Recurrent, Moderate Hypoglycemia Ameliorates Brain Damage and Cognitive Dysfunction Induced By Severe Hypoglycemia

Running Title: Preconditioning Limits Hypoglycemic Brain Injury

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**Objective:** Although intensive glycemic control achieved with insulin therapy increases the incidence of both moderate and severe hypoglycemia, clinical reports of cognitive impairment due to severe hypoglycemia have been highly variable. It was hypothesized that recurrent moderate hypoglycemia “preconditions” the brain and protect against damage caused by severe hypoglycemia.

**Research Design and Methods:** Nine-week old male Sprague-Dawley rats were subjected to either three consecutive days of recurrent, moderate (25-40 mg/dl) hypoglycemia (RH) or saline injections. On the fourth day, rats were subjected to a hyperinsulinemic (0.2 U/kg/min) severe hypoglycemic (~11 mg/dl) clamp for 60 or 90 minutes. Neuronal damage was subsequently assessed by H&E and Fluoro-Jade B staining. The functional significance of severe hypoglycemia induced brain damage was evaluated by motor and cognitive testing.

**Results:** Severe hypoglycemia induced brain damage and striking deficits in spatial learning and memory. Recurrent moderate hypoglycemia pretreated rats had 62-74% less brain cell death and were protected from most of these cognitive disturbances.

**Conclusions:** Antecedent recurrent moderate hypoglycemia “preconditioned” the brain and markedly limited both the extent of severe hypoglycemia induced neuronal damage and associated cognitive impairment. In conclusion, changes brought about by recurrent moderate hypoglycemia can be viewed, paradoxically, as providing a beneficial adaptive response in that there is mitigation against severe hypoglycemia induced brain damage and cognitive dysfunction.
Hypoglycemia is the major obstacle in achieving tight glycemic control in people with diabetes (1). Intensive insulin therapy increases the risk of iatrogenic hypoglycemia (2). Episodes of both moderate and severe hypoglycemia have long-term clinical consequences. Recurrent moderate hypoglycemia induces a maladaptive response that limits symptoms of hypoglycemia (hypoglycemia unawareness), limits the counterregulatory response to subsequent hypoglycemia (hypoglycemia associated autonomic failure), and thus jeopardizes patient safety (1). By depriving the brain of glucose, more severe hypoglycemia causes brain damage in animal studies and leads to long-term impairments in learning and memory (3;4). However, studies examining the effect of severe hypoglycemia in humans are conflicting. Severe hypoglycemia alters brain structure (5-7) and causes significant cognitive damage in many (5;7-12) but not all (13-16) studies. Reasons for the discrepancy between human and animal studies are unknown but a major contributing factor may be the extent of glycemic control (including recurrent hypoglycemia) prior to the episode of severe hypoglycemia.

In other models of brain damage such as ischemic stroke, brief, mild episodes of antecedent brain ischemia causes a beneficial adaptation that protects the brain against a subsequent episode of more severe ischemia (a phenomena known as ischemic preconditioning) (17). In a similar fashion, antecedent, recurrent episodes of moderate hypoglycemia was hypothesized to protect the brain against damage caused by a subsequent episode of more severe hypoglycemia.

To investigate this hypothesis, recurrent moderately hypoglycemic (25-40 mg/dl) rats and control saline injected rats were subjected to hyperinsulinemic, severe hypoglycemic clamps (10-15 mg/dl). One group of rats were sacrificed one week after severe hypoglycemia to quantify brain damage while a second group of rats were evaluated by behavioral and cognitive tests 6-8 weeks after the severe hypoglycemia. The results demonstrated that recurrent antecedent moderate hypoglycemia “preconditioned” the brain and protected it against neurological damage and cognitive defects induced by an episode of severe hypoglycemia.

**RESEARCH DESIGN AND METHODS**

**Animals.** Nine week old male Sprague-Dawley rats (Charles River Laboratories) were individually housed in a temperature and light controlled environment maintaining the animal’s diurnal cycle (12hrs light, 12hrs dark) with an ad lib standard rat chow diet. All studies were done in accordance with the Animal Studies Committee at the Washington University School of Medicine.

**Implantation of arterial and venous catheters.** Micro-renathane® (Braintree Scientific) catheters were inserted into the left carotid artery and into the right jugular vein of anesthetized rats (ketamine 40-80 mg/kg with xylazine 5-8 mg/kg). To maintain patency, catheters were filled with 40% polyvinylpyrrolidone (Sigma) in heparin (1000 USP U/ml) (Baxter Healthcare Corporation).

**Recurrent Moderate Hypoglycemia (Hypoglycemic Preconditioning).** One week after catheter implantation, recurrent moderate hypoglycemia (RH) was induced in non-fasted rats with injections of subcutaneous regular human insulin (Lilly) [6 U/kg, day 1; 5 U/kg, day 2; and 4 U/kg, day 3] while control rats (CON) were given equal volume saline injections for three consecutive days. Food was withheld and tail vein blood glucose values were measured hourly. For insulin treated rats, recurrent hypoglycemia resulted in blood glucose levels of 25-40
mg/dl for three hours. To terminate moderate hypoglycemia, rats were given a subcutaneous injection of dextrose (Hospira) and were allowed free access to food.

**Hyperinsulinemic-Severe Hypoglycemia Clamp.** Animals were fasted overnight after the third day of injections and the following morning, were subjected to a hyperinsulinemic (0.2 U/kg/min) severe hypoglycemic clamp (Figure 1). Rats were awake, unrestrained, and had free access to water. Arterial blood glucose was measured every 15 min with Ascensia Contour glucose monitors which are reported to have accurate blood glucose readings in the hypoglycemic range although accuracy in the severe hypoglycemic range has not been reported (Ascensia Contour, Bayer HealthCare, LLC). Insulin and glucose were co-infused intravenously to lower blood glucose to 10-15 mg/dl, as this level of severe hypoglycemia was necessary to induce neuronal damage (3;18). Severe hypoglycemia (SH) was maintained between 10-15 mg/dl for either 60 min (CON-SH60, n=6; RH-SH60, n=10) or 90 min (CON-SH90, n=20; RH-SH90, n=18) for the saline injected controls (CON) and recurrently hypoglycemic (RH) treated rats respectively. To terminate hypoglycemia, insulin infusion was stopped and infusions of dextrose were given until animals could maintain euglycemia. Additional blood samples were obtained during the basal period and 2 hours into the hyperinsulinemic clamp when severe hypoglycemia had been reached for 30 minutes for epinephrine measurements, as determined by single isotope derivative method (19).

Tonic-clonic seizure-like behavior was visually noted by characteristic brief (5-10 seconds) neck extensions, tonic stretching, uncontrolled limb movements, and spontaneous spinning (18;20). The number of episodes of seizure-like behavior during the clamp was quantified for each rat and was later correlated with histological and cognitive findings.

Two other groups of rats were made either recurrently hypoglycemic or given saline injections as described above, and on the fourth day, underwent a 90 minute hyperinsulinemic (0.2 U/kg/min) euglycemic clamp (CON-EUG, n=9; RH-EUG, n=11). These two additional groups served as euglycemic control rats treated in the same fashion except that they were not exposed to severe hypoglycemia.

The first grouping of rats that underwent hyperinsulinemic severe hypoglycemic clamps or hyperinsulinemic euglycemic clamps was analyzed for brain damage. The second grouping of rats was subjected to the same hyperinsulinemic clamp protocols except they underwent sensorimotor and behavioral testing (Figure 1).

**Histology.** One week after the severe hypoglycemic or euglycemic clamps, anesthetized rats were intracardially perfused with 0.01 M PBS (Sigma) followed by 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA). Brains were immersed in 4% paraformaldehyde overnight and then cryoprotected in 30% sucrose. Beginning at 2.8 mm posterior to the bregma, coronal cryostat sections (20 µm) were collected on superfrost coated slides (VWR). Four coronal sections, 120 µm apart, were analyzed for neuronal damage by Fluoro-Jade B (Chemicon International, Inc.) and hematoxylin and eosin (H&E, Sigma) staining, according to manufacturer’s protocol. Fluoro-Jade B is a well characterized stain for degenerating neurons (21). Fluorescent cells (Fluoro-Jade positive cells) were quantified in both hemispheres of the cortex and of the hippocampal structures, CA1 and dentate gyrus. For each region of interest, data is expressed as the average number of Fluoro-Jade B positive (FJB+) cells per section. (CON-SH90, n=9; RH-SH90, n=8).
Behavioral Testing. Consistent with other protocol designs (4;22;23), while histopathological outcomes are assessed one week following the hypoglycemic neuronal insult, cognitive studies are performed 6-8 weeks later in a separate group of similarly treated rats. This later assessment of cognitive function is a more useful measure of clinical outcome and a better functional index of neuroprotection because it allows for a complete and integrated evaluation of ongoing damage and/or possible recovery (24). As the Morris water maze test is a measure of hippocampal dependent spatial learning/memory and since the rats that underwent 60 minutes of severe hypoglycemia had little damage in the hippocampus, cognitive testing was not performed in this group. Cognitive testing was performed in the rats that underwent 90 min of severe hypoglycemia since these animals had marked damage in the hippocampus. After a 6-8 week recovery from the severe hypoglycemic (CON-SH90, n=11; RH-SH90, n=9) and euglycemic clamps (CON-EUG, n=7; RH-EUG, n=9), rats were transferred to the behavioral testing facility and allowed one week to acclimate before locomotor activity, sensorimotor measures, and Morris maze tests were performed under euglycemic conditions.

1-h Locomotor activity test and sensorimotor battery. General locomotor activity and exploratory behavior were evaluated for one hour using a computerized system (MotorMonitor, Kinder Scientific, LLC) of photobeam pairs to quantify ambulations (whole body movements) and rearing frequency. The ledge, platform, 90° inclined screen, and walking initiation tests were conducted to measure balance, strength, coordination and initiation of movement, as previously described (25).

Water maze cognitive testing. Spatial learning and memory were assessed using the Morris water maze test, similar to previously published methods (25). Briefly, a computerized tracking program (Polytrack, San Diego Instruments) recorded the swim path lengths and time required to find the platform. For the cued trials, rats were trained to swim to the submerged platform (1.5 cm below the surface) marked (cued) by a visible pole. Spatial learning capabilities of the rats were tested during the place trials. In the place trials, rats were trained to learn the position of a submerged and non-visible platform which remained in the same location across all trials. To evaluate memory retention of the platform location, a probe trial was conducted 1 hr after the last place trial which involved removing the platform from the pool and quantifying rats' search behaviors for 30 s. Probe trial performance indices included: the number of times a rat passed directly over the platform location (platform crossings); the time spent in the target quadrant versus the time spent in each of the other pool quadrants (spatial bias), and average proximity (distance to the platform location sampled and averaged across 1-s epochs throughout the trial).

Statistical Analysis. All data are expressed as mean ± SEM. Statistical analyses were performed by either Student t-tests or analysis of variance (ANOVA). Quantification of brain damage and behavioral assessments were made by investigators blinded to treatment conditions.

RESULTS

Recurrent hypoglycemia reduced cortical brain damage induced by 60 min of severe hypoglycemia. No significant difference in blood glucose was observed before, during, or after the 60 min severe hypoglycemic clamps between RH and CON rats (Figure 2A). As expected, recurrently hypoglycemic rats (RH-SH60) had an attenuated epinephrine response to hypoglycemia compared to control saline injected rats (CON-SH60) (2001±241 and
3487 +474 pg/ml, p < 0.01) (Supplementary Figure 1 which can be found in an online appendix at http://diabetes.diabetesjournals.org).

Importantly, RH-SH60 had 64% less neuronal damage, as assessed by the number of Fluoro-Jade B positive (FJB+) cells, in the cortex than controls (173 ± 64 vs. 479 ± 170 cells, p<0.05) (Figure 2B and 2C). To note, 60 min of severe hypoglycemia did not induce significant damage in the hippocampus in either RH-SH60 or CON-SH60.

**Recurrent hypoglycemia attenuated cortical and hippocampal brain injury after 90 minutes of severe hypoglycemia.**

To consistently induce hypoglycemic brain damage in the hippocampus, the above experiments were repeated except that the duration of severe hypoglycemia was extended to 90 min. The average blood glucose during 90 min of severe hypoglycemia was 10.9±0.2 versus 11.0±0.3 mg/dl in the saline injected (CON-SH90) and recurrently hypoglycemic (RH-SH90) rats, respectively (p=NS) (Figure 3C). As an additional set of experimental controls, euglycemic hyperinsulinemic clamps were also performed in recurrently hypoglycemic (RH-EUG, n=2) or saline injected control (CON-EUG, n=2) rats. Blood glucose was maintained at 76±5 and 84±6 mg/dl in the CON-EUG and RH-EUG, respectively (p=NS) (Figure 3C).

Again validating the model of HAAF, RH reduced the epinephrine response to hypoglycemia (CON-SH90: 3175±516 and RH-SH90: 2077±426 pg/ml, p<0.05) (Supplementary Figure 1). Severe hypoglycemia of 90 min induced significant cellular damage in the cortex, as evidenced by the presence of pyknotic cells observed with H&E staining (Figure 3A) and the marked number of fluorescent cells with Fluoro-Jade B staining (Figure 3B). Interestingly, 90 min of severe hypoglycemia induced 6-fold greater cortical neuronal damage than 60 min of severe hypoglycemia (Figure 3D and 2C). Recurrent antecedent moderate hypoglycemia decreased cortical brain damage induced by 90 min of severe hypoglycemia by 62% (RH-SH90: 1107 ± 428 and CON-SH90: 2918 ± 615 FJB+ cells, p<0.05). Unlike 60 minutes of severe hypoglycemia, 90 min of severe hypoglycemia did induce hippocampal brain damage (Figure 3). Recurrent antecedent hypoglycemia resulted in less hippocampal brain damage following 90 min of severe hypoglycemia as compared to CON-SH90 (Figure 3).

Specifically, RH-SH90 had decreased FJB+ cells in the CA1 region by 74% (RH-SH90: 88±56 vs. CON-SH90: 334±91 cell, p<0.05) and by 67% in the dentate gyrus (RH-SH90:274±119 vs. CON-SH90: 833 ± 148, p<0.05) compared to CON-SH90 (Figure 3D). No damage was observed in the hypothalamus in both CON-SH90 and RH-SH90 rats (Supplementary Figure 2).

Interestingly, recurrent hypoglycemia also reduced the episodes of seizure-like behavior observed during severe hypoglycemia (RH-SH90: 2.0±0.3 vs. CON-SH90: 3.4±0.3, p<0.01) (Figure 3E). There was a significant correlation between the number of episodes of seizure-like behavior and number of FJB+ cells (R=0.572, p< 0.05) (Figure 3F).

In the absence of severe hypoglycemia, virtually no Fluoro-Jade positive cells (Fluoro-Jade B staining) nor pyknotic cells (H&E) were observed in the cortex and hippocampus of either the euglycemic CON-EUG or RH-EUG groups (Figure 3).

**Preserved cognitive function in recurrently hypoglycemic rats.** General activity was not different between groups (Supplementary Figure 3). The severe hypoglycemic groups (both CON-SH90 and RH-SH90) exhibited significantly (p=0.02) more rearings than the two groups of EUG rats (Supplementary Figure 3B). Data from the walking initiation, ledge, platform, and...
90° inclined screen were not significantly different between groups (Supplementary Figure 3C-F).

During the cue (Figure 4A) and place (Figure 4B) trials, the CON-SH90 rats performed worse than the other three groups in spite of having normal swimming speeds (Supplementary Figure 4). In the cue trials, CON-SH90 had significantly longer path lengths across the blocks of trials compared to the CON-EUG (P=0.0002). Importantly, RH-SH90 had significantly shorter path lengths relative to the CON-SH90 (P=0.0025), while no differences were observed between RH-SH90 versus CON-EUG nor between the two EUG control groups.

During the place (spatial learning) trials, the CON-SH90 rats again showed significant performance deficits. CON-SH90 had significantly (P=0.0001) longer path lengths across the blocks of trials compared to the CON-EUG rats (Figure 4B). Notably, RH-SH90 had significantly shorter path lengths compared to CON-SH90 (p = 0.0006) (Figure 4B). Again, no differences were observed between RH-SH90 and CON-EUG or between the two euglycemic groups.

During the probe trial, CON-SH90 rats made significantly fewer platform crossings relative to the CON-EUG controls (P=0.014), though no differences in platform crossings between CON-SH90 and RH-SH90 were observed (Figure 4C). However, with regard to spatial bias and average proximity to the platform location, RH-SH90 did have improved performance compared to CON-SH90. In spatial bias analysis, RH-SH90, CON-EUG, and RH-EUG all exhibited spatial bias for the target quadrant whereby each group spent significantly more time in the target quadrant compared to the other pool quadrants (P < 0.0025). CON-SH90 did not show significant spatial bias (Figure 4D). Further, CON-SH90 had significantly higher average proximity scores compared to CON-EUG (P = 0.014) and to RH-SH90 (p = 0.014). RH-SH90 performed similarly to CON-EUG (Figure 4E). In summary, during the probe trial, severe hypoglycemia (CON-SH90) significantly impaired all three tests of memory retention, and antecedent recurrent moderate hypoglycemia pretreatment (RH-SH90) significantly improved memory performance on 2 out of 3 measures.

Interestingly, the number of episodes of seizure-like behavior during severe hypoglycemia positively correlated with performance during Morris water maze testing (Figure 3F). Specifically, increases in the number of episodes of seizure-like behavior were associated with longer average path lengths (R=0.685, p<0.001) (Figure 3F).

**DISCUSSION**

Since severe hypoglycemia affects 40% of insulin treated people with diabetes (26), concern regarding the hazardous potential for severe hypoglycemia to cause “brain damage” continues to be a very real barrier for patients intent on realizing the full benefits of intensive glycemic control (27). Patients with the highest incidence of severe hypoglycemia are most often those who maintain intensive glycemic control, and hence are likely to have had recurrent bouts of moderate hypoglycemia. In this study, recurrent moderate hypoglycemia “preconditioned” the brain and protected it against brain damage and cognitive dysfunction induced by severe hypoglycemia.

In these experiments, severe hypoglycemic brain injury was consistently induced with hyperinsulinemic hypoglycemic (<15 mg/dl) clamps that carefully controlled the depth and duration of severe hypoglycemia and avoided the confounding effects of anesthesia (28-31). The amount and distribution of neuronal damage was markedly different between the 60 minute and 90 minute clamp studies (Figures 2 and 4). In spite of similar degrees of hypoglycemia (10-15 mg/dl), the extra 30 minutes of severe
hypoglycemia induced a 6-fold increase in cortical brain damage and markedly increased hippocampal brain damage (which was minimal in the 60 minute clamp). These findings emphasize the importance of the duration of severe hypoglycemia, and not hypoglycemic nadir alone, as a critically important component in determining the extent of brain damage and cognitive dysfunction (22). Of note, the lack of brain damaged cells in the euglycemic controls indicated that experimental conditions other than severe hypoglycemia (i.e. catheter implantation surgery, recurrent moderate hypoglycemia, hyperinsulinemic clamp, and glucose infusion) did not cause significant brain damage.

The most notable findings were that rats exposed to three days of recurrent, moderate hypoglycemia, had less brain injury associated with severe hypoglycemia in both the cortex and hippocampus. Thus, as with ischemic preconditioning (17), hypoglycemic preconditioning attenuated brain damage by 62-74%. Although hypoglycemia-induced neuronal damage in the hypothalamus has been noted (32), other reports (33) as well as this study observed no severe hypoglycemia induced neuronal injury in the hypothalamus.

In spite of the marked degree of cortical neuronal damage induced by severe hypoglycemia, the rats had no meaningful deficit in sensorimotor function as measured by the locomotor activity and sensorimotor tests. Further supporting the absence of gross motor deficits following severe hypoglycemia was the observation of no differences between groups in swimming speeds (Supplementary Figure 4). Importantly, rats exposed to the severe hypoglycemia showed no signs of sensorimotor impairments which could have affected interpretation of cognitive function as measured during in the Morris Water maze.

Cognitive assessment with water maze testing documented severe cognitive performance deficits induced by severe hypoglycemia, and these impairments were prevented by antecedent recurrent moderate hypoglycemia. Specifically, analysis of the escape path length data showed that severe hypoglycemia significantly impaired performance relative to euglycemic controls during both the cued and place trials, and recurrent hypoglycemia completely prevented the impaired performance induced by severe hypoglycemia (Figure 4). For the probe trial, three measures of memory performance were evaluated: platform crossings, spatial bias toward the target quadrant, and average proximity (Figure 4). Severe hypoglycemia again induced significant memory impairment in all three measures. Antecedent recurrent hypoglycemia prevented these impairments in two of those measures (spatial bias and average proximity). Regarding platform crossings, recurrent hypoglycemia tended to improve performance but was not significant (RH-SH90 vs CON-SH90), indicating that recurrent hypoglycemia was unable to completely reverse the retention deficits concerning the exact location of the platform. However, analysis of the spatial bias and average proximity data demonstrated that recurrent hypoglycemia did preserve retention of a more general platform location. Specifically, RH-SH90 exhibited a spatial biasness for the target quadrant while CON-SH90 did not, and CON-SH90 had an average proximity that was farther away from the platform location than RH-SH90 and the euglycemic controls. These findings indicate that memory retention was impaired due to severe hypoglycemia relative to euglycemic controls in all probe trial variables, and recurrent hypoglycemia prevented severe hypoglycemia induced impairments in 2/3 probe trials indices.

Consistent with the notion that recurrent hypoglycemia induces an adaptive brain response is the observation that RH-SH90 rats had less seizure-like behavior during severe hypoglycemia (Figure 3E),
suggesting the RH treated brain better tolerated severe hypoglycemia. A novel finding of this study is that the number of episodes of seizure-like behavior observed during severe hypoglycemia also correlated with cognitive performance (Figure 4F). As in the real world setting, witnessed hypoglycemic seizures were defined clinically. In the absence of electroencephalogram (EEG) monitoring, the effect of subclinical seizures (i.e. seizures not associated with noticeable motor activity) on brain damage and cognition could not be assessed. Nonetheless in these experimental conditions, observable instances of seizure-like behavior correlated with the extent of neuronal damage and long-term cognitive function, and while not causative, the number of seizures during hypoglycemia was a marker for the extent of neuronal injury and was prognostic of long-term cognitive outcomes. Indeed, clinical studies support these finding because the presence of hypoglycemic seizures, even more than severe hypoglycemia per se, correlate more closely with impaired cognitive function (10;12).

Independent of episodes of severe hypoglycemia, previous studies have shown that recurrent moderate hypoglycemia can alter cognitive function. Recurrent moderate hypoglycemia did not cause neuronal damage in the hippocampus (as confirmed in this study) but did impair hippocampal long-term potentiation (LTP), a cellular mechanism believed to be involved in learning and memory (34). Conversely, recurrent hypoglycemia improved cognitive ability in rats tested in an euglycemic state (35;36). In the current study, recurrent moderate hypoglycemic control rats not exposed to severe hypoglycemia did not have impaired or improved cognitive ability during Morris water maze testing. Since 2-3 weeks of scrupulous avoidance of hypoglycemia reverses the hypoglycemia unawareness associated with recurrent hypoglycemia (37;38), it is presumed that any effect of antecedent recurrent hypoglycemia on cognition may have dissipated during the recovery 6-8 weeks prior to cognitive testing.

Although recurrent moderate hypoglycemia leads to maladaptive responses resulting in hypoglycemia unawareness and hypoglycemia associated autonomic failure (HAAF), the mechanism(s) by which recurrent hypoglycemia leads to these adaptations remain elusive. Similarly, the current experiments do not identify the mechanisms by which recurrent moderate hypoglycemia: [1] protected against severe hypoglycemia induced neuronal damage, [2] limited severe hypoglycemia induced neurocognitive dysfunction, and [3] increased thresholds for hypoglycemic seizures. Putative mechanisms for these beneficial adaptations could include glycogen supercompensation—increased brain glycogen content above pre-hypoglycemic levels (39-43). By keeping a higher level of stored fuel units, increased brain glycogen content has been shown to reduce hypoglycemic neuronal injury by maintaining brain electrical activity and forestalling EEG isoelectricity (44). Enhanced nutrient transport may also contribute to the neuroprotective effects of recurrent hypoglycemia (45;46). Monocarboxylate acid transport is increased during hypoglycemia in patients with well-controlled type 1 diabetes (45;46). Increased transport of monocarboxylate acids (e.g. lactate) could provide an alternative energy source that maintains neuronal function (4). Other possibilities could account for the neuroprotective effect such as altered brain metabolism or neuronal activity(39;47-49). Recurrent hypoglycemia enhances the inhibitory neurotransmitter, GABA, which could reduce neuronal activity and limit excitotoxic damage (48). Further studies on the precise mechanisms of how recurrent
hypoglycemia exerts its neuroprotective effects are warranted. These studies demonstrate that recurrent moderate hypoglycemia preconditions and protects the brain against severe hypoglycemia induced neuronal damage and its associated cognitive deficits. These intriguing findings suggest that recurrent bouts of moderate hypoglycemia that occur with intensive glycemic control might, paradoxically, render an individual more prone to, but less vulnerable to, an episode of severe hypoglycemia. If the current data indicating a neuroprotective preconditioning effect of recurrent moderate hypoglycemia were to be extrapolated to the clinical setting, it could explain the apparent divergent findings between animal and clinical studies and may also explain the seemingly incongruous clinical findings that intensively treated patients who experience recurrent moderate and severe hypoglycemia may be paradoxically protected from severe hypoglycemia induced brain damage and (fortunately) may not suffer from associated long-term cognitive damage (13;50).

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**Figure 1. Experimental Protocol.** Arterial and venous catheters were implanted into 9 week-old Sprague Dawley rats. After one week of recovery, animals were either given an insulin injection daily for three consecutive days to induce moderate hypoglycemia (25-40 mg/dl) or they were given saline injections as a control. On the fourth day, rats underwent a severe hypoglycemic (10-15 mg/dl) hyperinsulinemic (0.2 U/kg/min) clamp for either 60 or 90 min, or alternatively, underwent a 90 min euglycemic (~80mg/dl) hyperinsulinemic (0.2 U/kg/min) clamp. Animals were either sacrificed one week later to assess neuronal damage by H&E and Fluoro-Jade B staining, or animals underwent sensorimotor and cognitive testing 6-8 weeks following the clamp.

**Figure 2. Recurrent Hypoglycemia attenuates brain damage after 60 minutes of severe hypoglycemia.** (A) Blood glucose levels are shown in rats subjected to a 60 minute severe hypoglycemic (SH) (10-15 mg/dl) hyperinsulinemic (0.2 U/kg/min) clamp. Blood glucose was not significantly different between saline-treated (CON-SH6, open circles, n=6) and recurrently hypoglycemia pre-treated rats (RH-SH60, closed circles, n=10) during 60 minutes of severe hypoglycemia. (B) Representative hematoxylin and eosin (H&E -top) and Fluoro-Jade B positive (bottom) staining of the cortex of saline-treated (CON-SH60) and recurrently hypoglycemic (RH-SH60) rats one week following 60 min of severe hypoglycemia. Neuronal damage is indicated by pyknotic cells (H&E staining, green arrows) or with Fluoro-Jade B positive cells (green fluorescence). Scale bar = 100 μm. (C) Quantification of Fluoro-Jade B staining in CON-SH60 (white bar, n=6) and in RH-SH60 (black bar, n=10). Following severe hypoglycemia, RH rats had significantly less degenerating cells in the cortex compared to CON rats (* p < 0.05, by Student t-test)

**Figure 3. Recurrent hypoglycemia limits brain cell death one week following 90 min of severe hypoglycemia.** (A) Representative hematoxylin and eosin staining of the cortex and hippocampal structures, CA1 and the dentate gyrus (DG), one week following 90 minute severe hypoglycemic clamps (SH) or euglycemic clamps (EUG) in antecedent recurrently hypoglycemic (RH-SH90 and RH-EUG, respectively) and antecedent saline injected rats (CON-SH90 and CON-EUG). Rats that underwent severe hypoglycemia had damaged neurons characterized by pyknotic nuclei (green arrows) Scale bar = 100 μm (B) Fluoro-Jade B positive cells (FJB+, green fluorescence) in the cortex, hippocampal CA1 region and dentate gyrus (DG) of the same four treatment groups. Scale bar = 100 μm. (C) Blood glucose was not significantly different between saline-treated (CON-SH90, open circles, n=9) and recurrently hypoglycemic rats (RH-SH90, closed circles, n=8) during 90 minutes of severe hypoglycemia. (D) Following 90 minutes of severe hypoglycemia, the markedly increased number of FJB+ cells
in the cortex, CA1, and dentate gyrus observed in the CON-SH90 (diagonal hatch) was significantly (*p<0.05) reduced by antecedent recurrent moderate hypoglycemia (RH-SH90, grey horizontal hatch). Bars representing Fluoro-Jade B in CON-EUG and RH-EUG groups are not visible in this figure as no appreciable brain damage was observed in euglycemic control rats. (E) Euglycemic rats (CON-EUG and RH-EUG) experienced no seizure-like behavior. Rats exposed to 90 min severe hypoglycemia exhibited seizure-like behavior, although RH-SH90 had significantly less seizure-like behavior than CON-SH90 (p<0.01). (F) In rats that experienced severe hypoglycemia (RH-SH90 and CON-SH90), seizure-like behaviors positively correlated with the amount of Fluoro-Jade B cells in the hippocampus (R=0.572, P<0.05).

Figure 4. Antecedent recurrent hypoglycemia mitigated cognitive dysfunction induced by severe hypoglycemia. Morris water maze testing was performed 6-8 weeks following severe hypoglycemic or euglycemic clamps. (A) During the cue trial, control rats exposed to 90 min of severe hypoglycemia (CON-SH90, open circles, n=11) performed worse as evidenced by higher escape path lengths compared to euglycemic control (CON-EUG, open triangles, n=7) (*p=0.002). Notably, rats exposed to recurrent moderate hypoglycemia before severe hypoglycemia (RH-SH90, closed circles, n=9) had shorter escape path lengths than CON-SH90 (*p=0.0025) and performed similarly to CON-EUG and RH-EUG (closed triangles, n=9). (B) A similar pattern was observed during the place trials as CON-SH90 had significantly higher escape path lengths compared to CON-EUG (*p=0.0001) and RH-SH90 (*p=0.0006). (C) CON-SH90 (diagonal hatch) had significantly less platform crossings than CON-EUG (white bar) (p=0.014). No significant difference was observed between CON-SH90 and RH-SH90 (grey horizontal hatch) or between CON-EUG and RH-EUG (black bar). (D) RH-SH90, CON-EUG, and RH-EUG had a spatial bias towards the target quadrant while CON-SH90 did not (*p<0.0025). (E) During the probe trial, CON-SH90 rats showed an average proximity to the platform location that was significantly farther away than that of the CON-EUG (p=0.014). RH-SH90 rats swam significantly closer to the platform location than CON-SH90 (p=0.014), similar to euglycemic controls. (F) The number episodes of seizure-like behaviors observed during severe hypoglycemia 6-8 weeks prior positively correlated with average path length during the place trials (R=0.685, P<0.001, n=20).
**Figure 1**

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**Figure 2**

**A.**

![Blood Glucose (mg/dl) vs Time (min)](image)

**B.**

CON-SH60 | RH-SH60

**C.**

![Fluo-Red Positive Cells](image)
Figure 3

A. CON-EUG  RH-EUG  CON-SH90  RH-SH90
   Cortex  
   CA1 
   DG

B. CON-EUG  RH-EUG  CON-SH90  RH-SH90
   Cortex  
   CA1 
   DG

C. Blood Glucose (mg/dL)
   Basal 0 30 60 90 120 150

D. Fluoro-Jade positive cells
   Cortex CA1 DG

E. Episodes of Seizure-like Behavior
   CON-EUG  RH-EUG  CON-SH90  RH-SH90

F. Fluoro-Jade Positive Cells
   Episodes of Seizure-like Behavior
Figure 4

A. 

Cue Trial

Escape path length (cm)

1600
1400
1200
1000
800
600
400
200

1 2 3 4

Blocks of Trials

1600
1400
1200
1000
800
600
400
200

1 2 3 4 5

Blocks of Trials

B. 

Place Trial

Escape path length (cm)

1600
1400
1200
1000
800
600
400
200

1 2 3 4 5

Blocks of Trials

C. 

Platform Crossings

CON-EUG RH-EUG CON-SHRH RH-SHRH

D. 

Spatial Bias

Quadrant Time (sec)

Opposite Right Left Target

E. 

Average Proximity to Platform

Distance (cm)

CON-EUG RH-EUG CON-SHRH RH-SHRH

F. 

Average Path Length (cm)

Episodes of Seizure-like Behavior

PRECONDITIONING LIMITS HYPOGLYCEMIC BRAIN INJURY