



Impaired Glutamatergic Neurotransmission in the Ventromedial Hypothalamus May Contribute to Defective Counterregulation in Recurrently Hypoglycemic Rats

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The objectives of this study were to understand the role of glutamatergic neurotransmission in the ventromedial hypothalamus (VMH) in response to hypoglycemia and to elucidate the effects of recurrent hypoglycemia (RH) on this neurotransmitter. We 1) measured changes in interstitial VMH glutamate levels by using microdialysis and biosensors, 2) identified the receptors that mediate glutamate's stimulatory effects on the counterregulatory responses, 3) quantified glutamate metabolic enzyme levels in the VMH, 4) examined astrocytic glutamate reuptake mechanisms, and 5) used ¹H-¹³C-nuclear magnetic resonance (NMR) spectroscopy to evaluate the effects of RH on neuronal glutamate metabolism. We demonstrated that glutamate acts through kainic acid receptors in the VMH to augment counterregulatory responses. Biosensors showed that the normal transient rise in glutamate levels in response to hypoglycemia is absent in RH animals. More importantly, RH reduced extracellular glutamate concentrations partly as a result of decreased glutaminase expression. Decreased glutamate was also associated with reduced astrocytic glutamate transport in the VMH. NMR analysis revealed a decrease in [4-¹³C]-glutamate but unaltered [4-¹³C]glutamine concentrations in the VMH of RH animals. The data suggest that glutamate release is important for proper activation of the counterregulatory response to hypoglycemia and that impairment of glutamate metabolic and

resynthetic pathways with RH may contribute to counterregulatory failure.

Hypoglycemia is the major barrier to attaining and maintaining good glycemic control in patients with diabetes (1). The imperfections of current insulin treatment regimens, coupled with a compromised ability to counter falling glucose levels, increase patient susceptibility to hypoglycemic events (2,3). In addition, antecedent hypoglycemia can impair the sympathoadrenal response to hypoglycemia, leading to what has been termed hypoglycemia-associated autonomic failure (HAAF). The mechanisms underlying HAAF have yet to be fully identified, but impairments in the brain's ability to detect hypoglycemia may be one factor.

The ventromedial hypothalamus (VMH) is a key brain region involved in glucose sensing and metabolic regulation (4–7). Although VMH GABAergic neurotransmission has been studied more extensively with respect to its role in glucose sensing (8–10), less is known about VMH glutamatergic neurotransmission. In the VMH, glutamate serves multiple purposes, including the regulation of feeding, reproductive function, and sympathetic outflow (11–13). Previous studies showed that mice lacking the vesicular glutamate transporter 2 (*Vglut2*) in VMH Sf1

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neurons have attenuated glucagon and epinephrine responses to hypoglycemia (14). In contrast, glutamate levels in the VMH were reported to decline during hypoglycemia in rats (15). Differences in glutamate responses between these studies are likely due to the location of glutamate action, glutamate release kinetics, and/or clearance from the synapse. These data suggest that VMH glutamate is an integral feature of the counterregulatory response.

Once released, glutamate is sequestered through transporters located in presynaptic terminals and astrocytes (16). The reuptake of glutamate 1) clears the synapse of high glutamate levels, preventing excitotoxicity; 2) replenishes neurotransmitter glutamate stores; and 3) provides oxidative fuel substrate for astrocytes. In the hypothalamus, the main astrocytic glutamate transporter is GLT-1 (17), which removes 85–90% of the released glutamate (16). Once in the astrocytes, glutamate either enters the tricarboxylic acid cycle (TCA) to be oxidized or is converted to glutamine by glutamine synthetase (GS) for export to neighboring glutamatergic neurons. In the neuron, glutamine is converted back into glutamate for release. This latter process, called the glutamate-glutamine cycle, accounts for most of the resynthesized glutamate.

Magnetic resonance spectroscopy studies in healthy human subjects have shown that the total glutamate:creatinine ratio in the occipital cortex decreases during moderate hypoglycemia (18). In contrast, glutamate concentrations in the occipital cortex of patients with type 1 diabetes with HAAF do not decline significantly during hypoglycemia, suggesting that antecedent hypoglycemia alters central glutamatergic function (19). Together, these data suggest that antecedent hypoglycemia impairs glutamate neurotransmission.

We currently lack a clear understanding of the role of VMH glutamatergic neurons in the counterregulatory response to hypoglycemia and the molecular mechanisms involved in this process. In this study, we investigated key elements involved in glutamate functionality within the VMH by using a combination of approaches: microdialysis and biosensor measurements of interstitial glutamate, ^{13}C -labeled glucose infusion and nuclear magnetic resonance (NMR) spectroscopy *ex vivo*, and molecular assessments of enzyme and transporter expression. We hypothesized that recurrent hypoglycemia (RH) impairs VMH glutamatergic neurotransmission, which in turn contributes to counterregulatory failure. Hence, the main focus of these studies was to understand 1) how VMH glutamate neurotransmission influences hypoglycemic counterregulatory responses, 2) how RH affects VMH glutamate release during hypoglycemia, and 3) what underlying mechanisms contribute to defective glutamate neurotransmission. We report for the first time in our knowledge that RH reduces glutamatergic neuronal metabolism and interstitial glutamate in the VMH, blunting counterregulation in part by reducing the resynthesis of glutamate from glutamine in neurons.

RESEARCH DESIGN AND METHODS

Animals

Male Sprague-Dawley rats (Charles River) weighing ~250–280 g were housed in the Yale Animal Resources Center in temperature- (22–23°C) and humidity-controlled rooms with free access to food and water. The animals were acclimated to handling and a 12-h light cycle (lights on at 0700 h) for 1 week before experimental manipulation. All animal experiments were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, and procedures were approved by the institutional animal care and use committee at Yale (New Haven, CT).

Surgery

Vascular catheters were surgically placed into the left-side carotid artery and right-side jugular vein on day 0 as described previously (8). Animals that underwent microdialysis or biosensor measurements were fitted with intracranial guide cannulas after placement of the catheters. Stainless steel microdialysis guide cannulas (Eicom) targeting the VMH were implanted by using the stereotaxic coordinates from Paxinos and Watson (from bregma: –2.6 mm anteroposterior, ± 3.8 mm medial lateral, –8.8 mm dorsal ventral, 16° angle) (8). One biosensor guide cannula (Pinnacle Technology) was implanted (from bregma: –2.6 mm anteroposterior, +0.6 mm medial lateral, –8.6 mm dorsal ventral) and encased in plastic housing (11). The animals were then allowed 1 week to recover from surgery before undergoing the RH phase of the experiment.

RH Protocol

For 3 consecutive days, the RH group received an injection of regular human insulin (5–10 units/kg *i.p.*; Eli Lilly). Blood glucose, as assessed from a tail nick every 30 min for 3 h with an AlphaTRAK2 glucometer (Abbott Laboratories), was maintained between 30 and 40 mg/dL for at least 2 h. At the end of each bout of hypoglycemia, food was returned to the rats, and glucose levels were recovered to normal. Controls were given a saline injection and monitored under similar conditions. After the last bout of hypoglycemia and after recovery of glucose levels, the animals were fasted overnight for a clamp study the next day.

Study 1: VMH Glutamate Responses

To evaluate VMH glutamate dynamics during hypoglycemia, we used two complementary techniques: microdialysis to quantify and biosensors to monitor real-time changes in VMH glutamate levels during hypoglycemia. A schematic of the clamp procedure is presented in Fig. 1. In microdialysis studies, control and RH rats were connected to infusion pumps as described previously and allowed 2.5 h to recover from handling stress (10). Baseline blood and microdialysate samples were subsequently collected and constant-rate insulin (50 mU/kg/min) and variable-rate 20% glucose

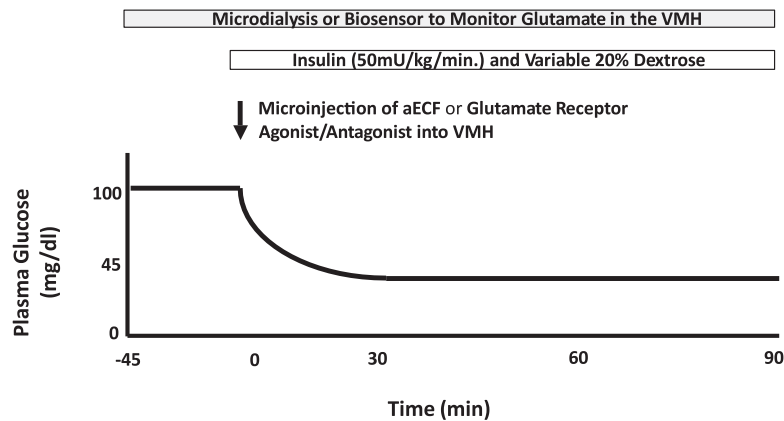


Figure 1—Schematic diagram outlining the general hypoglycemic clamp protocol. Baseline measurements are collected 30 min before the start of the insulin infusion for biosensor studies, whereas for the microdialysis studies, baseline measurements started 45 min before microinjection and/or the start of the hypoglycemic clamp.

infusions were used to lower plasma glucose levels and maintain them at 45 ± 5 mg/dL. Blood samples were collected every 30 min until 90 min to assess plasma glucagon, catecholamine, and insulin concentrations. Blood samples were centrifuged and the plasma aliquoted into Eppendorf tubes for storage at -20°C (or -80°C for catecholamines). The erythrocytes were resuspended in artificial plasma and reinfused into the animal (20). Microdialysate samples were collected at 10-min intervals throughout the study.

For biosensor studies, *in vitro* calibrations were performed the day before the clamp on each 1-mm glutamate biosensor (11). After the sensors were calibrated, the animals were briefly anesthetized with isoflurane (<2 min), and the probes were inserted into the VMH and connected to the circuit board in the cap housing. After recovered from anesthesia, the animals were returned to their home cages and fasted overnight. The next day, the animals were connected to infusion pumps for insulin and glucose, and 2.5 h later, baseline blood samples were collected and data collection initiated from the biosensors for 30 min to establish a stable baseline reading before the start of the hypoglycemic clamp as described for microdialysis. At the conclusion of the clamp, the animals from the biosensor and microdialysis studies were euthanized and the brains removed and frozen. Coronal sections were taken through the VMH to verify probe position (Supplementary Fig. 1). Only animals with correctly positioned probes were analyzed.

Study 2: Glutamate Receptor Identification

These studies identified the class of glutamatergic receptors that enhanced counterregulatory hormone release during hypoglycemia. Normal, hypoglycemia-naïve rats were implanted with vascular catheters and bilateral VMH microinjection guide cannulas as above. One week after surgery, animals were fasted overnight for a hypoglycemic clamp study the next day. Unless otherwise stated, the compounds were obtained from Sigma-Aldrich. In these studies, $0.1 \mu\text{L}$ of a glutamate receptor agonist ($100 \mu\text{mol/L}$ AMPA, $100 \mu\text{mol/L}$ *N*-methyl-*D*-aspartate [NMDA], or $100 \mu\text{mol/L}$

kainic acid) or antagonist (100 nmol/L YM90K [Tocris], $100 \mu\text{mol/L}$ AP5, or $100 \mu\text{mol/L}$ 6,7-dinitroquinoxaline-2,3-dione [DNQX]) was microinjected into the VMH immediately before initiation of the hypoglycemic clamp as described above. Controls were microinjected with artificial extracellular fluid (aECF).

Study 3: Glutamate Metabolic Enzymes and Astrocytic GLT-1 Expression

Glutamate uptake through astrocytic glutamate transporters represent the major pathway through which released glutamate is sequestered. We evaluated whether RH influences the expression of GLT-1 and two glutamate metabolizing enzymes. Control and RH animals were euthanized and their brains collected and frozen. Frozen VMH micropunches were homogenized in lysis buffer and GS, and phosphate-activated glutaminase (PAG) and GLT-1 protein levels were quantified by Western blot. Briefly, $5 \mu\text{g}$ of protein was loaded onto a 4–20% SDS gradient gel, run at 85 V for 1–1.5 h, and transferred onto polyvinylidene fluoride membrane overnight at 4°C . The membrane was then blocked with Tris-buffered saline with Tween containing 5% nonfat milk for 1 h at 22°C . Subsequently, the membrane was incubated with primary anti-GS (1:5,000; Abcam), anti-PAG (1:1,000; Abcam), anti-GLT-1 (1:1,000; Millipore), anti-GAPDH (1:1,000; Millipore), or anti- β -actin (1:5,000; Sigma) overnight at 4°C . The next day, the membrane was washed in Tris-buffered saline with Tween and incubated with secondary anti-rabbit (1:5,000; PerkinElmer), anti-goat (1:20,000; Millipore), or anti-mouse (1:20,000; Sigma) antibody for 1 h at 22°C . After a second series of washes, the membrane was exposed to chemiluminescent reagent and autoradiographic film. GS, PAG, and GLT-1 protein were standardized to GAPDH or β -actin levels.

GLT-1 knockdown studies were done to understand the nature of the reduced GLT-1 protein levels in RH animals, we used an adenoassociated viral vector (AAV) (rAAV2; GeneDetect) to express a short hairpin RNA (shRNA) against GLT-1 under the control of a GFAP promoter to

reduce the expression of astrocytic GLT-1 protein levels in the VMH of RH rats. Details of the viral construct are described elsewhere (9). Three weeks before the study, 1.5 μ L of the AAV was bilaterally microinjected into the VMH over the course of 15 min. Control animals were microinjected with an AAV expressing a scrambled RNA sequence. Immediately after viral inoculation, microdialysis probes were implanted. Two weeks after stereotaxic surgery, the animals underwent a second surgery to have vascular catheters implanted. One week after vascular surgery, the animals were fasted overnight and subjected to a hypoglycemic clamp with microdialysis the next day as described above.

Study 4: Astrocytic Glutamate Uptake

To assess the effect of recurrent glucose deprivation on astrocytic glutamate uptake, primary rat cortical astrocytes (Invitrogen) were cultured in DMEM containing high glucose, 10% FBS, and 1% penicillin/streptomycin. The cells were grown until they reached 90% confluence. Subsequently, 1×10^6 cells were transferred to six-well plates with fresh medium containing 2.5 mmol/L glucose. Controls were incubated in 2.5 mmol/L glucose medium. Additional wells of cells were initially incubated in 2.5 mmol/L glucose medium before undergoing the recurrent glucose deprivation protocol, wherein the medium was changed to 0.2 mmol/L glucose for 3 h/day before being changed back to fresh DMEM containing 2.5 mmol/L glucose. This procedure was repeated for 3 consecutive days. On day 4, glutamate uptake was evaluated in the astrocyte cultures under baseline conditions, where the astrocytes were incubated in 2.5 mmol/L glucose medium or after a low-glucose challenge by switching to medium containing 0.2 mmol/L glucose for 90 min. To assess glutamate uptake, the astrocytes were washed twice with PBS that was warmed to 37°C for 10 min. The PBS was removed and cells incubated for 5 min in 0.5 mL fresh PBS containing 100 μ mol/L unlabeled glutamate and 0.2 μ Ci/mL 14 C-labeled glutamate for 5 min. After 5 min, the reaction was stopped by washing the cells twice in ice-cold PBS. Subsequently, the cells were solubilized in 0.5 mL 1% SDS/0.1 N NaOH solution and counted (Tri-Carb 2800TR LSC; PerkinElmer). Radioactivity count was standardized to total protein content. A second subset of cells was treated as described above (without undergoing the uptake assay) and harvested on day 4 for quantification of GLT-1 mRNA with quantitative RT-PCR.

Study 5: NMR Spectroscopy

1 H- 13 C-NMR spectroscopy of VMH extracts was used to examine the effects of acute and RH on VMH glucose metabolism and glutamate-glutamine neurotransmitter cycling. To investigate which aspects of glutamate metabolism were affected by RH, long-term catheterized rats underwent the RH treatments described above before undergoing one of the two NMR studies. For the baseline studies, a bolus-continuous infusion of 0.3 mol/L [1,6- 13 C₂]-glucose to produce steady-state plasma glucose levels was

administered through the jugular vein catheter (21), and blood samples were collected at 1, 3, 6, and 15 min after the start of the labeled glucose infusion to assess plasma levels and enrichment. After collection of the final blood sample, the animals were briefly (30 s) anesthetized with isoflurane. The label infusion was stopped, and the brain was fixed by using focused-beam microwave irradiation (\sim 4.5 kW for <1.5 s) (Model TMW-4012C; Muromachi Kikai). This method quickly arrests metabolism, allowing brain tissue to be removed for assessment of amino acids and metabolites with negligible postmortem changes (22). The VMH was then dissected out, frozen in liquid nitrogen, and stored at -80°C until extraction and NMR analysis.

For hypoglycemic glucose clamp studies, a constant-rate insulin infusion was started at 50 mU/kg/min, and plasma glucose was lowered to 45 ± 5 mg/dL with an infusion of unlabeled 20% glucose. When plasma glucose was stable at the hypoglycemic target, the unlabeled 20% glucose infusion was switched to 20% [1,6- 13 C₂]glucose infusion. The labeled glucose infusion was timed such that it reached the animal at precisely 40 min after the start of the insulin infusion. The labeled glucose was then used to maintain the clamp for the remainder of the study. From here, blood samples were collected at 41, 45, 49, and 53 min to assess plasma enrichment. After collecting the final blood sample, the brains were fixed by using microwave irradiation as described above.

NMR Spectroscopy Analysis

Ex vivo 1 H- 13 C-NMR spectroscopy of VMH extracts and plasma samples were performed as described previously (23,24).

Hormone and Microdialysate Analysis

Plasma catecholamine concentrations were analyzed by high-performance liquid chromatography by using electrochemical detection, whereas plasma hormone concentrations were determined by using commercially available radioimmunoassay kits. VMH glutamate concentrations from microdialysate samples were determined by using a photometric assay on the ISCUS^{flex} Microdialysis Analyzer (M Dialysis).

Statistical Analysis

Treatment effects were analyzed by using one- or two-way ANOVA for independent or repeated measures as appropriate followed by post hoc analysis with GraphPad Prism 6.0 software. $P < 0.05$ was set as the criterion for statistical significance.

RESULTS

Interstitial Glutamate Is Reduced by RH

During the clamp, plasma glucose and insulin concentrations were matched between the control and RH groups. Biosensor traces revealed that VMH glutamate levels increased transiently during the initial bout of hypoglycemia (Fig. 2A, left panel) before declining below baseline

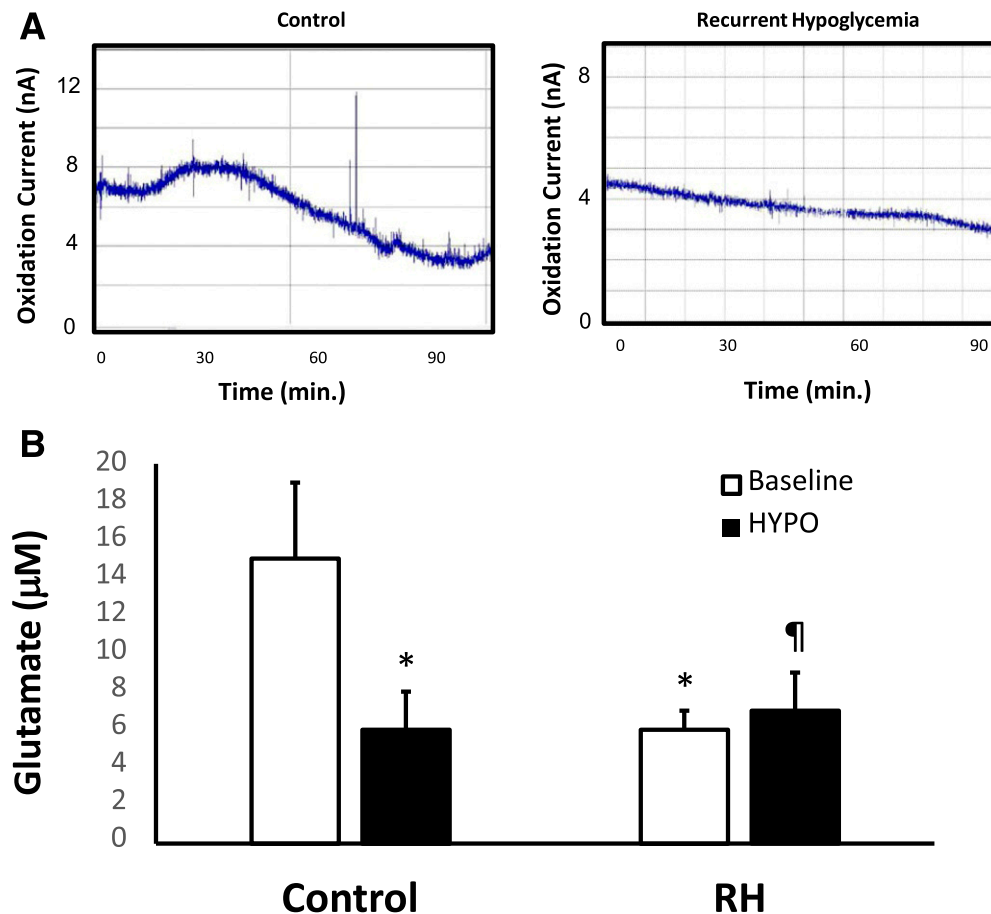


Figure 2—Biosensor and microdialysis measurements of VMH glutamate levels. *A*: Representative glutamate biosensor traces from control ($n = 4$) (left panel) and RH ($n = 4$) (right panel) animals, showing changes in VMH glutamate levels in response to a hypoglycemic clamp. RH resulted in loss of the initial rise in glutamate levels during the onset of hypoglycemia. *B*: Average extracellular glutamate concentrations in the VMH of control ($n = 10$) and RH ($n = 9$) animals measured during baseline and the final 30 min of the hypoglycemic clamp (HYPO) through microdialysis. In controls, extracellular VMH glutamate concentrations were reduced by the end of the hypoglycemic clamp. In contrast, baseline VMH glutamate levels were lower in RH rats, and these levels remained the same throughout the hypoglycemic clamp. Data are mean \pm SEM. * $P < 0.03$ vs. control baseline; ¶ $P < 0.05$ vs. control baseline.

levels. Through microdialysis, control animals showed that VMH glutamate concentrations decreased to ~50% of their baseline levels by the end of the hypoglycemic clamp (Fig. 2B). In contrast, RH resulted in a loss of the initial rise in glutamate and a small, but insignificant decline in VMH glutamate levels over the course of the clamp (Fig. 2A, right panel). Baseline extracellular glutamate concentrations in microdialysate samples were about one-half of those in the control group, and these levels remained similar by the end of the hypoglycemic challenge.

Kainic Acid-Type Receptors Mediate the Counterregulatory Response to Hypoglycemia

Neither activation nor inhibition of NMDA or AMPA receptors in the VMH affected the counterregulatory response to hypoglycemia (Tables 1 and 2). However, activation of VMH kainic acid receptors was significantly ($P < 0.05$) amplified, whereas inhibition of VMH kainic acid receptors with DNQX suppressed the hormone responses to hypoglycemia in normal, hypoglycemia-naive animals (Fig. 3).

Neuronal Glutaminase and Astroglial GLT-1 Is Reduced by RH

RH resulted in a 39% decrease in PAG expression in the VMH ($P < 0.03$) (Fig. 4A). GS levels were unaltered (Fig. 4B). GLT-1 protein levels in the VMH of RH rats were ~20% lower ($P < 0.05$) compared with controls (Fig. 5A).

Table 1—Peak plasma glucagon and epinephrine responses during the hypoglycemic clamping period after local injection of NMDA and AMPA receptor agonists and antagonists

	Glucagon (pg/mL)		Epinephrine (pg/mL)	
	Basal	Hypoglycemia	Basal	Hypoglycemia
Control ($n = 6$)	76 \pm 11	402 \pm 58	100 \pm 41	4,957 \pm 557
NMDA ($n = 6$)	92 \pm 13	543 \pm 152	102 \pm 11	4,516 \pm 486
AP5 ($n = 5$)	49 \pm 27	576 \pm 49	135 \pm 29	5,146 \pm 754
AMPA ($n = 5$)	53 \pm 3	583 \pm 118	94 \pm 18	5,847 \pm 183
YM90K ($n = 6$)	61 \pm 7	491 \pm 85	78 \pm 22	5,150 \pm 345

Data are mean \pm SEM.

Table 2—Glucose infusion rates during the final 30 min of the hypoglycemic clamp

	Glucose infusion rate (mg/kg/min)
Study 1	
Control (<i>n</i> = 10)	6.2 ± 0.8
RH (<i>n</i> = 9)	10.3 ± 0.5*
Study 2	
Control (<i>n</i> = 6)	9.1 ± 0.7
Kainic acid (<i>n</i> = 6)	7.1 ± 0.5¶
DNQX (<i>n</i> = 6)	12.2 ± 1.1*
NMDA (<i>n</i> = 6)	9.2 ± 1.4
AP5 (<i>n</i> = 5)	9.4 ± 1.9
AMPA (<i>n</i> = 5)	9.8 ± 1.3
YM90K (<i>n</i> = 6)	8.7 ± 1.2
Study 3	
Control (<i>n</i> = 7)	7.2 ± 1.1
RH (<i>n</i> = 7)	12.8 ± 1.7**
RH + GLT-1 KD (<i>n</i> = 6)	7.4 ± 0.5

Data are mean ± SEM. KD, knockdown. **P* < 0.05 vs. control; ***P* < 0.02 vs. control; ¶*P* < 0.04 vs. control.

Administration of the GLT-1 shRNA AAV to animals exposed to RH further reduced VMH GLT-1 levels by another 20% (Fig. 5A) and increased extracellular glutamate concentrations, improving the counterregulatory hormone response to hypoglycemia (Fig. 5B–D and Table 2).

Astrocytic Glutamate Uptake Is Reduced by Glucose Deprivation In Vitro

In cultured cortical astrocytes, recurrent glucose deprivation led to a significant reduction in GLT-1 mRNA expression (Fig. 6A). Although the decrease in GLT-1 expression did not affect glutamate uptake under baseline euglycemic conditions, glutamate transport was significantly impaired under low glucose conditions. During the low glucose challenge, glutamate uptake increased more than twofold in the control group, whereas no significant change was seen in the RH group (Fig. 6B).

Glucose Metabolism and Glutamate-Glutamine Cycling Is Reduced by RH

Glucose metabolism and cycling in the VMH were estimated by brief (8-min) intravenous infusions of [1,6-¹³C₂]glucose in separate groups of RH and control animals at baseline euglycemia and at the end (last 8 min) of the hypoglycemic clamp. No significant differences were seen in blood plasma (concentration and ¹³C enrichment) and the total (¹³C + ¹²C) concentrations of glutamate and glutamine in the VMH between these groups at euglycemia (glutamate *P* > 0.2, glutamine *P* > 0.3) or hypoglycemia (glutamate *P* > 0.8, glutamine *P* > 0.5). The baseline [4-¹³C]glutamate concentration, which is primarily of neuronal origin and is a measure of TCA flux (21), was significantly lower in the VMH of RH animals (Fig. 7A) compared with controls (*P* < 0.01). In contrast, [4-¹³C]glutamine concentration, which is produced in astrocytes and reflects both neurotransmitter cycling and glial metabolism, was similar

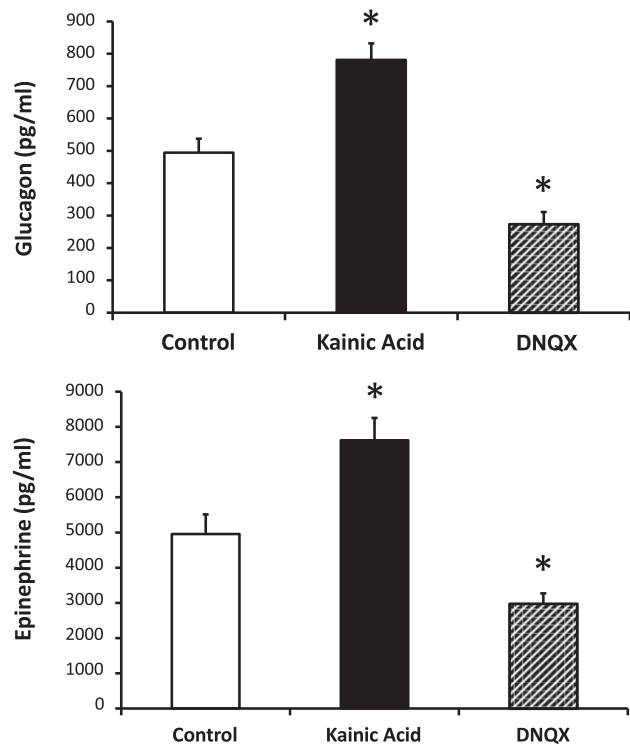


Figure 3—Counterregulatory hormone responses to a hypoglycemic clamp. Peak plasma glucagon (top panel) and epinephrine (bottom panel) responses from hypoglycemia-naïve animals microinjected with aECF (controls, *n* = 6), kainic acid (*n* = 6), or DNQX (*n* = 6) during the hypoglycemic clamp. Data are mean ± SEM. **P* < 0.05 vs. control.

between the two groups under baseline conditions (Fig. 7B). In response to hypoglycemia, VMH [4-¹³C]glutamate decreased significantly (*P* < 0.001) in both control and RH animals, but the decrease in the RH group was much less (Fig. 7A). Hypoglycemia led to a similar decline in [4-¹³C]glutamine concentration in controls (*P* < 0.01), whereas no significant change was observed in RH animals. From these changes, we infer that glutamatergic neuronal metabolic activity is lower in the VMH of RH animals at baseline, and their metabolic response to hypoglycemia is markedly blunted to nil. The relative maintenance of labeled glutamine in astrocytes suggests that glial metabolism is less affected (or possibly augmented) by RH.

DISCUSSION

VMH glutamatergic neurotransmission has been shown to be important for enhancing counterregulatory responses to hypoglycemia (14). However, the postsynaptic receptors that mediate these stimulatory effects and how RH affects glutamate neurotransmission have not been investigated in detail. We demonstrate for the first time in our knowledge that RH leads to significant alterations in astrocytic and neuronal metabolism of glutamate within the VMH, which reduces glutamatergic neurotransmission and impairs glucose counterregulatory mechanisms.

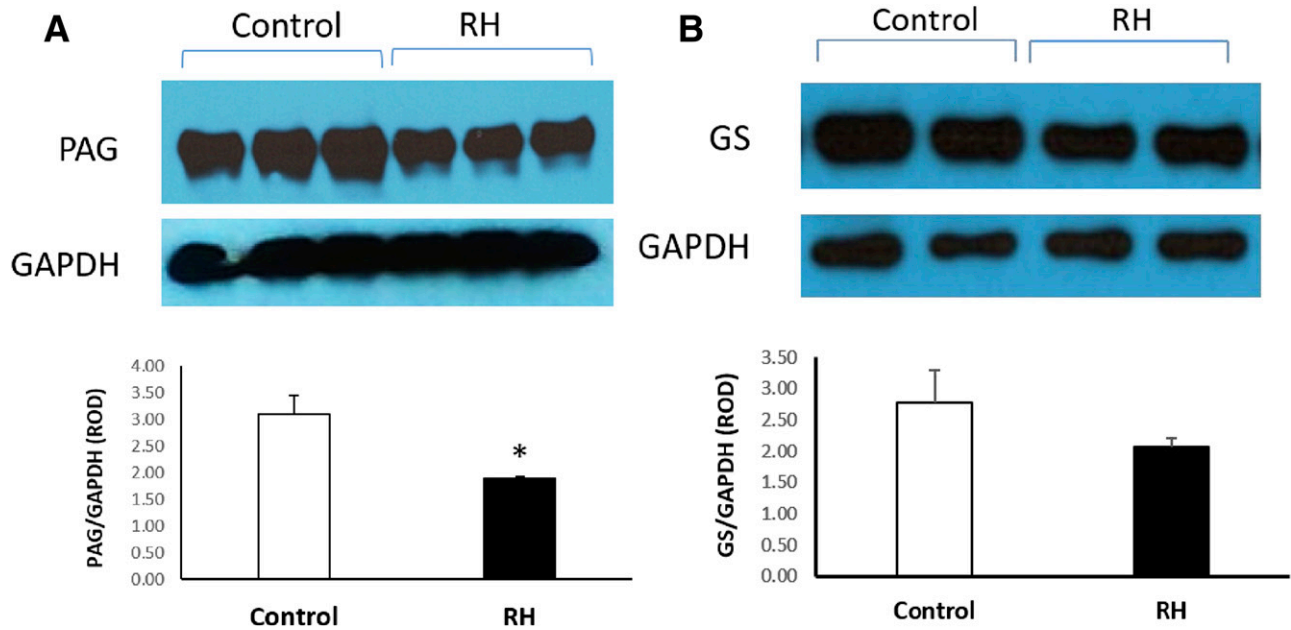


Figure 4—Protein quantification of glutamate synthetic enzymes in the VMH. Representative immunoblots and quantitative densitometric analysis showing PAG (A) and GS (B) protein levels in the VMH of control ($n = 7$) and RH ($n = 7$) animals standardized to the corresponding GAPDH loading control. RH resulted in a significant reduction in PAG protein levels in the VMH but no change in GS levels. Values are expressed as the ratio of the protein of interest/GAPDH expressed as mean relative optical density (ROD) \pm SEM. * $P < 0.03$ vs. control.

The first set of studies examined the extent to which glutamate responses in the VMH are affected by RH. Biosensors that monitor real-time changes in VMH extracellular glutamate levels revealed that glutamate rises transiently as plasma glucose levels begin to fall, reaching peak levels at the start of the hypoglycemic nadir before falling below baseline levels toward the end of the clamp. By using microdialysis to quantify extracellular glutamate concentrations, we confirmed that glutamate concentrations decline to 50% of the baseline concentrations by the end of the clamp. The rise in glutamate is consistent with the observation that VMH glutamate release is an important activator of the counterregulatory hormone responses to hypoglycemia (14). After demonstrating that glutamate levels change within the VMH, we identified the subclass of glutamatergic receptors through which the stimulatory effects are facilitated. Glutamate acts through both metabotropic and ionotropic subtypes, and we focused on the VMH ionotropic receptors because they influence glucose homeostatic mechanisms (13,25). Pharmacological activation of kainic acid receptors enhanced (while inactivation suppressed) the counterregulatory responses, whereas modulation of VMH AMPA or NMDA receptors had no effect. Thus, kainic acid receptors likely are the predominant subclass of glutamate receptors in the VMH that stimulate the counterregulatory responses. This finding is consistent with previous reports that showed that activation of VMH kainic acid receptors enhances sympathetic drive to endocrine and neuroendocrine systems, helping to raise plasma glucose concentrations (13,26–29). Although the agonists used in the current study are selective

for their respective targets, some overlap exists in the selectivity of the AMPA and kainic acid receptor antagonists. At the concentrations used, YM90K has a 22- to 37-fold greater affinity for AMPA than either kainic acid or NMDA receptors (30). In contrast, DNQX is a nonselective AMPA/kainic acid receptor antagonist. That we observed an inhibitory effect on the hormone responses with DNQX but not with the more selective YM90K suggests that kainic acid receptors are the predominant receptors mediating the effects of glutamate in the VMH. Together, these findings indicate that the hypoglycemia-induced transient surge in VMH synaptic glutamate, as mediated by kainic acid receptors, is required for proper activation of the counterregulatory responses to hypoglycemia. Rapid sequestration of released glutamate may underlie the decrease in extracellular glutamate observed in the biosensor traces and lower microdialysate concentrations at the end of the hypoglycemic clamp.

In contrast to control animals, baseline interstitial VMH glutamate concentrations were \sim 50% lower in RH animals. Furthermore, the early rise and fall in glutamate levels observed in controls during hypoglycemia were absent in RH animals. Microdialysis measurements confirmed that interstitial glutamate concentrations in the RH animals were similar at baseline and during the hypoglycemic clamp. Moreover, RH animals showed lower VMH metabolic activity at baseline than controls, as demonstrated by the lower isotopic enrichment of amino acids and metabolites from ^{13}C -labeled glucose. The reduced ^{13}C labeling in the RH animals reflects lower rates of glucose oxidation in the TCA

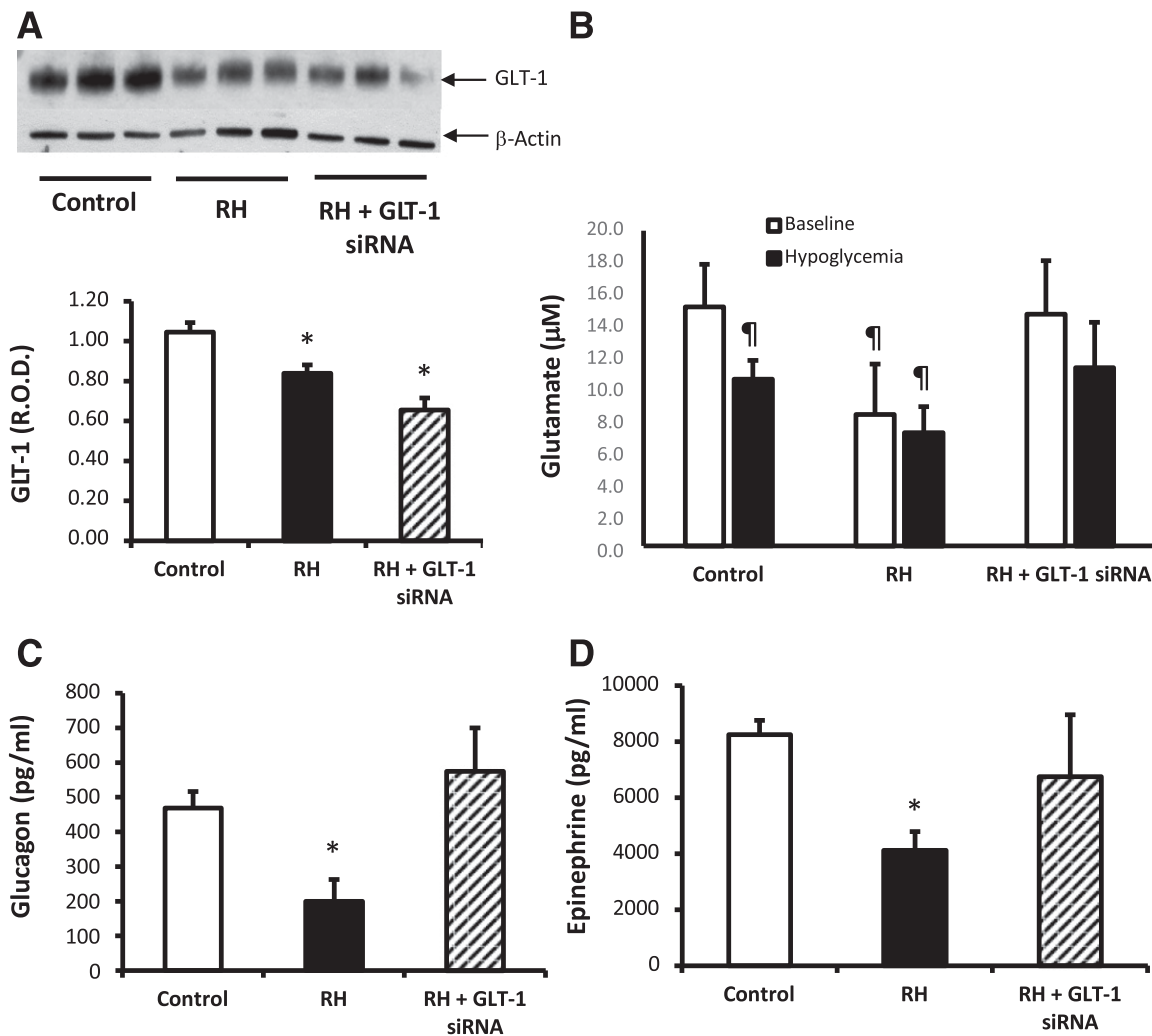


Figure 5—VMH GLT-1 knockdown studies. *A*: GLT-1 protein levels in the VMH of control ($n = 7$), RH ($n = 7$), and RH animals that received the GLT-1 small interfering RNA (RH + GLT-1 siRNA) ($n = 6$) standardized to β -actin. *B*: Extracellular glutamate concentrations in the VMH of control, RH, and RH + GLT-1 siRNA under baseline (white bars) and hypoglycemic clamp (black bars) conditions. *C* and *D*: Peak plasma glucagon and epinephrine concentrations of control, RH, and RH + GLT-1 siRNA during the hypoglycemic clamp. Further reducing GLT-1 protein levels in the VMH of RH animals increased VMH extracellular glutamate and improved the counterregulatory hormone responses to hypoglycemia. Data are mean \pm SEM. * $P < 0.05$ vs. control; ¶ $P < 0.05$ vs. control. R.O.D., relative optical density.

of VMH glutamatergic neurons and glutamate-glutamine neurotransmitter cycling. Taken together, the findings of lower baseline (and hypoglycemia-stimulated) interstitial glutamate and lower neuronal metabolic activity and glutamate-glutamine cycling in the VMH strongly support the conclusion that antecedent hypoglycemia reduces VMH glutamatergic neurotransmission. The observation that extracellular glutamate concentrations remained relatively constant in RH animals throughout hypoglycemia, in contrast to the transient glutamate surge seen in the controls, suggests that RH impairs the activation of VMH glutamatergic neurons and/or neurotransmitter glutamate resynthesis. The alternate possibility that the lower interstitial glutamate in the VMH of RH animals resulted from increased glutamate clearance into astrocytes can be discounted because GLT-1, the major astroglial glutamate transporter in the hypothalamus in vivo (31), was

substantially lower in RH animals, and glutamate uptake in cultured astrocytes was reduced—not increased—by repeated bouts of glucose deprivation in vitro. These observations suggest that the lower GLT-1 transporter concentration may be an adaptive response to lower synaptic glutamate levels and glutamatergic activity in RH animals. Indeed, when GLT-1 protein was further reduced by shRNA, interstitial glutamate was increased, indicating that GLT-1 expression levels were sufficient to maintain pre-existing neuronal glutamate release rates and homeostasis at the synapse. Of note, we found that further reducing astrocytic glutamate uptake in the RH group by 20% raises extracellular glutamate and restores counterregulation, suggesting that functionality of VMH glutamate neurotransmission under these conditions may be critically influenced by astroglia. GLT-1 is also expressed on neurons, and although we cannot fully exclude the contribution of the

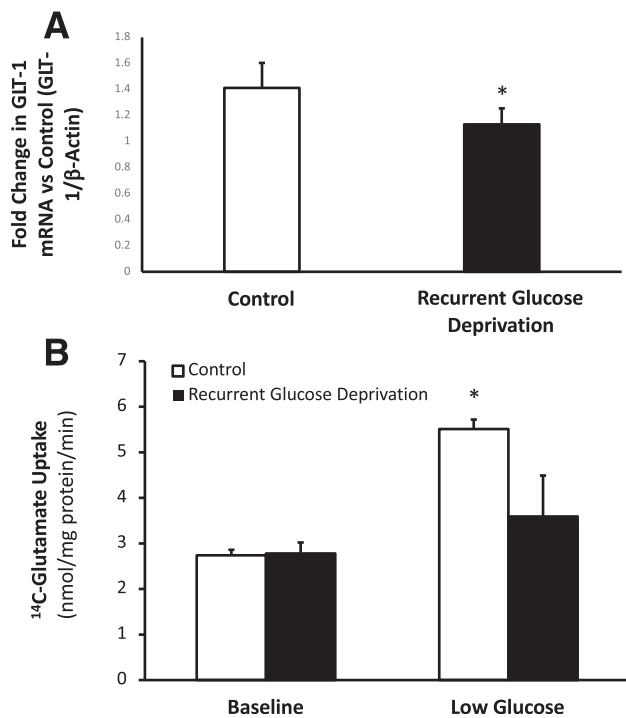


Figure 6—Glutamate uptake in primary rat astrocyte cultures. *A*: GLT-1 mRNA expression in rat cortical astrocytes cultured ($n = 3$ –4/treatment) in 2.5 mmol/L glucose (white bars) or exposed to repeated bouts of low-glucose (0.2 mmol/L) medium for 3 h/day for 3 consecutive days (black bars). *B*: Uptake of ^{14}C -labeled glutamate in rat cortical astrocyte cultures under baseline conditions (2.5 mmol/L glucose medium) and after a low-glucose challenge (0.2 mmol/L glucose medium). Recurrent glucose deprivation decreased GLT-1 mRNA expression in cultured rat astrocytes, which reduced glutamate during a low-glucose challenge but not under normal glucose conditions. Data are mean \pm SEM. * $P < 0.05$ vs. control (baseline).

neuronal transporters toward the observed effects on VMH glutamate levels, we saw major improvements in glutamate neurotransmission and the counterregulatory responses with astrocyte-specific knockdown of GLT-1, which suggests that the astrocyte transporter is likely the more important contributor. We note that GLAST (glutamate/aspartate transporter), the other major astroglial glutamate transporter, was not measured in the current study. However, any potential compensatory increase in GLAST with GLT-1 reduction, as seen in astrocytes of the hypothalamus in response to heart failure (32), was insufficient to prevent the rise in interstitial glutamate.

In the RH animals, we found that PAG, which regenerates neurotransmitter glutamate from glutamine, was significantly reduced. Reducing PAG activity pharmacologically reduces neuronal glutamate concentration and ultimately vesicular release (33), whereas knockdown of *GLS1* (the gene for PAG synthesis), which decreased whole-brain PAG activity in heterozygous mice by $\sim 50\%$, led to modest (13–16%) reductions in glutamate levels in the frontal cortex and hippocampus and increased glutamine in these regions and hypometabolism by functional imaging of regional cerebral blood volume (34).

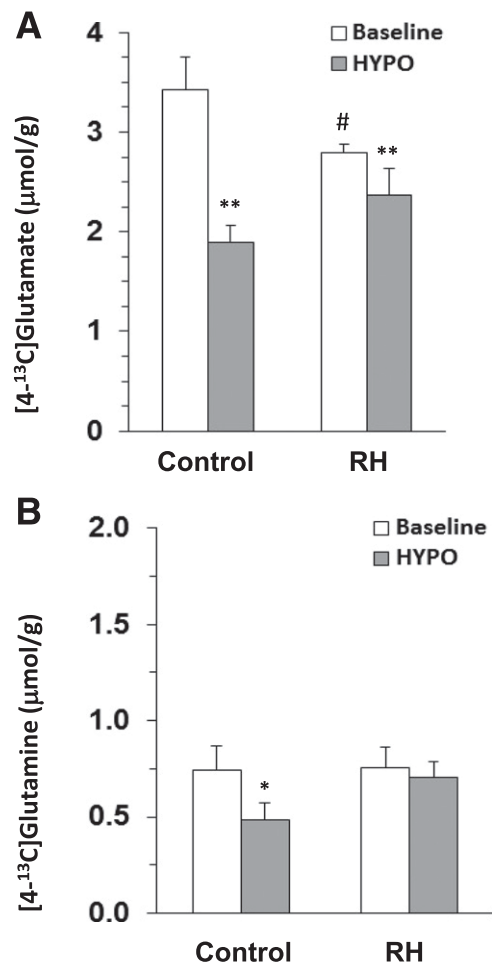


Figure 7— ^{13}C effects of acute hypoglycemia and RH on glutamate release and recycling into glia (glutamate cycling) and neuronal and glial energy metabolism in the VMH of the rat after $[1,6\text{-}^{13}\text{C}_2]$ glucose infusions of 15 min. *A*: $[4\text{-}^{13}\text{C}]$ glutamate concentration of baseline and hypoglycemia (HYPO). *B*: $[4\text{-}^{13}\text{C}]$ glutamine concentration of baseline and HYPO. ^{13}C concentrations were determined ex vivo in the tissue extract by using $^1\text{H}\text{-}[^{13}\text{C}]$ -NMR spectroscopy. Concentrations were normalized to the plasma glucose-C1 enrichment of individual animals for each group. Data are mean \pm SD ($n = 6$ /group). * $P < 0.01$ vs. control baseline; ** $P < 0.001$ vs. baseline; # $P < 0.01$ vs. control baseline.

Electrophysiological recordings in hippocampal slices from these mice (34) revealed reduced excitatory neurotransmission, and relative cerebral blood volume functional imaging showed blunted frontal cortical responses to low-dose ketamine compared with wild-type mice, which has been shown to stimulate glutamate release (35), glutamatergic metabolism, and glutamate-glutamine cycling (21,36). Of note, although VMH PAG protein in RH animals was $\sim 40\%$ of that of controls in the current study, VMH tissue levels of glutamate (and glutamine) were similar to control levels, suggesting that glutamate release and resynthesis were balanced at the lower metabolic rate at least after 3 days of RH. Whether the reduction in PAG activity in RH animals leads to reduced glutamatergic function at baseline, prevents the surge in glutamate release seen in

hypoglycemia-naïve controls during hypoglycemia, or is an adaptive response to RH is presently unknown. Future studies (e.g., overexpressing PAG in RH animals, use of pharmacological PAG activators) are needed to address the specific role of PAG in this context.

In conclusion, we show that a surge in synaptic glutamate, acting through kainic acid receptors in the VMH, is necessary to optimize the counterregulatory hormone response to hypoglycemia. However, RH compromises VMH glutamatergic neurotransmission by reducing the capacity for glutamate resynthesis from glutamine in neurons (PAG reduction) which ultimately reduces neurotransmitter glutamate release. In addition to the changes in neurons, RH led to significant reductions in the astroglial transporter GLT-1, which may be an adaptation to reduced interstitial glutamate levels. Taken together, the findings indicate that reduced VMH glutamatergic neurotransmission contributes to the development of defective counterregulation in RH animals. Treatments that normalize interstitial glutamate levels by augmenting neuronal PAG or enhancing glutamatergic function could be of potential therapeutic value. Although these studies identified defects in presynaptic glutamatergic neurons, we have not identified the origins of these neurons or the postsynaptic targets of VMH kainic acid receptors, which will be the focus of future studies.

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