

Key Role for AMP-Activated Protein Kinase in the Ventromedial Hypothalamus in Regulating Counterregulatory Hormone Responses to Acute Hypoglycemia

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OBJECTIVE—To examine in vivo in a rodent model the potential role of AMP-activated protein kinase (AMPK) within the ventromedial hypothalamus (VMH) in glucose sensing during hypoglycemia.

RESEARCH DESIGN AND METHODS—Using gene silencing technology to selectively downregulate AMPK in the VMH, a key hypothalamic glucose-sensing region, we demonstrate a key role for AMPK in the detection of hypoglycemia. In vivo hyperinsulinemic-hypoglycemic (50 mg dl⁻¹) clamp studies were performed in awake, chronically catheterized Sprague-Dawley rats that had been microinjected bilaterally to the VMH with an adeno-associated viral (AAV) vector expressing a short hairpin RNA for AMPK α .

RESULTS—In comparison with control studies, VMH AMPK downregulation resulted in suppressed glucagon (~60%) and epinephrine (~40%) responses to acute hypoglycemia. Rats with VMH AMPK downregulation also required more exogenous glucose to maintain the hypoglycemia plateau and showed significant reductions in endogenous glucose production and whole-body glucose uptake.

CONCLUSIONS—We conclude that AMPK in the VMH plays a key role in the detection of acute hypoglycemia and initiation of the glucose counterregulatory response. *Diabetes* 57:444–450, 2008

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ACTH, adrenocorticotrophin hormone; AAV vector, adeno-associated viral vector; AICAR, 5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside; AMPK, AMP-activated protein kinase; Arc, Arcuate nucleus; CAG, chicken β -actin promoter; CRH, corticotrophin-releasing hormone; CRR, counterregulatory response; DMH, dorsomedial hypothalamus; GFP, green fluorescent protein; GIR, glucose infusion rate; NIDDK, National Institute of Diabetes and Digestive and Kidney Diseases; PBT, 0.04% Triton X-100 in PBS; PVN, paraventricular nucleus; R_a , rate of endogenous glucose production; R_u , rate of whole-body glucose uptake; VMH, ventromedial hypothalamus; VMN, ventromedial nucleus; WPRE, woodchuck posttranscriptional regulatory element.

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Insulinogenic hypoglycemia remains one of the most serious complications of insulin therapy and is the major limiting factor to optimal glycemic management in type 1 diabetes (1). Key to developing therapies designed to minimize the impact of severe hypoglycemia in type 1 diabetes will be to achieve a greater understanding of the molecular mechanisms through which humans can detect a falling glucose and initiate a counterregulatory defense response designed to restore glucose homeostasis.

It is now well established that falling glucose levels are detected by specialized glucose-sensing neurons that, to date, have been demonstrated in the brain (2–8) and periphery (9–11). Of the several brain glucose-sensing areas identified, the ventromedial hypothalamus (VMH) in particular has been shown to play a key role in the detection of hypoglycemia and generation of a counterregulatory response (CRR) (2–4,12–18). The VMH contains neurons that are activated in response to an increase in circulating glucose (glucose-excited neurons) and others that are activated in response to a fall in circulating glucose (glucose-inhibited neurons) (19). The mechanisms through which these specialized neurons detect changes in the glucose levels to which they are exposed remain to be fully determined.

Recent evidence has emerged to suggest that the serine/threonine kinase AMP-activated protein kinase (AMPK) may play a role in the sensing of hypoglycemia within the VMH. AMPK is activated during cellular energy depletion and acts to suppress ATP-consuming pathways and to activate ATP-generating pathways, which has led to it being called an intracellular "fuel gauge" (20). Hypothalamic AMPK has been implicated in the regulation of food intake (21–25) and is activated in response to fasting or central glucoprivation (22–24). Hypothalamic AMPK has also been implicated in the regulation of CRRs to acute hypoglycemia (14,15,26), and 5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside (AICAR), a pharmacological activator of AMPK, mimics the effect of low glucose to stimulate neuronal activity in VMH glucose-inhibited neurons (27). Although these studies provide good evidence of a role for AMPK in glucose sensing, they are limited because of the use of pharmacological interventions to manipulate hypothalamic AMPK or having examined for changes in AMPK activity in response to a systemic or intracerebroventricular manipulation. In the current manuscript, gene silencing technology is used to selectively downregulate AMPK in

the VMH of Sprague-Dawley rats. We then determine in vivo the effect of AMPK downregulation on CRRs to subsequent controlled hypoglycemia.

RESEARCH DESIGN AND METHODS

Male Sprague-Dawley rats (weight 250–350 g) were housed in the Yale Animal Resource Center, fed a standard pellet diet (Harlan 2018; Harlan, Boston, MA), and maintained on a 12-h/12-h day/night cycle. The animal care and experimental protocols were reviewed and approved by the Yale Animal Care and Use Committee.

One week before each study, the rats were anesthetized with an intraperitoneal injection (1 ml/kg) of a mixture of xylazine (20 mg/ml AnaSed; Lloyd Laboratories, Shenandoah, IA) and ketamine (100 mg/ml Ketaset; Aveco, Fort Dodge, IA) in a ratio of 1:2 (vol:vol). The rats initially underwent vascular surgery for the implantation of chronic vascular catheters followed by the stereotaxic insertion of VMH (anterior-posterior -2.6 mm, medio-lateral ± 3.8 mm, and dorsal-ventral -8.3 mm at an angle of 20°) microinjection guide cannulas, as described previously (15). The stereotaxic coordinates used in this study are designed to target the center of the ventromedial nucleus (VMN) (28). However, because microinjection of a compound or viral vector is unlikely to be fully contained within the VMN, we have referred to the region of injection as the VMH, which includes the VMN and Arcuate nucleus (Arc). After insertion of the guide cannulas, each rat was then injected bilaterally with either adeno-associated viral (AAV) vector containing a DNA template encoding the AMPK shRNA ($n = 14$) or scrambled shRNA as a control ($n = 14$), at a rate of 50 nl/min for 30 min (total dose 1.5 μ l). On completion of the hypoglycemia study (7–9 days after microinjection), rats were killed, and their brains were removed rapidly. VMH micropunches were then obtained from 600- μ m sections taken through the hypothalamus.

Infusion protocol

All animals were fasted overnight. On the morning of the study the vascular catheters were opened, and the animals were allowed to settle and recover from any stress of handling for at least 90 min. Thereafter, a hyperinsulinemic-hypoglycemic clamp technique as adapted for the rat (29) was used to provide a standardized hypoglycemic stimulus. At time = 0, a 2-h 10 mU \cdot kg $^{-1}$ \cdot min $^{-1}$ infusion of human regular insulin (Eli Lilly, Indianapolis, IN) was begun. The plasma glucose was allowed to fall to ~ 2.8 mmol/l (aiming to achieve target glucose levels within 30–40 min) and was then maintained at this level for 120 min using a variable rate 10% dextrose infusion based on frequent plasma glucose determinations. Samples for glucose, insulin, epinephrine, and glucagon were obtained at regular intervals during the baseline and hypoglycemic states.

In addition, in a subset of rats (VMH-shAMPK, $n = 11$; controls, $n = 7$), a primed infusion of [3 H]glucose was started at $t = -120$ min and continued throughout the hypoglycemia studies to measure rates of endogenous glucose production (R_a) and peripheral glucose utilization (R_d) during insulin-induced hypoglycemia. Glucose turnover was calculated according to the method of Wall et al. (30). Endogenous production was calculated by subtracting the exogenous glucose infusion rate (GIR) from total R_a .

shRNA design and validation

A candidate shRNA sequence was identified within the encoded protein kinase domain of the α -subunit of the AMPK gene. A DNA template encoding the shRNA equivalent of this sequence was cloned into the Ambion pSilencer 2.1-U6 neo vector. The upper strand of the AMPK α_2 shRNA template was 5'-GTATGATGTCAGATGGTGAATT CTCAAGAGA AATTCACCATCTGACAT-CATACTT TTTTGGAA-3', where the sequences in italics correspond to the sense and antisense shRNA templates, respectively (in which the underlined G at the beginning of the sequence is an additional nucleotide inserted to improve RNA polymerase III transcription, the underlined sequence between the sense and antisense templates corresponds to the loop joining the shRNA template strands, and the terminal, nonunderlined sequence corresponds to the RNA polymerase III terminator sequence and "gene silencing" element). The efficacy of the shRNA sequence was tested in BHK, PC12, and HeLa cells and was shown to achieve strong downregulation of the AMPK- α subunit (31). A negative control plasmid containing a shRNA cassette with no known similarity to the human, rat, or mouse genomes was provided by Ambion. The sequence of this cassette is as follows: 5'-ACTACCGTTGTTATAGGTTTCAAGAGA CACCTATAACACGGTAGT TTTTGGAA-3'.

Viral vectors

Adeno-associated viral (AAV) vectors (serotype 2) carrying the DNA templates for AMPK- α or negative control shRNAs were produced using a kit from Stratagene (AAV Helper-Free System no. 240071). The AAV vector was altered to carry shRNAs by replacing the complete gene expression cassette in the pAAV-MCS plasmid with the respective shRNA expression cassette (specific for AMPK- α or negative control shRNAs) of the pSilencer 2.1-U6 neo vectors.

Vectors were purified and concentrated following the method of Auricchio et al. (32). The viral vector suspension in PBS was filter-sterilized before infusion into the rat brain. Viral stocks for injection were titrated by capture ELISA (AAV Titration ELISA, Progen PRATV) and used at a concentration of $\sim 10^{12}$ transduction units/ml.

To locate viral presence and effective transduction within the hypothalamus, in addition to mRNA analysis, we conducted a further study in a separate group of Sprague-Dawley rats ($n = 4$). In this study, a reporter gene was added to the original adenoviral vector, such that the new viral expression construct was rAAV2-CAG-AMPK α -GFP-WPRE (GeneDetect, Auckland, New Zealand). The viral cassette contained a hybrid cytomegalovirus enhancer/chicken β -actin promoter (CAG), a woodchuck posttranscriptional regulatory element (WPRE), and a bovine growth hormone polyadenylation sequence. The viral vectors were packaged and affinity purified (GeneDetect). Rats were microinjected bilaterally to the VMH (as described above) and killed after 7–9 days for later immunohistochemistry.

Analytical procedures

Immunohistochemistry. The immunohistochemistry protocol used in the present study was modified from previous studies (33,34). Briefly, sections taken from stock were washed in PBS for 15 min, before being incubated overnight at room temperature with 1:2000 rabbit anti-green fluorescent protein (anti-GFP) antibody (Invitrogen, Carlsbad, CA) in 0.3% fresh normal donkey serum and 0.02% sodium azide plus 0.04% Triton X-100 in PBS (PBT). The following day, sections were incubated for 1 h with Alexa Fluor488-conjugated donkey anti-rabbit (1:400; Invitrogen) in 0.3% fresh donkey serum and PBT. After mounting on polylysine slides, sections were coverslipped with antifade mount medium for fluorescence (Vectashield; Vector, Burlingame, CA) analysis.

mRNA analysis. mRNA levels were evaluated with a Brilliant SYBR Green QRT-PCR master mix kit (Qiagen). Total cellular RNA from rats' whole hypothalamus was obtained using TRIzol reagent (Invitrogen) and cleaned up with Rneasy Mini kit (Qiagen) according to the manufacturer's instructions. VMH micropunches were obtained using 18-gauge needles. Subsequently, total cellular RNA from punches was extracted with PicoPure RNA isolation kit (Arcturus, Mountain View, CA). The forward primer for rat AMPK α_1 consisted of 5'-atccgcagagatccagaa-3', and the reverse primer consisted of 5'-cgctc-gactctcctttctgctc-3'. The forward primer for rat AMPK α_2 consisted of 5'-gctgtggatgccaaattat-3', and the reverse primer consisted of 5'-gcatacagcagatggccaata-3'. Quantitative RT-PCR reactions were made by combining 12.5 μ l 2 \times SYBR RT-PCR Master Mix, 0.5 μ l upstream primer (5 μ mol/l), 0.5 μ l downstream primer (5 μ mol/l), 0.0625 μ l StrataScript RT/Rnase block enzyme mixture, 1.4375 μ l Rnase-free water, and 10 μ l (20 ng/ μ l) RNA template. No-template controls and no-RT controls were introduced in each run. The quantitative RT-PCR was performed using DNA Engine Opticon 2 (MJ Research), in which the mixture was heated to 50°C for 30 min for reverse transcription, heated to 95°C for 10 min, and then cycled 40 times at 95°C for 30 s, 56°C for 1 min, and 72°C for 50 s. To verify the specificity of the amplification reaction, melting-curve analysis was performed. The size of the amplified product was confirmed by electrophoresis. The level of rat β -actin mRNA was determined in all of the samples, and the expression of AMPK was normalized to rat β -actin. The threshold cycle (C_T) value is taken as the fractional cycle number at which the emitted fluorescence of the sample passes a fixed threshold above the baseline. The 2 $^{-C_T}$ method was used to calculate the relative differences between experimental and control groups as fold change in gene expression (35). All of the reactions were run in a minimum of four independent assays.

Plasma levels of glucose were measured by the glucose oxidase method (Beckman, Fullerton, CA). Catecholamine analysis was performed by high-performance liquid chromatography using electrochemical detection (ESA, Acton, MA); plasma insulin and glucagon were measured by radioimmunoassay (Linco, St. Charles, MO). All data are expressed as the means \pm SE and were analyzed statistically using either Student's t test or repeated measures ANOVA followed by post hoc testing to localize significant effects as indicated (SPSS 11.0 for Windows; SPSS).

RESULTS

Localization of AAV-shAMPK microinjection. To determine the relative distribution of AAV-shAMPK, bilateral VMH microinjections were performed with a GFP-tagged AAV-shAMPK. Figure 1A–D illustrates the distribution of the AAV using this approach. The majority of GFP-positive neurons were located in the VMH, with some spread outside of this region to the dorsomedial hypothalamus (DMH). Within the VMH, the distribution of GFP-positive

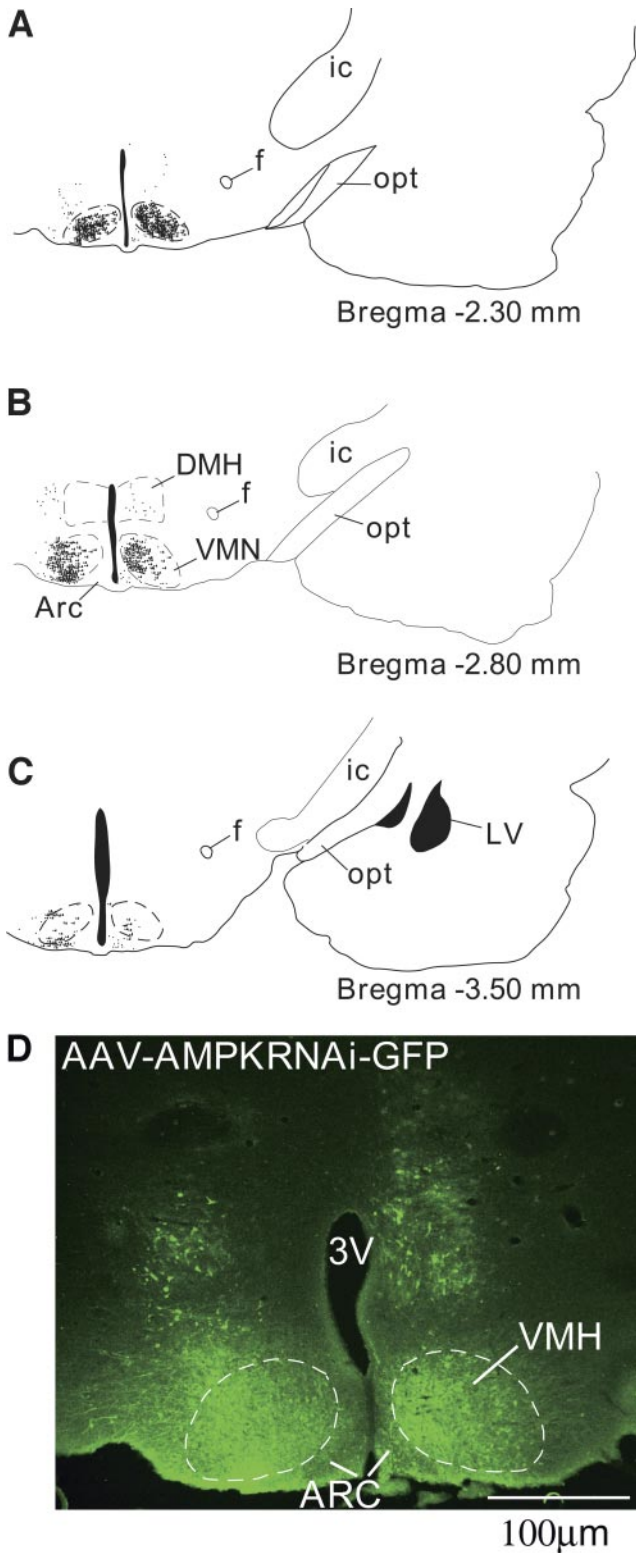


FIG. 1. Localization studies of AAV microinjection, showing rostro-caudal distribution of GFP-positive neurons within the hypothalamus (A–C), and a representative microphotograph of the VMH showing GFP-positive neurons taken 7 days after microinjection of rAAV2-CAG-AMPK α -GFP-WPRE. The white bar represents 100 μ m. 3V, third ventricle. (Please see <http://dx.doi.org/10.2337/db07-0837> for a high-quality digital representation of this figure.)

neurons was predominantly the medial portion of the VMN with only a small amount of staining in the Arc (Fig. 1A–D).

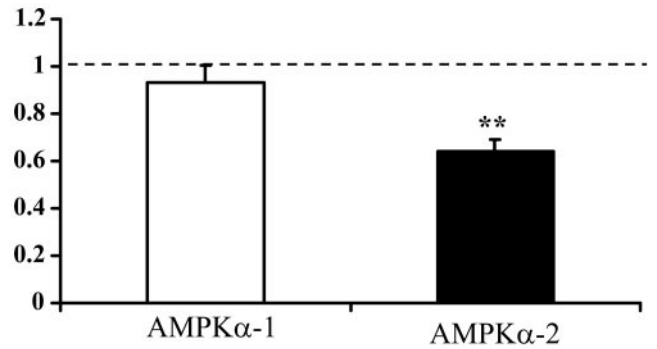


FIG. 2. Microinjection of AMPK-RNAi results in a significant reduction in AMPK α -2 isoform (black bar) but not AMPK α -1 isoform (gray bar) in micropunches of VMH from frozen sections. ** $P < 0.01$.

In addition, quantitative real-time PCR was used to determine AMPK α ₁ and α ₂ gene expression in VMH micropunches from rats at 7–9 days after injection. In comparison with control RNAi-injected rats, AMPK mRNA from VMH showed significant decreases in AMPK α ₂ but not AMPK α ₁ gene expression (Fig. 2).

Hypoglycemia studies. During the hypoglycemic clamp, plasma glucose profiles in the two animal groups did show some differences over the time course of the study ($F = 15.17$, $P < 0.05$). Post hoc testing showed that this resulted from a significantly faster fall in plasma glucose in the VMH-shAMPK group that reached lower glucose levels in response to the initial insulin infusion at the 10-, 20-, and 30-min time points (each $P < 0.05$; Fig. 3A). After these time points, plasma glucose did not differ significantly, and over the course of the hypoglycemic plateau, plasma

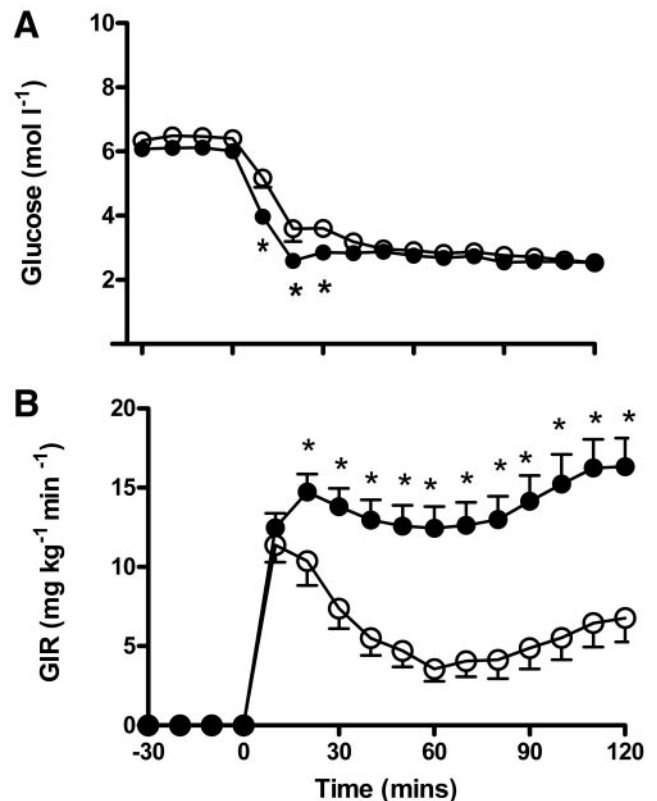


FIG. 3. Plasma glucose profiles (A) and GIRs (B) during the hyperinsulinemic clamp procedure (AMPK-RNAi, ●; control, ○). Values are means \pm SE. * $P < 0.05$.

glucose (mean \pm SE) achieved in each group (60–120 min) was 2.6 ± 0.1 mmol/l for the VMH-shAMPK group and 2.7 ± 0.1 mmol/l for controls. GIRs (for whole group; $n = 14$ in each), however, differed markedly between groups with the VMH-shAMPK rats requiring significantly more exogenous glucose to maintain the hypoglycemic plateau (Fig. 3B). Over the last 60 min of the hypoglycemic clamp, the mean GIR was 14.3 ± 1.6 vs. 5.1 ± 1.2 $\text{mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ in the VMH-shAMPK versus control rats ($F = 24.17$, $P < 0.0001$).

Glucose dynamics. In a subgroup of rats (VMH-shAMPK, $n = 11$; controls, $n = 7$), tracer studies were performed to determine the nature of the differences between groups in GIR during hypoglycemia. Glucose specific activity was stable over the 60- to 120-min portion of the clamp, and so steady-state equations were applied. Basal measures of glucose uptake and production did not differ significantly between groups (Fig. 4). In these subgroups, GIR during hypoglycemia also differed markedly (13.4 ± 1.7 vs. 2.2 ± 1.6 $\text{mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$, VMH-shAMPK vs. controls, respectively, $P < 0.001$). VMH-shAMPK rats were found to differ from controls in terms of both glucose production and uptake (Fig. 4). After AMPK knockdown in the VMH, R_a was significantly less (8.1 ± 1.1 vs. 14.2 ± 1.2 $\text{mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$, VMH-shAMPK vs. controls, respectively, $F = 10.44$, $P < 0.01$), and R_d was significantly increased (21.9 ± 1.7 vs. 16.6 ± 0.8 $\text{mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$, VMH-shAMPK vs. controls, respectively, $F = 13.4$, $P < 0.005$) during acute hypoglycemia.

Glucoregulatory hormones. Basal levels of the counter-regulatory hormones (epinephrine, norepinephrine, glucagon, and corticosterone) and of insulin and C-peptide before the initiation of the hyperinsulinemic-hypoglycemic clamp procedure did not differ significantly between groups. During the hyperinsulinemic glucose clamp procedure, plasma insulin (mean \pm SE levels stabilized at similar levels in both groups; $2,635 \pm 387$ and $2,696 \pm 380$ pmol/l in VMH-shAMPK and $2,149 \pm 247$ and $2,428 \pm 257$ pmol/l in controls, 60 and 120 min after the start of the insulin infusion; $F = 0.56$, NS). C-peptide during basal and hyperinsulinemic-hypoglycemic conditions did not differ significantly (181 ± 29 , 49 ± 5 , and 43 ± 3 vs. 252 ± 70 , 49 ± 5 , and 44 ± 4 pmol/l, respectively; $F = 2.7$, NS).

In contrast, VMH-shAMPK resulted in a suppressed CRR to acute hyperinsulinemic hypoglycemia. Mean \pm SE plasma epinephrine during hypoglycemia was reduced by $\sim 42\%$ (5.7 ± 0.7 vs. 9.8 ± 1.2 nmol/l, respectively; $F = 8.89$, $P < 0.005$; Fig. 5) and plasma glucagon by $\sim 59\%$ (111.6 ± 19.0 vs. 270.0 ± 42.5 ng/l, respectively; $F = 14.1$, $P < 0.001$; Fig. 5). No significant differences were seen in plasma norepinephrine (2.4 ± 0.3 vs. 2.4 ± 0.3 nmol/l, respectively; $F = 0.17$, NS) or plasma corticosterone (1.52 ± 0.08 vs. 1.55 ± 0.08 mmol/l, respectively; $F = 0.47$, NS).

DISCUSSION

We have previously shown that pharmacological activation of AMPK in the VMH, using AICAR, led to an amplification of the glucose CRR to acute hypoglycemia in normal Sprague-Dawley rats and in rats with defective counterregulation induced by prior recurrent hypoglycemia (14,15). Although these studies provided support for a role for AMPK in glucose sensing, they were limited by the potential for AICAR to activate other AMP-regulated kinases and/or to act through the adenosine receptor (36). In the current study, we show that selective downregulation of the α -catalytic subunit of AMPK in the VMH leads to a

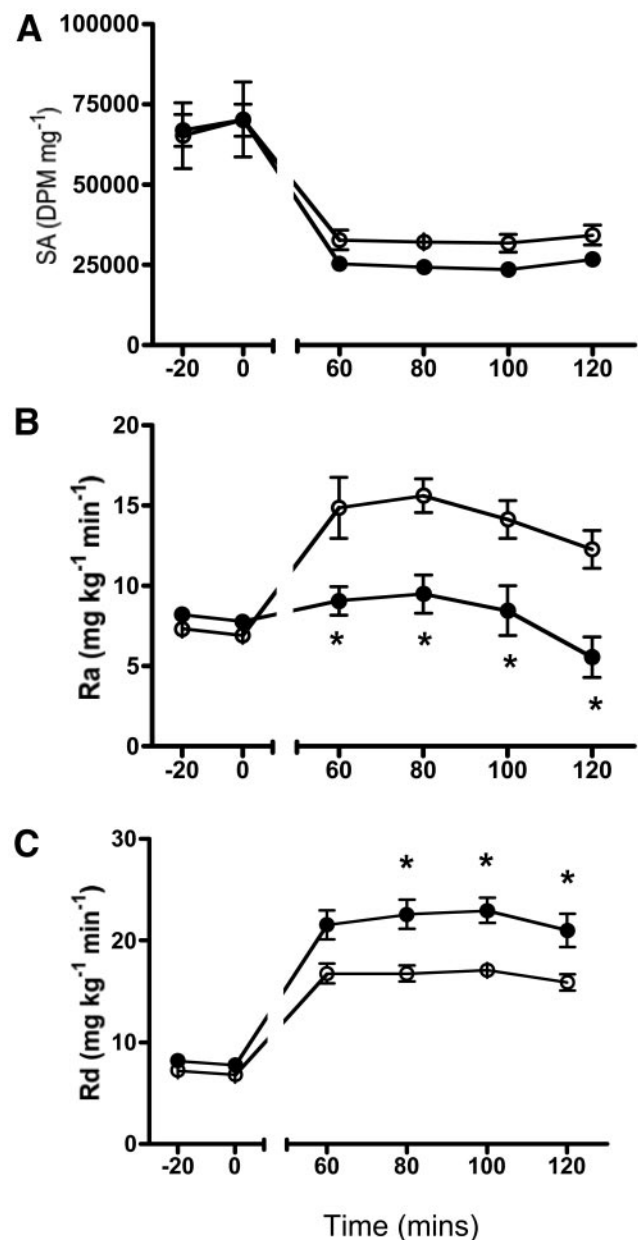


FIG. 4. In a subgroup of rats, an infusion of [^3H]glucose was started at $t = -120$ min and continued throughout the hyperinsulinemic-hypoglycemic clamp studies to allow calculation of glucose kinetics. Graphs show specific activity (SA) during hypoglycemia (A), rates of R_a (B), and R_d (C) are shown for control (\circ) and AMPK-RNAi (\bullet) groups at each point of measurement during basal and hypoglycemic periods. Values are means \pm SE. * $P < 0.05$ vs. control.

suppressed glucose-CRR to subsequent hypoglycemia. Namely, we observed an increase in the GIR required to maintain equivalent hypoglycemia to control animals, which reflected a reduction in R_a and in an increase in R_d and significantly suppressed glucagon and epinephrine secretory responses to the hypoglycemic stimulus. Taken together with our previous work, the current findings provide robust evidence for a key role for AMPK in the mechanism through which a falling glucose is detected by specialized neurons in the VMH and that subsequently leads to the generation of a full counterregulatory defense response.

The aim of this study was to more specifically alter AMPK function within the VMH and to test the impact of

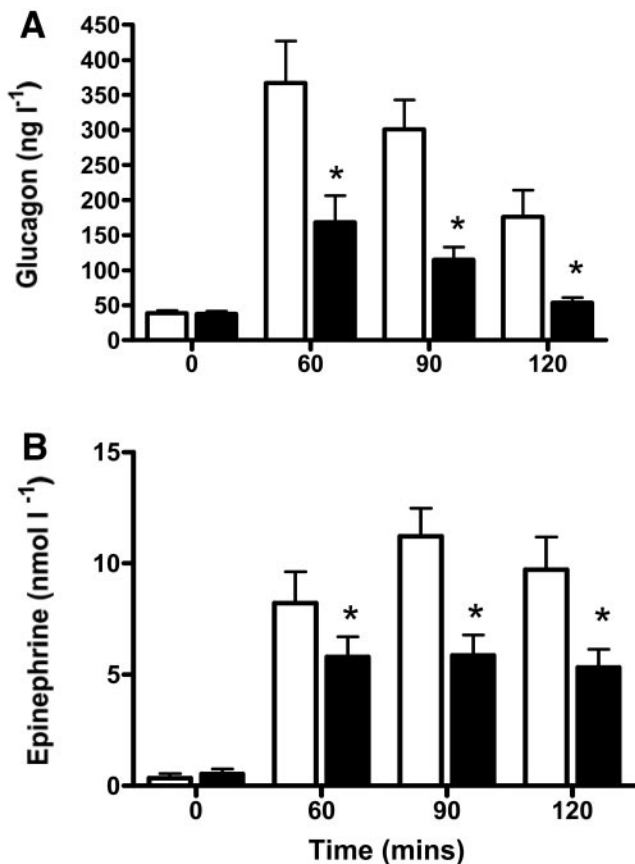


FIG. 5. Counterregulatory hormone response to acute hypoglycemia after VMH microinjection of AMPK-RNAi (black bars) or control (white bars). Samples for plasma epinephrine and glucagon were taken at baseline ($t = 0$ min) and during hypoglycemia ($t = 60, 90,$ and 120 min). Values are means \pm SE. * $P < 0.05$ vs. control.

this manipulation on glucose CRRs to acute hypoglycemia. To do this, we developed a short-hairpin RNA against AMPK and delivered this, using an AAV vector, directly to the VMH. Initial in vitro studies in a variety of cell types confirmed that the shRNA for AMPK we had developed induced a strong downregulation of AMPK expression (both AMPK- $\alpha 1$ and - $\alpha 2$ subtypes [31]). AAV vectors (serotype 2) carrying the DNA templates for AMPK- α or scrambled shRNAs were then developed. Recombinant AAV technology allows for the long-term expression of transgenes and offers considerable advantages of non-pathogenicity and nonimmunogenicity. After microinjection of the AAV-shAMPK, we were able to produce a 30–40% reduction in the expression of AMPK- $\alpha 2$ isoform in VMN micropunches but no significant impact on AMPK- $\alpha 1$ isoform. In vitro, the shRNA did not show such selectivity, however, the brain predominantly expresses the AMPK- $\alpha 2$ isoform with the AMPK- $\alpha 1$ isoform expressed at much lower levels (37). As such, it may not be possible to detect difference in AMPK- $\alpha 1$ after microinjection. To address the spread of the AAV-shAMPK from the injection site, we subsequently microinjected at an identical volume and rate an AAV containing both the shAMPK and GFP (Fig. 1A–D). We found that staining for GFP-positive neurons was primarily within the VMH with a small amount of spread to the DMH, the latter predominantly via the needle track. The DMH is a glucose-sensing region, however, its chemical inactivation in vivo with lidocaine results in a mild impairment of adrenocortico-

trophin hormone (ACTH) and corticosterone response to acute hypoglycemia only (38). In contrast, in the present study, AMPK downregulation in the VMH resulted in reduced epinephrine and glucagon responses to acute hypoglycemia and had no effect on the corticosterone response. Thus, the primary focus of the effects observed are most likely to have resulted from AMPK downregulation in the VMH.

Within the brain, AMPK- $\alpha 2$ expression is thought to be predominantly neuronal, with very little expression evident in astrocytes (37). The question then arises as to which neuronal population(s) is being affected by the AMPK downregulation. Although it is not possible to answer this question directly from our study, recent data suggest that AMPK may be predominantly acting within glucose-inhibited neurons in the VMH, at least with regard to the detection of hypoglycemia. Canabal et al. (27) demonstrated that in VMN glucose-inhibited neurons exposed to 2.5 mmol/l glucose, AICAR (an activator of AMPK) mimicked the excitatory effect of low glucose (0.5 mmol/l) on action potential frequency. Both low glucose and AICAR were shown to mediate their effects through an increase in nitric oxide (NO) production in glucose-inhibited neurons, and the increase in NO production in response to a low glucose was blocked by Compound C, an inhibitor of AMPK (27). In a related series of studies, Mountjoy et al. (39) reported that activation of AMPK with AICAR or inhibition with Compound C altered neuronal activity in glucose-inhibited but not glucose-excited neurons in an ex vivo hypothalamic cell culture system obtained from the mediobasal (including VMN and Arc) hypothalamus. Taken together, these studies provide evidence for AMPK playing a key role in the glucose-sensing mechanism used by VMH glucose-inhibited neurons to detect a falling glucose. Our finding that AMPK downregulation in the VMH in vivo leads to a marked suppression of the glucose CRR to hypoglycemia would therefore be consistent with the hypothesis that the CRR is triggered by an increase in neuronal activity within VMH glucose-inhibited neurons.

Hypoglycemia stimulates, directly or indirectly, the release of corticotrophin-releasing hormone (CRH) from the paraventricular nucleus (PVN) of the hypothalamus, which subsequently induces pituitary corticotrophes to produce ACTH, which in turn act on the adrenal cortex to evoke corticosteroid release. In the present study, no effect of VMH AMPK knockdown was seen on the corticosterone response to acute hypoglycemia, a finding that is consistent with previous work from our laboratory in which the role of VMH GABA and urocortin on CRRs to acute hypoglycemia were examined (16,40). In rodent models, hypoglycemia induces neuronal activation in the PVN (as measured by cFOS) (e.g., 41), and inactivation of the PVN during hypoglycemia with a chronic lidocaine infusion has been shown to impair peak epinephrine, ACTH, but not corticosterone responses (42). In contrast, local administration of the CRH-1 receptor antagonist antalarmin to the PVN suppressed corticosterone but not epinephrine or glucagon secretory responses to acute hypoglycemia, whereas local administration of antalarmin to the VMH did the converse (43). The findings from the present study suggest that corticosteroid release during acute hypoglycemia may be regulated by a different mechanism and one that does not directly involve the VMH. Interestingly, increased duration of disease and recurrent hypoglycemia in human subjects with type 1 diabetes is

predominantly associated with defective glucagon and epinephrine responses but not cortisol responses to hypoglycemia (rev. in 44). Taken together with the rodent studies, this implies an alteration in VMH glucose sensing may be involved in the genesis of defective counterregulation in type 1 diabetes.

In human subjects, insulin per se has been shown in most studies to amplify the CRR to acute hypoglycemia (45–47), and mice with a brain/neuron-specific insulin receptor knockout have suppressed CRRs to acute hypoglycemia (48). In the present study, although no overall significant differences in plasma insulin levels were seen between groups, the levels at 60 min were higher in VMH-shAMPK rats than in controls. On the basis of previous studies, this would have amplified early CRRs in VMH-shAMPK rats and could potentially contribute to our findings. However, because VMH-shAMPK significantly suppressed the CRR in the present study, any confounding effect of this difference is likely to have been small and, if anything, would have acted against the effect of AMPK downregulation. Moreover, insulin levels between groups in the present study differed by a much smaller amount than in previous studies (45,46,49). Thus, overall it seems unlikely that insulin per se has contributed to the findings in this study.

Although the precise role for AMPK in glucose sensing during hypoglycemia remains to be fully elucidated, there is now reasonably robust evidence indicating that it plays a key role in this process. In vivo activation of AMPK in a key brain glucose-sensing region, the VMH, significantly amplifies (14,15) the glucose CRR to acute hypoglycemia. Whereas, as we show in the present study, downregulation of AMPK in the VMH significantly suppresses the CRR to acute hypoglycemia. In addition, ex vivo studies (27,39,50) show a direct action of AMPK on neuronal activity in populations of glucose-inhibited neurons tested under low glucose conditions. We conclude that AMPK is a key part of the glucose-sensing mechanism operative under conditions of hypoglycemia in the VMH.

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REFERENCES

- Cryer PE: Banting Lecture. Hypoglycemia: the limiting factor in the management of IDDM. *Diabetes* 43:1378–1389, 1994
- 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
- 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
- Borg WP, Sherwin RS, During MJ, Borg MA, Shulman GI: Local ventromedial hypothalamus glucopenia triggers counterregulatory hormone release. *Diabetes* 44:180–184, 1995
- Frizzell RT, Jones EM, Davis SN, Biggers DW, Myers SR, Connolly CC, Neal DW, Jaspan JB, Cherrington AD: Counterregulation during hypoglycemia is directed by widespread brain regions. *Diabetes* 42:1253–1261, 1993
- Oomura Y, Ono T, Ooyama H, Wayner MJ: Glucose and osmosensitive neurones of the rat hypothalamus. *Nature* 222:282–284, 1969
- Ritter S, Dinh TT, Zhang Y: Localization of hindbrain glucoreceptive sites controlling food intake and blood glucose. *Brain Res* 856:37–47, 2000
- Sanders NM, Ritter S: Repeated 2-deoxy-D-glucose-induced glucoprivation attenuates Fos expression and glucoregulatory responses during subsequent glucoprivation. *Diabetes* 49:1865–1874, 2000
- Donovan CM: Portal vein glucose sensing. *Diabetes Nutr Metab* 15:308–312; discussion 313–304, 2002
- Donovan CM, Halter JB, Bergman RN: Importance of hepatic glucoreceptors in sympathoadrenal response to hypoglycemia. *Diabetes* 40:155–158, 1991
- Hevener AL, Bergman RN, Donovan CM: Novel glucosensor for hypoglycemic detection localized to the portal vein. *Diabetes* 46:1521–1525, 1997
- Evans ML, McCrimmon RJ, Flanagan DE, Keshavarz T, Fan X, McNay EC, Jacob RJ, Sherwin RS: Hypothalamic ATP-sensitive K⁺ channels play a key role in sensing hypoglycemia and triggering counterregulatory epinephrine and glucagon responses. *Diabetes* 53:2542–2551, 2004
- McCrimmon RJ, Evans ML, Fan X, McNay EC, Chan O, Ding Y, Zhu W, Gram DX, Sherwin RS: Activation of ATP-sensitive potassium channels in the ventromedial hypothalamus amplifies counterregulatory responses to acute hypoglycemia in normal and recurrently hypoglycemic rats. *Diabetes* 54:3169–3174, 2005
- McCrimmon RJ, Fan X, Cheng H, McNay E, Chan O, Shaw M, Ding Y, Zhu W, Sherwin RS: Activation of AMP-activated protein kinase within the ventromedial hypothalamus amplifies counterregulatory hormone responses in rats with defective counterregulation. *Diabetes* 55:1755–1760, 2006
- McCrimmon RJ, Fan X, Ding Y, Zhu W, Jacob RJ, Sherwin RS: Potential role for AMP-activated protein kinase in hypoglycemia sensing in the ventromedial hypothalamus. *Diabetes* 53:1953–1958, 2004
- McCrimmon RJ, Song Z, Cheng H, McNay EC, Weikart-Yeckel C, Fan X, Routh VH, Sherwin RS: Corticotrophin-releasing factor receptors within the ventromedial hypothalamus regulate hypoglycemia-induced hormonal counterregulation. *J Clin Invest* 116:1723–1730, 2006
- Routh VH: Glucosensing neurons in the ventromedial hypothalamic nucleus (VMN) and hypoglycemia-associated autonomic failure (HAAF). *Diabetes Metab Res Rev* 19:348–356, 2003
- 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
- Routh VH: Glucose-sensing neurons: are they physiologically relevant? *Physiol Behav* 76:403–413, 2002
- Hardie DG, Carling D: The AMP-activated protein kinase: fuel gauge of the mammalian cell? *Eur J Biochem* 246:259–273, 1997
- Kim EK, Miller I, Aja S, Landree LE, Pinn M, McFadden J, Kuhajda FP, Moran TH, Ronnett GV: C75, a fatty acid synthase inhibitor, reduces food intake via hypothalamic AMP-activated protein kinase. *J Biol Chem* 279:19970–19976, 2004
- Kim MS, Park JY, Namkoong C, Jang PG, Ryu JW, Song HS, Yun JY, Namgoong IS, Ha J, Park IS, Lee IK, Viollet B, Youn JH, Lee HK, Lee KU: Anti-obesity effects of alpha-lipoic acid mediated by suppression of hypothalamic AMP-activated protein kinase. *Nat Med* 10:727–733, 2004
- Lee K, Bing L, Xi X, Suh Y, Martin RJ: The role of neuronal energy status in the regulation of AMPK-activated protein kinase, orexigenic neuropeptides expression and feeding behavior. *Endocrinology* 146:3–10, 2005
- Minokoshi Y, Alquier T, Furukawa N, Kim YB, Lee A, Xue B, Mu J, Foufelle F, Ferre P, Birbaumer MJ, Stuck BJ, Kahn BB: AMP-kinase regulates food intake by responding to hormonal and nutrient signals in the hypothalamus. *Nature* 428:569–574, 2004
- Andersson U, Filipsson K, Abbott CR, Woods A, Smith K, Bloom SR, Carling D, Small CJ: AMP-activated protein kinase plays a role in the control of food intake. *J Biol Chem* 279:12005–12008, 2004
- Han SM, Namkoong C, Jang PG, Park IS, Hong SW, Katakami H, Chun S, Kim SW, Park JY, Lee KU, Kim MS: Hypothalamic AMP-activated protein kinase mediates counter-regulatory responses to hypoglycaemia in rats. *Diabetologia* 48:2170–2178, 2005
- Canabal DD, Song Z, Potian JG, Beuve A, McArdle JJ, Routh VH: Glucose, insulin, and leptin signaling pathways modulate nitric oxide synthesis in glucose-inhibited neurons in the ventromedial hypothalamus. *Am J Physiol Regul Integr Comp Physiol* 292:R1418–R1428, 2007
- Paxinos G, Watson C: *The Rat Brain in Stereotaxic Co-Ordinates*. San Diego, CA, Academic Press, 1997
- Powell AM, Sherwin RS, Shulman GI: Impaired hormonal responses to hypoglycemia in spontaneously diabetic and recurrently hypoglycemic

- rats: reversibility and stimulus specificity of the deficits. *J Clin Invest* 92:2667–2674, 1993
30. Wall JS, Steele R, DeBodo RD, Altszuler N: Effect of insulin on utilization and production of circulating glucose. *Am J Physiol* 189:43–50, 1957
 31. Shaw MM, Gurr WK, McCrimmon RJ, Schorderet DF, Sherwin RS: 5'AMP-activated protein kinase alpha deficiency enhances stress-induced apoptosis in BHK and PC12 cells. *J Cell Mol Med* 11:1582–1838, 2007
 32. Auricchio A, Hildinger M, O'Connor E, Gao GP, Wilson JM: Isolation of highly infectious and pure adeno-associated virus type 2 vectors with a single-step gravity-flow column. *Human Gene Ther* 12:71–76, 2001
 33. Zhou L, Furuta T, Kaneko T: Neurokinin B-producing projection neurons in the lateral stripe of the striatum and cell clusters of the accumbens nucleus in the rat. *J Comp Neurol* 480:143–161, 2004
 34. Zhou L, Furuta T, Kaneko T: Chemical organization of projection neurons in the rat accumbens nucleus and olfactory tubercle. *Neuroscience* 120:783–798, 2003
 35. Livak K, Schmittgen T: Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻ΔΔCT method. *Methods* 25:402–408, 2001
 36. Gadalla AE, Pearson T, Currie AJ, Dale N, Hawley SA, Sheehan M, Hirst W, Michel AD, Randall A, Hardie DG, Frenguelli BG: AICA riboside both activates AMP-activated protein kinase and competes with adenosine for the nucleoside transporter in the CA1 region of the rat hippocampus. *J Neurochem* 88:1272–1282, 2004
 37. Turnley AM, Stapleton D, Mann RJ, Witters LA, Kemp BE, Bartlett PF: Cellular distribution and developmental expression of AMP-activated protein kinase isoforms in mouse central nervous system. *J Neurochem* 72:1707–1716, 1999
 38. Evans SB, Wilkinson CW, Gronbeck P, Bennett JL, Zavosh A, Taborsky GJ Jr, Figlewicz DP: Inactivation of the DMH selectively inhibits the ACTH and corticosterone responses to hypoglycemia. *Am J Physiol Regul Integr Comp Physiol* 286:R123–R128, 2004
 39. Mountjoy PD, Bailey SJ, Rutter GA: Inhibition by glucose or leptin of hypothalamic neurons expressing neuropeptide Y requires changes in AMP-activated protein kinase activity. *Diabetologia* 50:168–177, 2007
 40. Chan O, Zhu W, Ding Y, McCrimmon RJ, Sherwin RS: Blockade of GABA(A) receptors in the ventromedial hypothalamus further stimulates glucagon and sympathoadrenal but not the hypothalamo-pituitary-adrenal response to hypoglycemia. *Diabetes* 55:1080–1087, 2006
 41. Evans SB, Wilkinson CW, Bentson K, Gronbeck P, Zavosh A, Figlewicz DP: PVN activation is suppressed by repeated hypoglycemia but not antecedent corticosterone in the rat. *Am J Physiol Regul Integr Comp Physiol* 281:R1426–R1436, 2001
 42. 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 Regul Integr Comp Physiol* 284:R57–R65, 2003
 43. Cheng H, Zhou L, Zhu W, Wang A, Tang C, Chan O, Sherwin RS, McCrimmon RJ: Type 1 corticotrophin releasing factor receptors (CRFR1) in the ventromedial hypothalamus (VMH) promote hypoglycemia-induced hormonal counterregulation. *Am J Physiol* 293:E705–E712, 2007
 44. Gerich JE, Bolli GB: Counterregulatory failure. In *Hypoglycaemia and Diabetes: Clinical and Physiological Aspects*. Frier BM, Fisher BM, Eds. London, Edward Arnold, 1993
 45. Davis SN, Goldstein RE, Jacobs J, Price L, Wolfe R, Cherrington AD: The effects of differing insulin levels on the hormonal and metabolic response to equivalent hypoglycemia in normal humans. *Diabetes* 42:263–272, 1993
 46. Davis SN, Goldstein RE, Price L, Jacobs J, Cherrington AD: The effects of insulin on the counterregulatory response to equivalent hypoglycemia in patients with insulin-dependent diabetes mellitus. *J Clin Endocrinol Metab* 77:1300–1307, 1993
 47. Davis SN, Shavers C, Collins L, Cherrington AD, Price L, Hedstrom C: Effects of physiological hyperinsulinemia on counterregulatory response to prolonged hypoglycemia in normal humans. *Am J Physiol* 267:E402–E410, 1994
 48. Fisher SJ, Bruning JC, Lannon S, Kahn CR: Insulin signaling in the central nervous system is critical for the normal sympathoadrenal response to hypoglycemia. *Diabetes* 54:1447–1451, 2005
 49. Davis SN, Dobbins R, Tarumi C, Colburn C, Neal D, Cherrington AD: Effects of differing insulin levels on response to equivalent hypoglycemia in conscious dogs. *Am J Physiol* 263:E688–E695, 1992
 50. Fioramonti X, Contie S, Song Z, Routh VH, Lorsignol A, Penicaud L: Characterization of glucose-sensing neuron subpopulations in the arcuate nucleus. *Diabetes* 56:1219–1227, 2007