Antecedent Hindbrain Glucoprivation Does Not Impair the Counterregulatory Response to Hypoglycemia

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RESEARCH DESIGN AND METHODS

Adult male Sprague-Dawley rats (Simonsen Laboratories, Gilroy, CA) weighing between 350 and 380 g were used for these experiments. Rats were housed individually and maintained on a 12-h light-dark schedule (lights on at 6:00 A.M., off at 6:00 P.M.) at 22–23°C with ad libitum access to food (Purina rat chow #5001) and water, except where otherwise specified. All procedures were approved by the Animal Studies Subcommittee of the Veterans Affairs Puget Sound Health Care System, Seattle, Washington; the Department of Psychiatry and Behavioral Sciences, University of Washington, Seattle, Washington; the Department of Medicine, University of Washington, Seattle, Washington; the Division of Endocrinology, Education and Clinical Center, Veterans Affairs Puget Sound Health Care System, Seattle, Washington; and the Seattle Institute for Biomedical and Clinical Research, Seattle, Washington.

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Received for publication 24 July 2006 and accepted in revised form 12 October 2006.

2DG, 2-deoxy-D-glucose; 5TG, 5-thio-glucose; CRR, counterregulatory response; HAAF, hypoglycemia-associated autonomic failure; RHI, recurrent hypoglycemia; VMN, ventromedial hypothalamic nucleus.

DOI: 10.2337/db06-1025

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TABLE 1
Day 1 plasma glucose levels in response to saline or insulin

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Drug</th>
<th>First bout</th>
<th>Second bout</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>t = 0</td>
<td>t = 60</td>
</tr>
<tr>
<td>Single hypoglycemia (SH)</td>
<td>Saline 1 ml/kg, s.c.</td>
<td>98 ± 5</td>
<td>100 ± 4</td>
</tr>
<tr>
<td>Recurrent hypoglycemia (RH)</td>
<td>Insulin 2.5 units/kg, s.c.</td>
<td>107 ± 6</td>
<td>34 ± 2*</td>
</tr>
</tbody>
</table>

Data are means ± SE. Plasma glucose, mg/dl. *P ≤ 0.05 vs. t = 0.

Sound Health Care System Research and Development Committee, Seattle Division.

Surgery. All rats were surgically implanted with a Silastic intravenous catheters under ketamine/xylazine (80 mg/kg ketamine; KetaFlo; Abbott Laboratories, Chicago, IL, and 12.9 mg/kg xylazine; Xyla-ject; Phoenix Pharmacetical, St. Joseph, MO) anesthesia as previously described (22). The catheter was tunneled subcutaneously and exteriorized through a midline incision in the scalp. In addition, some rats received either a 20G stainless steel–guide cannula (Plastics One, Roanoke, VA) aimed at the caudal dorsal medial medulla in the hindbrain using coordinates previously described (17) or the third ventricle using the stereotaxic coordinates (−2.2 bregma; 0 midline; −7.5 dura) according to the atlas of Paxinos and Watson (23). The guide cannula and/or intravenous catheter were held in place with four skull screws (Small Parts, Miami Lakes, FL) and acrylic cement (Lang Dental, Wheeling, IL). Rats received subcutaneous 3 ml lactated Ringer solution (Baxter Pharmaceutical Products, New Providence, NJ) and buprenorphine (0.05 mg/kg) and were maintained on a circulating water heating pad until recovery from anesthesia. Catheter lines were filled with 60% polyvinylpyrrolidone (PVP10; Sigma, St. Louis, MO)/heparin (1,000 units/ml; Elkins-Sinn, Cherry Hill, NJ) and kept patent by a heparin (10 units/ml) flush every 3 days. All rats regained weight to at least the presurgical level (−7 days) before the onset of the study.

Experimental procedures. Rats were divided into two groups: one receiving only an intravenous catheter and the other an intravenous catheter and a guide cannula implanted into either the caudal dorsal medial medulla hindbrain site or the third ventricle. All experiments began at 9:00 a.m. Nonfasted rats were subjected to a 2-day testing procedure that was conducted in square, acrylic testing chambers (−30 × 30 × 30 cm) to allow for remote blood collection. Before initiating the experiment, rats were familiarized with the testing chambers (4 h/day for 4 days). During this time, rats were extensively handled and mock central and subcutaneous injections administered to mimic the experimental procedure. The concentration of insulin and saline used in all experiments was 2.5 units/kg s.c. (Novolin R, regular humulin insulin, recombinant human origin; Novo Nordisk, Princeton, NJ) and 1 ml/kg s.c., respectively. The concentration of 5TG (Sigma) injected into the hindbrain was 24 μg/200 nl (17), and 150 μg/5 μl (24) was injected into the third ventricle. Saline was used as a vehicle control for hindbrain (200 nl) and third ventricle (5 μl) injections.

On the 1st day of testing (day 1), rats surgically fitted with an intravenous catheter were anesthetized with 1.5 ml/kg ketamine/xylazine and received either insulin to induce hypoglycemia (two 2-h bouts of hypoglycemia separated by a 60-min interval) or saline vehicle. Food was available during the 60-min interval. To verify induction of hypoglycemia, blood (0.1 ml) was drawn immediately before each insulin or saline injection and 60 min thereafter for measurement of plasma glucose. On the 2nd day of testing (day 2), all rats were subcutaneously injected with insulin to induce hypoglycemia. Blood was collected (1.5 ml) immediately before insulin injection (t = 0) and 60 and 120 min thereafter for subsequent measurement of plasma glucose, glucagon, epinephrine, norepinephrine, adrenocorticotropic hormone (ACTH), and corticosterone. Blood was immediately replaced with donor blood drawn from unstressed rats before the experiment. Thus, there were two distinct protocols implemented in this study. The recurrent hypoglycemia protocol consisted of two bouts of systemic hypoglycemia on day 1, whereas the central glucoprivation protocol consisted of a single 5TG injection on day 1. In a pilot study, no differences in day 2 hypoglycemia hormonal CRRs were observed in rats that received one versus two central injections of 5TG on day 1 of testing (N.M.S., unpublished data). In addition, since the hyperglycemic response induced by the first injection of 5TG persisted at the onset of the second 5TG injection, we chose the single injection protocol for this study.

Positive cannula placements were verified by a hyperglycemic response to hindbrain (17) or third ventricular 5TG stimulation (25) (determined on day 1 of testing). For the vehicle control groups receiving saline on day 1 of testing (HB Sal-Hypo and 3v Sal-Hypo groups), 5TG was administered at the end of the experiment to verify cannula placement. Only rats that exhibited a statistically significant increase in plasma glucose above their baseline values were included in the study. In response to either hindbrain or third ventricular 5TG injection, plasma glucose levels were increased above baseline values by ~100 mg/dl.

Plasma assays. Blood samples were obtained for the measurement of hormonal CRRs to insulin-induced hypoglycemia and stored at −80°C until assayed. Blood for the catecholamine assays was collected on EGTA-glutathione (2.3:1.5 mg/ml; Sigma). Tubes for glucagon assays contained 50 μl of 1 mol/l benzamide (Sigma) and 1 unit heparin. Blood for glucose, ACTH, and corticosterone assays was collected on EDTA and aprotinin (1.7 tissue inhibitor unit; Sigma). The assays have been previously described (22). Briefly, a radioenzymatic method as described by Evans et al. (26) was used for determination of plasma epinephrine and norepinephrine. A radioimmunoassay procedure was used for plasma corticosterone measurement, as described by Van Dijk et al. (27). Plasma glucose was measured using the Beckman glucose analyzer. Glucagon was assayed by the Linco glucagon radioimmunoassay kit (Linco Research). Measurements of ACTH were made using the Nichols Institute Diagnostics immunoradiometric assay kit (Nichols Institute Diagnostics, San Juan Capistrano, CA).

Statistical analysis. Data from the plasma assays were analyzed using repeated-measures ANOVA with time as the repeated measure and treatment (SH, RH, HB 5TG-Hypo, 3v 5TG-Hypo, HB Sal-Hypo, and 3v Sal-Hypo groups) as the between-groups factor. In the event of significant effects, Bonferroni’s post hoc tests were used to determine significant differences, and t tests were used where appropriate. Significance levels for all tests were taken as P < 0.05.

TABLE 2
Day 1 plasma glucose levels in response to hindbrain or third ventricular 5TG

<table>
<thead>
<tr>
<th>Injection site</th>
<th>Drug</th>
<th>t = 0</th>
<th>t = 60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hindbrain</td>
<td>5TG 24 μg/200 nl</td>
<td>112 ± 5</td>
<td>221 ± 22*</td>
</tr>
<tr>
<td>Third ventricle</td>
<td>5TG 150 μg/5 μl</td>
<td>90 ± 5</td>
<td>189 ± 15*</td>
</tr>
</tbody>
</table>

Data are means ± SE. Plasma glucose, mg/dl. *P ≤ 0.05 vs. t = 0.
TABLE 3
Plasma glucose and hormone responses during day 2 insulin-induced hypoglycemia

<table>
<thead>
<tr>
<th>Time point (min)</th>
<th>Glucose (mg/dl)</th>
<th>Glucagon (pg/ml)</th>
<th>Epinephrine (pg/ml)</th>
<th>Norepinephrine (pg/ml)</th>
<th>ACTH (pg/ml)</th>
<th>Corticosterone (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single hypoglycemia (n = 11)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>104 ± 7</td>
<td>62 ± 13</td>
<td>120 ± 25</td>
<td>363 ± 30</td>
<td>11 ± 3</td>
<td>37 ± 6</td>
</tr>
<tr>
<td>60</td>
<td>37 ± 3</td>
<td>494 ± 56</td>
<td>3,811 ± 540</td>
<td>925 ± 91</td>
<td>226 ± 30</td>
<td>223 ± 12</td>
</tr>
<tr>
<td>120</td>
<td>25 ± 2</td>
<td>428 ± 40</td>
<td>3,640 ± 525</td>
<td>1,022 ± 85</td>
<td>200 ± 19</td>
<td>250 ± 21</td>
</tr>
<tr>
<td>Recurrent hypoglycemia (n = 12)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>0</td>
<td>118 ± 3</td>
<td>70 ± 15</td>
<td>130 ± 19</td>
<td>311 ± 40</td>
<td>9 ± 2</td>
<td>26 ± 7</td>
</tr>
<tr>
<td>60</td>
<td>38 ± 3</td>
<td>222 ± 43*</td>
<td>1,470 ± 325*</td>
<td>744 ± 75*</td>
<td>181 ± 19</td>
<td>190 ± 20</td>
</tr>
<tr>
<td>120</td>
<td>25 ± 1</td>
<td>231 ± 34*</td>
<td>2,500 ± 490</td>
<td>1,322 ± 180</td>
<td>192 ± 20</td>
<td>223 ± 22</td>
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<tr>
<td>Antecedent hindbrain 5TG (n = 11)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>112 ± 4</td>
<td>69 ± 13</td>
<td>129 ± 30</td>
<td>393 ± 44</td>
<td>13 ± 3</td>
<td>36 ± 5</td>
</tr>
<tr>
<td>60</td>
<td>41 ± 3</td>
<td>513 ± 56</td>
<td>3,806 ± 344</td>
<td>1,154 ± 92</td>
<td>243 ± 42</td>
<td>208 ± 9</td>
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<tr>
<td>120</td>
<td>27 ± 2</td>
<td>403 ± 71</td>
<td>4,248 ± 618</td>
<td>1,602 ± 170</td>
<td>165 ± 17</td>
<td>231 ± 10</td>
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<tr>
<td>Antecedent third ventricular 5TG (n = 11)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>105 ± 7</td>
<td>65 ± 6</td>
<td>142 ± 18</td>
<td>352 ± 41</td>
<td>9 ± 3</td>
<td>28 ± 7</td>
</tr>
<tr>
<td>60</td>
<td>37 ± 3</td>
<td>210 ± 21*</td>
<td>1,162 ± 120*</td>
<td>723 ± 70*</td>
<td>220 ± 30</td>
<td>180 ± 17</td>
</tr>
<tr>
<td>120</td>
<td>28 ± 2</td>
<td>290 ± 53</td>
<td>3,325 ± 460</td>
<td>1,205 ± 180</td>
<td>211 ± 35</td>
<td>195 ± 19</td>
</tr>
</tbody>
</table>

Data are means ± SE; n, no. of rats. *P ≤ 0.05 vs. SH.

RESULTS

Day 1 plasma glucose responses. Table 1 presents the plasma glucose data for the experimental groups injected with saline or insulin on day 1 of testing. In the SH group, plasma glucose levels did not change in response to the first or second injection of saline. In response to insulin injection in the RH group, plasma glucose levels were significantly reduced from baseline (t = 0) values (P < 0.05). Table 2 presents the plasma glucose results for the experimental groups receiving a single injection of 5TG into the hindbrain or third ventricle. Both hindbrain and third ventricular delivery of 5TG resulted in a significant increase above baseline plasma glucose at t = 60 (P < 0.05). Vehicle control injection of saline into either the hindbrain (100 ± 4 [t = 0] and 90 ± 5 mg/dl [t = 60]) or third ventricle (92 ± 5 [t = 0] and 102 ± 4 mg/dl [t = 60]) on day 1 did not change plasma glucose levels.

Day 2 counterregulatory hormones. Table 3 presents all the raw data for all time points, while Fig. 1 summarizes glucagon, epinephrine, and norepinephrine responses to day 2 hypoglycemia. Baseline (t = 0) values of glucose, glucagon, epinephrine, norepinephrine, ACTH, and corticosterone were not different among the experimental groups (Table 3). In response to insulin-induced hypoglycemia, all experimental groups demonstrated significant decreases in plasma glucose from baseline values (main effect of time: $F_{2,580} = 1398.570, P < 0.001$ (Table 3). The declines in plasma glucose levels did not differ among the treatment groups (no interaction between time and treatment: $F_{6,880} = 3.082, P = 0.91$).

FIG. 1. Day 2 plasma glucagon (A), epinephrine (B), and norepinephrine (C) levels (t = 60) in response to insulin-induced hypoglycemia after antecedent (day 1) saline (□), antecedent hindbrain glucoprivation (■), antecedent recurrent hypoglycemia (●), or antecedent third ventricular glucoprivation (○). Data are expressed as means ± SE. *P < 0.05 vs. SH.
There was a significant main effect of treatment on glucagon secretion during day 2 of insulin-induced hypoglycemia (treatment effect: $F_{2,34} = 12.5, P = 0.001$; time × treatment interaction: $F_{5,82} = 3.603, P = 0.0032$). Recurrent hypoglycemia significantly blunted the glucagon response to day 2 hypoglycemia 60 and 120 min after insulin injection compared with SH-treated rats ($P = 0.003$ and $P = 0.001$, respectively) (Table 3 and Fig. 1A). Antecedent third ventricular glucoprivation also significantly suppressed glucagon secretion in response to day 2 hypoglycemia at $t = 60$ ($P = 0.003$) (Table 3 and Fig. 1A). In marked contrast, antecedent hindbrain glucoprivation had no effect on glucagon secretion during day 2 of hypoglycemia (Table 3 and Fig. 1A).

There was a significant treatment effect on epinephrine secretion during day 2 of insulin-induced hypoglycemia (treatment effect: $F_{2,35} = 5.7, P = 0.0027$; time × treatment interaction: $F_{6,70} = 3.6, P = 0.0036$) (Table 3 and Fig. 1B). Compared with the SH group, RH- and 3v 5TG-Hyp0–treated rats exhibited a significantly reduced epinephrine response 60 min after insulin injection ($P = 0.0001$ and $P = 0.001$, respectively). In contrast, plasma epinephrine levels in HB 5TG-Hypo rats were not different from epinephrine values in the SH group ($P = 0.91$ and $P = 0.478$, respectively). There was also a significant treatment effect on the norepinephrine response (treatment effect: $F_{2,34} = 4.15, P = 0.0132$; time × treatment interaction $F_{6,68} = 2.74, P = 0.0191$). Norepinephrine secretion was reduced in response to hypoglycemia at $t = 60$ in both RH and 3v 5TG-Hyp0 groups ($P = 0.014$ and $P = 0.009$, respectively) but not in the HB 5TG-Hypo group (Table 3 and Fig. 1C).

All treatment groups exhibited significant increases in plasma ACTH in response to insulin-induced hypoglycemia (main effect of time: $F_{5,58} = 86.9, P = 0.0001$) (Table 3). The increases in plasma ACTH levels did not differ among the treatment groups (no interaction between time and treatment: $F_{5,58} = 6.007, P = 0.7235$). Plasma corticosterone levels also increased in all experimental groups across the 120-min hypoglycemia session (main effect of time: $F_{5,58} = 86.7, P = 0.0001$), with no treatment effect ($F_{2,42} = 2.094, P = 0.1147$; no time × treatment interaction: $F_{5,68} = 6.007, P = 0.7235$).

**Control injections do not blunt counterregulatory responses.** Data for hindbrain and third ventricular vehicle control injections are presented in Table 4. As is evident, vehicle injection into the hindbrain or third ventricle on day 1 did not blunt hormonal CRRs as compared with control SH-treated rats (for HB time × treatment: glucagon, $F_{2,42} = 1.491, P = 0.2368$; epinephrine, $F_{2,32} = 0.750, P = 0.4803$; norepinephrine, $F_{2,34} = 2.099, P = 0.1382$; ACTH, $F_{2,30} = 0.951, P = 0.3976$; corticosterone, $F_{2,32} = 4.667, P = 0.167$; for third ventricle time × treatment: glucagon, $F_{2,42} = 1.7, P = 0.1962$; epinephrine, $F_{2,32} = 2.072, P = 0.1425$; norepinephrine, $F_{2,34} = 1.951, P = 0.1577$; ACTH, $F_{2,30} = 1.664, P = 0.2063$; corticosterone, $F_{2,38} = 0.340, P = 0.7137$).

**DISCUSSION**

This study evaluates the potential contribution of two distinct brain glucose sensing regions to the development of defective hypoglycemia counterregulation. Our findings demonstrate that a hindbrain glucoreceptive site makes little, if any, contribution to impairments in hormonal CRRs that occur following recurrent bouts of hypoglycemia. Thus, we find that antecedent delivery of 5TG into a hindbrain glucose sensing site does not impair the hormonal CRRs to subsequent hypoglycemia. In contrast, antecedent delivery of 5TG into the third ventricle does reproduce the impairments in hormonal CRRs observed in recurrent hypoglycemic rats.

It has long been known that glucoreceptive sites exist within the hindbrain, and it has been suggested that glucoreceptors exist only in the hindbrain (15,16). For example, acute cerebral aqueduct blockade was shown to abolish hyperglycemic and feeding responses to lateral but not fourth ventricular injection of 5TG (15), demonstrating that 5TG must be transported to caudal hindbrain glucoreceptive sites to initiate these responses. More recently, cannula mapping studies have identified the hindbrain sites from which stimulation of food intake and hyperglycemia could be elicited by local glucoprivation (17). In that study, multiple hindbrain sites were identified as glucoreceptive. However, unilateral injection of 5TG into 61 different hypothalamic sites only revealed 1 site that was positive for stimulation of feeding (17). In the present study, we evaluated the role of the caudal dorsomedial hindbrain glucoreceptive site because delivery of 5TG into this site was shown to elicit the largest feeding and sympathoadrenal response of all the hindbrain sites evaluated (17).

Despite the ability of the caudal hindbrain site to detect glucoprivation and elicit CRRs, we find that a single antecedent bout of 5TG-induced glucoprivation localized to this site does not by itself produce defective hormonal CRRs to a subsequent bout of systemic insulin-induced hypoglycemia. In fact, we find that antecedent glucoprivic stimulation of this hindbrain site resulted in hormonal...
responses to subsequent hypoglycemia that closely matched the magnitude of hormonal responses elicited by a single bout of hypoglycemia. In marked contrast to antecedent hindbrain glucoprivation, antecedent delivery of 5TG into the third ventricle did impair hormonal CRRs to a subsequent bout of hypoglycemia, which mimicked the effects of recurrent systemic hypoglycemia on hormonal CRRs. Similarly, Marin-Spiotta et al. (28) reported that 2-deoxy-D-glucose (2DG) delivered into the third ventricle also attenuated the epinephrine response to a subsequent bout of hypoglycemia. However, in that study there was no deficit in the glucagon response as we found here for third ventricular 5TG. This discrepancy may be due to the greater potency of 5TG in producing glucoprivation compared with 2DG (29).

Although third ventricular delivery of 5TG would be expected to reach the hindbrain, we believe our findings were due to the actions of 5TG within the third ventricle and surrounding hypothalamic nuclei for the following reasons. First, and most importantly, direct glucoprivic stimulation of the caudal hindbrain glucose sensing site did not impair CRRs during subsequent hypoglycemia. Second, 5TG, like glucose, is actively transported into neurons and glia. Thus, it does not seem likely that an adequate concentration of 5TG would remain in the cerebrospinal fluid once it reached the hindbrain to elicit a CRR. Finally, the majority of cerebrospinal fluid exits the ventricular system via the lateral apertures, located at the pontomedullary junction (30), a site rostral to this hindbrain glucoreceptive site, which is located deep within the ventral medulla (17).

The fact that third ventricular glucoprivation blunted CRRs to subsequent hypoglycemia reinforces the idea that hypothalamic sites are critical not only for the expression of CRRs to single hypoglycemia, but also for the blunting of CRRs with recurrent hypoglycemia. For example, bilateral dialysis of 2DG into the ventromedial hypothalamus (VMH), including the ventromedial hypothalamic nucleus (VMN) and arcuate nucleus, stimulates a robust hormonal CRR (8). Conversely, VMH lesions (7) or bilateral VMH glucose perfusion during systemic hypoglycemia (9) effectively block the hormonal CRRs. In addition, glucose sensing neurons exist in the VMN (14), arcuate (31), and lateral hypothalamus (6). These specialized neurons alter their firing rate in response to physiological changes in glucose availability (14) and are hypothesized to initiate CRRs (32). The VMH is also an important brain site in the HAAF pathway: Recurrent hypoglycemia impairs the activation of CRRs induced by bilateral VMH glucoprivation (10). Further, it was recently demonstrated that recurrent hypoglycemia reduces the glucose sensitivity of glucose sensing neurons residing in the VMN (33), a potential mechanism contributing to the development of defective CRRs. Taken together with our current findings, these studies suggest that altered function of neurons surrounding the third ventricle, particularly in the VMH, are primarily responsible for much of the reduced CRRs to hypoglycemia.

Whether or not antecedent third ventricular glucoprivation specifically impaired hypothalamic glucose sensing mechanisms cannot be determined from our findings. Although impairments in afferent mechanisms are possible, it is also possible that impairments in efferent responses contributed to defective CRRs. Indeed, this has been suggested by previous studies. For example, local administration of lidocaine into key hypothalamic auto-
glucose sensing mechanisms may provide insight into why this glucose sensing site remains responsive under recurrent glucoprivic conditions and why other hypothalamic sites do not.

In summary, we find that hindbrain and hypothalamic glucose sensing sites do not contribute equally to the development of HAAF. Prior glucoprivic stimulation of a hindbrain glucoreceptive site did not impair subsequent hypoglycemic CRRs. Thus, this hindbrain glucoreceptive site may not play a role in the development of HAAF. In marked contrast, antecedent third ventricular glucopriva-

tion impaired CRRs to a subsequent bout of hypoglycemia, thus reproducing the effects of recurrent systemic hypo-
glycemia. These findings demonstrate that hindbrain glu-
cose sensing sites are not subject to the pathogenic mechanisms that result in defective hypoglycemia CRRs, whereas those surrounding the third ventricle are.

ACKNOWLEDGMENTS
These studies were supported by the Research Services of the VA, National Institutes of Health grants (DK 40963 to D.P.F. and DK 50154 to G.J.T.), the American Diabetes Association (Junior Faculty Award to N.M.S.).

We gratefully acknowledge the technical expertise of Aryana Zavosh, Pam Gronbeck, Carl Sikkema, and the Metabolism Lab for excellent technical support with as-
says and surgical preparations.

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