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Role of Synaptic Plasticity and EphA5-EphrinA5 Interaction Within the Ventromedial Hypothalamus in Response to Recurrent Hypoglycemia

COMPLICATIONS

Hypoglycemia stimulates counterregulatory hormone release to restore euglycemia. This protective response is diminished by recurrent hypoglycemia, limiting the benefits of intensive insulin treatment in patients with diabetes. We previously reported that EphA5 receptor-ephrinA5 interactions within the ventromedial hypothalamus (VMH) influence counterregulatory hormone responses during acute hypoglycemia in nondiabetic rats. In this study, we examined whether recurrent hypoglycemia alters the capacity of the ephrinA5 ligand to activate VMH EphA5 receptors, and if so, whether these changes could contribute to pathogenesis of defective glucose counterregulation in response to a standard hypoglycemic stimulus. The expression of ephrinA5, but not EphA5 receptors within the VMH, was reduced by antecedent recurrent hypoglycemia. In addition, the number of synaptic connections was increased and astroglial synaptic coverage was reduced. Activation of VMH EphA5 receptors via targeted microinjection of ephrinA5-Fc before a hyperinsulinemic hypoglycemic clamp study caused a reduction in the glucose infusion rate in nondiabetic rats exposed to recurrent hypoglycemia. The increase in the counterregulatory response to insulin-induced hypoglycemia was associated with a 150% increase in glucagon release ($P < 0.001$). These data suggest that changes in ephrinA5/EphA5 interactions and synaptic plasticity within the VMH, a key glucose-sensing region in the brain, may contribute to the impairment in glucagon secretion and counterregulatory responses caused by recurrent hypoglycemia.

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Frequent episodes of acute hypoglycemia represent the principal obstacle to achieving optimal glycemic control during insulin treatment in patients with type 1 diabetes and long-standing type 2 diabetes (1,2). This problem is further magnified by the loss of an appropriate counterregulatory response to hypoglycemia that results as a consequence of frequent episodes iatrogenic insulin-induced hypoglycemia (3,4). The molecular mechanisms underlying this phenomenon remain uncertain but are likely to involve the key brain glucose–sensing region, the ventromedial hypothalamus (VMH) (5,6).

We have previously reported that local stimulation of VMH EphA5 receptors by microinjection of ephrinA5-Fc or ephrinA5 overexpression increased, whereas knockdown of VMH ephrinA5 reduced, counterregulatory

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responses to hypoglycemia. Furthermore, overexpression of VMH ephrinA5 transiently increased local glutamate concentrations, whereas ephrinA5 knockdown produced profound suppression of VMH interstitial fluid glutamine concentrations in the basal state and during hypoglycemia. These data suggest that the activation of VMH EphA5 receptors by ephrinA5 may act in concert with β-cell Eph receptor forward signaling to restore glucose homeostasis during acute hypoglycemia via alterations in glutamate/glutamine cycling (7,8).

Within the central nervous system, Eph receptors and their ligands, the ephrins, play a key role in cell-to-cell communication as well as in synaptic structure and function. Eph receptors function as transmembrane receptor tyrosine kinases and are divided by sequence similarity and ligand affinity into an A and a B subclass. Their ligands, the ephrins, are also divided into an A and a B subclass: the A subclass is tethered to the cell membrane by a glycosylphosphatidylinositol anchor, and members of the B subclass have a transmembrane domain and as a short cytoplasmic region. For the most part, A-type receptors bind to most or all A-type ligands, and B-type receptors bind to most or all B-type ligands (9). As for many other receptor tyrosine kinases, ligand-binding induces "forward signaling," mostly through phosphotyrosine-mediated pathways. However, ephrins can also signal into their host cell—referred to as "reverse signaling'"(10). Eph receptors and their ephrin ligands are present in the adult brain and are specifically enriched in glutamate excitatory synapses (11). Moreover, Eph receptor tyrosine kinases are mainly expressed in synaptic terminals where they influence synaptic plasticity via binding to ephrins found on astrocytic processes that surround the synapse or on neuronal synapses (12,13).

Several observations suggest that changes in hypothalamic synaptic plasticity may play a significant role in the regulation of energy balance. For example, peripheral signals, such as leptin, ghrelin, and estrogen, induce synaptic adaptations that serve as dynamic regulators of neuronal activity in the arcuate nucleus, the hypothalamic center for feeding control (14,15). Whether hypoglycemia per se induces local changes in the VMH affecting both neuronal synapses and/or surrounding glia cells is unknown, but such alterations could potentially modulate neurotransmission within VMH, thereby altering brain glucose sensing. This study tests whether recurrent hypoglycemia alters EphA5 receptor–ephrinA5 interactions within the VMH, which might contribute to diminished activation of counterregulatory responses to acute hypoglycemia.

RESEARCH DESIGN AND METHODS

Animals

Male Sprague-Dawley rats (Charles River, Wilmington, MA) weighing 300–350 g were individually housed in the Yale Animal Resource Center in temperature- (22–23°C) and humidity-controlled rooms. Animals were fed rat chow (Prolab 3000; Agway, Syracuse, NY) and water ad libitum and were acclimatized to a 12-h light-dark cycle. The Yale University Institutional Animal Care and Use Committee approved the experimental protocols. Different sets of animals were used for the clamp and electron microscopy (EM) experiments described below.

Vascular and Stereotaxic Surgery

Animals were anesthetized ~7–10 days before study and underwent aseptic surgery in which vascular catheters were implanted into the left carotid artery for blood sampling and into the right jugular vein for infusion, as previously described (8). These catheters were tunneled subcutaneously and exteriorized at the back of the neck between the scapulae. Wound staples were used to close the incision. For stereotaxic surgery, animals were placed into a stereotaxic frame (David Kopf Instruments, Tujunga, CA), and stainless steel guide cannulas (Plastics One, Roanoke, VA) were inserted into the brain and secured in place with screws and dental acrylic (coordinates from Bregma: anteroposterior −2.6 mm, mediolateral ± 0.8 mm, and dorsoventral −8.0) for microinjection.

Recurrent Hypoglycemia Protocol

Rats received intraperitoneal injections of insulin (10 units/kg) for 3 consecutive days. After each injection, food was withheld to allow plasma glucose to fall into the hypoglycemic range (30–50 mg/dL); throughout, animals were monitored with tail vein glucose measurements every 30 min using the AlphaTRAK rodent glucometer (Abbott Animal Health, Chicago, IL) to ensure sustained hypoglycemia and to avoid glucose reduction sufficient to cause seizure activity. At the end of this period, the rats were given free access to food again. Control rats received an injection of 0.9% saline under the same conditions.

Microinjection of EphrinA5-Fc or Control-Fc

On the morning of the study, awake, overnight-fasted rats were connected to infusion pumps ~90 min before the start of the experiment and then left undisturbed to recover from handling stress. After the recovery period, 22-gauge microinjection needles (Plastics One), designed to extend 1 mm beyond the tip of the guide cannula, were inserted bilaterally through the guide cannula into each VMH region. Rats then received a microinjection of recombinant human ephrinA5-Fc or recombinant human IgG1-Fc (control-Fc protein; both R&D Systems, Minneapolis, MN; catalog number: 374-EA-200) in a concentration of 0.3 μg/μL dissolved in artificial extracellular fluid delivered at a rate of (0.1 μL/min) over 60 min (dose: 1.8 μg for each side). After the microinjection, needles were left in place for 30 min before being removed. Immediately thereafter, a hyperinsulinemic hypoglycemic clamp study was performed. These compounds have been previously administered into the central nervous system in in vivo studies (16) and in

vitro in brain slices (17), without adverse effects. In addition, ephrinA5-Fc has been shown to specifically bind EphA5 receptors (18).

Hyperinsulinemic-Hypoglycemic Clamp

A primed continuous infusion of 20 mU · kg^{-1} · min^{-1} insulin (Humulin R; Eli Lilly, Indianapolis, IN) was given, and a variable infusion of 20% dextrose was adjusted at 5- to 10-min intervals based on glucose measurements (Analox Instruments, Lunenburg, MA) designed to maintain plasma glucose at 50 mg/dL from 30 to 90 min (19). Additional blood was drawn at baseline and at 30, 60, and 90 min for measurement of insulin, glucagon, epinephrine, and norepinephrine. Rats were killed at study termination, and the probe position was confirmed histologically.

Hormone and Neurotransmitter Analyses

Plasma catecholamine measurements were analyzed by high-performance liquid chromatography using electrochemical detection, and plasma insulin and glucagon concentrations were determined by radioimmunoassay (Linco, St. Charles, MO).

Immunoblot Analysis

Frozen tissue micropunches from VMH and control regions were homogenized in buffer containing 1% NP40, 150 mmol/L NaCl, 50 mmol/L Tris (pH 7.4), 1 mmol/L Na_3VO_4, 1 mmol/L phenylmethylsulfonyl fluoride, and protease inhibitor (Roche Diagnostics) using a plastic pestle and ultrasonicator. Protein content was assessed with the Bradford protein assay. Protein samples were fractioned under reducing conditions on SDS-9% PAGE (Bio-Rad). After electrophoresis, proteins were electroblotted onto nitrocellulose membranes, blocked with 5% nonfat dry milk in PBS, probed with first antibody (α-tubulin; Cell Signaling, cat. 2125S; ephrin-A5; R&D Systems, cat. AF3743) and EpA5 receptor (EphA5; Sigma-Aldrich, cat. P8651), and incubated with the appropriate secondary antibody conjugated to peroxidase by horseradish peroxidase–linked protein A (1:2000; Sigma-Aldrich). The immunoblots were developed using an enhanced chemiluminescence detection system (Amersham Biosciences).

Electron Microscopy

Briefly, animals were perfused with paraformaldehyde fixative and ultrathin brain sections were cut on a Leica ultramicrotome, collected on Formvar-coated single-slot grids, and analyzed with a Tecnai 12 Biotwin EM (FEI). Glia coverage of the cell membrane of random VMH cells was performed using ImageJ software. EM photographs (original magnification ×11,500) were used to first measure the perimeter of each VMH neuron analyzed, followed by determination of the amount of membrane covered by glia (in nanometers). Results are reported as glia coverage/perimeter of the VMH neurons.

Characteristics of synaptic contacts were defined, as previously described (20). They were collected from serial sections of the cell membrane of random VMH neurons. Synapse characterization was performed at original magnification ×20,000, and quantitative measurements were performed at original magnification ×11,500. Results are reported as synapses number/perimeter of the VMH neurons (21).

Statistics

Data are expressed as the means ± SEM. Analysis was performed by one-way ANOVA or the Student *t* test, as appropriate. Statistical analysis was then performed by two-way ANOVA for repeated measures, followed by post hoc analysis using GraphPad Prism 4.0 software (GraphPad Software, Inc., San Diego, CA). $P < 0.05$ was considered statistically significant.

RESULTS

Recurrent Hypoglycemia and EphrinA5 Expression

As shown in Fig. 1, rats exposed to recurrent hypoglycemia for 3 days exhibit a 25% reduction in ephrinA5 expression in the VMH (Fig. 1*A* and *B*). In contrast, no significant change in the expression of the EphA5 receptor in the VMH was detected (Fig. 1*C* and *D*).

Stimulation of VMH EphA5 Receptors in Rats Exposed to Recurrent Hypoglycemia

To assess the biological consequences of the reduction of ephrinA5 expression, we microinjected the Eph receptor agonist, ephrinA5-Fc, into the VMH before conducting a hyperinsulinemic-hypoglycemic clamp study in rats previously exposed to three episodes of insulin-induced hypoglycemia. Schematic representation of the experimental protocol is presented in Fig. 2*A*. Body weight and plasma levels of glucose, insulin, glucagon, epinephrine, and norepinephrine were indistinguishable at baseline and immediately after completion of the VMH microinjection of ephrinA5-Fc or control-Fc (Table 1). Subsequently, during the hypoglycemic clamp study, plasma glucose (Fig. 2*B*) and insulin (Fig. 2*C*) were indistinguishable between the two groups. EphrinA5-Fc delivery, however, significantly reduced within 15 min the glucose infusion rate required to maintain hypoglycemia (Fig. 2*D*). This was accompanied by a rapid 150% ($P < 0.001$) increase in glucagon release (Fig. 2*E*). As was observed in our previous study (8) in rats not exposed to antecedent hypoglycemia, neither plasma epinephrine (Fig. 2*F*) nor norepinephrine (Fig. 2*G*) responses to hypoglycemia were significantly altered by VMH delivery of ephrinA5-Fc compared with the control-Fc microinjection.

Effect of Recurrent Hypoglycemia on Glia Ensheetment and Synaptic Input Organization

Next, we assessed if recurrent hypoglycemia affected the VMH synaptic organization and glia ensheetment in rats exposed for 3 days to recurrent hypoglycemia compared with controls, a model we have previously shown suppresses hypoglycemic counterregulation (22). Figure 3*A* and *B* compares representative EMs of glia ensheetment

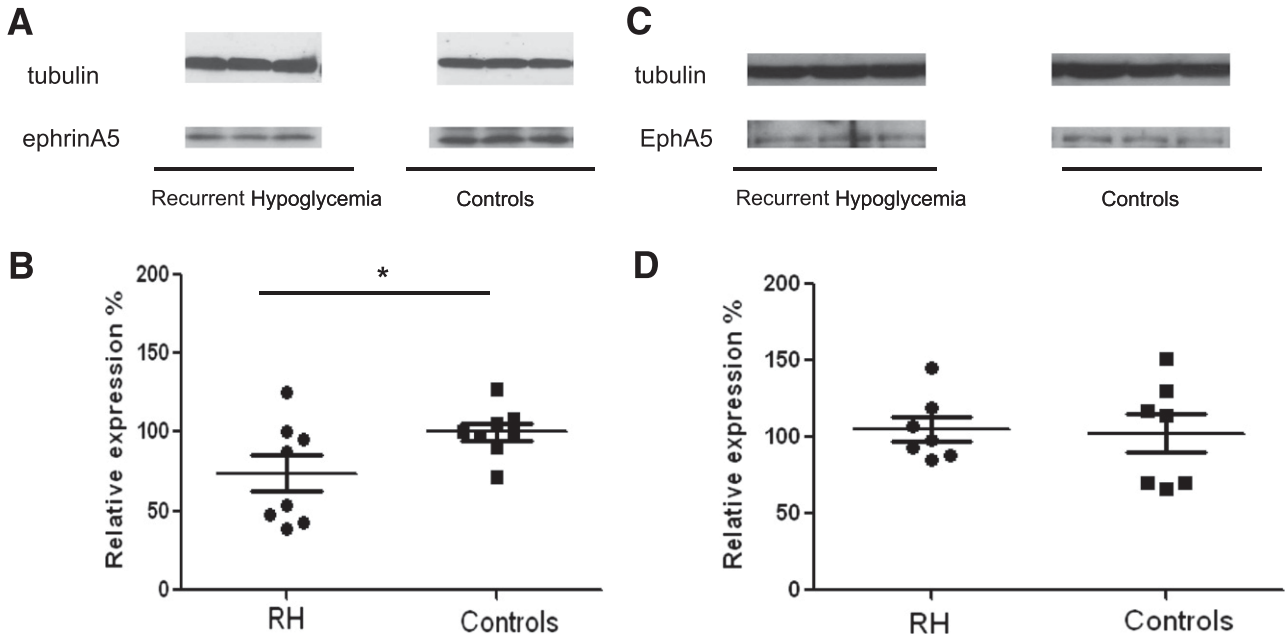

Figure 1—Effect of recurrent hypoglycemia (RH) on ephrinA5 and EphA5 expression. *A*: Representative data showing expression level of ephrinA5 in the VMH in rats after ~3 days of RH as determined by Western blot analysis. Tubulin served as the loading control. *B*: Relative VMH expression of ephrinA5 in control (n = 8) and RH (n = 8) rats. Data are presented as means ± SEM. Statistical analysis by Student t test: *P < 0.05 vs. control. *C*: Representative data showing expression level of the EphA5 in VMH in rats after ~3 days of RH as determined by Western blot analysis. Tubulin served as the loading control. *D*: Relative VMH expression of EphA5 in control (n = 7) and RH (n = 7) rats. Data are presented as means ± SEM. Statistical analysis by Student t test.

in random VMH neuron perikarya in control and recurrent hypoglycemic rats. The rats exposed to recurrent hypoglycemia exhibited reduced glial coverage of neurons ($P < 0.001$) (Fig. 3*C*) and, as a result, more total synaptic contacts in random VMH neurons ($P < 0.05$) (Fig. 3*D*).

DISCUSSION

The current study demonstrates that exposure of nondiabetic rats to recurrent hypoglycemia for 3 consecutive days diminishes ephrinA5 expression within the VMH in association with reduced glial coverage of VMH neurons and synaptic remodeling. In addition, targeted VMH delivery of the EphA5 receptor ligand ephrinA5-Fc enhances glucose counterregulation and glucagon release in rats exposed to recurrent hypoglycemia. These findings are consistent with the possibility that recurrent hypoglycemia diminishes EphA5 receptor forward signaling in the VMH, which in turn reduces the magnitude of glucagon secretion.

Signaling via the EphA/ephrinA receptor system has been reported to regulate neuron-astrocyte interactions that cause rapid changes in synaptic structural and functional plasticity (17,23). It has been proposed that the loss of ephrinA alters astrocytic-neuronal contacts (24), whereas application of ephrinA3-Fc or endogenous ephrin induces rapid growth of the astrocytes processes and growth of new filopodia (17). In addition, activation of EphA4 by ephrinA3 has been shown to induce spine retraction (25). The current finding that recurrent hypoglycemia decreases ephrinA5 expression and produces diminished glia coverage and more synaptic connections within the VMH raises the question of a possible relationship. The fact that bypassing ephrinA5 using a targeted VMH ephrinA5-Fc microinjection can increase counterregulatory responses in animals exposed to recurrent hypoglycemia is in keeping with the hypothesis that reduced VMH ephrinA5 expression might induce alterations in VMH synaptic plasticity that in turn contribute to the development of disordered glucose counterregulation. However, a direct link between the observed changes in ephrinA5 and in glia coverage as well as synaptic connection rearrangements remains to be established in future studies.

Previous studies have reported rapid changes in synaptic network connectivity and glia morphology in the VMH in response to alterations in energy substrate bioavailability (15,26–30). Acute hypoglycemia has been shown to alter synaptophysin expression, findings consistent with a rapid alternation in synaptic morphology (31,32), whereas insulin-deficient diabetic rats display a decrease in the number of hypothalamic astrocytes as a consequence of increased death and decreased proliferation (33). Given that diabetes and recurrent glucose deprivation are both accompanied by impaired counterregulation (22), these observations are consistent with the hypothesis that synaptic connectivity and the

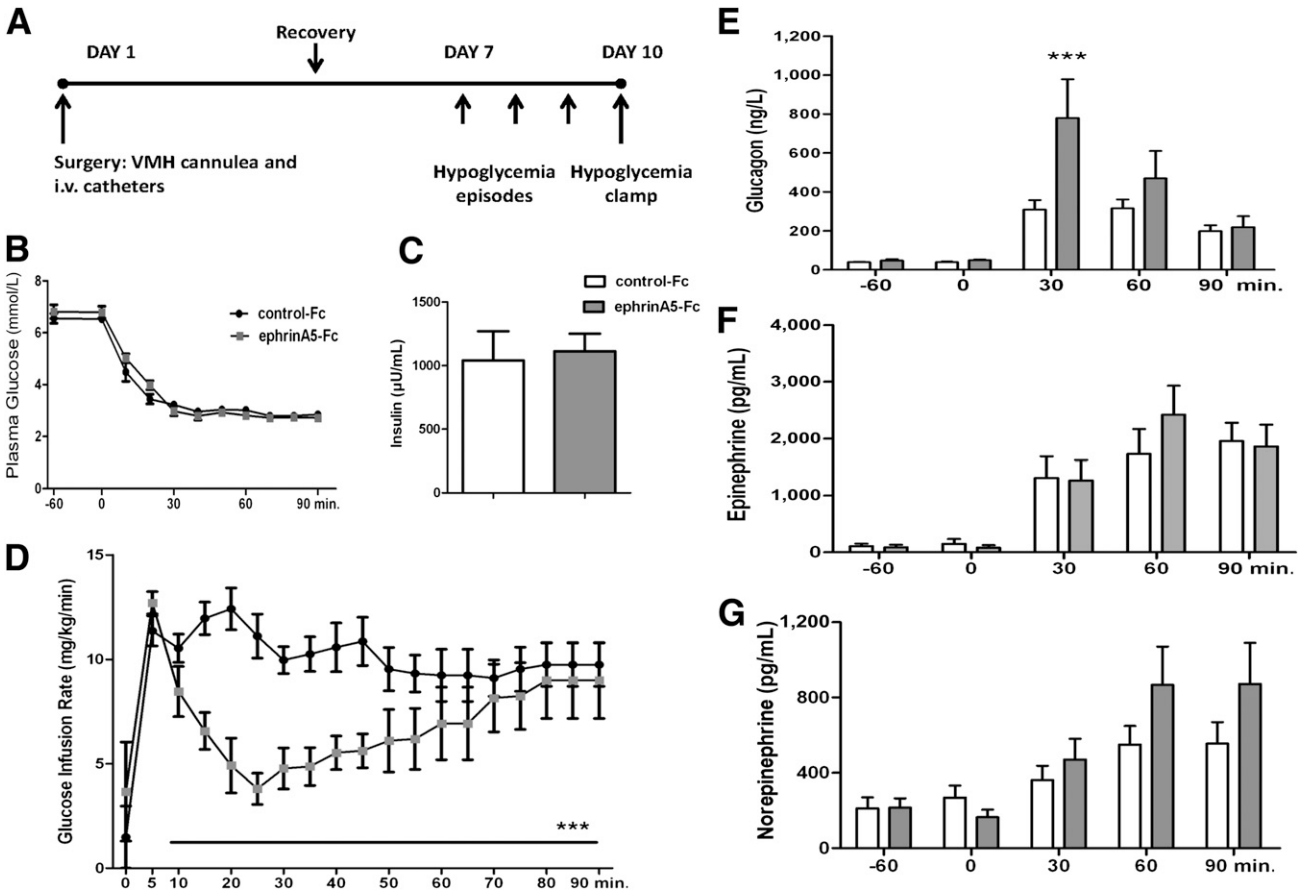

Figure 2—Effect of acute stimulation of VMH EphA5 receptors with ephrinA5-Fc on glucose counterregulation in rats exposed to recurrent hypoglycemia. The components are a schematic representation of the experimental protocol (*A*); plasma glucose (*B*); mean plasma insulin from 30, 60, 90 min time points (*C*); glucose infusion rate (*D*); plasma glucagon (*E*); plasma epinephrine (*F*); and plasma norepinephrine (*G*) during the hypoglycemic clamp study (n = 7 for ephrinA5-Fc and n = 9 for control-Fc). Data are presented as means ± SEM. Statistical analysis used mixed-model ANOVA with the Bonferroni post hoc test: ***$P < 0.001$ vs. control-Fc. i.v., intravenous.

function of glia in the VMH play a significant role in supporting the neurotransmission required for proper counterregulatory responses to acute hypoglycemia.

It is noteworthy that the principal effect of VMH microinjection of the ephrinA5 ligand on hypoglycemia-induced counterregulatory hormone release was on glucagon, whereas recurrent hypoglycemia normally leads to a suppression of glucagon and epinephrine levels in nondiabetic animals. These findings are consistent with previous studies in mice showing that knockdown of VGLUT2 (vesicular glutamate transporter) selectively in SF1 VMH neurons predominately inhibited the secretion of glucagon in response to acute hypoglycemia (34). It should be noted, however, that alterations in EphA receptor/ephrinA signaling appear to influence epinephrine responses as well. Using a targeted gene expression manipulation approach to chronically alter VMH ephrinA5 expression, we observed that overexpression of ephrinA5 in the VMH stimulates, whereas targeted VMH knockdown of ephrinA5 inhibits, epinephrine as well as glucagon responses to acute hypoglycemia. These effects also appear to be mediated by alterations in glutamate–glutamine cycling (8). In this study, we did not include a control group not subjected to recurrent hypoglycemia, and thus, the extent that acute delivery of the EphA receptor agonist restored the glucagon response to hypoglycemia in rats exposed to recurrent hypoglycemia cannot be determined. However, results from previous studies from our laboratory using a similar hypoglycemic clamp protocol show the effect of the EphA receptor agonist on glucagon responses appears to be at least as great as the suppressive effect of recurrent hypoglycemia on glucagon levels (8).

Given that the EphA5/ephrinA5 system is mainly localized on glutamatergic synapses (12,25), it is intriguing to speculate that the stimulation of glucagon produced by the EphA5 receptor agonist in the current study is most likely mediated by the augmented VMH glutamatergic neurotransmission. The maintenance of VMH glutamate neurotransmission during acute hypoglycemia is supported by the transport of glutamate into astrocytes, resulting in the production of glutamine for delivery to

Table 1—Characteristics for the rats with recurrent hypoglycemia at baseline and after microinjection of ephrinA5-Fc in the VMH

	Control-Fc (n = 9)		EphrinA5-Fc (n = 7)	
	Basal	60 min postinjection	Basal	60 min postinjection
Body weight (g)	305 ± 6.7		308 ± 5.8	
Plasma glucose (mmol/L)	6.5 ± 0.2	6.5 ± 0.1	6.8 ± 0.3	6.8 ± 0.2
Insulin (μU/mL)	9.6 ± 1.8	10.1 ± 2.4	11.7 ± 4.1	8.0 ± 1.6
Glucagon (ng/L)	39 ± 1.9	39 ± 4	47 ± 8	49 ± 4
Epinephrine (pg/mL)	107 ± 43	148 ± 87	88 ± 45	80 ± 46
Norepinephrine (pg/mL)	212 ± 57	268 ± 64	215 ± 50	164 ± 41

Data are means ± SEM.

neurons and glutamate–glutamine cycle activation (35). Previous studies have shown that astrocyte synaptic coverage is linked to glutamate clearance and the activation of metabotropic glutamate receptors (36), and this has been proposed to alter synaptic and astroglia organization, and in turn, neurotransmission (37,38). Thus, the reduced glia coverage of the VMH neurons observed in the current study in rats exposed to recurrent hypoglycemia may have produced a deficit in astroglial function to support proper glutamate neurotransmission during hypoglycemia. Interestingly, this was associated with more neuronal synaptic contacts in the VMH (Fig. 3*D*), which appeared to be in large part symmetric and thus potentially γ-aminobutyric acid (GABA) inhibitory in nature (21). An increase in the VMH GABA tone has been shown to be an important contributor to the development of impaired glucose counterregulation in response to recurrent hypoglycemia (39).

Taken together, our data demonstrate that recurrent hypoglycemia alters neuron-glia plasticity in VMH nuclei

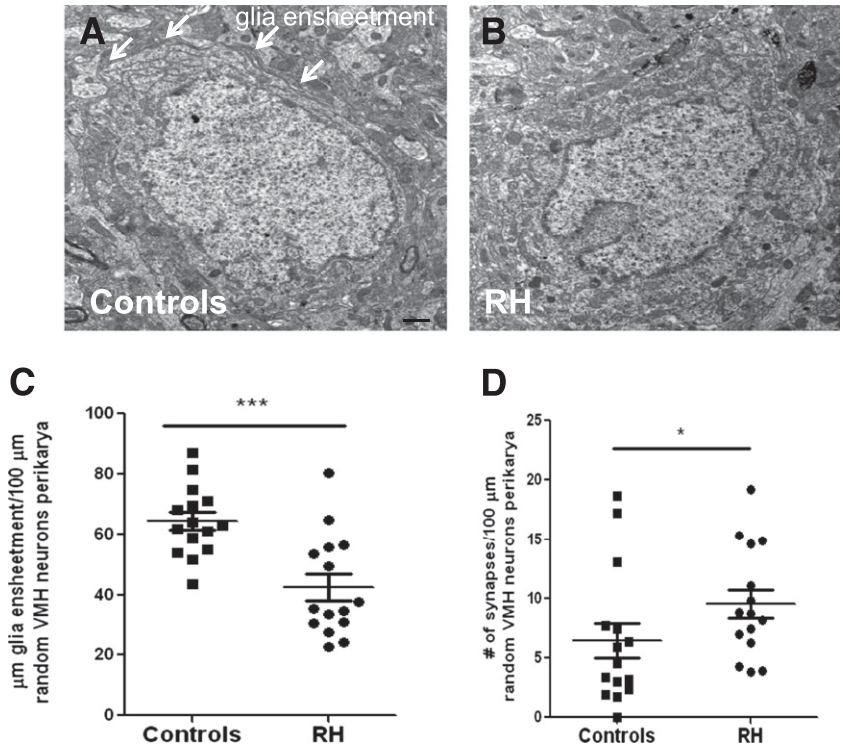

Figure 3—Effect of recurrent hypoglycemia (RH) on glia ensheetment and synaptic input organization. Representative electron micrographs show random VMH neuron and glia ensheetment (white arrow) in a control rat (*A*) and in a rat after RH (*B*). *C*: Graph showing glia ensheetment in rats after repeated episodes of insulin-induced hypoglycemia and in controls. *D*: Graph showing the number of total synaptic connections in rats after repeated episodes of insulin-induced hypoglycemia and in controls. All data are expressed as means ± SEM. Student *t* test or one-way ANOVA. $^{*}P < 0.05$, $^{***}P < 0.001$.

and diminishes ephrinA5 ligand expression within the VMH. It is thus possible decreased ephrin-induced activation of Eph receptors in VMH glutamate neurons may contribute to the impairment in glucose counterregulation in response to recurrent antecedent hypoglycemia.

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Author Contributions. B.S. designed the study, performed animal surgery and studies, analyzed data, and wrote the manuscript. T.L.H. designed EM experiments. R.S.S. designed the study, reviewed data, and revised the manuscript. B.S. and R.S.S. are the guarantors of this work, and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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