EphA5-EphrinA5 Interactions Within the Ventromedial Hypothalamus Influence Counterregulatory Hormone Release and Local Glutamine/Glutamate Balance During Hypoglycemia

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Activation of β-cell EphA5 receptors by its ligand ephrinA5 from adjacent β-cells has been reported to decrease insulin secretion during hypoglycemia. Given the similarities between islet and ventromedial hypothalamus (VMH) glucose sensing, we tested the hypothesis that the EphA5/ephrinA5 system might function within the VMH during hypoglycemia to stimulate counterregulatory hormone release as well. Counterregulatory responses and glutamine/glutamate concentrations in the VMH were assessed during a hyperinsulinemic-hypoglycemic glucose clamp study in chronically catheterized awake male Sprague-Dawley rats that received an acute VMH microinjection of ephrinA5-Fc, chronic VMH knockdown, or overexpression of ephrinA5 using an adenoassociated viral construct. Local stimulation of VMH EphA5 receptors by ephrinA5-Fc or ephrinA5 overexpression increased, whereas knockdown of VMH ephrinA5 reduced counterregulatory responses during hypoglycemia. 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. Changes in ephrinA5/EphA5 interactions within the VMH, a key brain glucose-sensing region, act in concert with islets to restore glucose homeostasis during acute hypoglycemia, and its effect on counterregulation may be mediated by changes in glutamate/glutamine cycling. *Diabetes* **62:1282–1288, 2013**

L owering glucose levels toward normal in insulin-treated patients with type 1 and type 2 diabetes diminishes the risk of long-term complications (1,2). The degree to which this can be achieved in clinical practice is often limited by the increased risk of hypoglycemia (3). In nondiabetic individuals, a fall in blood glucose is rapidly detected, and a series of compensatory responses occur to prevent or limit hypoglycemia and to restore euglycemia (4–6). These responses include the secretion of glucagon, epinephrine, and norepinephrine along with the suppression of endogenous insulin secretion, which together promote endogenous glucose production, reduce glucose utilization, and generate

typical warning symptoms. These protective responses are often disrupted in type 1 diabetic patients receiving intensive insulin therapy who have a history of hypoglycemia (7). As a result, the fear of hypoglycemia is the major factor limiting the benefits of intensive insulin treatment (8).

Activation of counterregulation requires effective detection of falling glucose levels. Although a complex network of glucose sensors has been described in the central nervous system (9–11) and peripherally (12), the brain appears to have the dominant role during hypoglycemia and, specifically, the ventromedial region of the hypothalamus or VMH (13,14). Interestingly, VMH neurons contain much of the same glucose-sensing machinery (e.g., glucokinase [15], ATP-sensitive K^+ channels [16–18]) as pancreatic β-cells, suggesting that parallels exist between the molecular mechanisms used by them and those used by β-cells. In keeping with this idea, EphA5/ephrinA5, members of synaptically localized cell adhesion molecules (19), are also specifically expressed in β-cells and have been shown to regulate insulin secretion (20).

The Eph receptor tyrosine kinases and their membrane-anchored ephrin ligands play a critical role in modulating neuronal synaptic structure and its physiological properties (21). Eph receptors and their ligands, the ephrins, are membrane-bound proteins that have been divided into A and B subclasses that preferentially bind to their corresponding subclass. In the brain they play an important role in cell–cell interactions (22). Eph/ephrin interactions are bidirectional (23). Ligand binding to the Eph receptor induces "forward signaling," mostly through phosphotyrosine-mediated pathways; however, ephrins can also signal into their host cell via receptor binding, which is referred to as "reverse signaling" (19,24).

Historically, these proteins were thought to mainly function as regulators of nervous system development (25). Specifically, they were thought to primarily play a role in axon guidance during the assembly of the neural circuitry (26,27). However, many Eph receptors and their ephrin ligands are present in the adult brain and are enriched in glutamate excitatory synapses (28). Moreover, a growing body of evidence now indicates that Eph receptors are expressed in synaptic terminals where they influence synaptic plasticity via binding to glial-derived ephrins (21). These interactions between neurons and glia at the level of the synapse may serve to modulate the transmission of neurochemical signals at the synapse (29). Whether hypoglycemia per se induces local changes in the VMH affecting both neuronal synapses and surrounding glia cells is unknown, but alterations in neuron–glia interactions could potentially modulate neurotransmission within brain glucose-sensing regions. Expression of ephrinA5 has

been shown to be present in the VMH (30) as well as in a variety of other brain regions (31,32).

This study tests the hypothesis that stimulation of crosstalk between EphA5 receptors and ephrinA5 within the VMH might regulate the magnitude of counterregulatory responses to hypoglycemia and that reductions in the capacity of ephrinA5 to activate EphA5 receptors in the VMH might impair glucose counterregulation. It is noteworthy in this regard that in β-cells, EphA/ephrinA is also bidirectional; EphA5 forward signaling inhibits insulin secretion, whereas ephrinA5 reverse signaling stimulates insulin secretion after glucose stimulation (20).

RESEARCH DESIGN AND METHODS

Animals. Male Sprague-Dawley rats (Charles River Laboratories International, Inc., Wilmington, MA) weighing 300–350 g were individually housed in the Yale Animal Resource Center in rooms controlled for temperature (22–23°C) and humidity. Animals were fed rat chow (Agway Prolab 3000; Syracuse, NY) and water ad libitum and were acclimatized to a 12-h light-to-dark cycle. Experimental protocols were approved by the Yale University Institutional Animal Care and Use Committee.

Vascular and stereotaxic surgery. Animals were anesthetized ~7–10 days before study and underwent aseptic surgery to have vascular catheters implanted into the left carotid artery for blood sampling and right jugular vein for infusion, as previously described (33). These catheters were tunneled subcutaneously and exteriorized at the back of the neck between the scapulae. The incision was closed using wound staples. For stereotaxic surgery, animals were placed into a stereotaxic frame (David Kopf Instruments, Tujunga, CA), and stainless steel guide cannulas were bilaterally inserted into the brain and secured in place with screws and dental acrylic for *1*) microinjection (Plastics One, Roanoke, VA; coordinates from bregma: anteroposterior –2.6, mediolateral ± 0.8, and dorsoventral –8.0 mm), or *2*) microdialysis (coordinates from bregma: anteroposterior –2.6, mediolateral ± 3.8, and dorsoentral –8.3 mm at the angle of 20°; Eicom Corp., Kyoto, Japan). Adenoassociated virus (AAV) constructs were microinjected bilaterally into the VMH with a Hamilton glass syringe located in a Harvard Apparatus microinjection pump with arms fixated to Bregma apparatus for proper brain coordination (anteroposterior –2.6, mediolateral ± 0.8, and dorsoventral –8.8 mm) at the rate 0.1 μL/min per 10 min.

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 a 7-day 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. Rats then received a microinjection of recombinant human ephrinA5-Fc (R&D systems, Minneapolis, MN; catalog number 374-EA-200) or recombinant human IgG1-Fc; control-Fc protein (R&D Systems; 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 were previously used in the central nervous system in vivo (34) and well as in vitro (35). The timeline of the protocol is presented in Fig. 1*A*.

AAV construction. AAV constructs carrying RNA interference or full-length cDNA sequences to knockdown or overexpress ephrinA5, respectively, were packaged by GeneDetect (Auckland, New Zealand). The ephrinA5 overexpression construct (AAV2-CAG-rat ephrinA5-internal ribosome entry site [IRES]-enhanced green fluorescent protein [EGFP]-woodchuck hepatitis posttranscriptional regulatory element [WPRE]-bovine growth hormone polyadenylation [BGH-polyA]) was driven by a CAG promoter (consisting of the chicken β-actin promoter hybridized with the cytomegalovirus immediate early enhancer sequence) and contained an IRES cassette for simultaneous expression of EGFP protein. The construct used to knockdown expression of ephrinA5 (AAV2-U6-rat ephrinA5.short hairpin (sh)RNA-terminator-CAG-EGFP-WPRE-BGH-polyA) contained a shRNA driven by the U6 promoter; expression of EGFP was independently driven by the CAG promoter.

The AAV–small interfering RNA viral vector for AAV controls was AAV2-CAG-null/empty-IRES-EGFP-WPRE-BGH-polyA. It has been suggested that AAV serotype 1 to 9 predominantly effect neurons or both neurons and glia (36) All viral stocks (approximately 10^{12} viral particles/mL) were validated in vivo before microinjection of the AAV construct. Subsequently, animals were returned to their home cages, and was performed 7 days later vascular

surgery. Clamp studies were performed ~7 days after recovery from vascular surgery. The timeline of the protocol is presented in Fig. 2*A*.

Microdialysis. At ~14 days after AAV injection and ~7 days after vascular catheter surgery, animals were fasted overnight in microdialysis cages to allow sufficient time for acclimation. The next morning, the animals were connected to infusion pumps, and bilateral microdialysis probes were inserted down to the level of the VMH. Artificial extracellular fluid was perfused through the microdialysis probes at a constant rate of 1.5 μL/min for 2.5 h to allow glutamate levels to stabilize before the collection of microdialysate and baseline blood samples. Thereafter, microdialysate samples were collected at 20-min intervals for the duration of the hyperinsulinemic-hypoglycemic clamp study.

Hyperinsulinemic-hypoglycemic clamp. A primed-continuous infusion of 20 mU kg^{-1} · min^{-1} insulin (Humulin R; Eli Lilly & Co., 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. Additional blood was drawn at baseline and at 30, 60, and 90 min for measurement of insulin, glucagon, epinephrine, and norepinephrine concentrations. At study termination rats, were killed and probe position was confirmed histologically.

Hormone and neurotransmitter analyses. Plasma hormones were determined, and catecholamine concentrations were analyzed by high-performance liquid chromatography using electrochemical detection, and plasma insulin and glucagon were determined by radioimmunoassay (Linco, St. Charles, MO). The VMH glutamate and glutamine from microdialysate samples were determined using liquid chromatography–mass spectrometry.

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$_3$VO$_4$, 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 a SDS-9% PAGE (Bio-Rad). After electrophoresis, proteins was electroblotted onto nitrocellulose membranes, blocked with 5% nonfat dry milk in PBS, probed with first antibody (α-tubulin; Cell Signaling, catalog 2125S), (ephrin-A5; R&D Systems, catalog AF3743), and incubated with the appropriate secondary antibody conjugated to peroxidase by horseradish peroxidase–linked protein A (Sigma; 1:2000). The immunoblots were developed using an enhanced chemiluminescence detection system (Amersham Biosciences).

Statistics. Data are expressed as the means ± SEM. Baseline 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 Prism 4.0 software (GraphPad Software, San Diego, CA). $P < 0.05$ was considered statistically significant.

RESULTS

Effect of acute stimulation of VMH EphA5 receptors on glucose counterregulation. Body weight and plasma levels of glucose, insulin, glucagon, epinephrine, and norepinephrine were indistinguishable at baseline and immediately after VMH microinjection of ephrinA5-Fc or control-Fc (Table 1). Subsequently, after the start of the hyperinsulinemic-hypoglycemic clamp study, plasma glucose (Fig. 1*B*) and insulin (Fig. 1*C*) levels were indistinguishable between the two groups. Nevertheless, as shown in Fig. 1*D*, ephrinA5-Fc–stimulated activation of VMH EphA5 receptors reduced glucose infusion rates (GIR) required to maintain steady-state hypoglycemia by 45% (9.9 ± 2.1 in the control-Fc group vs. 5.4 ± 0.8 mg/kg/min in the ephrinA5-Fc group; $P < 0.05$). This was associated with a nearly twofold greater glucagon response at 30 min (883 ± 128 in the VMH-ephrinA5-Fc group vs. 482 ± 92 ng/L in controls, $P < 0.001$; Fig. 1*E*). However, neither plasma epinephrine (Fig. 1*F*) nor norepinephrine (Fig. 1*G*) responses to hypoglycemia differed significantly between the groups.

Effect of chronic changes in VMH ephrinA5 expression on glucose counterregulation. Body weight was not significantly different in the animal groups given the three different AAV constructs (ephrinA5 overexpression, ephrinA5 knockdown, or control). Baseline plasma glucose,
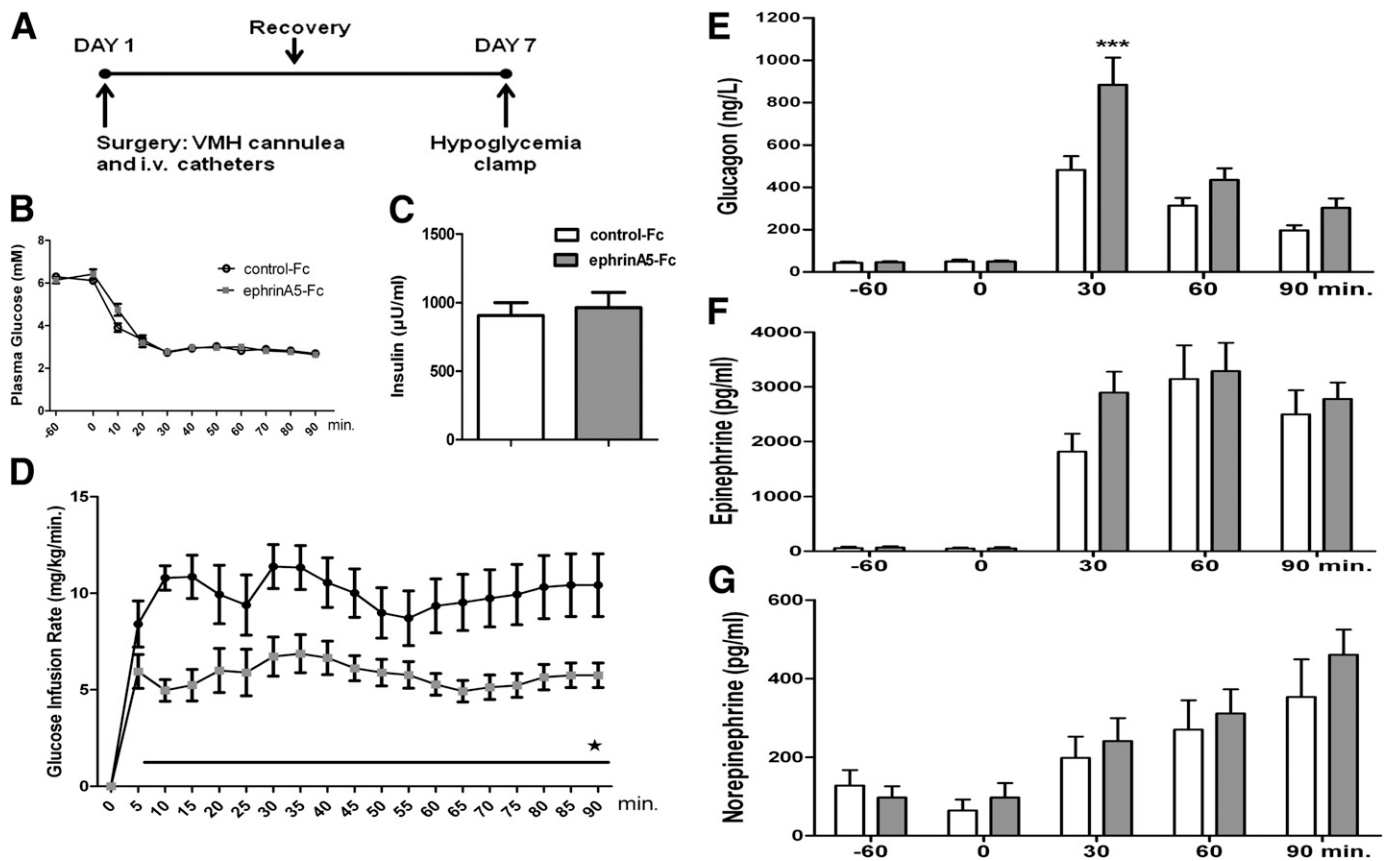
FIG. 1. Effect of acute stimulation of VMH EphA5 receptors with ephrin5A-Fc on glucose counterregulation during hypoglycemia in rats. The components include a schematic representation of the experimental protocol (*A*); plasma glucose (*B*); mean plasma insulin at 30, 60, and 90 min (*C*); glucose infusion rate (*D*); plasma glucagon (*E*); plasma epinephrine (*F*); and plasma norepinephrine during the hypoglycemic clamp study (*G*) (*n* = 12 for ephrin-Fc and *n* = 12 for ephrinA5-Fc). Data are presented as means ± SEM. Statistical analysis used mixed-model ANOVA with Bonferroni post hoc test: *$P < 0.05$, *** $P < 0.001$ vs. control-Fc.

insulin, glucagon, epinephrine, and norepinephrine levels in rats given ephrinA5 shRNA were also not significantly different from rats given the control AAV constructs. In contrast, ephrinA5 VMH overexpression produced significantly higher levels of glucose, insulin, glucagon, and norepinephrine at baseline compared with the control animals. Moreover, the levels of glucose and glucagon in the rats with VMH ephrinA5 overexpression were greater than those with VMH ephrinA5 knockdown (Table 2).

During the hyperinsulinemic-hypoglycemic clamp studies, neither VMH overexpression nor knockdown of ephrinA5 had a significant effect on either plasma glucose (Fig. 2*B*) or insulin concentrations (Fig. 2*C*). As shown in Fig. 2*D*, VMH overexpression of ephrinA5 was significantly reduced, whereas knockdown of ephrinA5 raised the exogenous glucose requirement throughout the hypoglycemic period. During last 60 min of the clamp study, GIR was 6.8 ± 0.6 in controls, 13.0 ± 1.6 in VMH ephrinA5 knockdown ($P < 0.01$) rats, and 2.8 ± 0.4 mg/kg/min in the VMH ephrinA5 overexpression group ($P < 0.01$) compared with controls. These changes were associated with alternations in glucagon and epinephrine release. At 30 min, plasma glucagon levels rose more than twofold higher in the ephrinA5 overexpression group ($P < 0.001$) but were diminished by 30% in the ephrinA5 knockdown group ($P < 0.001$) compared with controls (Fig. 2*E*). Similarly, the increase in plasma epinephrine was 65–70% greater in the ephrinA5 overexpression group ($P < 0.01$) and suppressed by 65–70% in the ephrinA5 knockdown group ($P < 0.01$)

compared with controls (Fig. 2*F*). Norepinephrine levels were also increased at 30 min only in the group with VMH ephrinA5 overexpression ($P < 0.05$; Fig. 2*G*). Western blot analysis of VMH micropunches obtained from rats 14 days after they were given AAV constructs designed to knockdown or overexpress ephrinA5 demonstrated an ~60% reduction in ephrinA5 expression after knockdown and an ~90% increase in VMH ephrinA5 after targeted overexpression of the ephrinA5 gene (Fig. 3). Taken together, these observations are consistent with the possibility that the ephrinA5 ligand, via its interaction with Eph5A receptors, has a role in modulating counterregulatory responses to hypoglycemia.

The VMH glutamate/glutamine concentration during hypoglycemia. Previous studies have shown that the EphA5 receptor and its ligand ephrinA5 are primarily localized on glutamatergic synapses (25). Given that alterations in VMH glutamate neurotransmission modulate counterregulatory responses to hypoglycemia (37), we used microdialysis in the next set of experiments to determine whether alterations in ephrinA5 gene expression cause changes in the local concentrations of glutamate and glutamine in VMH interstitial fluid. Overexpression of VMH ephrinA5 transiently increased local glutamate concentrations when blood glucose reached hypoglycemic levels at 40 min ($P < 0.05$; Fig. 4*A*). In striking contrast, knockdown of VMH ephrinA5 expression produced the profound suppression of glutamine concentrations in VMH interstitial fluid in the basal state ($P < 0.001$) and during
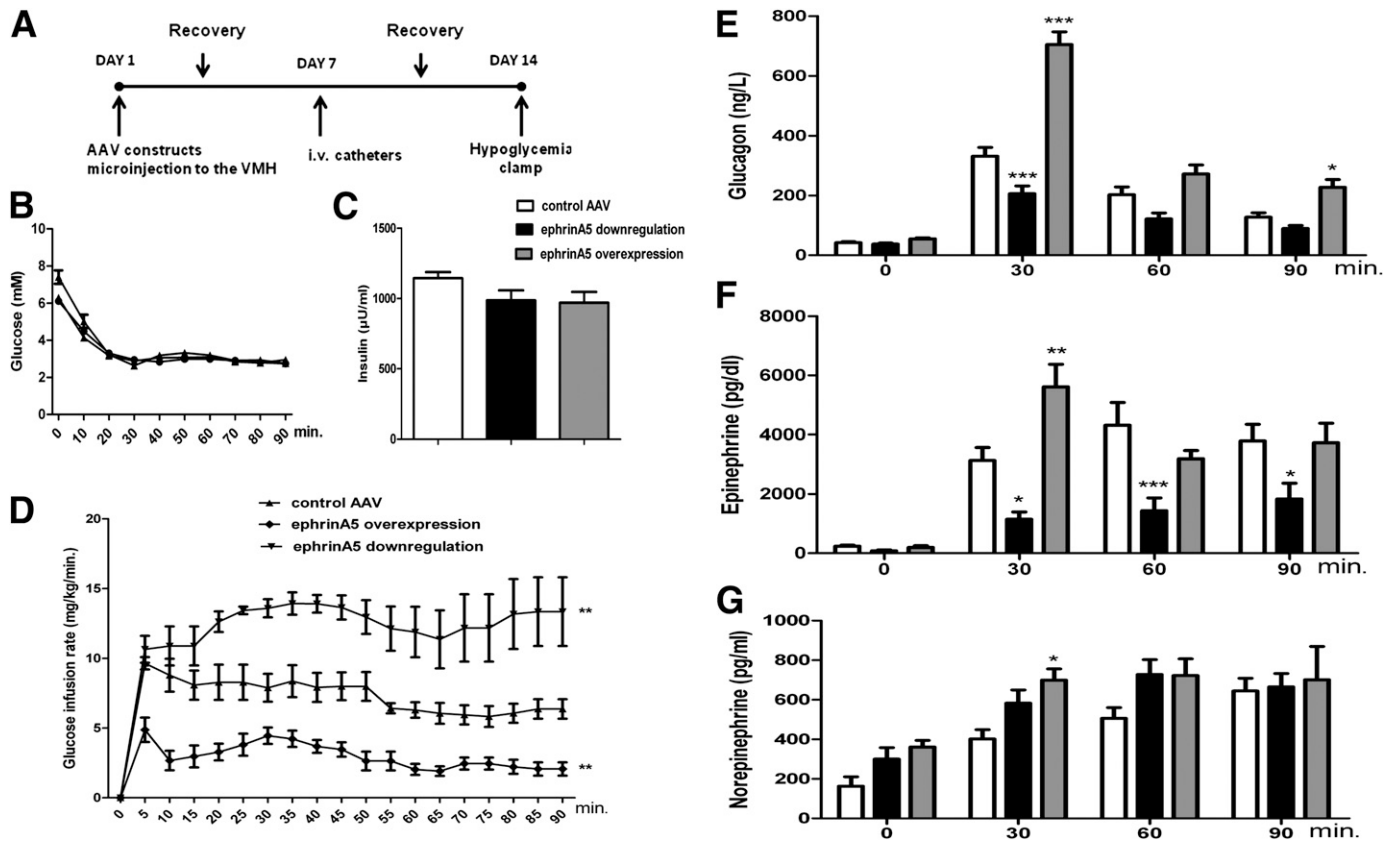
FIG. 2. Long-term effect of chronic changes in VMH ephrinA5 expression on glucose counterregulation during hypoglycemic clamp study. The components include schematic representation of the experimental protocol (*A*); plasma glucose (*B*); mean plasma insulin at 30, 60, and 90 min (*C*); glucose infusion rate (*D*); plasma glucagon (*E*); plasma epinephrine (*F*); and plasma norepinephrine during the hypoglycemic clamp (*G*) (*n* = 8 for control AAV, *n* = 7 for ephrinA5 downregulation AAV, and *n* = 7 for ephrinA5 overexpression AAV). Data are presented as means ± SEM. Statistical analysis used mixed-model ANOVA with Bonferroni post hoc test: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. control AAV.

hypoglycemia ($P < 0.01$; Fig. 4*B*). We observed similar effects on counterregulatory responses to hypoglycemia with this group of rats (data are not shown). The latter observation is consistent with the possibility that reduced VMH ephrinA5 expression diminishes glutamate/glutamine cycling via inhibition of local astroglial glutamine synthesis or release.

DISCUSSION

There are two dominant centers responsible for glucose sensing and the regulation of glucose homeostasis: the pancreatic islets peripherally and the VMH centrally.

Specialized glucose-sensing cells in both regions are able to detect small decrements in circulating glucose levels and alter the release of glucoregulatory hormones (38). Interestingly, recent data suggest that there are parallels between the molecular mechanisms used by the VMH and islet β-cells (39). With regard to the current work, activation of pancreatic β-cell EphA receptors by the ephrin ligand on adjacent β-cells have been reported to inhibit insulin release (20). Here we demonstrate that similar activation of EphA5 receptors using VMH delivery of an exogenous ligand or local overexpression of ephrinA5 increases counterregulatory hormone responses to hypoglycemia.

TABLE 1
Effect of VMH microinjection of ephrin5A-Fc on baseline levels of plasma glucose and counterregulatory hormones

	Control-Fc		EphrinA5-Fc	
n	12		12	
Body weight (g)	327 ± 13		330 ± 12	
	Basal	60 min postinjection	Basal	60 min postinjection
Plasma glucose (mmol/L)	6.3 ± 0.3	6.1 ± 0.2	6.3 ± 0.2	6.4 ± 0.3
Insulin (μU/mL)	7.5 ± 2.7	6.2 ± 1.7	8.2 ± 1.9	9.7 ± 1.7
Glucagon (ng/L)	46 ± 4	49 ± 12	44 ± 4	49 ± 6.4
Epinephrine (pg/mL)	115 ± 61	48 ± 28	129 ± 60	52 ± 36
Norepinephrine (pg/mL)	207 ± 51	64 ± 39	174 ± 36	97 ± 51

Data are presented as means ± SEM analyzed by one-way ANOVA.

TABLE 2
Long-term effect of VMH changes in ephrinA5 expression on baseline fasting levels, body weight, glucose, and counterregulatory hormones characteristics

	AAV		
	Control	EphrinA5 downregulation	EphrinA5 overexpression
n	8	7	7
Body weight (g)	331 ± 8.2	340 ± 4.6	349 ± 12
Plasma glucose (mmol/L)	6.1 ± 0.3	6.3 ± 0.1	7.4 ± 0.4*#
Insulin (μU/mL)	8.5 ± 1.1	11.7 ± 1.4	15 ± 2.6*
Glucagon (ng/L)	42 ± 4.5	37 ± 3.8	55 ± 3.7*#
Epinephrine (pg/mL)	237 ± 36	82 ± 30	194 ± 61
Norepinephrine (pg/mL)	163 ± 47	300 ± 57	361 ± 33*

Data are presented as mean ± SEM. Statistical analysis was performed using one-way ANOVA. *$P < 0.01$ vs. controls and # $P < 0.01$ vs. ephrinA5 downregulation.

Conversely, local VMH knockdown of ephrinA5 gene expression suppresses counterregulatory hormone responses. Thus, EphA/ephrinA signaling within the islet and VMH could potentially act in concert in these two glucose-sensing centers to defend against hypoglycemia and contribute to defective counterregulation after intensive insulin treatment.

Communication between neurons at synapses is mediated primarily by neurotransmitter release and by the gating of postsynaptic receptor ion channels, but a growing body of evidence indicates that adhesion molecules that interact in the synaptic cleft also mediate signaling (25,29). These synaptically localized cell adhesion molecules are not only structural components but also often act as dynamic regulators of synaptic function (40). EphA receptors are present in axon terminals, dendritic spines, and astrocytes, predominantly in glutamatergic synapses (31,41,42), and serve to regulate synaptic plasticity by acting as a binding partner for ephrins. Nestor et al. (35)

demonstrated that the interaction between EphA receptors and ephrinA influences the release of glutamate from glial cells. In addition, Filosa et al. (43) observed upregulation of astroglia glutamate transporters in ephrinA3 and in EphA4 knockout mice, raising the possibility that EphA-ephrinA interactions may modulate glutamate/glutamine cycling and, thereby, synaptic function. This may have important effects on glucose counterregulation.

Previous studies have shown that glutamatergic neurotransmission in the VMH influences the magnitude of counterregulatory responses to hypoglycemia. Mice with impaired glutamatergic neurotransmission secondary to the loss the glutamate transporter, VGLUT2, selectively in SF1 VMH neurons exhibit a modest reduction in fasting glucose levels, marked suppression of glucagon secretion, and diminished catecholamine responses during insulin-induced hypoglycemia (37). In the current study, targeted overexpression of ephrinA5 caused an increase in glucagon and
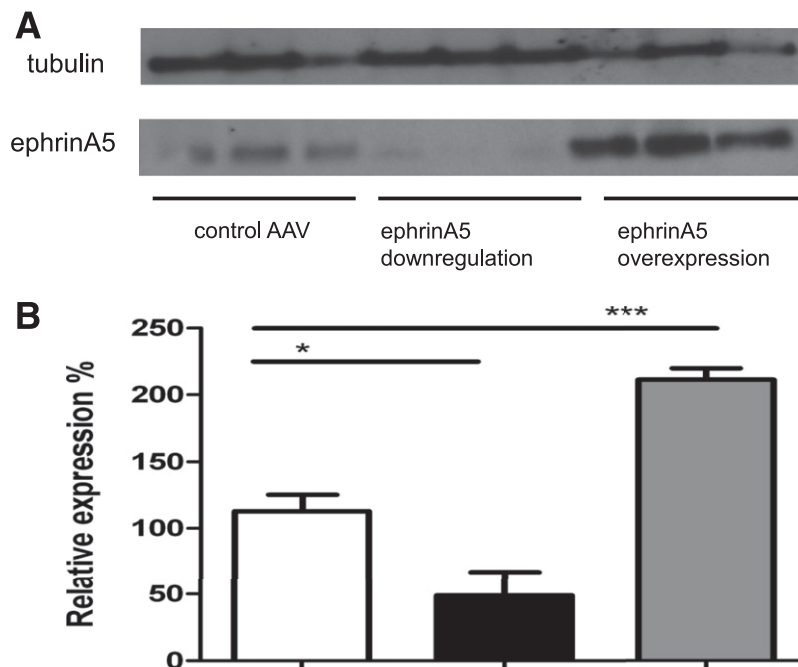

FIG. 3. A: Representative data show expression level of ephrinA5 in the VMH in rats after ~14 days after VMH delivery of AAV constructs as determined by Western blood analysis. Tubulin served as the loading control. B: Relative VMH expression of ephrinA5 after VMH administration of control AAV ($n = 6$; white bars), ephrinA5 knockdown ($n = 6$; black bar), and ephrinA5 overexpression ($n = 5$; gray bar). Data are presented as means ± SEM. Statistical analysis used one-way ANOVA: *$P < 0.05$, ***$P < 0.001$ vs. control AAV.
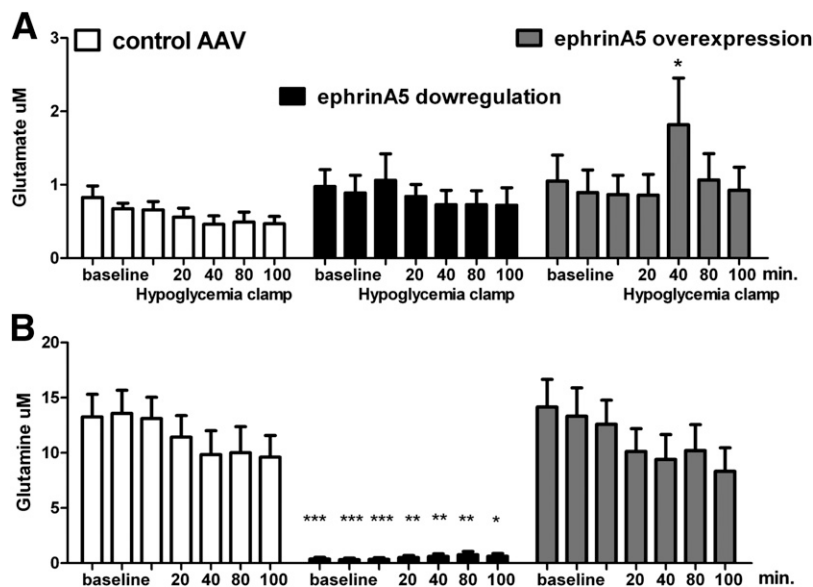
FIG. 4. Effect of ephrinA5 knockdown and overexpression on the levels of glutamate and glutamine in VMH interstitial fluid during hypoglycemia in rats given control AAV (n = 6), ephrin5A knockdown AAV (n = 9), or ephrinA5 over-expression AAV (n = 9). Collections of dialysis fluid were obtained over 20 min intervals beginning at −60 to −40 min and ending at 80–100 min. Data are expressed as means ± SEM. Statistical analysis used mixed-model ANOVA with Bonferroni post hoc test: *P < 0.05, **P < 0.01, *** P < 0.001 vs. control AAV.

epinephrine secretion that was maximal at the same time that an increase in glutamate levels occurred in VMH interstitial fluid during hypoglycemia. However, downregulation of ephrinA5 within the VMH produced a sustained reduction in glucagon and epinephrine release that occurred in conjunction with a profound and sustained decrease in the levels of glutamine in VMH interstitial fluid. These observations are consistent with the possibility that Eph5A/ephrin5A interaction influences glutamate neurotransmission.

We used a microinjection protocol to deliver the viral construct into the VMH that we have previously shown leads to minimal spread to the remainder of the hypothalamus (41). This provided the means to specifically manipulate the VMH expression level of ephrinA5. The expression of ephrinA within the brain is believed to be mostly in astrocytes (44), whereas EphA receptors are located mostly on the plasma membrane of axons, dendritic spines, and axon terminals, supporting its availability for surface interactions with ephrins (41). Nevertheless, our experimental approach does not allow us to draw conclusions regarding the specific cellular compartments (neurons vs. astrocytes) affected. More specific viral promoters and viral vectors designed to target neurons or astrocytes will be required to address this issue. Also noteworthy is that the changes in glucose infusion rate observed during our hypoglycemia experiments may not be totally mediated by changes in counterregulatory hormone release. An additional effect on insulin-stimulated glucose disposal cannot be excluded.

Taken together, these data suggest that bidirectional EphA5/ephrinA5 interaction within the VMH influences the response of glucose-sensing neurons to changes in the level of circulating glucose. This bidirectional signaling is localized on synaptic terminals where it modulates interactions between glia and neurons, which in turn modulates the magnitude of counterregulatory responses to hypoglycemia by glutamate neurotransmission and glutamate/glutamine cycling. One might thus speculate that alterations in the capacity of ephrinA5 to activate EphA5

receptors within the VMH might be a contributory factor to the development of impaired glucose counterregulation.

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