Role of synaptic plasticity and EphA5-ephrinA5 interaction within the ventromedial hypothalamus in response to recurrent hypoglycemia.

Synaptic plasticity in response to recurrent hypoglycemia

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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. Previously we have reported that EphA5 receptor-ephrinA5 interactions within the ventromedial hypothalamus (VMH) influence counterregulatory hormone responses during acute hypoglycemia in non-diabetic 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 found to be reduced by antecedent recurrent hypoglycemia. In addition, there was an increase in the number of synaptic connections as well as reduced astroglial synaptic coverage. Activation of VMH EphA5 receptors via targeted microinjection of ephrinA5-Fc prior to hyperinsulinemic hypoglycemic clamp study caused a reduction in the glucose infusion rate in non-diabetic 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 brain glucose-sensing region, 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 over-expression increased, whereas knockdown of VMH ephrinA5 reduced counter-regulatory responses to hypoglycemia. Furthermore, over-expression of VMH ephrinA5 transiently increased local glutamate concentrations, whereas ephrinA5 knockdown produced profound suppression of VMH interstitial fluid glutamine concentrations both in the basal state and during hypoglycemia. These data suggest that the activation of VMH EphA5 receptors by ephrinA5 may play an important role in promoting the restoration of 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-cell communication as well as synaptic structure and function. Eph receptors function as transmembrane receptor tyrosine kinases and are divided on the basis of 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 as well 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 so-called ‘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 like 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.
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 (Agway Prolab 3000; 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 EM experiments described below.

Vascular and Stereotaxic Surgery: Approximately 7-10 days before study, animals were anaesthetized 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 [8]. These catheters were tunnelled 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 (Plastics One, Roanoke, VA) were inserted into the brain secured in place with screws and dental acrylic (coordinates from Bregma: anterior-posterior – 2.6 mm, medio-lateral ±0.8 mm, and dorso-ventral –8.0) for microinjection.

Recurrent hypoglycemia protocol: For 3 consecutive days rats received intraperitoneal injections of insulin (10 units/kg). 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 minutes 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 prior to the start of the experiment and then left undisturbed to recover from handling stress. Following the recovery period, 22-gauge microinjection needles (Plastics One, Roanoke, VA), 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 either 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, Minneapolis, MN catalog number: 374-EA-200) in a concentration of 0.3 ug/ul dissolved in artificial extracellular fluid (aECF) delivered at a rate of (0.1 µl/min) over 60 min (dose 1.8 ug for each side). Following 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 vivo studies [16] as well as in vitro in brain slices [17] without adverse effects. Additionally, 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 (Analogx Instruments, Lunenburg, MA) designed to maintain plasma glucose at 50 mg/dl from 30-90 min [19]. Additional blood was drawn at baseline and at 30, 60 and 90 min, for measurement of insulin, glucagon, epinephrine and norepinephrine. At study termination rats were sacrificed and probe position confirmed histologically.

Hormone and neurotransmitter analyses: Plasma catecholamine measurements were analyzed by HPLC 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, 150mM NaCl, 50 mMTris, (pH 7.4), 1 mM Na3VO4, 1mM phenylmethylsulfonyl fluoride (PMSF) 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 sodium dodecylsulfate-9% polyacrylamide gel (Bio-Rad). After electrophoresis, proteins was electro-blotted onto nitrocellulose membranes, blocked with 5% nonfat dry milk in PBS, probed with first antibody (alpha-Tubulin; Cell
Signaling cat.2125S), (ephrin-A5; R&D systems cat. AF3743), and EpA5 receptor (EphA5; Sigma cat # P8651) and incubated with the appropriate secondary antibody conjugated to peroxidase by horseradish peroxidase (HRP)-linked protein A (Sigma) (1:2000). The immunoblots were developed using an ECL detection system (Amersham Biosciences).

**Electron microscopy (EM)** Briefly, animals were perfused with paraformaldehyde fixative and ultrathin brain sections were cut on a Leica ultra-microtome, collected on Formvar-coated single-slot grids, and analyzed with a Tecnai 12 Biowin electron microscope (FEI). Glia coverage of the cell membrane of random VMH cells was performed using ImageJ. EM photographs (11,500x) 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 20,000 magnification, while quantitative measurements were performed at a magnification of 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, t- student test, as appropriate. Statistical analysis was then performed by two-way ANOVA for repeated measures, followed by post hoc analysis using (Prism 4.0; GraphPad Software, San Diego, CA), p<0.05 was considered statistically significant.
RESULTS-

Recurrent hypoglycemia and ephrinA5 expression

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

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 prior to conducting a hyperinsulinemic hypoglycemic clamp study in rats previously exposed to 3 episodes of insulin-induced hypoglycemia. Schematic representation of the experimental protocol is presented on Fig. 2a. 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. 2b) and insulin (Fig. 2c) were indistinguishable between the 2 groups. EphrinA5-Fc delivery, however, significantly reduced within 15 minutes the glucose infusion rate required to maintain hypoglycemia (Fig. 2d). This was accompanied by a rapid 150% (p<0.001) increase in glucagon release (Fig. 2e). As was observed in our previous study [8] in rats not exposed to antecedent hypoglycemia, neither plasma epinephrine (Fig. 2f) nor norepinephrine (Fig. 2g) responses to hypoglycemia were significantly altered by VMH delivery of ephrinA5-Fc as compared to the control–Fc microinjection.

Effect of Recurrent hypoglycemia on glia ensheathment and synaptic input organization

Next we assessed if recurrent hypoglycemia affected the VMH synaptic organization and glia ensheathment in rats exposed for 3 days to recurrent hypoglycemia as compared to controls, a model we have previously shown suppresses hypoglycemic counterregulation [22]. Fig. 3a and 3b compares representative electron micrograph of glia ensheathment in random ventromedial hypothalamic neuron perikarya in control and recurrent hypoglycemic rat. The rats exposed to RH exhibited reduced glial coverage of neurons (p<0.001) (Fig. 3c), and as a result more total synaptic contacts in random VMH neurons (p<0.05) (Fig. 3d).
DISCUSSION:

The current study demonstrates that exposure of non-diabetic 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 was shown to enhance glucose counter-regulation 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 as well as producing 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 possibility 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 ventromedial hypothalamus 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 both increased death and decreased proliferation [33]. Given that both diabetes and recurrent glucose deprivation are accompanied by impaired counterregulation [22], these observations are consistent with the hypothesis synaptic connectivity and the 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 EphrinA5 ligand on hypoglycemia-induced counter-regulatory hormone release was on glucagon, whereas RH normally leads to a suppression of both glucagon and epinephrine levels in non-diabetic 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 over-expression 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 RH and thus the extent that acute delivery of the EphA receptor agonist restored the glucagon response to hypoglycemia in rats exposed to RH cannot be determined. However, based on previous studies from our laboratory using a similar hypoglycemic clamp protocol the impact of the EphA receptor agonist on glucagon responses appears to be at least as great as the suppressive effect of RH 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 augmented the VMH glutamatergic neurotransmission. During acute hypoglycemia the maintenance of VMH glutamate neurotransmission is supported by the transport of glutamate into astrocytes, resulting in the production of glutamine for delivery to 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. 3d), which appeared to be in large part symmetric and thus potentially in GABA inhibitory in nature [21]. Increased in the VMH GABA tone has been shown to be an important contributor to the development of impaired glucose counterregulation in response to RH [39]. Taken together, our data demonstrate that recurrent hypoglycemia alters neuron-glia plasticity in VMH nuclei as well as 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.
Table 1. Characteristics for the rats with recurrent hypoglycemia at baseline and after microinjection of ephrinA5-Fc in the VMH. Data are means ± SEM.

|                      | control-Fc | ephrinA5-Fc |
|----------------------|------------|-------------|
| **n**                | 9          | 7           |
| **Body weight (g)**  | 305 ± 6.7  | 308 ± 5.8   |
| **Plasma glucose (mmol/l)** | 6.5 ± 0.2 | 6.5 ± 0.1  |
|                      | basal      | 60 min post injection | basal | 60 min post injection |
| **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    |
Figure 1. Effect of Recurrent hypoglycemia on ephrinA5 and EphA5 expression.
   a) Representative data showing expression level of ephrinA5 in the VMH in rats after ~ 3 days of recurrent hypoglycemia as determined by western blood analysis. Tubulin served as the loading control.
   b) Relative VMH expression of ephrinA5 in control rats (n=8); RH (n=8). Data are presented as means ± SEM. Statistical analysis by T student test: *p<0.05 vs. control.
   c) Representative data showing expression level of the EphA5 in VMH in rats after ~ 3 days of recurrent hypoglycemia as determined by western blood analysis. Tubulin served as the loading control.
   d) Relative VMH expression of EphA5 in control rats (n=7); RH (n=7). Data are presented as means ± SEM. Statistical analysis by T student test.

Figure 2. Effect of acute stimulation of VMH EphA5 receptors with ephrin5A-Fc on glucose counter-regulation in rats exposed to recurrent hypoglycemia. The components are: a) Schematic representation of the experimental protocol; b) Plasma glucose; c) Mean plasma insulin: from 30, 60, 90 minutes time points; d) Glucose infusion rate; e) Plasma glucagon f) Plasma epinephrine; and g) Plasma norepinephrine during the hypoglycemic clamp study (n=7 for ephrinA5-Fc and n=9 for control-Fc). Data presented as means ± SEM. Statistical analysis employed mix model ANOVA with Bonferroni post hoc test *p<0.05 **<0.01 *** p<0.001 vs. control-Fc.

Figure 3. Effect of Recurrent hypoglycemia on glia ensheetment and synaptic input organization. All data are expressed as means ± SEM. Student’s t test or one-way ANOVA * p<0.05 *** p<0.001.
   a) Representative electron micrograph showing random ventromedial hypothalamic neuron and glia ensheetment (white arrow) in (a) control rat
   b) in rat after recurrent hypoglycemia.
   c) Graph showing glia ensheetment in rats after repeated episodes of insulin-induced hypoglycemia and controls.
   d) Graph showing the number of total synaptic connections in rats after repeated episodes of insulin-induced hypoglycemia and controls.
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Author Contributions: B.Sz. designed study, performed animal surgery and studies, analyzed data and wrote the manuscript. T.L.H. designed electron microscopy experiments. R.S.S. designed study, reviewed data and revised the manuscript.

Drs. Sherwin and Szepietowska are the guarantors of this work, had full access to all the data, and take full responsibility for the integrity of data and the accuracy of data analysis.
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Fig. 1

(a) Tubulin and ephrinA5 expression in Recurrent Hypoglycemia compared to Controls.

(b) Box plots showing relative expression percentages for Tubulin and EphA5. Asterisk (*) indicates a significant difference.

(c) Tubulin and EphA5 expression in Recurrent Hypoglycemia compared to Controls.

(d) Box plots showing relative expression percentages for Tubulin and EphA5 in Recurrent Hypoglycemia and Controls.
Fig. 3

glia ensheathing

Controls

RH

C

Rum glia ensheathment/100 μm
random VMH neurons perikarya

***

D

# of synapses/100 μm
random VMH neurons perikarya

*