Eight days after 3v injections, 3-h food intake was assessed in response to an overnight (1800–0800) fast. Prior to the onset of the feeding test, tail nip blood (20 μl) was collected for glucose determination. At 14 and 16 days after 3v injections, 2DG-induced food intake and hyperglycemic responses, respectively, were reassessed. Food intake in response to an overnight fast was reassessed 19 days after injections. The experiment was terminated at 22 days, and rats were decapitated and their brains were immediately removed, frozen on dry ice, and stored at −80°C for subsequent in situ hybridization processing (see below).

Experiment 2. In the first group of rats, saline (n = 6) or alloxan (n = 6) was directly injected into the 4v. Rats were tested at 3 days for food intake in response to saline and at 4 and 6 days for 2DG-induced feeding and hyperglycemic responses, respectively, as described above. In the second group of rats, saline (n = 6) or alloxan (n = 6) was injected into the 4v and the rats were tested for food intake responses to saline (3 days), 2DG (4 days), and 2DG-induced hyperglycemia (6 days), as well as overnight food deprivation at 7 days. Rats were retested at 14 and 16 days for 2DG responses and at 20 days for refeeding in response to an overnight fast. The experiment was terminated 22 days after injection.

Experiment 3. The purpose of this experiment was to determine if the glucoprivic hyperglycemic response to 5TG injected into the caudal dorsomedial medulla was dependent on intact hypothalamic sites. In two separate tests on separate days, rats (n = 16) were injected with sterile saline (200 nl) or 5TG (24 μg/200 nl; Aldrich Chemical) into the dorsomedial medulla using an injector fabricated from 33G stainless steel tubing connected to a Hamilton syringe with PE 20 tubing. Fifteen minutes before injection and 30, 60, 90, and 120 min after injection, tail nip blood (20 μl) was assayed for blood glucose to verify positive cannula placement. Three days later, rats were injected with alloxan (n = 8; 120 μg in 5 μl saline, pH 3.0) or saline (n = 8; 5 μl, pH 3.0) into the 3v. Five and 12 days after 3v injections, 5TG was reinjected and hyperglycemic responses were reassessed. To reduce tissue damage at the cannula tip as a result of multiple injections, only 5TG-induced hyperglycemia was evaluated, although both feeding and hyperglycemia can be elicited by 5TG from this hindbrain site (18).

In situ hybridization. Frozen brains were sectioned at 15 μm on a cryostat through the rostrocaudal extent of the ARC at the level of the compact subnucleus of the DMN. Sections were processed for in situ hybridization by minor modifications of a previously described method (15) on frozen brain sections using cRNA synthesized and radiolabeled from probes for NPY (511 bp), POMC (923 bp), and GK (1422 bp) (10). Hybridized slides were opposed to SB-5 X-ray film (Kodak, Rochester, NY) for 24–48 h (NPY and POMC) or for ~3 weeks (GK).

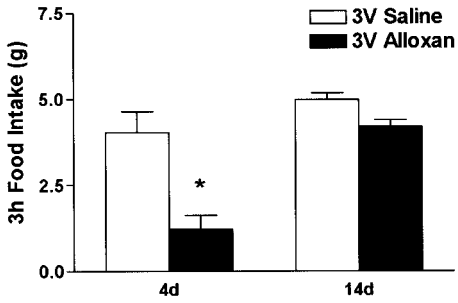

FIG. 1. Food intake (g) over 3 h after systemic 2DG (250 mg/kg, s.c.) administered to nonfasted rats 4 or 14 days after 3v alloxan or saline injection. Data are means ± SEM. *$P \leq 0.01$ vs. 3v saline–injected rats tested at 4 days.

Immunohistochemistry. Hypothalamic glial fibrillary acidic protein (GFAP) and neuron-specific nuclear protein (NeuN) immunoreactivity were assessed in separate groups of rats injected with alloxan (n = 6) or saline (n = 6) into the 3v. Seven or 14 days after 3v injections, rats were anesthetized with chloropent (0.3 ml/100g, i.p.) and perfused transcardially with PBS followed by 4% paraformaldehyde (both pH 7.4). Brains were removed, postfixed at room temperature, and cryoprotected overnight in 25% sucrose. Coronal sections (15 μm) through the rostrocaudal extent of the ARC through the compact subnucleus of the DMN were collected and processed using previously described immunohistochemistry techniques (10). Sections were incubated overnight in rabbit anti-GFAP (1:2,500; Dako Laboratories) and mouse anti-NeuN (1:1,000; Chemicon), followed by incubation with appropriate secondary antibodies conjugated to either fluorescein (GFAP) or Cy3 (NeuN; TSA, Inc.) at room temperature for 2 or 1 h, respectively. Hypothalamic sections were stained with Harris hematoxylin and eosin Y (Poly Scientific, Bay Shore, NY) to view the cytoarchitecture.

Statistical analysis. For 2DG- or 5TG-induced glucose responses, data were analyzed by repeated-measures ANOVA, with post hoc analysis by Bonferroni correction. For these same data, the area under the curve (AUC) was calculated using GraphPad Prizm statistical analysis and then compared by the unpaired t test. Feeding data were analyzed using a paired t test. In situ hybridization autoradiographic films were read by an observer blind to the experiment, as previously described (10). Data were expressed as the area (mm^2) of exposed film in specific nuclei for each of the radiolabeled probes. The three largest areas from each film were averaged and compared among treatment groups by the unpaired t test.

RESULTS

Experiment 1: 3v alloxan: glucoprivic responses, hypothalamic neuropeptide expression, and 3v histology. Three days after 3v saline or alloxan injections, there

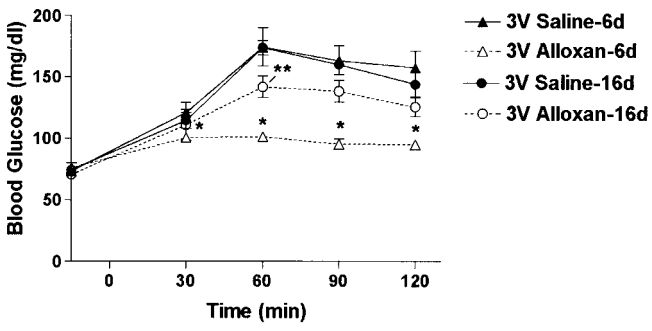

FIG. 2. Effect of systemic 2DG (250 mg/kg, s.c.) on blood glucose concentrations (mg/dl) 6 or 16 days following 3v alloxan or saline injection. The 2DG injections were administered to rats without food 15 min after the first blood sample was collected. Data are means ± SEM. *$P \leq 0.001$ vs. 3v saline–injected rats tested at 6 days; **$P \leq 0.05$ vs. 3v saline–injected rats tested at 16 days.

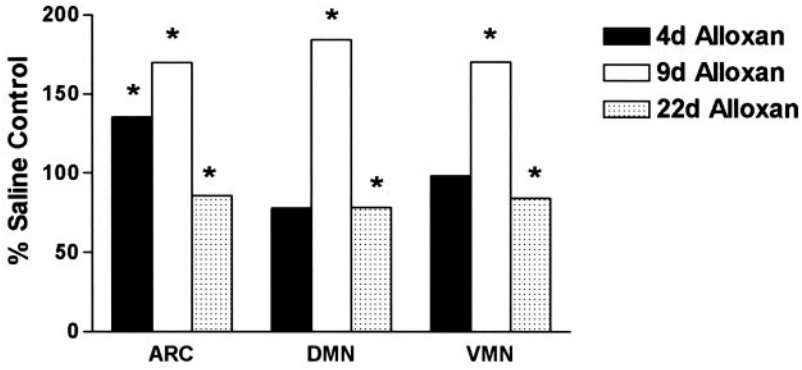

FIG. 3. GK mRNA expression in the VMN, DMN, and ARC hypothalamic nuclei 4, 9, and 22 days after 3v alloxan as a percent of their respective saline controls tested contemporaneously at each individual time point. *$P \leq 0.001$ vs. 3v saline–injected rats.

were no differences between the groups in food intake responses to systemic saline injection (1.4 ± 0.5 vs. 2.2 ± 0.5 g, respectively; $P = 0.3$). At 4 days, alloxan-injected rats ate only 30% as much food as saline rats over 3 h (Fig. 1). However, 14 days after 3v injections, 2DG-induced feeding in the alloxan group was not statistically different from that in the saline-injected group (Fig. 1). Body weights of the saline (372 ± 4 g) and alloxan (357 ± 10 g) rats did not differ at the time of the 2DG feeding test at 4 days. However, at 14 days, alloxan-treated rats weighed 10% less than saline-treated rats (439 ± 2 vs. 396 ± 8 g, respectively; $P = 0.04$).

Six days after 3v injections, systemic 2DG raised blood glucose levels in the 3v saline group from a baseline of 74 ± 3 mg/dl to a maximum of 174 ± 16 mg/dl (Fig. 2). In contrast, 2DG increased blood glucose levels in 3v alloxan rats from a baseline of 76 ± 4 mg/dl to a maximum of only 101 ± 3 mg/dl. Furthermore, the glucose AUC after 2DG injection in alloxan rats (2,385 ± 516 mg · dl^{-1} · min^{-1}) was only 28% of that in saline-treated rats (8,445 ± 1,448 mg · dl^{-1} · min^{-1}; $P = 0.001$). By 16 days, 2DG-induced glucose elevations (Fig. 2) and glucose AUC (7,142 ± 643 [alloxan] vs. 8,499 ± 492 [saline] mg · dl^{-1} · min^{-1}; $P = 0.164$) in the alloxan group were comparable with those in the saline group. However, blood glucose levels were slightly lower in the alloxan- vs. the saline-treated rats (142 ± 9 vs. 174 ± 6 mg/dl; $P = 0.03$) 60 min after 2DG injection (Fig. 2). Body weight did not differ significantly between saline- and alloxan-treated rats (441 ± 8 vs. 414 ± 11 g) at this time.

Eight days after 3v injections, fasting glucose levels were higher in alloxan-injected (71 ± 3 mg/dl) vs. saline-injected (60 ± 2 mg/dl; $P = 0.016$) and alloxan rats ate significantly more than saline rats (12 ± 0.9 vs. 8.2 ± 1.1 g; $P = 0.01$) over 3 h of refeeding after an overnight fast. Despite these differences, body weight gain did not differ between the two groups over the first 9 days following 3v saline (21 ± 2 g) and alloxan (26 ± 7 g) injections. By 19 days, however, neither fasting glucose levels (66 ± 3 vs. 60 ± 2 mg/dl) nor 3-h refeeding after an overnight fast (10 ± 0.7 vs. 9.8 ± 0.5 g) differed between the saline- and alloxan-treated groups, respectively. Similarly, neither body weight (saline: 458 ± 4 vs. alloxan: 436 ± 8 g) nor body weight gain (saline: 101 ± 6 vs. alloxan: 95 ± 9 g) differed at the termination of the experiment, 22 days after 3v injection.

In alloxan-injected rats killed 4 days after 3v injections, GK mRNA expression was significantly elevated only in the ARC nucleus (Fig. 3), whereas in rats killed at 9 days, GK mRNA expression was significantly elevated in the ARC, VMN, and DMN (Fig. 3). At 4 and 9 days postinjection, ARC NPY mRNA expression was 90 and 81% of saline controls, respectively (Fig. 4), while POMC mRNA expression was not significantly reduced until 22 days postinjection (Fig. 4). By 22 days postalloxan injection, GK mRNA expression in the ARC, DMN, and VMN was significantly reduced (Fig. 3). Likewise, ARC NPY and POMC mRNA expression were 62 and 73%, respectively, of saline controls 22 days postalloxan injection (Fig. 4).

Seven days after 3v alloxan injection, the ciliated ependymocytes lining the dorsal one-half of the 3v were denuded in most cases and the ependymocytes lining the roof of the 3v were absent in all cases. GFAP-ir astrocytes and their processes adjacent to these destroyed ependymal cells were reduced in number or totally missing. In some cases, only short strips of ependymocytes around the mid-portion of the 3v were missing. Tanycytes, the ependymal cells that line the lower ventrolateral two-thirds (α and β1) and floor (β2) of the 3v, were differentially affected by 3v alloxan. The cell bodies and processes

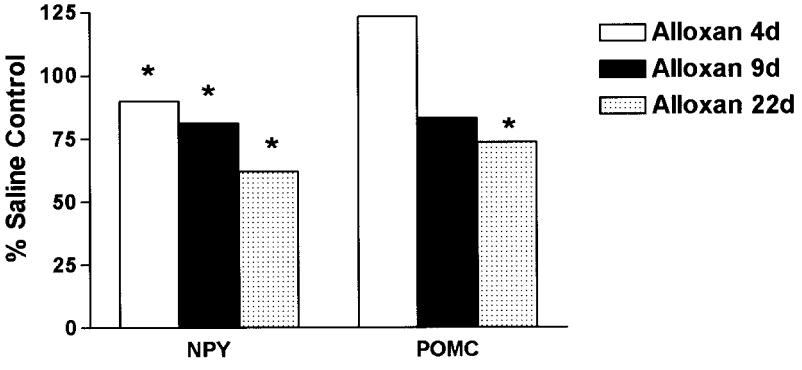

FIG. 4. ARC NPY and POMC mRNA expression 4, 9, and 22 days after 3v alloxan as a percent of their respective saline controls tested contemporaneously at each individual time point. *$P \leq 0.05$ vs. 3v saline–injected rats.

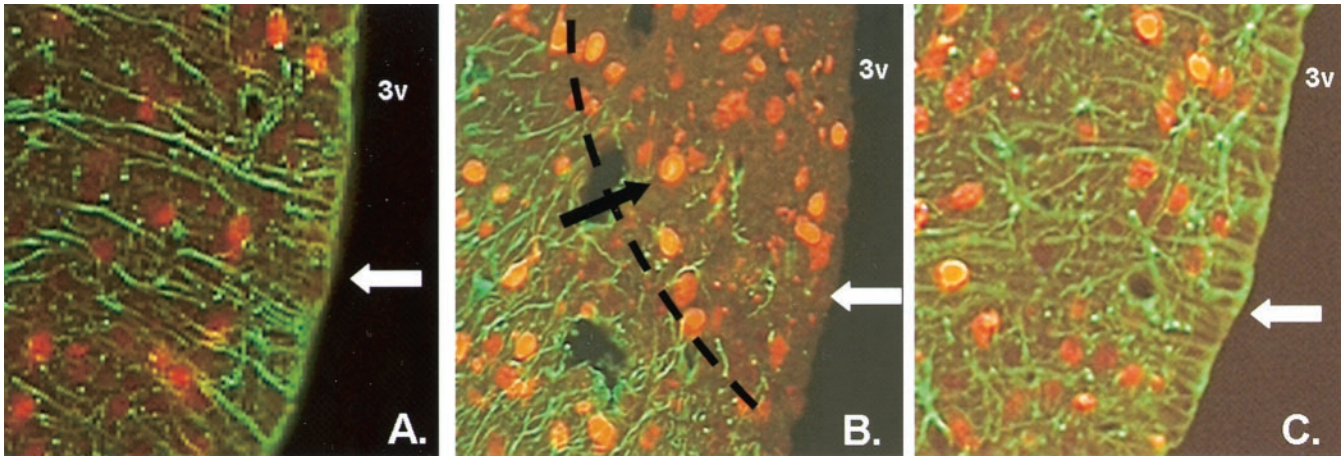

FIG. 5. Double-label immunohistochemistry for astrocytes (GFAP; green) and neurons (NeuN-ir; red) adjacent to the 3v and within the ventromedial hypothalamus in 3v saline–treated (*A*), 7-day post–3v alloxan–treated (*B*), and 14-day post–3v alloxan–treated (*C*) rats. Seven days following 3v alloxan injection, GFAP-ir cell bodies of β1 and α tanycytes lining the 3v wall were completely absent (*white arrow*, *B*) as compared with those in saline-treated rats (*white arrow*, *A*) and there was a circumscribed zone of absent GFAP-ir within the parenchyma surrounding the 3v (*dashed line*, *B*). Within this zone, NeuN-ir neurons appeared swollen (*black arrow*, *B*). By 14 days, GFAP-ir cell bodies lining the 3v and processes of α and β1 tanycytes extending into the parenchyma were restored (*white arrow*, *C*).

of α and β1 tanycytes were completely destroyed, and their GFAP-ir processes adjacent to the ventricular wall and surrounding parenchyma were absent (Fig. 5). GFAP-ir astrocytes were also absent within a circumscribed zone adjacent to the destroyed tanycytes, which included the dorsomedial portion of the VMN and portions of the dorsal and medial ARC. Within this zone, NeuN-ir neurons appeared swollen but, although not quantified, did not appear reduced in number in the alloxan-treated rats. On the other hand, GFAP-ir cell bodies and processes of the β2 tanycytes, which separate the median eminence from the 3v, were not affected by 3v alloxan. Commensurate with the return of glucoprivic responsiveness at 14 days, many or all of the ependymocytes lining the walls of the dorsal 3v of the alloxan-treated rats were restored. However, in most cases, there was little repair of the ependymocytes lining the roof of the 3v. Similarly, both GFAP-ir cell bodies and processes of the α and β1 tanycytes lining the ventrolateral walls of the 3v were partially to fully restored (Fig. 5). There was a general increase in the number of large, reactive GFAP-ir astrocytes and their processes opposite the previously damaged walls of the 3v. Where restoration of tanycytes was incomplete, GFAP-ir processes formed a layer lining the brain at the brain-cerebrospinal fluid (CSF) interface. Also, the previous neuronal swelling opposite the now-repaired tanycytes was largely reversed.

Experiment 2: 4v alloxan: feeding and blood glucose responses. There were no effects of 4v alloxan on systemic 2DG-induced food intake 4 days after injections (Fig. 6). Also, after 6 days, the saline and alloxan rats did not differ significantly in fasting blood glucose (60 ± 4 vs. 58 ± 3 mg/dl), 3-h refeeding after an overnight fast (7.2 ± 1.1 vs. 6.9 ± 0.3 g), or 2DG-induced glucose AUC (7,882 ± 522 vs. 6,520 ± 383 mg · dl^{-1} · min^{-1}). However, the 60-min glucose levels were slightly lower in alloxan- vs. saline-treated rats (145 ± 7 vs. 168 ± 6 mg/dl; $P = 0.04$) (Fig. 7) after systemic 2DG. Also, there were no differences between 4v saline- and alloxan-injected rats at 14–20 days postinjection for 2DG-induced food intake (3.1 ± 0.2 vs. 3.6 ± 0.4 g), 2DG-induced maximal blood glucose levels (140 ± 5 vs. 145 ± 12 mg/dl), glucose AUC (6,152 + 616 vs. 7,225 + 589 mg · dl^{-1} · min^{-1}), 3-h refeeding after an overnight fast (8.3 ± 0.3 vs. 7.7 ± 0.5 g), or fasting blood glucose levels (45.0 ± 2.8 vs. 45.2 ± 3.7 mg/dl), respectively. Finally, there were no differences in body weight after 4v alloxan versus saline injections at 4 days (385 ± 8

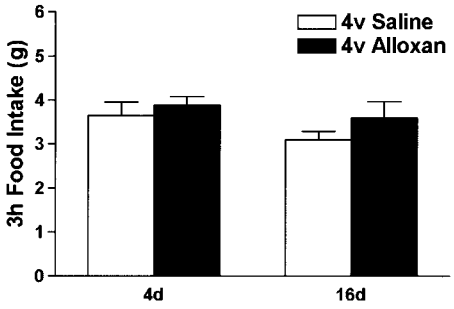

FIG. 6. Food intake in response to subcutaneous 2DG (250 mg/kg) administration 4 or 16 days following 4v alloxan or saline injection. 2DG injections were given immediately before presentation of food. Data are means ± SEM.

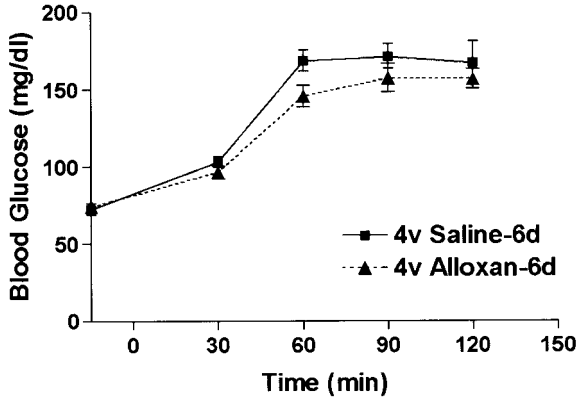

FIG. 7. Effect of systemic 2DG (250 mg/kg, s.c.) on blood glucose concentrations (mg/dl) 6 days following 4v alloxan or saline injection. The 2DG injections were given to rats without food 15 min after the first blood sample was collected. Data are means ± SEM.

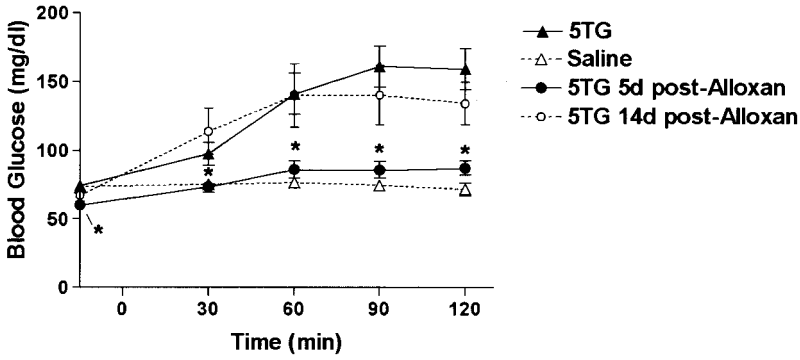

FIG. 8. Effect of 5TG (24 μg/200 nl) or saline injected into the caudal dorsomedial medulla on blood glucose concentrations (mg/dl) before and after 3v alloxan. Prior to 3v injections, saline or 5TG was injected into the dorsomedial medulla. 5TG-induced blood glucose responses were compared with blood glucose responses elicited by 5TG 5 days (5TG 5d post-Alloxan) and 14 days (5TG 14d post-Alloxan) after 3v alloxan. Data are means ± SEM. *$P \leq 0.05$ vs. 5TG responses prior to 3v alloxan injection.

vs. 395 ± 9 g) or 16 days (436 ± 9.5 vs. 442 ± 9.9 g). Because there were no deficits in glucoprivic responses at any time after 4v alloxan injection, hypothalamic NPY, POMC, and GK expression were not assessed in these experiments.

Experiment 3: effects of 3v alloxan on dorsomedial medulla 5TG-induced hyperglycemia. Prior to 3v injection of alloxan, 5TG injection into the dorsomedial medulla significantly elevated blood glucose above baseline levels (Fig. 8). As compared with 3v saline injections, the hyperglycemic response to focal hindbrain 5TG injection was impaired at 5 days, but was restored by 12 days after 3v alloxan injection (Fig. 8).

DISCUSSION

Existing studies support a critical role for both the VMH (VMH: the VMN and ARC) (28–31) and the hindbrain (18,32,33) in the detection of hypoglycemia and the expression of CRRs. The results of the present study support the idea that the feeding and sympathoadrenal components of glucoprivic CRRs are dependent on an integrated cross talk between these two critical brain areas. Thus, 3v alloxan produced a reversible impairment of both the feeding and hyperglycemic responses to systemic 2DG and the hyperglycemic response to localized glucoprivation produced by 5TG injected into the dorsomedial medulla. This demonstrates that the sympathoadrenal response to either focal hindbrain 5TG stimulation or systemic 2DG is dependent not only on spinally projecting hindbrain catecholamine neurons (35), but also on hypothalamic sites surrounding the 3v and/or substrates within the 3v. It is not clear from our results which hypothalamic sites contribute to the sympathoadrenal response, although there is evidence supporting a role for both the VMH (28–31) and the PVN (36). Because 3v alloxan caused both a transient and selective loss of the tanycytes and ependymocytes lining the 3v and a transient upregulation in GK mRNA expression, it is unclear whether these events were independently or additively responsible for the observed deficits in glucoprivic feeding and sympathoadrenal activation.

Alloxan is preferentially transported into the cell by GLUT2 (37). GLUT2-ir protein and mRNA are expressed both in the ependymocytes lining the dorsal 3v (17,38) and in α and β1, but not β2, tanycytes in the floor of the 3v (39). In addition to GLUT2, α and β1 tanycytes also express GK (17), ATP-sensitive K^+ channels, and the Kir6.1 subunit (39). These proteins (except Kir6.1) are present in pancreatic β-cells, where they may serve in glucosensing mechanisms (40–42). Since tanycytes and ependymocytes both express GLUT2 (17,39) and alloxan was administered into the 3v, it is likely that tanycytes and ependymocytes were the primary targets of alloxan, resulting in their destruction and the breakdown of the brain-CSF barrier. This is supported by the finding that β2 tanycytes, which line the floor of the 3v and do not express GLUT2 (39), were not affected by alloxan. Exactly how the loss of tanycytes and ependymocytes might contribute to glucoprivic deficits is not entirely clear. However, α and β1 tanycytes, located in the ventrolateral two-thirds of the 3v, form long processes that contact neurons in the VMN (α) (39) and ARC (β1) (43,44), while the proximal portion of tanycytes contact the 3v CSF (39,44). Thus, tanycytes may provide metabolic and/or physical support for adjacent astrocytes and neurons (39). If so, their destruction would disrupt critical astrocyte-neuron interactions, leading to defective neuronal glucosensing (45). In fact, the loss of glucoprivic CRRs was commensurate with the alloxan-induced loss of astrocytes and neuronal swelling, and the CRRs returned when the majority of the 3v lining was restored. This supports the hypothesis that 3v tanycytes participate in hypothalamic glucosensing mechanisms (39). Thus, their destruction may also have contributed to the transient changes in GK mRNA in hypothalamic neurons after alloxan administration.

Regardless of the mechanism, GK upregulation in alloxan-treated rats might have directly contributed to impaired glucoprivic CRRs if it were associated with increased GK enzyme activity. Such increased activity should increase the sensitivity to glucose in the alloxan-treated rats, allowing GK-expressing neurons to maintain ATP production despite glucoprivic conditions. The finding that GK expression was elevated when glucoprivic deficits were present, but then decreased when glucoprivic responses returned, supports such a role for GK. Similarly, upregulation of hypothalamic GK mRNA occurs in rats in association with blunted CRRs 48 h after a single bout of insulin-induced hypoglycemia. These rats also have reduced ARC NPY and POMC expression (46). Thus, hypothalamic GK may be a critical component for the expression of glucoprivic CRRs. In contrast, the finding that food intake and blood glucose levels were elevated in response to an overnight fast when hypothalamic GK expression was elevated speaks against a role for GK during fasting conditions. In rats with elevated GK expression, it could be predicted that both food intake and blood glucose levels would be decreased, and not elevated, due to an increased ability to utilize glucose at reduced levels. Thus, it appears that the role of

GK in glucoprivic-induced responses may be different from that of fasting-induced responses.

It is unlikely that decreased expression of either ARC NPY or POMC contributed to the alloxan-induced impairment of glucoprivic CRRs. Although NPY mRNA expression was reduced 4 days postalloxan injection when CRRs were impaired, both NPY and POMC expression was decreased at 14 days when glucoprivic responses returned to normal. However, impaired NPY signaling may be important for glucoprivic responses under some conditions, since glucoprivic feeding increases ARC NPY mRNA expression (47,48) and NPY antibodies block glucoprivic feeding (49). Finally, the earlier reduction in NPY expression is likely due to the fact that NPY neurons lie close to the tanycytes lining the 3v and would have been damaged before the more laterally placed POMC neurons by alloxan diffusing from the 3v. Thus, changes in hypothalamic GK mRNA expression and the integrity of the 3v tanycytes and adjacent glia showed the closest relationship with changes in glucoprivic CRRs after 3v alloxan injections.

While the hindbrain is clearly essential both for detecting glucoprivation and for the expression of CRRs (18,32,35), 4v alloxan injections produced no deficits in glucoprivic responses. This suggests that GK-expressing neurons within the diffusion radius of alloxan from the 4v are not involved in either glucoprivic hyperglycemia or feeding. These results differ from those of previous studies showing that 4v alloxan permanently impaired glucoprivic feeding, but not hyperglycemia (25). These apparent differences may be due to our use of lower doses or because glucoprivic responses were assessed at different times after alloxan injection. Although nuclei tractus solitarius neurons, which lie under the floor of the 4v, do express GK mRNA (N.M.S., B.E.L., A.A.D.-M., unpublished observations), the majority of hindbrain GK-expressing neurons are serotonergic and lie deep within the dorsomedial medulla in the raphe pallidus and obscurus (17). Direct injection of 5TG into the nucleus tractus solitarius evokes no substantial CRR (18), whereas 5TG injections into the dorsomedial GK-serotonin neurons, as carried out here, elicits both hyperglycemia and feeding responses (18). Thus, the lack of effect of alloxan, at the doses used here, is likely due to the fact that these dorsomedial GK-expressing neurons were not reached by diffusion of alloxan from the 4v. However, the fact that 3v alloxan transiently impaired the hyperglycemic response to dorsomedial medulla 5TG stimulation supports previous work demonstrating a critical interplay between hindbrain and hypothalamic sites in glucoprivic-induced responses (35,50,51).

In conclusion, 3v alloxan transiently increased hypothalamic GK mRNA expression, reversibly damaged 3v tanycytes and ependymocytes, and progressively decreased NPY and POMC mRNA expression. The timing of impaired glucoprivic responses correlated best with GK expression and tanycyte and ependymocyte damage. These two processes may be inextricably intertwined in this model of reversibly impaired glucoprivic CRRs. However, the results suggest that both may play critical roles in this attenuated responsiveness to glucoprivation. Hypothalamic GK mRNA is also increased when CRRs to insulin-induced hypoglycemia are impaired, while the ventricular lining is intact (10). Thus, it seems likely that hypothalamic GK expression is an important component of glucoprivic CRRs. However, it is unclear whether the changes in GK expression are a result of impaired glucoprivic responsiveness or simply a compensatory response to it. Lastly, our studies, in concert with previous studies (35), strongly suggest that behavioral and sympathoadrenal CRRs require coordinated input from both hypothalamic and hindbrain glucosensing sites.

ACKNOWLEDGMENTS

This study was supported by the Research Service of the Department of Veterans Affairs, the Juvenile Diabetes Research Foundation International (postdoctoral fellowship award to N.M.S.), and the National Institute of Diabetes and Digestive and Kidney Diseases (R01-53181 to B.E.L.).

The authors thank Ghoufeng Zhou, Matthew Klein, Charlie Salter, Eugenia Dziopa, and Antoinette Moralishvili for their expert technical assistance.

REFERENCES

1. Sokoloff L, Reivich M, Kennedy C, Des Rosiers MH, Patlak CS, Pettigrew KD, Sakurada O, Shinohara M: The [^{14}C]deoxyglucose method for the measurement of local cerebral glucose utilization: theory, procedure, and normal values in the conscious and anesthetized albino rat. *J Neurochem* 28:897–916, 1977
2. Ashford MLJ, Boden PR, Treherne JM: Glucose-induced excitation of hypothalamic neurones is mediated by ATP-sensitive K^+ channels. *Pflugers Arch* 415:479–483, 1990
3. Ashford MLJ, Boden PR, Treherne JM: Tolbutamide excites rat glucoreceptive ventromedial hypothalamic neurones by indirect inhibition of ATP-K^+ channels. *Br J Pharmacol* 101:531–540, 1990
4. Oomura Y, Ooyama H, Sugimori M, Nakamura T, Yamada Y: Glucose inhibition of the glucose-sensitive neurone in the rat lateral hypothalamus. *Nature* 247:284–286, 1974
5. Levin BE: Glucose-regulated dopamine release from substantia nigra neurons. *Brain Res* 874:158–164, 2000
6. Oomura Y, Ono T, Ooyama H, Wayner MJ: Glucose and osmosensitive neurons of the rat hypothalamus. *Nature* 222:282–284, 1969
7. Oomura Y: Glucose as a regulator of neuronal activity. In *Advances in Metabolic Disorders*. Szabo AJ, Ed. New York, Academic, 1983, p. 31–65
8. Kow LM, Pfaff DW: Actions of feeding-relevant agents on hypothalamic glucose-responsive neurons in vitro. *Brain Res Bull* 15:509–513, 1985
9. Minami T, Oomura Y, Sugimori M: Electrophysiological properties and glucose responsiveness of guinea-pig ventromedial hypothalamic neurones in vitro. *J Physiol* 380:127–143, 1986
10. Dunn-Meynell AA, Routh VH, Kang L, Gaspers L, Levin BE: Glucokinase is the likely mediator of glucosensing in both glucose excited and glucose inhibited central neurons. *Diabetes* 51:2056–2065, 2002
11. Song Z, Levin BE, McArdle JJ, Bakhos N, Routh VH: Convergence of pre- and postsynaptic influences on glucosensing neurons in the ventromedial hypothalamic nucleus (VMN). *Diabetes* 50:2673–2681, 2001
12. Yang XJ, Kow LM, Funabashi T, Mobbs CV: Hypothalamic glucose sensor: similarities to and differences from pancreatic β-cell mechanisms. *Diabetes* 48:1763–1772, 1999
13. Jetton TL, Liang Y, Pettepher CC, Zimmerman EC, Cox FG, Horvath K, Matschinsky FM, Magnuson MA: Analysis of upstream glucokinase promoter activity in transgenic mice and identification of glucokinase in rare neuroendocrine cells in the brain and gut. *J Biol Chem* 269:3641–3654, 1994
14. Kang L, Routh VH, Kuzhikandathil EV, Gaspers L, Levin BE: Physiological and molecular characteristics of rat hypothalamic ventromedial nucleus glucosensing neurons. *Diabetes* 53:549–559, 2004
15. Lynch RM, Tompkins LS, Brooks HL, Dunn-Meynell AA, Levin BE: Localization of glucokinase gene expression in the rat brain. *Diabetes* 49:693–700, 2000
16. Roncero I, Alvarez E, Vazquez P, Blazquez E: Functional glucokinase isoforms are expressed in rat brain. *J Neurochem* 74:1848–1857, 2000
17. Maekawa F, Toyoda Y, Torii N, Miwa I, Thompson RC, Foster DL, Tsukahara S, Tsukamura H, Maeda K: Localization of glucokinase-like

immunoreactivity in the rat lower brain stem: for possible location of brain glucose-sensing mechanisms. *Endocrinology* 141:375–384, 2000
18. Ritter S, Dinh TT, Zhang Y: Localization of hindbrain glucoreceptive sites controlling food intake and blood glucose. *Brain Res* 856:37–47, 2000
19. Lenzen S, Freytag S, Panten U: Inhibition of glucokinase by alloxan through interaction with SH groups in the sugar-binding site of the enzyme. *Mol Pharmacol* 34:395–400, 1988
20. Lenzen S, Tiedge M, Panten U: Glucokinase in pancreatic B-cells and its inhibition by alloxan. *Acta Endocrinol* 115:21–29, 1987
21. Takasu N, Asawa T, Komiya I, Nagasawa Y, Yamada T: Alloxan-induced DNA strand breaks in pancreatic islets: evidence for H_2O_2 as an intermediate. *J Biol Chem* 266:2112–2114, 1991
22. Kim HR, Rho HW, Park BH, Park JW, Kim JS, Kim UH, Chung MY: Role of Ca2+ in alloxan-induced pancreatic beta-cell damage. *Biochim Biophys Acta* 1227:87–91, 1994
23. Ritter S, Strang M: Fourth ventricular alloxan injection causes feeding but not hyperglycemia in rats. *Brain Res* 249:198–211, 1982
24. Murnane JM, Ritter S: Alloxan-induced glucoprivic feeding deficits are blocked by D-glucose and amygdalin. *Pharm Biochem Behav* 22:407–413, 1985
25. Ritter SS, Murnane M, Ladenheim EE: Glucoprivic feeding is impaired by lateral or fourth ventricular alloxan injection. *Am J Physiol* 243:R312–R317, 1982
26. Murnane JM, Ritter S: Intraventricular alloxan impairs feeding to both central and systemic glucoprivation. *Physiol Behav* 34:609–613, 1985
27. Woods SC, McKay LD: Intraventricular alloxan eliminates feeding elicited by 2-deoxyglucose. *Science* 202:1209–1211, 1978
28. Borg MA, Borg WP, Tamborlane WV, Brines ML, Shulman GI, Sherwin RS: Chronic hypoglycemia and diabetes impair counterregulation induced by localized 2-deoxy-glucose perfusion of the ventromedial hypothalamus in rats. *Diabetes* 48:584–587, 1999
29. Borg WP, Sherwin RS, During MJ, Borg MA, Shulman GI: Local ventromedial hypothalamic glucopenia triggers counterregulatory hormone release. *Diabetes* 44:180–184, 1995
30. Borg MA, Sherwin RS, Borg WP, Tamborlane WV, Shulman GI: Local ventromedial hypothalamus glucose perfusion blocks counterregulation during systemic hypoglycemia in awake rats. *J Clin Invest* 99:361–365, 1997
31. Borg WP, During MJ, Sherwin RS, Borg MA, Brines ML, Shulman GI: Ventromedial hypothalamic lesions in rats suppress counterregulatory responses to hypoglycemia. *J Clin Invest* 93:1677–1682, 1994
32. Ritter RC, Slusser PG, Stone S: Glucoreceptors controlling feeding and blood glucose: location in the hindbrain. *Science* 213:451–452, 1981
33. DiRocco RJ, Grill HJ: The forebrain is not essential for sympathoadrenal hyperglycemic response to glucoprivation. *Science* 204:1112–1114, 1979
34. Paxinos G, Watson C: *The Rat Brain in Stereotaxic Coordinates*. 2nd ed. New York, Academic, 1986
35. Ritter S, Bugarith K, Dinh TT: Immunotoxic destruction of distinct catecholamine subgroups produces selective impairment of glucoregulatory responses and neuronal activation. *J Comp Neurol* 432:197–216, 2001
36. Evans SB, Wilkinson CW, Gronbeck P, Bennett JL, Taborsky GJ Jr, Figlewicz DP: Inactivation of the PVN during hypoglycemia partially simulates hypoglycemia-associated autonomic failure. *Am J Physiol* 284: R57–R65, 2003
37. Elsner M, Tiedge M, Guldbakke B, Munday R, Lenzen S: Importance of the GLUT2 glucose transporter for pancreatic beta cell toxicity of alloxan. *Diabetologia* 45:1542–1549, 2002
38. Ngarmukos C, Baur EL, Kumagai AK: Co-localization of GLUT1 and GLUT4 in the blood-brain barrier of the rat ventromedial hypothalamus. *Brain Res* 900:1–8, 2001
39. Garcia MA, Millan C, Balmaceda-Aguilera C, Castro T, Pastor P, Montecinos H, Reinicke K, Zuniga F, Vera JC, Onate SA, Nualart F: Hypothalamic ependymal-glial cells express the glucose transporter GLUT2, a protein involved in glucose sensing. *J Neurochem* 86:709–724, 2003
40. Matschinsky FM, Glaser B, Magnuson MA: Pancreatic β-cell glucokinase: closing the gap between theoretical concepts and experimental realities. *Diabetes* 47:307–315, 1998
41. Newgard CB, McGarry JD: Metabolic coupling factors in pancreatic B-cell signal transduction. *Annu Rev Neurosci* 64:689–719, 1995
42. Matschinsky FM: Regulation of pancreatic β-cell glucokinase: from basics to therapeutics. *Diabetes* 51 (Suppl. 3):S394–S404, 2002
43. Peruzzo B, Pastor FE, Blazquez JL, Schobitz K, Pelaez B, Amat P, Rodriguez EM: A second look at the barriers of the medial basal hypothalamus. *Exp Brain Res* 132:10–26, 2000
44. Chauvet N, Parmentier ML, Alonso G: Transected axons of adult hypothalamo-neurohypophysial neurons regenerate along tanycytic processes. *J Neurosci Res* 41:129–144, 1995
45. Tsacopoulos M, Magistretti PJ: Metabolic coupling between glia and neurons. *J Neurosci* 16:877–885, 1996
46. Tkacs NC, Dunn-Meynell AA, Levin BE: Presumed apoptosis and reduced arcuate nucleus neuropeptide Y and proopiomelanocortin mRNA in non-coma hypoglycemia. *Diabetes* 49:820–826, 2000
47. Akabayashi A, Zaia CT, Silva I, Chae HJ, Leibowitz SF: Neuropeptide Y in the arcuate nucleus is modulated by alterations in glucose utilization. *Brain Res* 621:343–348, 1993
48. Minami S, Kamegai J, Sugihara H, Suzuki N, Higuchi H, Wakabayashi I: Central glucoprivation evoked by administration of 2-deoxy-D-glucose induces expression of the c-fos gene in a subpopulation of neuropeptide Y neurons in the rat hypothalamus. *Brain Res Mol Brain Res* 33:305–310, 1995
49. He B, White BD, Edwards GL, Martin RJ: Neuropeptide Y antibody attenuates 2-deoxy-D-glucose induced feeding in rats. *Brain Res* 781:348–350, 1998
50. Fraley GS, Dinh TT, Ritter S: Immunotoxic catecholamine lesions attenuate 2DG-induced increase of AGRP mRNA. *Peptides* 23:1093–1099, 2002
51. Fraley GS, Ritter S: Immunolesion of norepinephrine and epinephrine afferents to medial hypothalamus alters basal and 2-deoxy-D-glucose-induced neuropeptide Y and agouti gene-related protein messenger ribonucleic acid expression in the arcuate nucleus. *Endocrinology* 144:75–83, 2003