Prior Hypoglycemia Enhances Glucose Responsiveness in Some Ventromedial Hypothalamic Glucosensing Neurons

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ABSTRACT

Antecedent insulin-induced hypoglycemia (IIH) reduces adrenomedullary responses (AMR) to subsequent bouts of hypoglycemia. The ventromedial hypothalamus (VMH: arcuate [ARC] + ventromedial nuclei [VMN]) contains glucosensing neurons which are thought to be mediators of this AMR. Since type 1 diabetes mellitus often begins in childhood, we used juvenile (4-5 wk old) rats and demonstrated that a single bout of IIH (5 U/kg, s.c.) reduced plasma glucose level by 24% and peak epinephrine by 59% 1 d later. This dampened AMR was associated with 46% higher mRNA for VMH glucokinase (GK), a key mediator of neuronal glucosensing. Compared to neurons from saline-injected rats, VMN glucose excited (GE) neurons from insulin-injected rats demonstrated a left-shift in their glucose responsiveness (EC$_{50}$ saline- 0.45; insulin- 0.10 mmol/l; P=0.05) and a 31% higher maximal activation by glucose (P=0.05), although this maximum occurred at a higher glucose concentration (saline- 0.7; insulin- 1.5 mmol/l). While EC$_{50}$ values did not differ, ARC GE neurons had 19% higher maximal activation which occurred at a lower glucose concentration (saline- 2.5; insulin- 1.5 mmol/l) than those from saline-injected rats. In addition, ARC glucose inhibited neurons from insulin-injected rats were maximally inhibited at a five-fold lower glucose concentration (saline- 2.5; insulin- 0.5 mmol/l), although this inhibition declined above 0.5 mmol/l glucose. These data suggest that the increased VMH GK seen following antecedent IIH may contribute to the increased responsiveness of VMH glucosensing neurons to glucose and contribute to the associated blunting of the AMR.
Recurrent bouts of insulin-induced hypoglycemia (IIH) are common in patients with type 1 diabetes mellitus, especially in children (2, 4, 23, 43). Such recurrent bouts lead to severe dampening of the hormonal counterregulatory and adrenomedullary responses (AMR) to subsequent bouts of hypoglycemia, a component of the clinical syndrome known as hypoglycemia-associated autonomic failure (3, 13, 53). In non-diabetic adult rats we showed that a single bout of hypoglycemia produces impaired AMR in association with an upregulation of glucokinase (GK) mRNA expression in the ventromedial hypothalamus (VMH) (15, 53). This hypothalamic area, which includes the arcuate (ARC) and ventromedial nuclei (VMN), contains glucosensing neurons which we postulate to be critical elements in the detection of and response to hypoglycemia (8, 22, 25, 38, 48, 60). Unlike most neurons in the brain which utilize glucose to fuel their metabolic needs (47), glucosensing neurons utilize glucose as a signaling molecule to alter their membrane potential and firing rate (15, 22, 48, 60). Glucose-excited (GE) neurons increase, whereas glucose-inhibited (GI) neurons decrease their firing rate as ambient glucose levels rise (15, 22, 28, 38, 48). Conversely, during situations of low glucose availability, GI neurons are activated and GE neurons are inactivated.

There is good evidence that neurons within the VMH play a critical role in the initiation of the AMR to hypoglycemia and the development of a dampened AMR which occurs following antecedent IIH (6, 8, 9, 15, 33, 34, 53). A previous study in 2-3wk old rat pups showed that the development of a dampened AMR after 3 bouts of IIH was associated with a reduced responsiveness of VMN GI neurons to glucose (49). However, little is known about the actual mechanisms underlying such alterations in neuronal
glucosensing and whether they occur in VMN GE neurons or other neurons outside the VMN. Our work (15, 21, 22), as well as that of others (60), strongly supports a role for GK as a key regulator of glucosensing in some neurons as it is in pancreatic β-cell glucosensing (19, 31). Because a blunted AMR and increased VMH GK mRNA expression occur simultaneously at 48h, but not 24 h after a single bout of IIH in adult, non-diabetic rats (15, 53), we postulated that increased GK activity in hypothalamic glucosensing neurons might make them more responsive to low glucose levels and underlie the reduced AMR to subsequent bouts of IIH.

Since type I diabetes mellitus is common in children and tight regulation of blood glucose levels in these children is associated with a reduced AMR to IIH (2, 4, 20), we developed a model of an attenuated AMR following recurrent IIH in juvenile rats to investigate potential mechanisms underlying this phenomenon. We used 4-5 wk old rats to assess the timing of the occurrence of the blunted AMR, as well as changes in VMH expression of GK mRNA and the responsiveness of VMN and ARC glucosensing neurons to glucose which might accompany the blunted physiologic responses. In addition, since previous studies demonstrated the importance of AMP-activated protein kinase (AMPK) in the AMR to hypoglycemia (33, 34) and glucosensing in GI (10) and GE neurons (12), we also examined the mRNA expression of the AMPKα1 and AMPKα2 subunits of this enzyme in the VMH after a single bout of hypoglycemia. We predicted that prior hypoglycemia in juvenile rats would blunt the AMR in association with increased GK mRNA and enzyme activity which would lead to increased glucose responsiveness of VMH neurons.
RESEARCH DESIGN AND METHODS

Animals and husbandry

All work was carried out under a protocol approved by the Institutional Animal Care and Use Committees of the New Jersey Medical School and the E. Orange VA Medical Center and in accordance with the “Guiding Principles for Research Involving Animals and Human Beings” of the American Physiological Society (1). Male outbred 4-5 wk old (140-150 g) Sprague-Dawley rats (Charles River Labs) were used in all studies. They were housed on a 12 h light/dark schedule (lights on at 0700) and kept at 22–23°C. Food (Purina rat chow no. 5001) and water were available ad libitum except as noted below.

Assessment of the effect of a single episode of IIH on AMR to subsequent hypoglycemia

At 4 wk of age, rats (n=8) were surgically implanted with jugular venous catheters and were maintained and handled daily postoperatively until they regained preoperative weight (approximately 1 wk) as described previously (54). During this time, they were habituated to the experimental conditions in the procedure room. On the day of testing, food was withdrawn at 0800 and an initial blood sample (0.2ml) was collected to measure baseline (-15 min) levels of plasma glucose and epinephrine. An equal volume of donor blood collected from unstressed rats was infused after each blood collection to maintain hemodynamics. Next, rats were subcutaneously administered regular human insulin (Eli
Lilly, Indianapolis, USA) 5 U/kg in 0.5 ml saline. Blood samples (0.2 ml) for glucose were drawn at 30, 60, 90 and 120 min after insulin injection. After the 120 min blood sample was drawn, food was returned to all rats. Twenty-four hours after the initial hypoglycemic episode, all rats were subjected to a second bout of hypoglycemia using the same protocol as that used during the first bout.

Plasma for the catecholamine assays was vortexed with 5 N perchloric acid and then immediately frozen on dry ice and held at -70°C for further processing. After all rats completed the protocol, the samples were briefly thawed and centrifuged, and 100 µl of supernatant was mixed with the internal standard dihydroxybenzylamine. Catecholamines were assayed using HPLC with electrochemical detection as previously described (53). Plasma glucose was measured using a glucose analyzer (Analox Instruments, Lunenburg, MA, USA).

Assessment of changes in VMH GK mRNA expression in response to a single bout of hypoglycemia

Additional groups of rats were injected subcutaneously with insulin (n=6) as described above or with 0.5 ml saline (n=6). The insulin injections decreased tail blood glucose levels to 30-35 mg/dl over 120 min. After 24 h, animals were killed, their brains removed and coronal hypothalamic sections (350 µm) through the midpoint of the ARC and VMN from insulin- (n=6) or saline-injected (n=6) rats were cut on a vibratome and then immediately transferred to RNAlater® solution (Ambion, Austin, TX) to stabilize RNA within tissues. The VMH (ARC plus VMN) was hand dissected using a scalpel under a surgical microscope to cut a triangular wedge with the base of the triangle at the
ventral surface of the hypothalamus. The lateral border was the sulcus marking the lateral border of the ventral hypothalamic surface and the apex of the triangle was the midpoint of the third ventricle. We have confirmed that these dissection planes include both the ARC and VMN using cresyl violet stained sections in preliminary studies. RNA was extracted using RNeasy Protect Mini Kit (QIAGEN, Valencia, CA) and reverse transcribed with superscript III with oligo-dT priming and treated with RNase H (Invitrogen, Carlsbad, CA) (21, 27). The resultant cDNA was analyzed by real-time quantitative PCR on a Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA) using primer/probe and FAM-labeled probe sets targeting pancreatic GK (GenBank accession No. NM012565), cyclophilin (GenBank accession No. M19533), hexokinase I (GenBank accession No. NM012734) and the catalytic subunits of AMPKα1 (GenBank accession No NM019142) and AMPKα2 (GenBank accession No NM023991.1) as described previously (18, 21) (Table 1). Samples were compared against serially diluted reference standards prepared from pooled aliquots of cDNA from each sample. Data were expressed as the ratio of the standardized amount of the gene of interest to the standardized amount of cyclophilin which is constitutively expressed and unaltered by hypoglycemia (data not shown).

Assessment of changes in glucose concentration–dependent responses of VMH neurons following a single bout of hypoglycemia

Rats were injected subcutaneously with insulin (n=30) or saline (n=30) as described above. Glucose levels were measured at 120 min after insulin or saline injection in tail blood and fell to 30-35 mg/dl in all insulin-injected animals. Twenty-four
hours later, rats were decapitated and their brains were rapidly removed. After sectioning on a vibratome, 2 to 3, 350 µm sections through the VMH (-2.5 to -3.1 relative to bregma) were placed into a silicone coated Petri dish. Using a dissecting microscope, VMN (n=15 per treatment group) or ARC nuclei (n=15 per treatment group) were punched with a 500-µm blunt needle. The VMN was punched bilaterally while, for the ARC, a single punch centered on the midline of the lower 1/3 of the 3rd ventricular overlying the median eminence was made to include both nuclei. The resultant tissue punches were papain digested (2 mg/ml, 30 min, 37°C) and mechanically triturred. Dissociated cells were plated onto coverslips and allowed to adhere for 60 min before recording intracellular calcium ([Ca\(^{2+}\)]\(_i\)) imaging as described previously (15, 21, 22). Cells were loaded with the Ca\(^{2+}\) fluorophore fura-2 acetoxymethyl ester for 20 min in 2.5 mmol/l glucose Hanks’ balanced salt solution buffer (135 mmol/l NaCl, 5 mmol/l KCl, 1 mmol/l CaCl\(_2\), 1 mmol/l MgCl\(_2\), and 10 mmol/l HEPES, pH 7.4) at 37°C. Fura-2 fluorescence images were acquired every 5 s by alternating excitation at 340 and 380 nm, and emissions (420–600 nm) were collected using a cooled, charge-coupled device camera (15, 22).

Dissociated VMN and ARC neurons were first classified by their [Ca\(^{2+}\)]\(_i\) changes in response to a decrease in extracellular glucose (from 2.5 to 0.1 mmol/l) as GE if they had a >20% decrease, as GI if they had a >59% increase in area under the curve (AUC) for [Ca\(^{2+}\)]\(_i\) oscillations and as non-glucosensing (NG) if they did not meet either of these specific criteria which had been determined in our previous studies (22). Although our previous studies had been carried out between 0.3 and 2.5 mmol/l glucose, we used the lower level of 0.1 mmol/l glucose here to cover the increase in glucose responsiveness which we predicted would occur due to the increase in hypoglycemia-associated
elevations of GK expression. Then individual sets of neurons were exposed to various combinations of seven incremental changes (0.3, 0.5, 0.7, 1.0, 1.5, 2.0 and 2.5 mmol/l) of extracellular glucose concentrations. Because of the need to limit the duration of testing to avoid cell damage by the imaging laser, any given set of neurons was evaluated at only 2–3 of these increasing glucose concentrations. A minimum of 10 rats were used to assess neuronal responses for any set of 3 incremental glucose concentrations. Changes in [Ca\(^{2+}\)]\(_i\) oscillations from one condition to another were quantified as the area under curve (AUC) (22) and normalized by determining the percentage of responses to a given concentration of glucose relative to the initial response at the 2.5 mmol/l glucose level.

**Statistics**

Nadir plasma glucose and peak epinephrine levels, half-maximal effective concentration (EC\(_{50}\)), half-maximal inhibitory concentration (IC\(_{50}\)) and maximum activation and inhibition values for glucose concentration-response curves, VMH GK, AMPK\(\alpha1\) and \(\alpha2\) (expressed as a function of the constitutive gene, cyclophilin) were compared using an unpaired t test. Plasma glucose and epinephrine levels during IIH were also compared by one way repeated measures analysis of variance with post hoc Bonferroni test. For neuronal glucosensing, AUC of [Ca\(^{2+}\)]\(_i\) oscillations were calculated using Origin software (OriginLab) and GE and GI neurons were defined using criteria established in our prior studies (22). The EC\(_{50}\) and maximal activation values for GE neurons and the IC\(_{50}\) and maximal inhibition values for GI neurons were determined by nonlinear regression analysis (sigmoidal dose-response curve fit; GraphPad Prism). For statistical comparison of the maximal activation and inhibition values, data from each
RESULTS

A single bout of IIH impairs the AMR to subsequent hypoglycemia and alters VMH GK and AMPK mRNA expression

Insulin injections on day 1 caused blood glucose levels to decrease to a nadir of 34.5 ± 1.2 mg/dl and epinephrine levels to peak at 1802 ± 294 pg/ml (Figure 1). Following a second injection of insulin on day 2, glucose fell to a significantly lower nadir (26.3 ± 0.1 mg/dl; P=0.05) and peak epinephrine levels were significantly blunted (740 ± 81 pg/ml; P=0.05) compared to levels on day 1 (Figure 1). Also, repeated measures demonstrated that both glucose and epinephrine were lower from 30-120 min following the insulin injections on day 2 suggesting a significantly blunted AMR following repeated IIH.

In association with this blunted AMR, VMH GK mRNA was increased by 46% in insulin- compared to saline-injected rats 24 h after the first injections (Table 2). This was not a generalized or non-specific effect since hexokinase I and AMPKα1 mRNA’s expression were unchanged. Conversely, AMPKα2 expression was reduced by 18% in these same samples.

A single bout of hypoglycemia selectively increases the glucose responsiveness of some VMH glucosensing neurons
Here we identified ARC (and VMN; tracing not shown) GE neurons as those which decreased (Figure 2A) and GI neurons as those which increased (Figure 2B) their AUC for \([\text{Ca}^{2+}]_i\) fluctuations when glucose was decreased from 2.5 to 0.1 mmol/l. These responses were reversed in a concentration-dependent manner as glucose was raised in a stepwise fashion from 0.1 to 2.5 mmol/l (Figures 3 and 4). Overall, a total of 800–1442 neurons from 15 rats from each of the respective groups (VMN GE and GI, ARC GE and GI) were analyzed (Table 3). We previously demonstrated that VMN GE neurons from 4–5 wk old rats respond to such incremental changes in ambient glucose levels between 0.3 and 2.5 mmol/l with an EC$_{50}$ = 0.54 mmol/l, whereas GI neurons had an IC$_{50}$ = 1.12 mmol/l using calcium imaging (21). Compared with saline-injected rats, VMN GE neurons from insulin-injected rats showed significantly higher AUC of \([\text{Ca}^{2+}]_i\) fluctuations than in saline-injected rats at 0.1 (3103 ± 156 vs. 2756 ± 157), 0.5 (7282 ± 916 vs. 4900 ± 695), 0.7 (8681 ± 837 vs. 5617 ± 644), 1.5 (10104 ± 1260 vs. 5418 ± 976) and 2.0 mmol/l glucose (6664 ± 730 vs. 2158 ± 498), (P=0.05 or less) respectively. Thus, there was a left-shift of the glucose concentration-response curve for VMN GE neurons in insulin-injected (EC$_{50}$ = 0.10 mmol/l) as compared to saline-injected rats GE (EC$_{50}$ = 0.45 mmol/l; P=0.05; Figure 3A; Table 3). Also, the maximum activation above the initial 0.1 mmol/l glucose concentration was 31% higher in VMN GE neurons from insulin- than from saline-injected rats. On the other hand, the maximal activation occurred at a higher concentration (1.5 mmol/l) in neurons from insulin-treated rats than those from saline-treated rats (0.7 mmol/l glucose; Figure 3A; Table 3).

While there was a tendency for a left-shift in the responsiveness of ARC GE neurons from insulin-injected rats (EC$_{50}$ = 0.21 mmol/l), this difference did not reach
statistical significance compared to the responses of neurons from saline-injected rats
(EC$_{50}$ = 0.35 mmol/l; Figure 3B, Table 3). However, there was a significant 19% increase
in the level of maximal activation and this maximum occurred at 1.5 mmol/l glucose in
ARC GE neurons from insulin-injected rats vs. 2.5 mmol/l in saline-injected rats (Figure
3B; Table 3). This suggests that prior hypoglycemia did increase the glucose sensitivity
of ARC GE neurons. Also, we defined GE neurons as those having >20% decrease in
AUC of [Ca$^{2+}$]$\_i$ fluctuations when glucose was decreased from 2.5 to 0.1 mmol/l (22).
Thus, increased glucose sensitivity could result in fewer GE neurons from insulin-treated
rats being inhibited over this initial concentration range to explain why fewer ARC GE
neurons were identified from insulin- than from saline-treated rats (Table 3). However, if
this were the case, it cannot explain why we did not find fewer VMN GE neurons

By contrast to VMN GE neurons, VMN GI neurons from insulin-injected rats did
not differ significantly from those from saline-injected rats in their glucose
responsiveness as a function of their calculated IC$_{50}$ values or maximal inhibition by
glucose. In fact, maximal inhibition occurred at a slightly higher glucose levels in
neurons from insulin- (2.5 mmol/l) as opposed to saline-treated rats (2.0 mmol/l) (Figure
4A; Table 3). Of note, the calculated IC$_{50}$ values for both insulin- (0.12 mmol/l) and
saline-injected VMN GI neurons (0.11 mmol/l) assessed between 0.1 and 2.5 mmol/l
glucose here were an order of magnitude lower than those from untreated 4-5 wk old rats
tested previously using physiological glucose levels from 0.3 to 5 mmol/l glucose (IC$_{50}$=
1.12 mmol/l) (21). Also, while EC$_{50}$ values for VMN and ARC GE neurons from saline-
injected rats were comparable, the calculated IC$_{50}$ for ARC GI neurons (0.30 mmol/l) was
3-fold higher than that for VMN GI neurons (0.11 mmol/l) from saline-injected rats (Figures 4 A and B; Table 3).

The glucose concentration-response curves for ARC GI neurons from saline- vs. insulin-treated rats were more difficult to compare. While neurons from saline-injected rats followed the expected reduction in $[\text{Ca}^{2+}]_i$ AUC as glucose was raised from 0.1 to 2.5 mmol/l ($IC_{50} = 0.30$ mmol/l), ARC GI neurons from insulin-injected rats reached maximal inhibition at 0.5 mmol/l but, from 0.7 to 2.5 mmol/l, $[\text{Ca}^{2+}]_i$ AUC values were not inhibited with any predictable concentration-dependent relationship (Figure 4, Table 3). The $IC_{50}$ estimated for these neurons between 0.1 and 0.5 mmol/l was 0.18 mmol/l. While these data suggest that ARC GI neurons from insulin-injected rats were 2-5 fold more sensitive to inhibition by increasing glucose concentrations than those from saline-treated rats, the two sets of values could not be compared statistically because of the differing ranges of their responsiveness and the unexplained, partial loss of inhibition above 0.5 mmol/l glucose in the insulin-treated group.

**DISCUSSION**

These studies utilized a juvenile rat model that mimics the blunted AMR which occurs as a consequence of antecedent bouts of hypoglycemia in diabetic children undergoing intensive insulin therapy (2-4, 13, 23, 43). In this model, a single bout of IIH was followed 24 h later by a greater fall in glucose and a blunted epinephrine response to a second bout. We chose to assess the glucose responsiveness of VMN and ARC
glucosensing neurons since these two nuclei collectively compose the “VMH”, the hypothalamic area which is critical for the full expression of AMR to hypoglycemia (6, 8, 9, 55) and possibly for the blunted AMR which occurs after repeated bouts of IIH (33, 34). We found that the dampened AMR was associated with increased VMH GK and decreased AMPKα2 mRNA expression and increased responsiveness of some but not all VMH glucosensing neurons to glucose. Although no definitive causal link was established, these results suggest a possible sequence of events whereby antecedent IIH produces increases GK expression in VMH glucosensing neurons. This would increase their glucose responsiveness leading to a blunted AMR because they would not respond fully at plasma glucose levels which activate this response in animals undergoing their first bout of hypoglycemia.

The most robust evidence for increased glucose responsiveness was seen in VMN GE neurons from insulin-treated rats. These neurons had a four-fold reduction in the EC₅₀, and a 31% increase in their maximal glucose-induced activation, even though this maximum activation occurred at a higher glucose concentration than in saline-treated rats. In addition, although the EC₅₀ was not significantly altered in ARC GE neurons following antecedent hypoglycemia, maximal activation occurred at lower glucose levels and was 19% higher than were those in saline-treated rats. There was also a suggestion that ARC GI neurons might have become more sensitive to the inhibitory effects of glucose after prior hypoglycemia, but these data were more difficult to interpret. The fact that maximal inhibition occurred at a five-fold lower glucose concentration in ARC GI neurons from insulin- vs. saline-treated rats supports this contention. However, the maximal inhibition that occurred at 0.5 mmol/l was attenuated at higher glucose
concentrations. This loss of inhibition is unexplained but was quite reproducible. It occurred with repeated trials in neurons subjected to a range of various glucose concentrations from 0.1 to 2.5 mmol/l. Unfortunately, this curious response prevented direct comparison to the results from saline-treated rats. Therefore, our overall results support the finding the VMN GE, and possibly ARC GE neurons developed increased glucose responsiveness following a prior bout of hypoglycemia.

There are likely to be several different regulatory mechanisms utilized by neurons to sense glucose. Any of these might contribute to the altered neuronal responsiveness to glucose following recurrent hypoglycemia. As in the pancreatic β-cell (30), GK plays an important regulatory role in VMH neuronal glucosensing. For example, in the VMN, approximately 65% of GE and 45% of GI neurons express GK mRNA (22). Near-total inhibition of GK mRNA expression using RNA interference in primary VMH neuronal cultures almost completely abolishes glucosensing in both GE and GI neurons, while pharmacological inhibition of GK activity inhibits GE and activates GI VMN neurons at 2.5 mmol/l glucose. On the other hand, pharmacological activation of GK enzyme activity has the opposite effect at 0.5 mmol/l (15, 21, 22).

While such data implicate GK as a regulator of glucosensing in VMH neurons, it is likely that there are other mechanisms such as AMPK by which some neurons utilize glucose as a signaling molecule (10, 12), particularly during hypoglycemia (33, 34). The AMPKα2 isoform is particularly sensitive to alterations in brain glucose levels (37) and activation of AMPK is associated with an enhanced counterregulatory response to hypoglycemia (33, 34). Our finding of decreased expression of the AMPKα2 subunit in the VMH, in parallel with the development of a dampened AMR, suggests that these two
events might be causally linked. Yet, the fact that those VMH glucosensing neurons which did alter their glucose sensitivity following prior IIH, did so by increasing their responsiveness, makes it less likely that decreased AMPKα2 activity would underlie this change. On the other hand, adult rats have increased mRNA expression of both AMPKα1 and AMPKα2 and a dampened counterregulatory response after 3 d of recurrent bouts of IIH (33). However, these increases might represent a compensatory response occurring several days after the initial downregulation of counterregulatory responses to hypoglycemia which were seen here in juvenile rats after 24 h and in adult rats at 48h after a single bout of hypoglycemia (53). Thus, the increase in VMH GK mRNA associated with a possible parallel increase in GK activity is a likely explanation for the increased responsiveness of VMH glucosensing neurons to glucose after IIH.

An unexpected finding of the current studies was the leftward shift in responsiveness of VMN and ARC GI neurons isolated from saline-injected control rats as compared to our previous studies (21, 48, 59). One potential reason for this is that the original studies were carried out at glucose levels between 0.3 or 0.5 mmol/l to 2.5 mmol/l glucose. In fact, 0.1 mmol/l glucose to which neurons were exposed here is far below levels seen during even the most severe hypoglycemia (14). Thus, exposure of isolated GI neurons to such low glucose levels may have acutely altered their underlying glucosensing capacity and subsequent responses to raising glucose levels again. It is possible that GI neurons were selectively affected by such exposure because they normally respond to glucose at levels almost twice as high (IC₅₀ = 1.12 mmol/l) as those at which GE neurons respond (EC₅₀ = 0.54 mmol/l), at least in the VMN (21). Although, patch-clamped neurons in VMN slices from 2-3 wk old rats showed no such effect of
lowering glucose levels to 0.1 mmol/l glucose (49), those neurons could utilize lactate from adjacent astrocytes to fuel their metabolic needs (52), whereas neurons in our studies were isolated from any astrocytes. In addition, we utilized \([Ca^{2+}]_i\) fluctuations as our index of changes in glucose responsiveness. Although we previously demonstrated that such fluctuations correlate well with glucose-induced changes in membrane potential (21), it is still possible that this association breaks down after exposure of isolated neurons to very low levels of glucose. Altered neuronal function and/or a dissociation between \([Ca^{2+}]_i\) and membrane potential fluctuations to glucose might result from disordered mitochondrial electron transport chain function during hypoglycemia due to the decreased availability of reducing equivalents. This would increase mitochondrial production of free radicals and lead to mitochondrial oxidant injury (24).

The current studies also differ in other respects from the only other one in which VMH glucosensing neurons were examined after recurrent bouts of IIH (49). That study demonstrated that VMN GI neurons from recurrently hypoglycemic rats failed to respond at 0.5 mmol/l glucose but did respond at 0.1 mmol/l suggesting that recurrent hypoglycemia had made them less, rather than more responsive to glucose (49). However, there are several differences between these two studies. First, the previous study was carried out in 14-21d old pups subjected to 3 rather than 1 bout of antecedent IIH where glucose levels ranged from 1.4-2.3 mmol/l blood glucose. Also, as above, glucosensing neurons were assessed using patch-clamp technique in VMN slices which presumably had astrocytic glycogen available as a temporary buffer against severe hypoglycemia (49, 52). In addition, 14-21d is the age at which there is a transition from utilizing ketone bodies derived from maternal milk to glucose derived from ingested food as the primary
source of cerebral energy supply. This transition is paralleled by a switch from a heavy reliance on the transport of ketone bodies into the brain and neurons by monocarboxylate transporters to transport of glucose by glucose transporters (40, 56, 57). Thus, differences between the previous (49) and current results are not surprising given the differences in the age, degree of hypoglycemia, source of energy for neuronal metabolism and mode of testing.

One important caveat of the current studies is that we measured only GK and AMPK mRNA levels and did not assess the activity of either enzyme. This is particularly important with regard to GK for which we have been unable to find an experimentally reproducible activity assay. This is likely due to the extremely low abundance of GK mRNA expression in the VMH and individual VMH neurons (15, 22). In fact, these levels are so low that it has not been possible to reliably demonstrate the presence of GK protein in the VMH, as opposed to the hindbrain where GK immunoreactive neurons and GK mRNA have both been demonstrated (26, 29). Thus, we have not been able to establish a conclusive link between increased GK mRNA expression and a parallel increase in GK activity in our studies.

In conclusion, our findings indicate that a single bout of IHH in juvenile rats leads to a blunted AMR in association with increased VMH GK mRNA expression and increased glucose responsiveness of a subpopulation of VMH glucosensing neurons. The hypothetical model derived from these studies is that hypoglycemia induces some VMH glucosensing neurons to produce more GK mRNA, leading to increased GK activity. This predicts that these neurons would not respond until glucose levels fell to lower levels and/or would not be as strongly activated at comparable glucose levels as controls.
subjected to their first bout of IIH. These left and upward shifts in their responsiveness could contribute to the dampening of the AMR seen under such circumstances. However, it is also likely that there are many different types of VMH (and other brain area) glucosensing neurons which utilize additional regulatory mechanisms for neuronal glucosensing which might contribute to the blunting of AMR following antecedent hypoglycemia. Our current and previous data further demonstrate that hypoglycemia affects neurons in both the ARC and VMN (53), each of which has their own spectrum of neurotransmitters, neuropeptides and neuronal projections. The important implication of such results is that therapies aimed at preventing the development of hypoglycemia-associated autonomic failure or increasing the counterregulatory response to IIH in type 1 diabetic patients may have to take into account the heterogeneous characteristics of the neuronal populations in the critical hypothalamic area.
PERSPECTIVES AND SIGNIFICANCE

A comprehensive series of studies from investigators at Yale Medical School dating back to 1994 have established the VMH as a critical site for the control of the counterregulatory responses to IIH (6-9, 11, 16, 32-35). Although components of these responses can be elicited by glucoprivic stimuli applied to a variety of hindbrain sites (42), such responses do not occur without an intact VMH (44, 45). While we focused here on the alterations in glucose responsiveness of isolated VMH glucosensing neurons caused by antecedent hypoglycemia, the picture is obviously much more complicated. First, the function of these neurons cannot be separated from their critical symbiotic relationship with astrocytes which provide them with metabolic substrate and which, in turn, are provided with energy sources such as glutamate from neurons (39, 52). Second, glucosensing neurons in the VMH are quite heterogeneous with regard to the mechanisms that they utilize to sense glucose, their transmitter and peptide phenotypes and the myriad metabolic, neurohumoral and presynaptic inputs that impinge upon them (17, 22, 46, 48). Transmitters such as norepinephrine (5, 41), GABA (5, 11) and glutamate (55) and neuropeptides such as neuropeptide Y (59), CRF and urocortin (35) act as important modulators of neuronal activity in the VMH, the counterregulatory responses to hypoglycemia and the dampening of these responses that follows even a single bout of hypoglycemia. In addition, glucosensing neurons respond directly to a variety of hormones such as insulin (22, 51, 59), leptin (28, 50) and the availability of alternate fuels such as lactate (49) and fatty acids (36, 58). Thus, while we demonstrate here that antecedent hypoglycemia directly alters the glucose responsiveness of VMH glucosensing neurons, it also affects many of these other critical inputs to these neurons.
Whereas many of these changes represent physiological adaptations to prior hypoglycemia, the end result of dampened counterregulatory responses can be life threatening. Our challenge is to construct an integrated model of how the brain controls the counterregulatory responses to hypoglycemia with the aim of developing medical therapies to prevent the serious consequences of recurrent hypoglycemia in the diabetic patient.
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<td><strong>AMPKα1</strong></td>
<td>AAGATACTCAACCGGCAGAAGATTC</td>
<td>AAGATACTCAACCGGCAGAAGATTC</td>
</tr>
<tr>
<td><strong>AMPKα2</strong></td>
<td>CGGCAAAAGTGAAGATTTGGAGAAC</td>
<td>AACATCTAAAACGGTGAATCTTCTCTGTCT</td>
</tr>
<tr>
<td><strong>Cyclophilin</strong></td>
<td>AATGGCACTGGTGGCAAGTC</td>
<td>GCCAGGACCTGTATGCTTCAG</td>
</tr>
<tr>
<td><strong>Glucokinase</strong></td>
<td>CGAGGAGGCCAGTGAAAGATG</td>
<td>TCTCCGACTTCTGAGCCTCTCTG</td>
</tr>
<tr>
<td><strong>Hexokinase I</strong></td>
<td>CAGCCTCCGTCAAGATGC</td>
<td>CGAGATCCAGGGCAATGAAATC</td>
</tr>
</tbody>
</table>

Table 1: Primers for quantitative mRNA determinations
<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>Insulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexokinase I mRNA</td>
<td>1.86±0.09</td>
<td>1.73±0.05</td>
</tr>
<tr>
<td>Glucokinase mRNA</td>
<td>2.38±0.03</td>
<td>3.47±0.16 *</td>
</tr>
<tr>
<td>AMPKα1</td>
<td>0.60±0.03</td>
<td>0.60±0.02</td>
</tr>
<tr>
<td>AMPKα2</td>
<td>1.19±0.03</td>
<td>0.97±0.03 *</td>
</tr>
</tbody>
</table>

**Table 2:** VMH hexokinase, glucokinase, AMPKα1 and α2 mRNA expression (as a function of cyclophilin expression) 24 h after a single injection of saline or insulin (5 U/kg) which produced hypoglycemia. Data (mean ± SEM) are expressed as a function of cyclophilin mRNA values from the same micropunch samples. * P=0.05 when saline- and insulin-injected values were compared by un-paired t test.
<table>
<thead>
<tr>
<th>Type</th>
<th>Treatment</th>
<th>% of Total</th>
<th>EC$<em>{50}$/IC$</em>{50}$ (mmol/l)</th>
<th>Max activation/Inhibition (% of 0.1 mmol/l baseline)</th>
<th>Max activation/inhibition glucose (mmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VMN GE</td>
<td>Saline</td>
<td>13</td>
<td>0.45</td>
<td>74 ± 5</td>
<td>1.5</td>
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<tr>
<td></td>
<td>Insulin</td>
<td>15</td>
<td>0.10*</td>
<td>97 ± 5 *</td>
<td>0.7</td>
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<tr>
<td>ARC GE</td>
<td>Saline</td>
<td>20</td>
<td>0.35</td>
<td>100 ± 2</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>Insulin</td>
<td>8</td>
<td>0.21</td>
<td>119 ± 3 *</td>
<td>2.5</td>
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<tr>
<td>VMN GI</td>
<td>Saline</td>
<td>23</td>
<td>0.11</td>
<td>60 ± 6</td>
<td>2.0</td>
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<tr>
<td></td>
<td>Insulin</td>
<td>22</td>
<td>0.12</td>
<td>48 ± 5</td>
<td>2.5</td>
</tr>
<tr>
<td>ARC GI</td>
<td>Saline</td>
<td>12</td>
<td>0.30</td>
<td>59 ± 5</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>Insulin</td>
<td>13</td>
<td>0.18 *</td>
<td>54 ± 3</td>
<td>2.5</td>
</tr>
</tbody>
</table>

**Table 3:** Using a total of 3786 neurons from saline- or insulin-treated rats (VMN: 642 saline, 800 insulin; ARC 1442 saline, 902 insulin), neurons were classified as glucose excited (GE) or inhibited (GI) by their changes in [Ca$^{2+}$]$_i$ AUC when glucose was lowered from to 2.5 vs. 0.1 mmol/l glucose. They were then exposed to increasing concentrations of glucose from 0.1-2.5 mmol/l to calculate EC$_{50}$ and maximal (Max) activation (GE neurons) and IC$_{50}$ and maximal inhibition (GI neurons) values as described in Methods. % of Total = % of GE or GI neurons found in each area per treatment group. Data are mean ± SEM. *P=0.05 or less when data from insulin-treated rats were compared to those from saline-treated rats for each set of neurons from VMN or ARC. * Estimated IC$_{50}$ based on an incomplete data between 0.1 to 0.5 mmol/l glucose.
Figure 1: Blood glucose levels fell to lower levels (A) and plasma epinephrine (B) levels were significantly blunted to insulin induced hypoglycemia (5 U/kg, sc) on day 2 in 4-5 wk old rats (n=8) at 24 h after an initial bout on day 1 (* P<0.05).

Figure 2: Examples of glucose excited (GE) and glucose inhibited (GI) neurons dissociated from the arcuate nucleus of 4-5 wk old rats. The GE neuron (A) reduced its [Ca^{2+}]_i AUC when glucose was decreased from 2.5 (2.5G) to 0.1 (0.1G) mmol/l and then the AUC increased when glucose was raised incrementally to 0.7 (0.7G) and then 2.5 mmol/l. The GI neuron (B) increased its [Ca^{2+}]_i AUC when glucose was decreased from 2.5 to 0.1 mmol/l and decreased in a concentration-dependent manner as glucose was raised incrementally to 0.7 and then 2.5 mmol/l. Downward arrow heads denote change in glucose concentrations.

Figure 3: Glucose concentration-response curves for GE neurons from saline- vs. insulin-injected 4-5 wk old rats. Rats were injected with saline or insulin (5 U/kg, s.c.) and then sacrificed 24 h later. Dissociated VMN (A) and ARC (B) neurons were held in 2.5 mmol/l glucose and levels were decreased to 0.1 mmol/l glucose. Then sets of neurons had glucose concentrations increased in sets of 3 concentration increments between 0.3 and 2.5 mmol/l. Responsiveness was assessed by comparing differences in AUC of [Ca^{2+}]_i for individual neurons from insulin- (circles) vs. saline-injected (triangles) rats. Neurons in this figure were defined as GE by decreased [Ca^{2+}]_i AUC when glucose levels
were decreased from 2.5 to 0.1 mmol/l and then AUC increased with increasing concentrations from 0.1 to 2.5 mmol/l.

Figure 4: Glucose concentration-response curves for VMN (A) and ARC (B) GI neurons from insulin- vs. saline-injected rats. Conditions and data points are expressed as in Figure 2 except that IC₅₀ value for VMN GI neurons was calculated for inhibition of [Ca²⁺]ᵢ AUC as glucose concentrations were increased from 0.1 to 2.5 mmol/l while the IC₅₀ for ARC GI neurons was calculated only from 0.1 to 0.5 mmol/l glucose.
REFERENCES


lower brain stem: for possible location of brain glucose-sensing mechanisms.


Figure 1

A

- Day 1 Hypoglycemia
- Day 2 Hypoglycemia

Glucose (mg/dl)

Minutes

B

Epinephrine (pg/ml)

Minutes

* * *
Figure 3

A  
- Saline, EC$_{50}$=0.45 mmol/l, $R^2$=0.88
- Insulin, EC$_{50}$=0.10 mmol/l, $R^2$=0.89

B  
- Saline, EC$_{50}$=0.35 mmol/l, $R^2$=0.89
- Insulin, EC$_{50}$=0.21 mmol/l, $R^2$=0.88
Figure 4