Study Title: Measurement of Glucose/Glycogen Metabolism in Humans Using Magnetic Resonance at 4 or 7 Tesla

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NCT00786825

IRB 9412M09040

Revised: 9/30/2011

SPECIFIC AIMS

The fear of hypoglycemia and hypoglycemia unawareness limits the ability of patients with diabetes from achieving the level of glucose control known to reduce the risk of developing microvascular complications. The **long term goal** of this project is to define the pathogenesis of hypoglycemia-associated autonomic failure (HAAF) with the aim of helping patients better manage their diabetes. In HAAF, patients experience both *hypoglycemia unawareness* and *defective glucose counterregulation*. Increased glucose availability to the brain is a likely contributor to the development of *hypoglycemia unawareness* either by increased glucose transport, decreased glucose utilization or increased glycogen storage following repeated episodes of hypoglycemia. Work previously supported by this award provided evidence for elevated free glucose levels in cortical regions of patients with type 1 diabetes and hypoglycemia unawareness, but the impact of recurrent hypoglycemia on glucose levels and metabolism in the hypothalamus, the region in which both glucose sensing and coordination of the counterregulatory response are believed to occur, has not been evaluated. Growing evidence in animal models and humans also suggests that increased hypothalamic γ-aminobutyric acid (GABA) tone following recurrent hypoglycemia plays a central role in *defective glucose counterregulation*, but hypothalamic levels of GABA have not yet been measured in humans with and without HAAF. We recently developed in vivo proton magnetic resonance spectroscopy (^{1}H MRS) methodology to monitor glucose and GABA levels in the human hypothalamus at the ultra-high magnetic field of 7 tesla and to simultaneously measure all 4 kinetic parameters for cerebral glucose transport and utilization (K_M transport, V_{max} transport, K_M utilization, V_{max} utilization). These methods will now be employed in humans together with the ^{13}C-MRS protocol we have previously used to study brain glycogen to examine if changes in glucose availability (in the form of free glucose or glycogen) and GABA levels contribute to the development of *hypoglycemia unawareness* and *defective glucose counterregulation* in HAAF.

The **working hypotheses** for this project are: **1)** recurrent hypoglycemia, like that seen in subjects with type 1 diabetes and iatrogenic hypoglycemia, will lead to an upregulation of glucose availability for the brain (by increased transport, and/or increased glycogen storage), thereby facilitating maintenance of cerebral metabolism during subsequent episodes of hypoglycemia, and **2)** recurrent hypoglycemia will lead to an increased GABA tone in hypothalamus, thereby suppressing glucose counterregulation during subsequent episodes of hypoglycemia. To address these hypotheses we will examine cerebral metabolism in subjects with type 1 diabetes and hypoglycemia unawareness, as well as in healthy volunteers preconditioned with euglycemia vs. recurrent hypoglycemia to induce HAAF, thereby permitting us to understand the clinical syndrome as well as isolate the effects of recurrent hypoglycemia from those induced by diabetes.

Aim 1: To determine the contribution of increased glucose availability in the brain to HAAF

***Specific Aim 1a*:** To measure kinetic parameters for glucose transport and metabolism in the hypothalamus of subjects with type 1 diabetes and hypoglycemia unawareness and in healthy volunteers preconditioned with euglycemia vs. recurrent hypoglycemia to induce HAAF.

Hypothesis 1a.1: Subjects with type 1 diabetes and hypoglycemia unawareness will have a higher V_{max} transport than will healthy volunteers preconditioned with euglycemia.

Hypothesis 1a.2: Healthy volunteers will have a higher V_{max} transport following preconditioning with recurrent hypoglycemia than with euglycemia.

***Specific Aim 1b*:** To measure brain glycogen turnover and content by ^{13}C MRS in healthy volunteers preconditioned with euglycemia vs. recurrent hypoglycemia to induce HAAF.

Hypothesis 1b: Subjects will have higher glycogen content following preconditioning with recurrent hypoglycemia than with euglycemia.

Aim 2: To determine the contribution of increased GABA tone in the hypothalamus to HAAF

***Specific Aim 2*:** To measure euglycemic and hypoglycemic GABA levels in the hypothalamus of subjects with type 1 diabetes and HAAF and in healthy volunteers preconditioned with euglycemia vs. recurrent hypoglycemia to induce HAAF.

Hypothesis 2a: Hypothalamic GABA levels will be lower during hypoglycemia than during euglycemia in both normal controls and patients with type 1 diabetes.

Hypothesis 2b: Hypothalamic GABA levels will be higher both during euglycemia and during hypoglycemia in subjects with type 1 diabetes and HAAF than in matched controls preconditioned with euglycemia.

Hypothesis 2c: Hypothalamic GABA levels will be higher both during euglycemia and during hypoglycemia in healthy volunteers following preconditioning with recurrent hypoglycemia than with euglycemia.

A. SIGNIFICANCE

More than 25 million Americans have diabetes and hypoglycemia is a common event in the lives of patients who receive insulin or insulin secretagoges as therapy for their disease. Inconvenient and disruptive to daily activities, it can also lead to loss of consciousness, seizures, and death. Patients who experience recurrent episodes of hypoglycemia often develop hypoglycemia associated autonomic failure (HAAF); a clinical syndrome in which the first symptom of hypoglycemia is confusion or loss of consciousness (1). The fear of HAAF prevents many patients with diabetes from achieving the glycemic goals known to reduce the risk of developing the microvascular complications of the disease (2). Consequently, better ways to prevent HAAF are of critical importance in advancing care for patients with diabetes. To achieve this goal first requires an understanding of the pathogenesis of this syndrome, which is the purpose of the project outlined in this application.

Healthy humans have a robust and redundant counterregulatory response that prevents the development of hypoglycemia. In patients with type 1 diabetes and advanced type 2 diabetes, this response is impaired (3). Such patients are unable to prevent a fall in glucose by reducing their own insulin secretion because of the beta cell failure associated with their disease. They are also unable to release glucagon in response to hypoglycemia, likely due to a failure to reduce levels of insulin within the islet in response to the falling glucose (4). They come to rely on hypoglycemia-induced secretion of epinephrine from the adrenal medulla and norepinephrine from the sympathetic nerves to cause the adrenergic symptoms typically associated with a low blood sugar to signal the need to ingest carbohydrates. However, frequent episodes of hypoglycemia can lead to HAAF. In HAAF, the blood glucose level that triggers catecholamine release is reduced into the range at which humans experience neuroglycopenia. Patients with HAAF do not know they are experiencing hypoglycemia until they become confused or lose consciousness; a situation in which they cannot help themselves recover from the drop in blood sugar.

The mechanisms responsible for the development of HAAF remain uncertain (5). Clearly, upregulated fuel availability to the brain, either in the form of glucose (free or in glycogen reserves) or in the form of alternative fuels, can result in a failure to detect systemic hypoglycemia, the “unawareness” component of HAAF. While there is conflicting data if *global* cerebral glucose transport or metabolism are altered in HAAF (6-8), *regional* alterations seem likely, particularly in the hypothalamus (9; 10), the region that senses systemic glucose and coordinates the counterregulatory response (11). Our prior data using proton magnetic resonance spectroscopy (^{1}H MRS) demonstrated higher cortical glucose levels in patients with type 1 diabetes and hypoglycemia unawareness than controls at the same plasma glucose levels (12). Subsequently, we found that oxidative glucose consumption in this brain region was not different in patients with type 1 diabetes and hypoglycemia unawareness than controls (13), indicating that the elevated glucose levels we observed were due to increased glucose transport, rather than decreased metabolism, and that such upregulated glucose transport may provide additional fuel to the brain during subsequent episodes of hypoglycemia to maintain normal function, at least for a while. These studies had focused on the occipital cortex due to technical challenges with studying small and deep brain regions such as the hypothalamus. In fact, glucose content and metabolism have not been measured simultaneously in the human hypothalamus before. We have now overcome these challenges associated with acquiring MRS data from the human hypothalamus (14) (Appendix A, Preliminary studies)

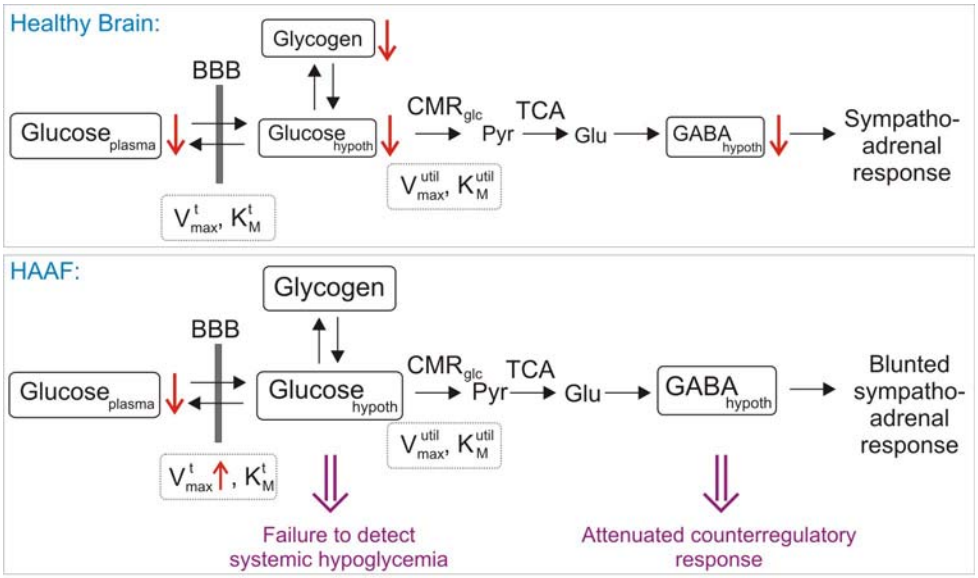

Figure 1. Working hypotheses shown with a “simplified” model of brain glucose, glycogen and GABA metabolism. The metabolic pools and kinetic parameters that will be measured are shown in boxes. The predicted relative pool sizes are reflected in the box sizes. BBB: blood brain barrier; CMR_{glc}: cerebral metabolic rate of glucose utilization; Pyr: pyruvate; TCA: tricarboxylic acid cycle; Glu: glutamate.

and further developed methodology to estimate kinetic parameters for glucose transport and utilization simultaneously (15) (Appendix B, Preliminary studies). Therefore we are now in a unique position to address the affects of HAAF on glucose transport and metabolism in this brain region critical in CNS regulation of glycemic control.

Regarding the second component of HAAF, the blunted counterregulatory hormonal response, recent investigations in rodents suggest a central role of an increased γ-aminobutyric acid (GABA) tone in the hypothalamus with recurrent hypoglycemia (16; 17). A fall in the hypothalamic levels of GABA, the main inhibitory neurotransmitter in the brain, appears necessary for activation of glucose counterregulatory responses during hypoglycemia. Recurrently hypoglycemic animals not only have elevated hypothalamic GABA levels, but they also fail to decrease these GABA levels in response to hypoglycemia (16). Furthermore, local application of glucose prevents this fall in a dose-dependent fashion, resulting in a positive correlation of extracellular glucose and GABA concentrations (17) and mechanistically linking increased glucose availability in this region to blunting of the GABA-regulated counterregulatory hormone release. With recently developed technology (14) (Appendix A, Preliminary studies) we can now test if the same sequence of events occurs in the human brain.

These prior data form the basis for our *working hypotheses* in the current application (Figure 1): In the healthy brain, a drop in plasma glucose results in a drop in brain glucose, as well as a reduction in glycogen (18) and GABA (see Preliminary studies). Following recurrent hypoglycemia, a drop in systemic glucose does not cause a similar drop in hypothalamic glucose levels due to upregulated transport capacity ($V^t_{\max}$), and thus the signal that leads to glycogen mobilization and a fall in hypoglycemic GABA levels is reduced, thereby blunting the hypoglycemia induced sympathoadrenal response. We will investigate these mechanisms both in healthy volunteers who have been preconditioned with recurrent hypoglycemia (a HAAF model), as well as in patients with type 1 diabetes, in order to distinguish the effects of HAAF alone from those of diabetes. With the elucidation of the contributions of glucose (Aim 1a), glycogen (Aim 1b) and GABA (Aim 2) to the pathogenesis of HAAF, new knowledge will be available that will permit the development of better strategies to prevent and treat this condition, such as pharmaceutical interventions to prevent an increase of hypothalamic GABA levels.

B. INNOVATION

This project is innovative because it will use ultra-high field MRS to examine in vivo cerebral metabolism in humans with and without diabetes. This is a significant advance because it will allow us to measure metabolites in a small, central structure like the hypothalamus, which is known to be critically important in recognizing and responding to hypoglycemia. In our previous work, we performed ^{1}H MRS at 4 tesla in the occipital cortex (12), but were unable to acquire sufficient signal in the hypothalamus to measure GABA and glucose concentrations in the hypothalamus. As is demonstrated in a recent publication from our group (14) (Appendix A), this problem is overcome with the methodology developed for use at 7 tesla. This technology will now allow us to test the elevated GABA tone hypothesis by direct measurements in the human hypothalamus for the first time.

This project is also innovative because we will use the recently developed MRS methodology that allows for the simultaneous measurement of kinetic parameters for cerebral glucose transport and utilization (K_M transport, V_{max} transport, K_M utilization, V_{max} utilization) with dynamic measurements of brain glucose levels as a function of plasma glucose (15). This methodology is a significant advance from previous investigations in humans which have relied on data collected under steady-state conditions and obtained kinetic parameters for glucose transport only by assuming a constant cerebral metabolic rate for glucose from literature. Using the new 7 tesla MRS methods developed in our lab combined with this new modeling approach, we will be able to investigate glucose content and metabolism simultaneously in the human hypothalamus for the first time.

C. APPROACH

In this section we will first present preliminary data that demonstrates the feasibility of our experiments and then discuss the approach we will use to address each of our aims.

C.1. PRELIMINARY STUDIES

C.1.1. Methodology to create a human model of HAAF (Aims 1 and 2):

Exposing healthy humans to recurrent hypoglycemia has repeatedly been shown to blunt the counterregulatory response to a subsequent episode of hypoglycemia (1; 2), just as observed in subjects with type 1 diabetes and HAAF (3). An examination of a human model of HAAF will allow us to isolate the effects of recurrent hypoglycemia on brain metabolism from any additional effects of diabetes we may have observed in our previous experiments in subjects with type 1 diabetes and hypoglycemia unawareness. To demonstrate

that we can create a human model of HAAF in our laboratory, four healthy humans participated in a two part study in which hypoglycemic clamp studies were performed from 9-11 AM and 1-3 pm on day 1 and again between 9-11 AM on day 3 (n=2) or day 5 (n=2). In each study, insulin was infused at a rate of 2.0 mU/kg/min for two hours and blood glucose was reduced to and then maintained at 50 mg/dl by the infusion of 20% dextrose, as guided by the measurement of blood sugars every 5 minutes. Blood was collected every 30 minutes during the clamp studies for the subsequent measurement of counterregulatory hormones in the Vanderbilt DRTC Core Laboratory. We observed the epinephrine secretory response during the third episode to be blunted to 46 ± 8% of the response measured during the first episode.

These data demonstrate that we can create a human model of HAAF in our laboratory and that healthy volunteers preconditioned with a repeated hypoglycemia protocol that involves 2 two-hour long hypoglycemic episodes on Day 1 have blunted counterregulatory response to hypoglycemia that lasts until Day 5 (critical for the design of the studies in Aim 1b).

C.1.2. Methodology to measure kinetics of cerebral glucose transport and utilization (Aim 1a):

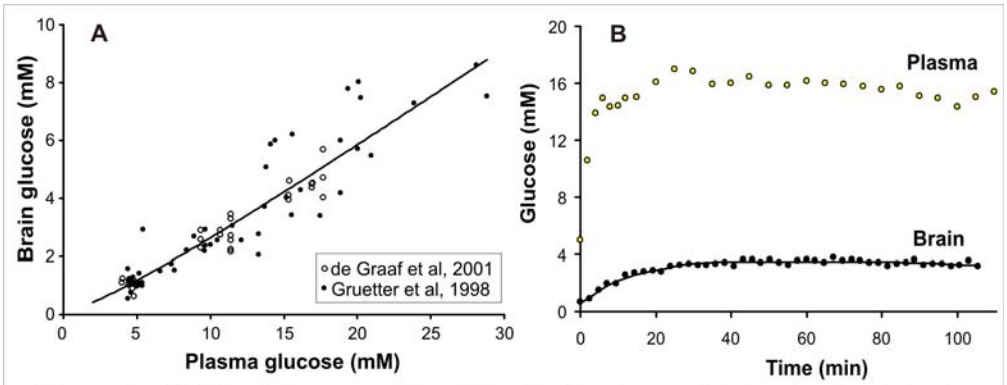

Figure 2. A) Fit of the reversible Michaelis Menten model to steady state data from literature. **B)** Glucose concentrations obtained in the plasma and occipital cortex of one volunteer, together with the fit of the non-steady state, reversible Michaelis-Menten model to brain glucose concentrations.

The kinetic parameters for glucose transport and the rates of its metabolism in the brain have so far been obtained from separate experiments, for example measurements of brain glucose concentrations by ^{1}H MRS as a function of blood glucose to extract transport parameters (19; 20) and MR and PET tracer studies to estimate net glucose uptake/oxidation (10; 21). In glucose transport measurements by ^{1}H MRS, the kinetic parameters of cerebral glucose transport are traditionally obtained by fitting steady-state Michaelis-Menten models to steady-state brain glucose concentrations obtained as a function of plasma glucose concentrations. This way, K_M for transport (K_M^t) and the ratio of V_{max} for transport ($V_{\max}^t$) to cerebral metabolic rate of glucose (CMR_{glc}) are obtained and, assuming a constant CMR_{glc} from prior work, $V_{\max}^t$ is calculated (19; 20). Such steady-state measurements do not take advantage of the additional information available in dynamic data. The feasibility of determining all four kinetic parameters for cerebral transport and utilization (K_M^t, $V_{\max}^t$, K_M^{util}, $V_{\max}^{util}$) by fitting both dynamic and steady-state data was demonstrated recently for acetate (22). To apply the same approach to glucose transport and utilization, we measured brain and plasma glucose time courses during glucose infusions to raise and maintain plasma concentration at ~17 mmol/l for ~2 hours in 5 healthy volunteers. Occipital cortex glucose levels were measured from a 22-27mL voxel using previously described methods (12; 23) at 4 tesla. We fitted a reversible, non-steady-state Michaelis-Menten model to these data (Fig. 2B) (15) combined with steady-state plasma vs. brain glucose concentrations taken from literature (Fig. 2A) (19; 20). In addition to providing simultaneous measurements of glucose transport and utilization and obviating assumptions for constant CMR_{glc}, this methodology does not necessitate infusions of expensive or radioactive tracers. Using this new methodology, we found that the maximum transport capacity for glucose through the blood-brain barrier ($V_{\max}^t$ = 0.94 ± 0.21 µmol/g/min) was nearly two-fold higher than maximum cerebral glucose utilization ($V_{\max}^{util}$ = 0.48 ± 0.12 µmol/g/min). Furthermore, the glucose transport and utilization parameters were consistent with previously published values for the occipital cortex. These results were presented at a conference (15) and a manuscript describing them was submitted.

These data demonstrate that the newly developed dynamic MRS methodology enables simultaneous measurements of the kinetic parameters for cerebral glucose transport and utilization (note that the steady state data of Fig. 2A will be obtained from the hypothalamus under the proposed studies by utilizing multiple hyperglycemic targets, unlike the preliminary studies in the occipital cortex that only utilized a single target, hence necessitating the use of literature data) and when tested in a widely studied region-of-interest, it provided transport parameters and a CMR_{glc} consistent with previously published values obtained in independent studies.

C.1.3. Methodology to measure glycogen turnover and content (Aim 1b):

We have a long track record in measuring glycogen turnover and content using ^{13}C MRS. We developed the technology for applications in the human brain (24; 25), measured glycogen metabolic rate and content (26), demonstrated glycogen mobilization during hypoglycemia and increased glycogen synthesis following a single episode of moderate hypoglycemia in the healthy human brain (18). In addition, we investigated glycogen content in the brains of patients with type 1 diabetes and hypoglycemia unawareness using the technology (see C.4. Progress Report). Therefore we have extensive experience in investigating glycogen metabolism using ^{13}C MRS in conjunction with IV infusions of ^{13}C-glucose.

C.1.4. Methodology to measure hypothalamic concentrations of GABA and glucose (Aims 1a and 2):

We have recently developed MRS methodology to obtain spectra of high quality (good signal-to-noise ratio and resolution) from any region in the cerebrum, cerebellum and brainstem at 7 tesla (14). We applied this technology to obtain neurochemical profiles, including GABA and glucose, from the human hypothalamus. GABA levels were quantified with Cramér-Rao lower bounds (CRLB, estimated errors (%SD) of metabolite quantification calculated by the quantification software LCModel) of 15% in these spectra obtained over 10 minutes at euglycemia (Fig. 3). Hypothalamic GABA levels were estimated at 3.5-4 μmol/g at euglycemia (Fig. 4), i.e. 3-4 fold higher than cortical GABA concentrations (27), in excellent agreement with biochemical literature (28). Glucose levels were quantified with CRLB of 33%, which was similar to the CRLB obtained at the euglycemic baseline measurements of cortical glucose levels at 4 tesla (Fig. 2B). Therefore these data demonstrate that we will be able to obtain kinetic parameters for glucose transport and utilization in the hypothalamus with the same reliability (measurement errors) as we have in the occipital cortex.

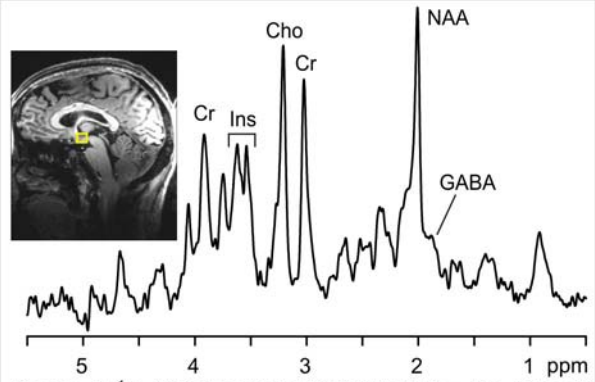

Figure 3. ^{1}H MR spectrum (STEAM, TR = 5 s, TE = 8 ms) obtained at 7T from the hypothalamus of a healthy volunteer. The voxel position (yellow) is shown on the T_1-weighted mid-sagittal image. The marked peaks are: N-acetylaspartate, creatine, choline, *myo*-inositol and γ-aminobyturic acid.

The CRLB are a good estimate of test-retest reproducibility of metabolite measurements, however we also obtained test-retest data from the hypothalamus at euglycemia in a healthy volunteer at 7 tesla (Fig. 4A, green trace). The coefficient-of-variance for the GABA measurement was 3% and for glucose 20% in this study. We further investigated the response of both neurochemicals to hypoglycemia in a patient with type 1 diabetes and hypoglycemia unawareness and an age-, gender- and BMI-matched healthy volunteer (Fig. 4). The reliability of the glucose measurement decreased as the hypothalamic glucose levels dropped, therefore we will conduct the glucose transport and metabolism studies by employing hyperglycemic targets as has been traditionally done to study cerebral glucose transport by MR (15; 19; 20). On the other hand, GABA levels were reliably quantified throughout the hypoglycemic clamp and we observed a decrease in GABA levels in response to hypoglycemia in both the healthy volunteer and the patient (Fig. 4). However, the GABA decrease from baseline to the last data point was 36% in the case of the healthy volunteer while it was 26% in the case of the patient.

These data demonstrate our ability to reliably measure GABA and glucose concentrations for the proposed studies in Aims 1a and 2 and support our hypothesis regarding an attenuated hypothalamic GABA response to hypoglycemia in type 1 diabetes and hypoglycemia unawareness.

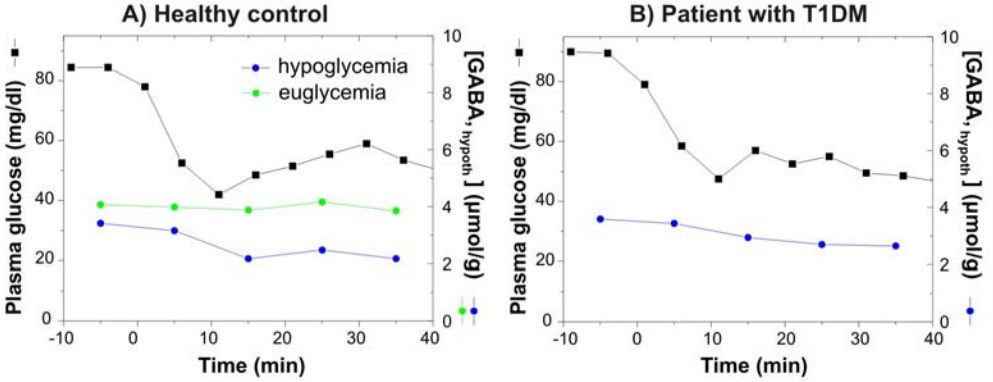

Figure 4. Plasma glucose and hypothalamic GABA concentrations in a healthy volunteer (A) and a patient with type 1 diabetes (B) during a hypoglycemic clamp study. Subjects received IV infusions of glucose and insulin. The glucose infusion was stopped at time 0 and restarted later at a lower rate to clamp subjects at 50mg/dl plasma glucose. The healthy volunteer was also studied in a separate session during euglycemia without IV infusions (green circles, A).

C.2. RESEARCH DESIGN

Aim 1a: To measure kinetic parameters for glucose transport and metabolism (K_M transport, V_{max} transport, K_M utilization, V_{max} utilization) in the hypothalamus of subjects with type 1 diabetes with hypoglycemia unawareness and in healthy volunteers preconditioned with euglycemia vs. recurrent hypoglycemia to induce HAAF.

In these experiments, subjects with type 1 diabetes with hypoglycemia unawareness as well as normal controls, preconditioned on separate occasions with euglycemia and with recurrent hypoglycemia, will have hypothalamic glucose concentrations measured continuously using ^{1}H MRS at 7 tesla over a ~1.5 hour period during which blood glucose concentrations will be experimentally increased from 95 mg/dl to one of 3 target hyperglycemic levels (200 mg/dl (11 mM), 300 mg/dl (16.5 mM), 400 mg/dl (22 mM)).

Study rationale:

We have previously demonstrated that steady state glucose concentrations in the occipital cortex are higher in subjects with type 1 diabetes and hypoglycemia unawareness than in matched controls studied under the same conditions (12), with no differences in oxidative glucose consumption (13). We have interpreted these data to suggest that brain glucose transport is upregulated in response to recurrent hypoglycemia and have proposed that this is one mechanism that contributes to the development of HAAF. However, these observations do not provide evidence of how recurrent hypoglycemia alters glucose transport or metabolism in the hypothalamus. In addition, the finding of higher steady state brain glucose concentrations in subjects with type 1 diabetes and hypoglycemia unawareness than in controls does not necessarily mean that this is purely a result of recurrent hypoglycemia, and not a confound of diabetes. To overcome these limitations, we will now directly examine hypothalamic glucose transport and metabolism by utilizing dynamic measurements of brain glucose levels as a function of plasma glucose. Such an approach will permit the simultaneous measurement of K_M transport, V_{max} transport, K_M utilization, V_{max} utilization in this critical region. We have selected 3 hyperglycemic targets to obtain steady-state brain vs. plasma glucose concentrations over a wide range of plasma glucose concentrations (as shown for occipital cortex in Fig. 2A). We will study subjects with type 1 diabetes and hypoglycemia unawareness, as well as a human model of HAAF. Note that we do not use the term “HAAF” in the case of the patients since we will not perform a hypoglycemic clamp in patients as part of this aim to confirm blunted counterregulatory response prior to the MR scan; however the presence of HAAF has been repeatedly demonstrated in subjects with type 1 diabetes and hypoglycemia unawareness (12), as assessed by a standardized questionnaire we will use. If healthy humans demonstrate an increase in the V_{max} transport in the hypothalamus following the induction of HAAF, it will provide firm support that this mechanism participates in the development of the syndrome.

Research Subjects:

Subjects with and without diabetes will be recruited from a registry maintained by the investigators of individuals interested in participating in this type of research. In addition, subjects with diabetes will be recruited from the Endocrine Clinic at the University of Minnesota and healthy controls will be recruited from the University of Minnesota community. Inclusion criteria for the subjects with diabetes include well controlled type 1 diabetes (hemoglobin A1C <7.5%), frequent episodic hypoglycemia (at least 2 episodes per week for the preceding two months) and hypoglycemia unawareness verified by a standardized questionnaire (29). Healthy controls will be age (within 5 years), gender, and body mass index (within 4 kg/m^2) matched to the subjects with diabetes. Exclusion criteria for both groups of subjects include history of stroke, seizures, neurosurgical procedures, or arrhythmias, and use of drugs that can alter glucose metabolism (other than insulin for the patients with diabetes). Subjects must also meet requirements for a study in the magnet, which includes weight less than 300 lbs and the absence of metallic substances in their body. After screening and consent, each patient will be randomized to one of three glucose targets (200, 300, 400 mg/dl); the patient’s matched control will be assigned to the same target.

Study Protocol:

Subjects with type 1 diabetes and hypoglycemia unawareness will report to the Center for Magnetic Resonance Research (CMRR) in the morning in the fasting state. They will be instructed to manage their diabetes during the day prior to the study in such a way as to minimize the amount of subcutaneous insulin present during the experiment while maintaining adequate control. Upon arrival, an intravenous catheter will be placed antegrade in a forearm for subsequent infusions and a catheter will be placed retrograde in the lower leg for blood sampling. An intravenous infusion of insulin will then be started and adjusted as necessary to bring blood glucose to 95 mg/dl. Blood will be collected every 10 minutes for measurement of glucose on a

nearby Analox machine to guide adjustments in the insulin infusion rate. The experiment will begin when the subject is at 95 mg/dl. At time 0, the intravenous insulin infusion will be fixed at 0.5 mU/kg/min, samples for blood glucose will be collected every 5 minutes and an intravenous infusion of glucose (20% dextrose) will be administered as necessary to maintain blood glucose at 95 mg/dl. At +5 minutes, subjects will be placed into the 7 tesla magnet (Fig. 5). Blood glucose will be maintained at 95 mg/dl during the next 25 minutes as the hypothalamic volume-of-interest (VOI) is selected and baseline MRS data are acquired. At + 30 min, subjects will be given an intravenous bolus injection of glucose over 1-2 minutes using the formula of administering 2 mg glucose/kg body weight for each 1 mg/dl increase desired that we have used with success previously (19). Immediately after the bolus injection, a continuous infusion of 20% dextrose will be started and the rate of administration will be adjusted to maintain the desired glucose target of 200 mg/dl, 300 mg/dl, or 400 mg/dl. MRS data will be collected continuously starting 10 minutes before the bolus injection (baseline) until the end of the study ~60 minutes after the bolus, which in our experience is sufficient to reach steady-state glucose concentrations in the brain and maintain them for at least 20 minutes (Fig. 2B). At the completion of the study, the infusions will be discontinued; the subject will resume their usual insulin regimen, and be fed a meal.

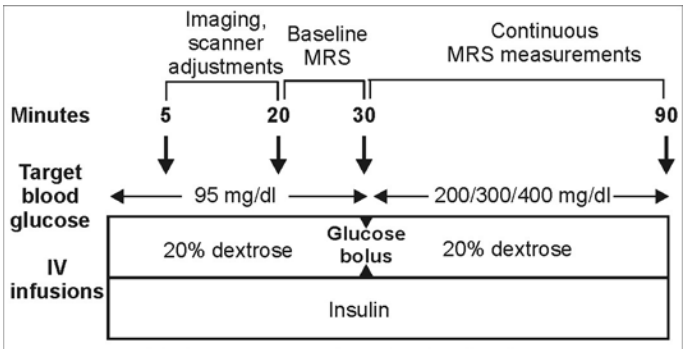

Figure 5. MRS protocol for Aim 1a. Scaling for the time periods is approximate.

Healthy controls will be studied both after preconditioning with euglycemia and after preconditioning with recurrent hypoglycemia to induce HAAF, done in random order separated by a minimum of 6 weeks (Fig. 6). Female controls will have their two studies two months apart in order to control for phase of menstrual cycle. We will follow a hypoglycemia preconditioning protocol we (see preliminary data) and others (30; 31) have found to induce HAAF.

For preconditioning, subjects will undergo hyperinsulinemic clamp studies between 8-10 AM on Day 1, 2-4 PM on Day 1, and 8-10 AM on Day 2. For hypoglycemia preconditioning, the target glucose during the clamp studies will be 50 mg/dl and for euglycemia preconditioning the target glucose will be 95 mg/dl. For each clamp study, subjects will be given intravenous insulin at a rate of 2.0 mU/kg/min for two hours and blood glucose will be maintained at target by the infusion of 20% dextrose, the rate of which will be adjusted based on blood glucose values measured on the Analox from samples collected every 5 minutes. Samples will also be collected every 30 minutes for later measurement of glucagon, catecholamines, cortisol, and growth hormone. Potassium phosphate will be infused at 4 mEq/hour as long as the insulin is being infused. At the completion of the two hour period, subjects will be given glucose as needed to restore euglycemia and then allowed to rest for two hours. On Day 2, subjects will go to the CMRR in the afternoon for assessment of hypothalamic glucose transport and metabolism using ^{1}H MRS. Upon arrival, an intravenous catheter will be placed antegrade in a forearm for subsequent infusions and a catheter will be placed retrograde in the lower leg for blood sampling. Subjects will be placed into the 7 tesla magnet and MRS data will be acquired from the hypothalamus with the same protocol as that for patients (Fig. 5). At + 30 min, subjects will be given a intravenous bolus injection of glucose over 1-2 minutes using the formula of administering 2 mg glucose/kg body weight for each 1 mg/dl increase desired that we have used with success previously (19). Following the bolus injection, the desired glucose target of 200 mg/dl, 300 mg/dl, or 400 mg/dl will be maintained with a continuous infusion of 20% dextrose at a varying rate based on glucose

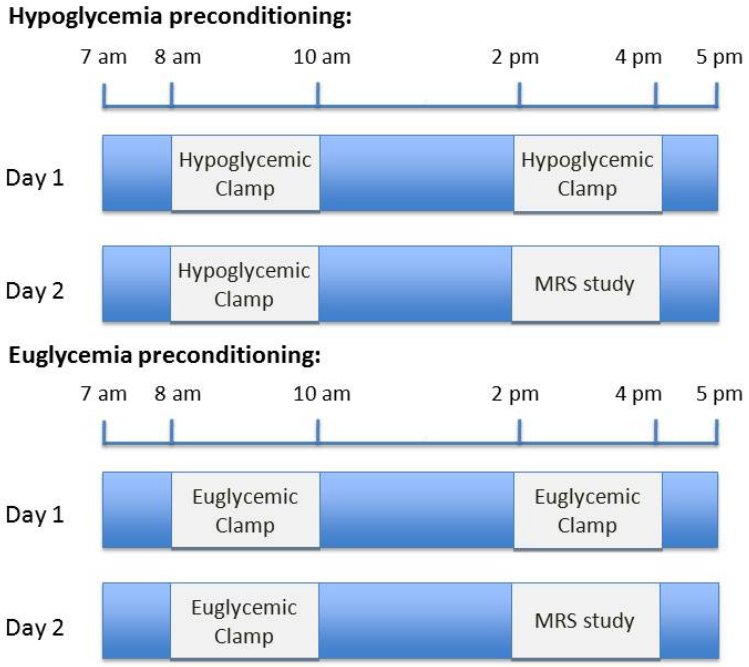

Figure 6. Study protocol for healthy controls in Aims 1a and 1b.

levels obtained in blood samples every 5 minutes. Insulin will not be administered due to the endogenous production of insulin in healthy volunteers and because plasma insulin concentrations up to 100 mU/l do not change brain glucose concentrations (32). At the completion of the study, the infusions will be discontinued and subjects will be fed a meal.

^{1}H MRS methods:

Studies will be performed on a 7T scanner operated with a Siemens console, using a 16-channel transmit/receive phased array coil (33). Methods to acquire the ^{1}H MRS data (including evaluation of the CSF contribution to each VOI) will be identical to those described before, using short echo (TE = 8 ms), single voxel stimulated echo acquisition mode (STEAM) spectroscopy (14, Appendix A, 34). Power deposition in each VOI will be optimized by B_1 shimming in each subject as described before (14; 35). Methods to quantify the neurochemical profiles using LCModel (36), and to assess the reliability of metabolite concentrations based on Cramér-Rao lower bounds, will be identical to those described before (14; 34; 37).

Modeling methods:

A kinetic model of glucose transport through the blood-brain-barrier (BBB) via reversible symmetric Michaelis-Menten kinetics and irreversible utilization in brain tissue will be utilized (15). In this model, the time-dependent brain glucose concentration Glc_{brain} is given by

$$\frac{d[Glc_{brain}]}{dt} = \frac{V^t_{\max}[Glc_{plasma}] - V^t_{\max}[Glc_{brain}]/V_d}{K^t_M + [Glc_{plasma}] + [Glc_{brain}]/V_d} - CMR_{glc} \qquad \text{with} \qquad CMR_{glc} = \frac{V^{util}_{\max}[Glc_{brain}]}{K^{util}_M \cdot V_d + [Glc_{brain}]}$$

where K^t_M is the Michaelis-Menten constant for transport of glucose through the BBB, $V^t_{\max}$ is the maximal transport rate of glucose, V_d is the physical distribution volume for brain glucose (0.77 ml/g) (38; 39) and K^{util}_M and $V^{util}_{\max}$ are the Michaelis-Menten constant and the maximal rate for glucose utilization. CMR_{glc} is the cerebral metabolic rate for glucose consumption and is modeled by irreversible Michaelis-Menten kinetics. The four unknown parameters in these equations are K^t_M, $V^t_{\max}$ and K^{util}_M and $V^{util}_{\max}$ and the measured data sets are $\left[Glc_{plasma}\right]$ and $\left[Glc_{brain}\right]$ under steady–state conditions and as a function of time in dynamic experiments. Simplex, Broyden-Fletcher-Goldfarb-Shanno and Levenberg-Marquart algorithms will be used to extract kinetic parameters by minimization of the cost function as defined by the least-square difference between model simulation and experimental data.

Sample size justification and data analysis plan:

$V^t_{\max}$ will be compared between patients and euglycemia conditioned controls with a paired t-test; if data are highly skewed, non-parametric alternatives will be considered. Similar comparisons will be carried out, within the controls, between the results following euglycemic preconditioning and following hypoglycemic conditioning. Pilot data standard deviation in $V^t_{\max}$ (section C1.2) indicate that with 12 participants per group we have 85% power to detect paired-group differences of 14% in $V^t_{\max}$ with type I error of 5%. We have budgeted for 15 per group to allow for some failed scans without sacrificing power. Our previous data in the occipital cortex demonstrated ~17% higher glucose concentrations in subjects with type 1 diabetes and hypoglycemia unawareness vs. controls. Therefore, we expect to be able to detect a similar effect size in the hypothalamus in the proposed studies with 12 subjects in each group. While our power calculations are based on SD in $V^t_{\max}$ because we hypothesize that unidirectional glucose transport is upregulated in HAAF, our studies will also be able to reveal a change in $V^{util}_{\max}$, if HAAF involves a change in glucose metabolism, thanks to the simultaneous measurement of these parameters with the new modeling approach.

Potential pitfalls and solutions:

Data will be collected as blood glucose is experimentally increased from 95 to 200, 300, or 400 mg/dl and we assume that the same kinetic constants will apply under hypoglycemia conditions. Under Aim 2, we will also obtain hypothalamic glucose data under hypoglycemia because we will be using the same MRS methods to obtain neurochemical profiles in these two aims. Since these data will also be available, we will assess the reliability of hypothalamic glucose measurements at hypoglycemia at a lower time resolution than that shown in Fig. 2B, e.g. in spectra acquired every 10-20 minutes instead of every 2.5 min. Because we save our MRS data in single shots, we can process these data to add over different time windows. Indeed, such lower time-

resolution data may also provide hypoglycemic glucose concentrations in hypothalamus. However, we chose to design the primary experiments under Aim 1a with hyperglycemic targets which we know will provide us with the kinetic parameters with certainty.

Another potential problem we may encounter is that the protocol we use to induce HAAF will not be effective for all subjects enrolled in the study. To ensure that HAAF is present at the time we do our experiment, we will measure counterregulatory hormones in blood collected from subjects during the first and third hypoglycemic clamps done before the MRS experiment. If the epinephrine response (the hormone most blunted during most protocols in which HAAF is induced in humans (30; 31)) during the third hypoglycemic clamp is $\geq$ 70% of the response measured during the first hypoglycemic clamp, we will conclude that HAAF was not induced and the data will not be used for analysis. If HAAF fails to be induced in 1/3, 2/5, 3/7, 4/9,… of the control studies (we will monitor in an ongoing way), we will change the protocol to induce HAAF to include more episodes of hypoglycemia before the MRS experiment.

Aim 1b: To measure brain glycogen turnover and content by ^{13}C MRS in healthy volunteers preconditioned with euglycemia vs. recurrent hypoglycemia to induce HAAF.

In these experiments, the impact of recurrent hypoglycemia on brain glycogen turnover and content will be measured using ^{13}C MRS in healthy humans.

Study rationale:

By monitoring incorporation of intravenously infused ^{13}C-glucose into glycogen by ^{13}C MRS, we have previously demonstrated increased brain glycogen synthesis following a single episode of moderate hypoglycemia in healthy humans, indicating glycogen supercompensation (18). On the other hand, we found that glycogen content in subjects with type 1 diabetes and hypoglycemia unawareness is not higher than controls (See C.4. Progress Report). We have interpreted these findings to indicate that supercompensated glycogen levels do not contribute to the pathogenesis of HAAF, perhaps because upregulation of glucose transport in these subjects ensures that they do not experience the reduction in brain glucose content necessary to stimulate glycogen supercompensation following the modest levels of hypoglycemia that routinely occur. However, it is also possible that diabetes itself alters brain glycogen metabolism in such a way as to confound the response to recurrent hypoglycemia. Therefore, we intend to examine the impact of recurrent hypoglycemia on brain glycogen metabolism in healthy volunteers without the confounding effects of diabetes. We will monitor ^{13}C-glucose incorporation into glycogen for up to 80 hours with the goal of turning over the entire glycogen pool for which shorter ^{13}C label infusion periods have been insufficient (18; 26). If we find that brain glycogen content is higher after induction of HAAF in normal volunteers, we can conclude that diabetes does alter brain glycogen metabolism in ways independent of recurrent hypoglycemia. If we find that brain glycogen content is the same or lower after induction of HAAF, we can conclude that supercompensated glycogen does not contribute to the development of HAAF.

Research Subjects:

Healthy controls will be recruited from the University of Minnesota community. Exclusion criteria will include history of stroke, seizures, neurosurgical procedures, or arrhythmias, and use of drugs that can alter glucose metabolism. Subjects must also meet requirements for a study in the magnet, which includes weight less than 300 lbs and the absence of metallic substances in their body.

Study Protocol:

We will examine the impact of repeated episodes of hypoglycemia on brain glycogen content and turnover in healthy controls. As in Aim 1a, subjects will be studied both after preconditioning with euglycemia and after preconditioning with recurrent hypoglycemia to induce HAAF, done in random order separated by a minimum of 6 weeks (Fig. 6). Female controls will have their two studies two months apart in order to control for phase of menstrual cycle.

The hypoglycemia preconditioning and euglycemia preconditioning protocols will be identical to those described in Aim 1a, with the first MRS session to measure ^{13}C-glycogen level in the afternoon on Day 2 (Fig. 6). At minute +30 following the end of the last hypoglycemic or euglycemic clamp, a bolus injection of 20 g of [1-^{13}C]glucose (prepared as 20% weight/volume D-glucose in water with 50% isotopic enrichment) will be administered to subjects regardless of their preconditioning protocol. Twenty minutes later a continuous infusion of 2 mg/kg/min of [1-^{13}C]glucose (prepared as 20% weight/volume D-glucose in water with 25% isotopic enrichment) will be started and then adjusted to maintain blood glucose at ~125 mg/dl based on samples collected every 10-60 minutes and measured on an automatic glucose analyzer. The infusion of ^{13}C-glucose will continue for ~82 hours. Cerebral ^{13}C-glycogen levels will be measured at ~5, 12, 22, 34, 46, 58,

70 and 82 hours after the end of the last hypoglycemic or euglycemic clamp. Blood samples will also be frozen for the later determination of plasma insulin concentrations by a chemiluminescent assay (Immulite, Diagnostic Products Corporation, Los Angeles, CA), and of the isotopic enrichment of the plasma glucose by gas-chromatography-mass spectroscopy (GC-MS) as described previously (40).

^{13}C MRS methods:

^{13}C-glycogen levels in the brain will be measured on a 4 T / 90 cm magnet (Oxford/Varian) using a quadrature 14 cm ^{1}H surface coil combined with a 9 cm diameter linear ^{13}C coil (41). The [1-^{13}C]glycogen NMR signal will be localized in a 7 × 5 × 6 cm^3 voxel in the occipital lobe as described before (25). The amount of ^{13}C label in the C1 position of glycogen will be quantified by the external reference method (23; 24). The [1-^{13}C]glycogen concentrations will be divided by the plasma glucose isotopic enrichment (IE) to correct for differences in IEs between subjects and to determine the newly synthesized glycogen concentrations. ^{13}C-glycogen levels will be corrected for the cerebrospinal fluid (CSF) content of the voxel (34).

Sample size justification and data analysis plan:

^{13}C-glycogen levels will be compared following euglycemic preconditioning to following hypoglycemic conditioning with a repeated measures model with effects for precondition, acquisition time (5, 12, etc. hours), and their interaction; we will also test for an effect of the order of preconditioning. Correlation structures appropriate for doubly-repeated measures (across acquisition times within preconditioning, for two preconditioning sessions) will be used. Previous work (18) shows standard deviations of the group difference ranging from 0.2 to 0.4 μmol/g across the acquisition times, indicating that with 5 participants we will have 85% power to detect paired-group differences between 0.36 and 0.73 μmol/g with type I error of 5%. Per-acquisition-time group comparisons will be adjusted for multiple comparisons using Sidak's method. We have budgeted for 6 participants to allow for one failed study without sacrificing power. Since we have detected highly significant differences during and following single hypoglycemia vs. euglycemia in our prior studies with 5 subjects per group (18), we expect to be able to also detect a difference with 5 subjects who will serve as their own controls, if recurrent hypoglycemia does affect glycogen content/metabolism.

Potential pitfalls and solutions:

We have had success in recruiting subjects to participate in similar protocols in the past (18; 26) and all of the methods are well established in our laboratory. Thus, we do not anticipate any problems with the completion of the work. As in our previous work (18; 24; 26) glycogen content and turnover will be studied in the occipital lobe because of the low cerebral glycogen concentrations, the large size of the molecule that broadens its MR signal and the resulting small ^{13}C MRS signal. It would be ideal to study hypothalamic glycogen levels and turnover; however this is not possible with the currently available technology and no other methods exist to monitor glycogen in the intact human brain.

As for aim 1a, we may find that the protocol we use to induce HAAF will not be effective for all subjects enrolled in the study. To ensure that HAAF is present at the time we examine glycogen metabolism, we will measure counterregulatory hormones in blood collected from subjects during the hypoglycemic clamps. If the epinephrine response (the hormone most blunted during most protocols in which HAAF is induced in humans (30; 31)) during the third hypoglycemic clamp is $\geq$ 70% of the response measured during the first hypoglycemic clamp, we will conclude that HAAF was not induced and the data will not be used for analysis. If HAAF fails to be induced in 1/3, 2/5, 3/7, 4/9,… of the studies (we will monitor in an ongoing way), we will change the protocol to induce HAAF to include more episodes of hypoglycemia before the glycogen experiment.

Aim 2: To measure euglycemic and hypoglycemic GABA levels in the hypothalamus of subjects with type 1 diabetes and HAAF and in healthy volunteers preconditioned with euglycemia vs. recurrent hypoglycemia to induce HAAF.

In these experiments, subjects with type 1 diabetes and HAAF (as confirmed by counterregulatory hormone measurements during a hypoglycemic clamp), as well as normal controls with and without HAAF, will have hypothalamic GABA levels measured at a blood glucose of 95 mg/dl and again at a glucose level of 50 mg/dl by ^{1}H MRS at 7 tesla over a ~one hour period.

Study rationale:

Recent studies in animals suggest that hypothalamic GABA may be important in regulating the counterregulatory response to hypoglycemia and that elevated GABA levels may contribute to the development of HAAF (17). Recurrent hypoglycemia leads to increased basal GABA inhibitory tone in the ventromedial hypothalamus (VMH) and suppresses the counterregulatory response to subsequent episodes of hypoglycemia (16). The role of GABA in regulating the counterregulatory response and in the development of

HAAF in humans is unknown. Therefore, we will use our newly developed ^{1}H MRS methods to measure hypothalamic GABA in subjects with type 1 diabetes and HAAF and in healthy controls preconditioned with euglycemia vs. recurrent hypoglycemia to induce HAAF. We predict that hypothalamic GABA will be lower during hypoglycemia than euglycemia in controls and subjects with type 1 diabetes, that induction of HAAF will lead to an increase in hypothalamic GABA under both euglycemic and hypoglycemic conditions, and that subjects with type 1 diabetes and HAAF will have higher levels of hypothalamic GABA under both euglycemic and hypoglycemic conditions than euglycemia preconditioned controls (Fig. 7). The MRS acquisition protocol will also provide concentrations of the precursor of GABA, glutamate (Fig. 1), which will potentially provide supporting information about GABA metabolism, however our hypotheses are based on GABA levels, which have been reported to change in prior animal work.

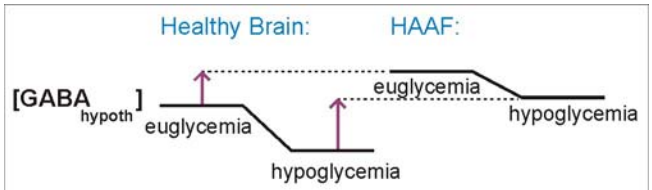

Figure 7. Predicted changes in eu- and hypoglycemic GABA levels in hypothalamus (as in Fig. 4) in the healthy brain and in HAAF.

Research Subjects:

Recruitment strategies and inclusion and exclusion criteria will be identical to those in Aim 1a, except we will also exclude subjects who take drugs that can alter GABA metabolism (such as benzodiazepines).

Study Protocol:

Subjects will report to the Center for Magnetic Resonance Research in the morning in the fasting state. *Subjects with diabetes* will be instructed to manage their diabetes in such a way as to minimize the amount of subcutaneous insulin present at the time of study while maintaining adequate control. *Healthy subjects* will serve as their own controls and be studied following the Day 1 hypoglycemia and euglycemia preconditioning protocols described for Aim 1 (Fig. 8). At least six weeks will separate the two studies, which will be done in random order. Female controls will have their two studies two months apart in order to control for phase of menstrual cycle.

On the day of the MRS experiment, an intravenous catheter will be placed antegrade in a forearm for subsequent infusions and a catheter will be placed retrograde in the lower leg for blood sampling. Patients with diabetes will be started on a variable rate infusion of intravenous insulin designed to bring their blood glucose to 95 mg/dl. To adjust the rate, blood will be collected every 5 minutes and tested on a nearby Analox machine. The experiment will begin, for both patients and controls, when the subject is at euglycemia. At time 0, insulin will be started at a rate of 2.0 mU/kg/min, potassium phosphate will be started at a rate of 4 mEq/hr, and glucose (20% dextrose) will be administered at a variable rate to maintain the blood glucose at 95 mg/dl. Subjects will then be placed into the 7 tesla magnet and hypothalamic GABA will be measured. At +60, blood glucose will be reduced to 50 mg/dl. The time course for hypothalamic GABA will be measured as shown in Fig. 4. Samples for the measurement of glucagon, epinephrine, norepinephrine, cortisol, and growth hormone will be collected at minutes 0, 60, 90, and 120. At the completion of the measurement, subjects will be taken out of the magnet, blood glucose will be restored, and subjects will be fed.

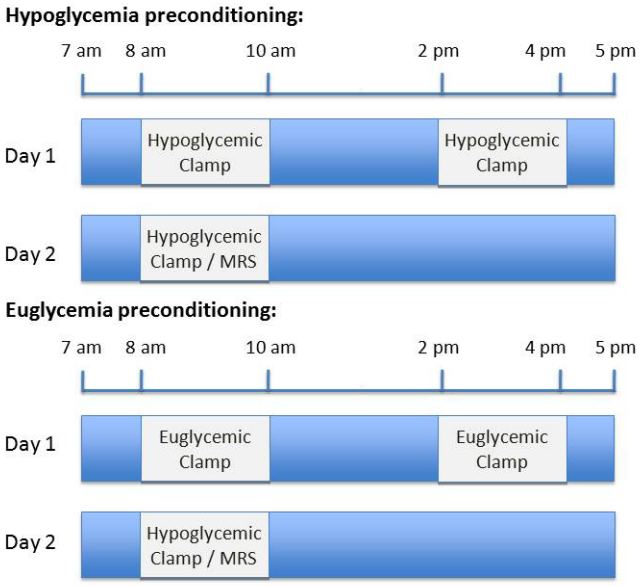

Figure 8. Study protocol for healthy controls in Aim 2.

^{1}HMRS methods:

Spectra will be acquired and quantified using identical methods to those described in Aim 1a. We chose to utilize STEAM spectroscopy to quantify GABA levels, as opposed to edited spectroscopy, since the GABA quantification using STEAM was validated against edited spectroscopy (42) and STEAM provides better signal-to-noise and additional metabolite concentrations such as glutamate (the precursor of GABA) and glucose (see Potential pitfalls for Aim 1a).

Sample size justification and data analysis plan:

Euglycemia vs. hypoglycemia states: After preconditioning with euglycemia among the controls, GABA levels will be compared during euglycemia to during hypoglycemia with a repeated measures model with effects for glycemic state, acquisition time (5, 15, etc. minutes, Fig. 4), and their interaction. Correlation structures appropriate for doubly-repeated measures (across acquisition times within glycemic states, for two glycemic states) will be used. These comparisons will be repeated among the controls after preconditioning with hypoglycemia, and among the patients. Pilot work (Fig. 4) shows a standard deviation of the control group difference between euglycemic states and hypoglycemic states of 0.52 μmol/g, indicating that with 10 participants we have 85% power to detect paired-group differences of 0.53 μmol/g with type I error of 5%. In the case that standard deviations are twice those observed in Figure 4, we have 85% power to detect differences of 1.10 μmol/g. Per-acqusition-time glycemic state comparisons will be adjusted for multiple comparisons using Sidak's method. We have budgeted for 12 participants to allow for two failed scans without sacrificing power. *Patients vs. controls:* Similar comparisons will be carried out between GABA levels in patient euglycemia states and in control euglycemia states after the controls are euglycemic preconditioned. This will be repeated for GABA in hypoglycemic states. Pilot work (Fig. 4) shows a standard deviation of the control group minus patient group difference during the hypoglycemic state of 0.25 μmol/g, indicating that with 10 participants we have 85% power to detect paired-group differences of 0.27 μmol/g with type I error of 5%. In the case that standard deviations are twice those observed in Figure 4, we have 85% power to detect differences of 0.53 μmol/g. *Preconditioning:* Similar comparisons will be carried out between GABA levels during control euglycemia states for euglycemic preconditioning compared to hypoglycemic preconditioning. This will be repeated for GABA during hypoglycemic states.

Potential pitfalls and solutions:

Prior rodent studies that demonstrated a role for increased hypothalamic GABA tone in blunting the counterregulatory response to hypoglycemia studied the VMH, while our spectroscopic voxel contains a larger portion of the hypothalamus. Despite this pitfall, our preliminary data demonstrate our ability to measure the hypothalamic GABA response to hypoglycemia both in controls and patients with type 1 diabetes. Therefore, we expect to be able to address our hypotheses regarding hypothalamic GABA despite these partial volume effects.

As discussed above, one potential problem we may encounter is that the protocol we use to induce HAAF will not be effective for all subjects enrolled in the study. To ensure that HAAF is present at the time we examine GABA metabolism, we will measure counterregulatory hormones in blood collected from subjects during hypoglycemia. If the epinephrine response (the hormone most blunted during most protocols in which HAAF is induced in humans) during the study done after hypoglycemic preconditioning is $\geq$ 70% of the response measured during the study after euglycemic preconditioning, we will conclude that HAAF was not induced and the data will not be used for analysis. If HAAF fails to be induced in 1/3, 2/5, 3/7, 4/9,... of the studies (we will monitor in an ongoing way), we will change the protocol to induce HAAF to include more episodes of hypoglycemia before the GABA experiment. In addition, in this aim the blunted counterregulation will also be confirmed in patients with type 1 diabetes by comparing their hormone measurements during the hypoglycemic clamp to those of the euglycemia-preconditioned controls.