JOURNAL OF
NEUROINFLAMMATION

Recurrent/moderate hypoglycemia induces hippocampal dendritic injury, microglial activation, and cognitive impairment in diabetic rats

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Abstract

Background: Recurrent/moderate (R/M) hypoglycemia is common in type 1 diabetes. Although mild or moderate hypoglycemia is not life-threatening, if recurrent, it may cause cognitive impairment. In the present study, we sought to determine whether R/M hypoglycemia leads to neuronal death, dendritic injury, or cognitive impairment.

Methods: The experiments were conducted in normal and in diabetic rats. Rats were subjected to moderate hypoglycemia by insulin without anesthesia. Oxidative stress was evaluated by 4-Hydroxy-2-nonenal immunostaining and neuronal death was determined by Fluoro-Jade B staining 7 days after R/M hypoglycemia. To test whether oxidative injury caused by NADPH oxidase activation, an NADPH oxidase inhibitor, apocynin, was used. Cognitive function was assessed by Barnes maze and open field tests at 6 weeks after R/M hypoglycemia.

Results: The present study found that oxidative injury was detected in the dendritic area of the hippocampus after R/M hypoglycemia. Sparse neuronal death was found in the cortex, but no neuronal death was detected in the hippocampus. Significant cognitive impairment and thinning of the CA1 dendritic region was detected 6 weeks after hypoglycemia. Oxidative injury, cognitive impairment, and hippocampal thinning after R/M hypoglycemia were more severe in diabetic rats than in non-diabetic rats. Oxidative damage in the hippocampal CA1 dendritic area and microglial activation were reduced by the NADPH oxidase inhibitor, apocynin.

Conclusion: The present study suggests that oxidative injury of the hippocampal CA1 dendritic region by R/M hypoglycemia is associated with chronic cognitive impairment in diabetic patients. The present study further suggests that NADPH oxidase inhibition may prevent R/M hypoglycemia-induced hippocampal dendritic injury.

Keywords: Recurrent/moderate hypoglycemia, Dendrite, Superoxide, Lipid peroxidation, Neuron death, Microglial activation, NADPH oxidase, Apocynin

Introduction

Recurrent/moderate (R/M) hypoglycemia, which is more common than severe hypoglycemia in type 1 diabetes patients (frequency is 0.1 to 0.3 episode/patient per day), is usually corrected by patients themselves or unrealized.

Therefore, these episodes may be easily forgotten and remain untreated [1-4]. Although moderate hypoglycemia is not life-threatening, if recurrent, it may have serious clinical implications. Poor academic performance in diabetic children and memory impairment in adults with diabetes are viewed as areas of increasing public concern. These problems are typically thought to result from hypoglycemic attacks, which occur more frequently during intensive insulin therapy [5-8].

The normal range for human blood glucose concentration is 3.9 to 7.1 mM (1 mM = approximately 18 mg/dl),

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and hypoglycemia is broadly defined as glucose concentrations that fall below this range. Reducing food intake does not reduce blood glucose levels below 2.8 mM, even with prolonged fasting [9], however, reductions below 1 mM can be induced by the administration of insulin or drugs such as sulfonylurea derivatives. Hypoglycemic brain injury occurs most frequently in diabetic patients attempting tight glucose control [10,11], and consequently the risk of hypoglycemic brain injury is the major factor limiting tight glucose control. Although there is no strict definition, hypoglycemia is generally classified as mild (3.2 to 3.6 mM), moderate (2.3 to 3.2 mM) and severe (<2 mM) hypoglycemia in humans [12]. While most diabetic patients experience mild to severe hypoglycemia at times, very few experience severe hypoglycemia to a degree that results in coma.

Studies using mice and rats indicate that widespread neuronal death does not occur unless blood glucose concentration falls below 1 mM and cortical electroencephalographic (EEG) activity remains isoelectric (silent) for at least 10 min. However, it is still possible to detect scattered neuronal death, even at early stages of electrocerebral silence or sustained blood glucose concentrations just in excess of 1 mM [13-16]. Case studies suggest that a similar degree of hypoglycemia is required to induce brain injury in humans, although this may vary in children, susceptible individuals with repeated hypoglycemia and the presence of co-morbid factors [17-22].

The main risk factors for cognitive impairment in diabetes are considered to be age, duration of diabetes, and coexistent microvascular and macrovascular complications [23]. Diabetes should be treated as a risk factor for cognitive impairment, and likewise, cognitive impairment is associated with poorer ability in diabetes self-care and decreased adherence to anti-diabetic treatment, setting the stage for a potential vicious cycle of decline in quality of life and disease management.

Children with type 1 diabetes who suffer recurrent and severe hypoglycemia while younger than 5 years old have impaired mental abilities later in life [6,24]. The combination of an early onset of diabetes and recurrent episodes of hypoglycemia appears to be associated with reduced attention and spatial memory in adolescence [24-27]. Within the diabetic group, verbal intelligence was reduced with increased exposure to hyperglycemia, but not to hypoglycemia. In contrast, spatial intelligence and delayed recall were reduced only with repeated hypoglycemia, particularly when hypoglycemic episodes occurred before the age of 5 years [28].

Recurrent episodes of mild/moderate hypoglycemia are associated with a decreased perception of the hypoglycemic state and blunted secretion of counter-regulatory hormones, phenomena termed 'hypoglycemia unawareness' and 'hypoglycemia-associated autonomic failure' (HAAF), respectively [29-31]. In diabetic patients, even mild to moderate hypoglycemia may produce a significant increase in low-frequency EEG activity [32] and impair cognitive function [33]. Several studies have sought to determine the cognitive impact of recurrent hypoglycemia, but results from clinical studies have been mixed and the question of whether recurrent exposure to severe hypoglycemia promotes long-term cognitive dysfunction is unresolved.

There have been several studies performed to determine whether neuronal death is induced after moderate hypoglycemia, which is defined as low blood glucose levels (below 2 mM blood glucose for more than 2 h) without the presence of isoelectric EEG (iso-EEG). These studies concluded that moderate hypoglycemia induced scattered neuronal death in the cerebral cortex layer 2–3 [34], but not in the hippocampus. Yamada et al. also found that moderate hypoglycemia led to no hippocampal neuronal death, however, they did observe significantly deteriorated synaptic plasticity, demonstrated by an inability to induce long term potentiation (LTP) at CA1 synapses [35]. Thus, we speculated that repetitive episodes of moderate hypoglycemia might lead to synaptic injury in the hippocampus in the absence of apparent neuronal somatic injuries in the hippocampus, and consequently, the development of cognitive impairments.

To date, several hypoglycemia experiments have been performed with normal (non-diabetic) adult rodents; therefore the clinical implication of these studies is not readily apparent since moderate hypoglycemia commonly occurs in juvenile type 1 diabetes patients. Therefore, the present study was conducted in 1-month-old young rats that were rendered diabetic by streptozotocin (STZ) injection, mimicking juvenile type 1 diabetes.

To test our hypothesis, the present study has addressed five questions. First, does a single episode of moderate hypoglycemia (without anesthesia and without iso-EEG) cause dendritic oxidative injury in the hippocampus? Second, if not, do repeated episodes of moderate hypoglycemia cause dendritic oxidative injury in the hippocampus? If so, third, do repeated episodes of moderate hypoglycemia cause cognitive impairment at a later time point? Fourth, is there a difference in cognitive function between non-diabetic and diabetic rats, with respect to the synaptic effects of R/M hypoglycemia? Fifth, is oxidative injury or microglial activation after R/M hypoglycemia mediated by NADPH oxidase?

Materials and methods

The surgical and animal care procedures were in accordance with the guidelines of the Institutional Animal Care and Use Committee of the San Francisco Veterans Affairs Medical Center (animal welfare assurance number A3476-01) and of the Hallym University (Hallym

2011–44). This manuscript was written up in accordance with the ARRIVE (Animal Research: Reporting In Vivo Experiments) guidelines [36].

Type 1 diabetes rat

For the type 1 diabetes model, rats received repeated intraperitoneal injections of streptozotocin (STZ). One-month-old male Sprague Dawley rats were injected with 50 mg/kg STZ (twice; second injection occurred 24 h after the first injection, i.p.). Rats displaying fasting blood glucose levels higher than 200 mg/dl 7 days after the first injection were used in this experiment. All rats injected with STZ showed typical symptoms of diabetes such as polydipsia, polyphagia, and polyuria. Although the STZ-induced diabetes mellitus (DM) model has certain caveats, this model offers a very effective technique that can be used in most rodents [37]. Fasting blood glucose levels in STZ-treated rats were left uncorrected and fell in the range of 10 to 20 mM.

R/M hypoglycemia

One week after STZ injection, R/M hypoglycemia was induced without anesthesia. After overnight fasting, rats were given an insulin injection (5 U/kg, i.p.) to induce moderate hypoglycemia, as previously described with minor modification [38]. After insulin injection, blood glucose was measured from the tail vein using ACCU-CHEK glucometer (Roche, Indianapolis, IN, USA) at 30-min intervals. Blood glucose levels between 1 and 2 mM were maintained for 1 h. Blood glucose level was increased by intraperitoneal injection of glucose (25%/ 1 mL/bolus) 1 h after moderate hypoglycemia (Table 1). In the present study we defined 'moderate hypoglycemia' as sustained (1 h) blood glucose concentrations between 1 to 2 mM without coma. This process was repeated for 5 consecutive days (Additional file 1: Figure S1). Rats were randomly assigned to two groups; one for studying the role of oxidative stress and the other group for cognitive testing 6 weeks post-insult. To test whether NADPH oxidase activation is involved in R/M hypoglycemia-induced oxidative injury in hippocampal dendrites, the NADPH oxidase assembly inhibitor, apocynin, was

Table 1 Change in mean arterial blood glucose levels before insulin injection, during moderate hypoglycemia and after glucose reperfusion in diabetic and non-diabetic rats (mM ± s.e.m.)

	Before insulin injection	During moderate hypoglycemia	After glucose reperfusion
Non-diabetic rats (n = 7)	4.72 ± 0.27	1.46 ± 0.04	5.61 ± 0.25
Diabetic rats (n = 7)	16.43 ± 0.81	1.48 ± 0.10	17.31 ± 0.77

injected intraperitoneally (15 mg/kg, Sigma, St Louis, MO, USA) immediately after R/M hypoglycemia. Since half-life of apocynin is about 1 h [39], we believe that apocynin may inhibit glucose reperfusion-induced NADPH oxidase activation for 2 to 3 h. Apocynin was dissolved in 1% DMSO (dimethylsulphoxide) and control rats for these studies received equal volumes of vehicle alone. This process was repeated for 5 consecutive days (Additional file 1: Figure S1).

Acute/severe (A/S) hypoglycemia

As a means of comparison with R/M hypoglycemia, A/S hypoglycemia with diabetic rats was also performed in the present study. Insulin-induced A/S hypoglycemia was induced in rats as previous described, with the period of severe hypoglycemia defined here as the interval during which the EEG exhibited an isoelectric state for 30 min [13,15]. Severe hypoglycemia was induced by intraperitoneal injection of 30 U/kg of regular insulin (Novolin-R, Novo Nordisk, Clayton, NC, USA) (Additional file 2: Figure S2).

Evaluation of neuron degeneration

Neuronal death after R/M or A/S hypoglycemia was evaluated after a 7-day survival period. Rats were intracardially perfused with 0.9% saline followed by 4% paraformaldehyde (PFA). The brains were post-fixed with 4% PFA for 24 h and then incubated with 20% sucrose for cryoprotection. For H&E staining, coronal 25 μm sections were conventionally stained [40]. Brain sections were also prepared for the Fluoro-Jade B staining (FJB) [15,41]. Degenerating cells were detected with 450 to 490 nm excitation and a 515 nm emission filter. Five coronal sections were collected from each animal by starting 4.0 mm posterior to Bregma, and collecting every third section until five sections were in hand. These sections were then coded and given to a blinded experimenter who counted the number of degenerating neurons in the hippocampal CA1.

Evaluation of oxidative stress

Oxidative injury was estimated by evaluating levels of the lipid peroxidation product, 4HNE (4-hydroxy-2-nonenal). Immunostaining with microtubule-associated protein 2 (MAP2; Millipore Co, Billerica, MA, USA) and 4HNE (Alpha Diagnostic Intl. Inc., San Antonio, TX, USA) antibodies was performed as described previously [42]. Three sections were analyzed from each brain, taken at 80 μm intervals to span the hippocampus. 4HNE signal intensity was expressed as the ratio of the mean fluorescence in SR of hippocampal CA1 to fluorescence in the lateral ventricles.

Immunohistochemistry

For evaluation of dendritic damage, the sections were immunostained with a rabbit antibody to rat MAP2 (Millipore Co, Billerica, MA, USA) at a 1:250 dilution. For evaluation of microglial activation, the sections were immunostained with a mouse antibody to rat CD11b (Serotec, Raleigh, NC, USA) at a 1:200 dilution. Microglia activation was evaluated by an observer blinded to experimental conditions. Three sections from each animal were evaluated for scoring. Microglia activation criteria were depending on number of CD11b immunoreactive cells and their morphology as published previously [43].

Behavioral assessments

Rats were handled for 5 min each per day and acclimated in the behavioral test room for 1 week prior to the start of behavior testing. Six weeks following the sham hypoglycemia or R/M hypoglycemia, rats were first tested in the novel open field test to evaluate the effects of diabetes and recurrent hypoglycemia on locomotor activity and exploratory behavior. In the open field test, rats were placed in a brightly lit, square Plexiglas enclosure (40 x 40 inches), surrounded by automated infrared photocells interfaced with a computer (Hamilton & Kinder, San Diego, CA, USA) to record the data. On each of 3 consecutive days, open field activity was recorded for 10 min after an initial 1-min adaptation period. For analysis of the exploratory behavior, the arena was divided on a zone map consisting of a center region (15 × 15 inches), four corner regions of 7.5 × 7.5 inches each, and a peripheral region (the remaining area).

Subjects then underwent testing in the Barnes maze test to evaluate spatial learning and memory [44]. Rats from each treatment group were randomly assigned to locate the escape tunnel from one of the three predetermined locations to rule out spatial preference. Moderately noxious stimuli, blowing fans and 500 LUX of bright light, were used to increase the incentive in finding the escape tunnel. Noldus EthoVision video tracking system (Noldus, Leesburg, VA, USA) was used to record and analyze the data. Rats were trained to locate the escape tunnel in two successive daily sessions for 5 days (three trials per session, 3 min per trial) with a 1-h intersession interval from different counterbalanced starting positions.
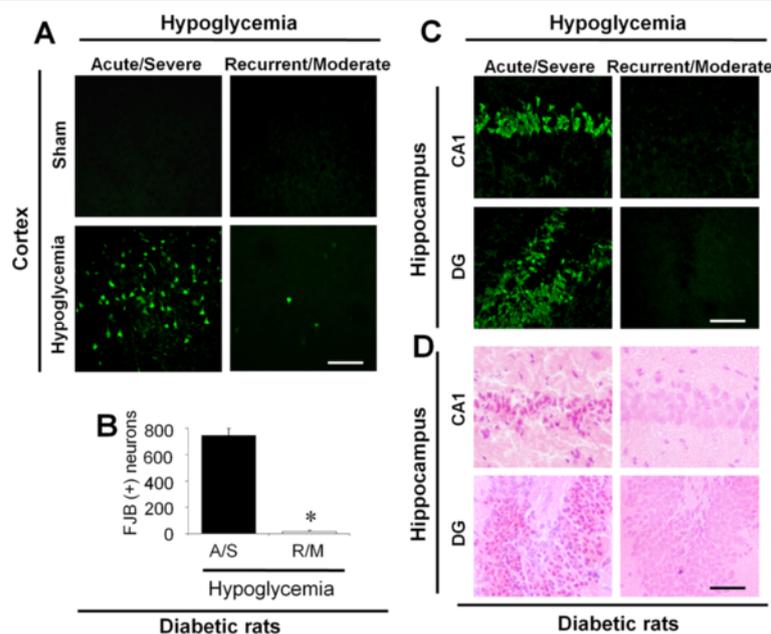
Figure 1 Neuronal death in the cerebral cortex and hippocampus after severe or moderate hypoglycemia in diabetic rats. (**A**) In sham hypoglycemia-operated rats, neuronal death (FJB (+) neurons) was not detected in diabetic rats. In both diabetic and non-diabetic rats, recurrent/moderate (R/M) hypoglycemia shows only occasional neuronal death in the cerebral cortex while acute/severe (A/S) hypoglycemia shows widespread neuronal death in the superficial layer of cortex at 7 days after hypoglycemia. Bar = 100 μm. (**B**) Bar graph represents counted degenerating neurons in the cerebral cortex. Data are mean + s.e.m.; $n = 5$ in R/M hypoglycemia, $n = 7$ in A/S hypoglycemia * $P < 0.05$. (**C**) R/M hypoglycemia shows no neuronal death (FJB (+) neurons) in the hippocampal CA1 and DG cell layer while severe hypoglycemia shows substantial neuron death in the hippocampus at seven days after hypoglycemia. Bar = 100 μm. (**D**) Light microscopic images represent hematoxylin & eosin (H&E) stained hippocampus from CA1 and DG cell layer. R/M hypoglycemia shows no neuronal death (eosinophilic) in the hippocampal CA1 and DG cell layer while severe hypoglycemia shows substantial neuronal death in the hippocampus at seven days after hypoglycemia. Bar = 100 μm.

Statistical analysis

Data are expressed as means ± s.e.m. and assessed by one-way ANOVA followed by either the Tukey-Kramer test for multiple comparisons between groups or the Dunnett's test for comparisons of multiple groups against a control group with Statview 5.0.1 software (SAS Institute Inc., Cary, NC, USA). Behavioral data were compared using two-way repeated measure ANOVA (RANOVAs) followed by *post hoc* pairwise comparisons using the Bonferroni test when appropriate. Values of $P < 0.05$ were considered as significant.

Results

A/S hypoglycemia induces cortical and hippocampal neuronal death in diabetic rats

Before initiating the R/M hypoglycemia experiments, diabetic rats were subjected to A/S hypoglycemia to test whether STZ-injected diabetic rats also have similar patterns of neuronal injury as seen in previous studies using non-diabetic rats (Figure 1). We found that, in the cerebral cortex, A/S hypoglycemia produced about twice as much neuron death in diabetic rats as in non-diabetic rats, compared to other historical controls [13,45] and our previous study [15,42]. However, in the hippocampus, diabetic and non-diabetic rats showed a similar degree of neuronal death after A/S hypoglycemia. This result is very similar to that reported by Bree *et al.* [46].

R/M hypoglycemia produces infrequent, sparse neuronal death in the cerebral cortex

Neuronal death was evaluated in the cerebral cortex of STZ-induced diabetic rats after R/M hypoglycemia (Figure 1A and B). Neuronal injury evaluated by FJB staining at 7 days after R/M hypoglycemia showed infrequent, sparse neuronal death in the parietal region of cerebral cortex. Only three out of five diabetic rats exhibited FJB(+) cells in the cerebral cortex.

R/M hypoglycemia does not produce neuronal death in the hippocampus

Neuronal injury evaluated by H&E or FJB staining at 7 days after A/S hypoglycemia showed widespread neuronal death in the hippocampus of type-1 diabetes mellitus model rats. However, R/M hypoglycemia produced no hippocampal neuronal death in the diabetic rats (Figure 1C and D).
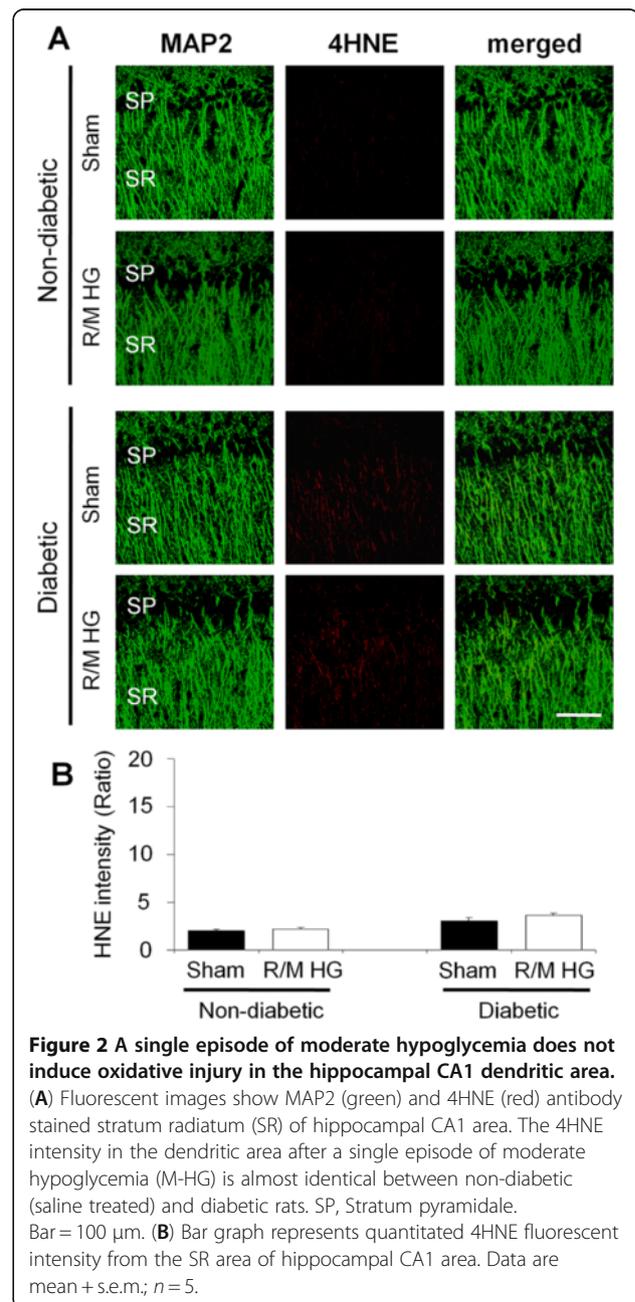
A single episode of moderate hypoglycemia does not induce oxidative injury in hippocampal CA1 dendrites

To test whether a single episode of moderate hypoglycemia induces oxidative injury at or proximal to hippocampal dendrites, rat hippocampus was evaluated at 24 h after a single episode of moderate hypoglycemia. Here we found only low levels of 4HNE fluorescent signal in SR area of hippocampus after a single episode of moderate hypoglycemia in either the non-diabetic or the diabetic rats (Figure 2).
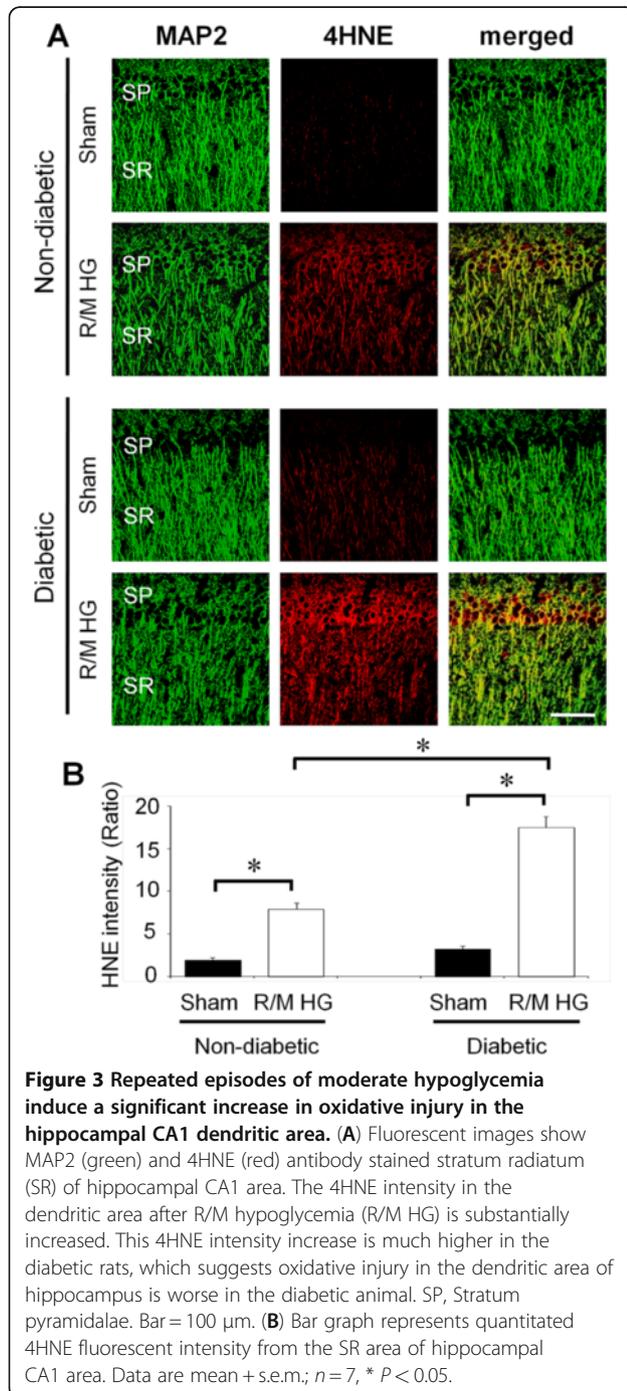
R/M hypoglycemia induces significant oxidative injury in the hippocampal stratum radiatum area

Since a single episode of moderate hypoglycemia induced no obvious oxidative injuries in the SR, rats were exposed to recurrent hypoglycemia for 5 consecutive days. One day after the final episode of R/M hypoglycemia (after five episodes of R/M hypoglycemia), brains were examined to determine if oxidative injury had occurred in

Figure 2 A single episode of moderate hypoglycemia does not induce oxidative injury in the hippocampal CA1 dendritic area.
(**A**) Fluorescent images show MAP2 (green) and 4HNE (red) antibody stained stratum radiatum (SR) of hippocampal CA1 area. The 4HNE intensity in the dendritic area after a single episode of moderate hypoglycemia (M-HG) is almost identical between non-diabetic (saline treated) and diabetic rats. SP, Stratum pyramidale. Bar = 100 μm. (**B**) Bar graph represents quantitated 4HNE fluorescent intensity from the SR area of hippocampal CA1 area. Data are mean + s.e.m.; *n* = 5.

hippocampal dendritic area. Compared with a single episode of moderate hypoglycemia, exposure to five consecutive episodes leads to significant oxidative injury in the hippocampal dendrite area. 4HNE fluorescent intensity was increased by 419% in the SR area of hippocampus of non-diabetic rats. 4HNE fluorescence intensity was increased by 549% in the diabetic rats over non-diabetic rats (Figure 3).

Figure 3 Repeated episodes of moderate hypoglycemia induce a significant increase in oxidative injury in the hippocampal CA1 dendritic area. (**A**) Fluorescent images show MAP2 (green) and 4HNE (red) antibody stained stratum radiatum (SR) of hippocampal CA1 area. The 4HNE intensity in the dendritic area after R/M hypoglycemia (R/M HG) is substantially increased. This 4HNE intensity increase is much higher in the diabetic rats, which suggests oxidative injury in the dendritic area of hippocampus is worse in the diabetic animal. SP, Stratum pyramidalae. Bar = 100 μm. (**B**) Bar graph represents quantitated 4HNE fluorescent intensity from the SR area of hippocampal CA1 area. Data are mean + s.e.m.; n = 7, * P < 0.05.
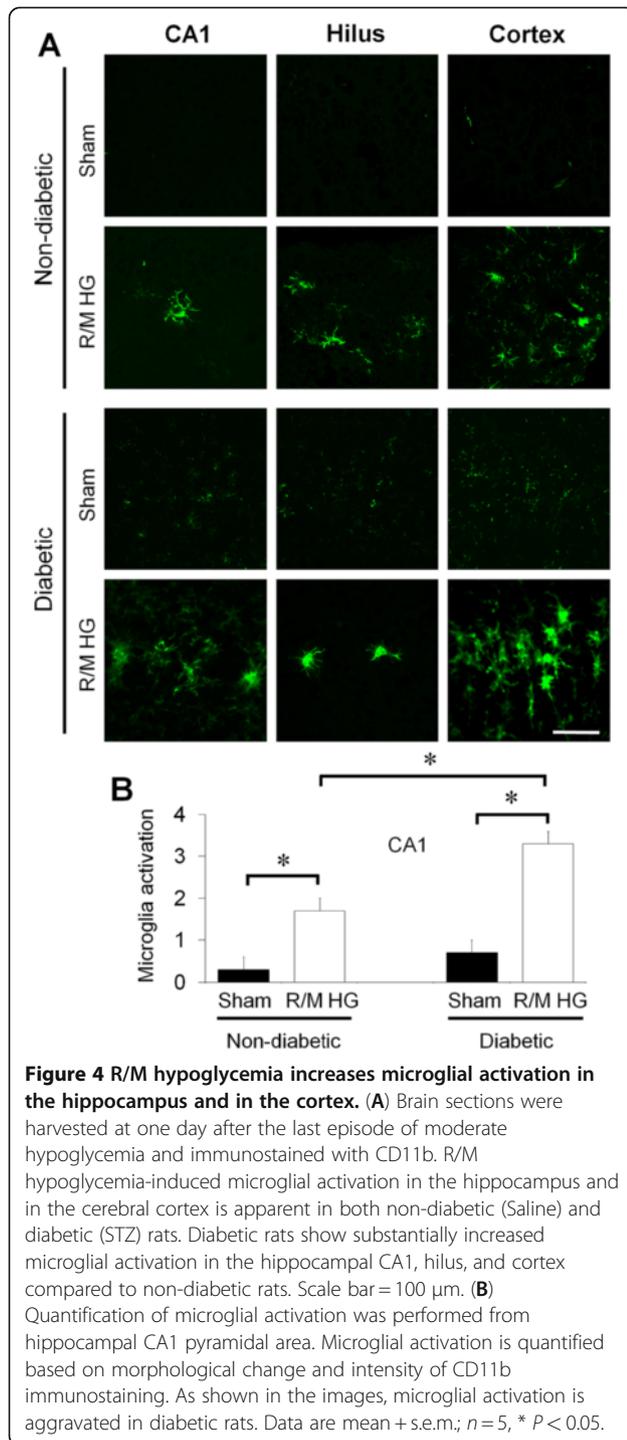
R/M hypoglycemia induces microglial activation

We tested whether R/M hypoglycemia also induces microglial activation in the cerebral cortex and the hippocampus. Rats were exposed for 5 days to R/M hypoglycemia and brains were harvested after the final episode of R/M hypoglycemia. Compared to sham hypoglycemia operated rats, microglia were activated in the cerebral cortex and in the hippocampus after R/M hypoglycemia in normoglycemic rats. The degree of microglial activation in the hippocampus of diabetic rats was significantly higher than in normoglycemic rats (>194%, Figure 4).

R/M hypoglycemia worsens impairment in spatial learning and memory in diabetic rats

We next examined whether hippocampal oxidative injury observed following R/M hypoglycemia correlated with the presence of memory deficits. Rats were subjected to testing designed to evaluate spatial learning in the Barnes maze that heavily relies on hippocampal function. Over the 5 days of training, all groups learned the spatial task as evidenced by a progressive reduction in the distance traveled to reach the escape tunnel in the Barnes maze test ($F_{4, 264} = 95.7$; $P < 0.01$). There was also a significant difference in the performance during the acquisition phase of the Barnes maze test between groups. In general, diabetic rats traveled significantly longer distances to reach the escape tunnel compared to non-diabetic rats (Two-way RANOVA, STZ effect: $F_{1, 66} = 17.5$; $P < 0.01$) (Figure 5A). *Post-hoc* analysis suggests that diabetic rats with R/M hypoglycemia performed significantly worse than either diabetic sham hypoglycemic rats ($P < 0.05$), or non-diabetic rats with R/M hypoglycemia ($P < 0.01$) (Figure 5A).

Diabetes, but not R/M hypoglycemia, alters exploratory behavior

To investigate whether R/M hypoglycemia induces activity changes in either non-diabetic or diabetic animals, rats were subjected to an open field test at 6 weeks after the final episode of R/M hypoglycemia. All groups were able to habituate over 3 days of testing in the novel open field (Figure 5B to D, total path length: $P < 0.01$; total active time: $P < 0.0001$; rearing: $P < 0.01$). Diabetes had a strong effect on locomotion (Figure 5B to D, total path length: $P < 0.01$; total active time: $P < 0.01$; rearing: $P < 0.01$), while R/M hypoglycemia did not reduce locomotion (5B to D, total path length: $P = 0.16$; total active time: $P = 0.49$; rearing: $P = 0.40$). Because the novel environment in the open field concurrently evokes both anxiety and exploration [47,48], an increase in time spent in the center of the open field suggests reductions in anxiety and/or increases in exploration [47]. Diabetic

Figure 4 R/M hypoglycemia increases microglial activation in the hippocampus and in the cortex. (**A**) Brain sections were harvested at one day after the last episode of moderate hypoglycemia and immunostained with CD11b. R/M hypoglycemia-induced microglial activation in the hippocampus and in the cerebral cortex is apparent in both non-diabetic (Saline) and diabetic (STZ) rats. Diabetic rats show substantially increased microglial activation in the hippocampal CA1, hilus, and cortex compared to non-diabetic rats. Scale bar = 100 μm. (**B**) Quantification of microglial activation was performed from hippocampal CA1 pyramidal area. Microglial activation is quantified based on morphological change and intensity of CD11b immunostaining. As shown in the images, microglial activation is aggravated in diabetic rats. Data are mean + s.e.m.; $n = 5$, * $P < 0.05$.

rats also displayed an anxiety-like behavior by reducing exploratory activity in the center zone in the open arena (Figure 5E and F, Z2 path length: $P < 0.01$; Z2 time: $P < 0.01$). Diabetic rats experiencing consecutive R/M hypoglycemia displayed an anxiety-like behavior compared to their non-diabetic counterparts (*post hoc*: Z2 path length: $P < 0.01$; Z2 time: $P < 0.01$).

R/M hypoglycemia leads to decreased dendritic density and reduced thickness of hippocampal CA1 at delayed time points

To evaluate long-term effects of R/M hypoglycemia on hippocampal dendritic structure, brains were histologically evaluated by MAP2 immunostaining 8 weeks after R/M hypoglycemia (Figure 6A). Here we found that R/M hypoglycemia reduced MAP2 intensity in the SR area of hippocampal CA1. STZ-induced diabetic rats showed significantly (31.65%) less MAP2 immunoreactivity in the SR area of the hippocampus compared with non-diabetic rats. R/M hypoglycemia in diabetic rats further decreases MAP2 intensity (29.03%) than in non-diabetic rats (10.15%) (Figure 6B). The thickness of the CA1 dendritic area was also decreased by 3.81% in non-diabetic rats but by 11.35% in diabetic rats, in concert with the decrease in MAP2 intensity (Figure 6C).

Inhibition of NADPH oxidase prevents oxidative injury and microglial activation after R/M hypoglycemia

To test whether NADPH oxidase activation contributes to the dendritic injury seen in the stratum radiatum of hippocampal CA1 area and microglial activation after R/M hypoglycemia, we injected an NADPH oxidase inhibitor, apocynin, into the intraperitoneal space. Apocynin treatment (15 mg/kg/day) reduced 4HNE fluorescence intensity by 51.30% in diabetic rats after hypoglycemia. Reduction of 4HNE fluorescent intensity represents that R/M hypoglycemia-induced oxidative injury may be caused by superoxide production through NADPH oxidase activation. R/M hypoglycemia-induced microglia activation was also reduced by 57.15% by apocynin treatment in diabetic rats. The degree of reduction of 4HNE fluorescent intensity and microglia activation by apocynin was similar in diabetic and non-diabetic rats (Figure 7A and B).

Discussion

The present study demonstrates that R/M hypoglycemia induces cognitive impairment associated with hippocampal dendritic injury. We found that R/M hypoglycemia induces oxidative injury in hippocampal dendrites and that R/M hypoglycemia induces microglial activation in the hippocampus and cerebral cortex. Both of these processes were more pronounced in diabetic rats. R/M hypoglycemia induces cognitive impairment 6 weeks after insult in diabetic rats, but not in non-diabetic rats. Finally; R/M hypoglycemia reduces dendritic density and thickness of the SR and SM of hippocampal CA1 area.

In the present study we tested if R/M hypoglycemia in diabetic rats could cause hippocampal dendritic injury and cognitive impairment. Severe hypoglycemia (that in which iso- EEG is present) induces neuronal death in the cerebral cortex and hippocampus, and cognitive
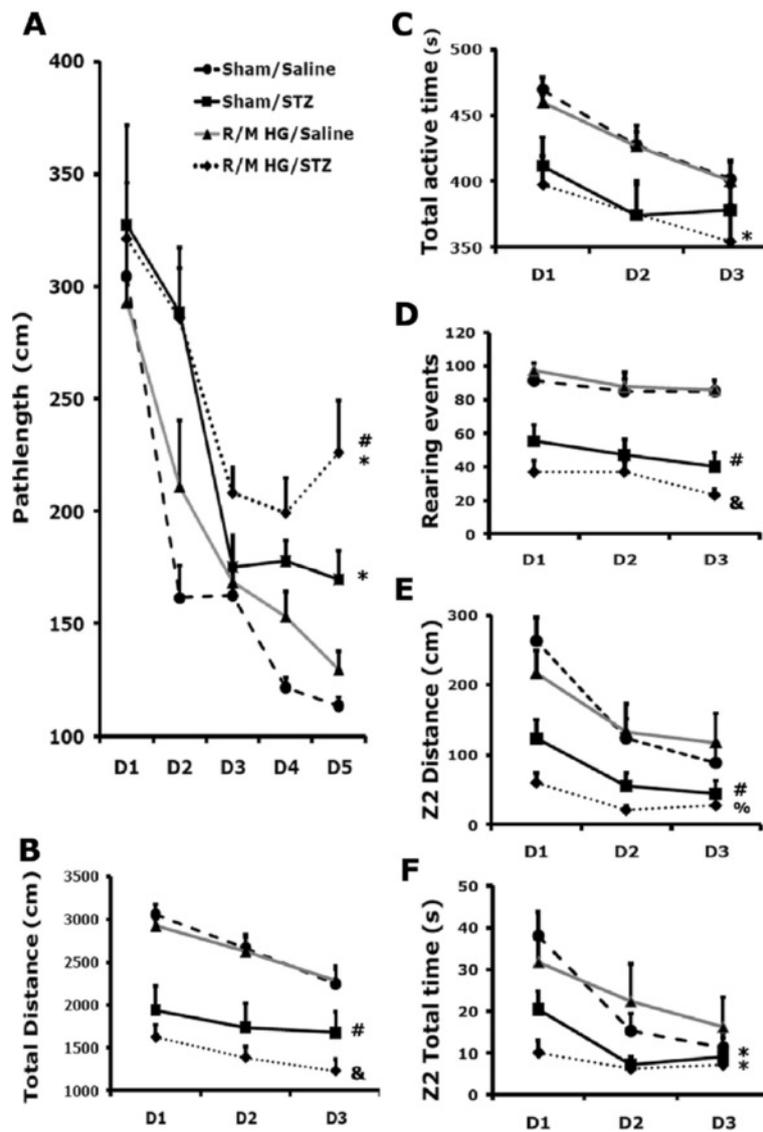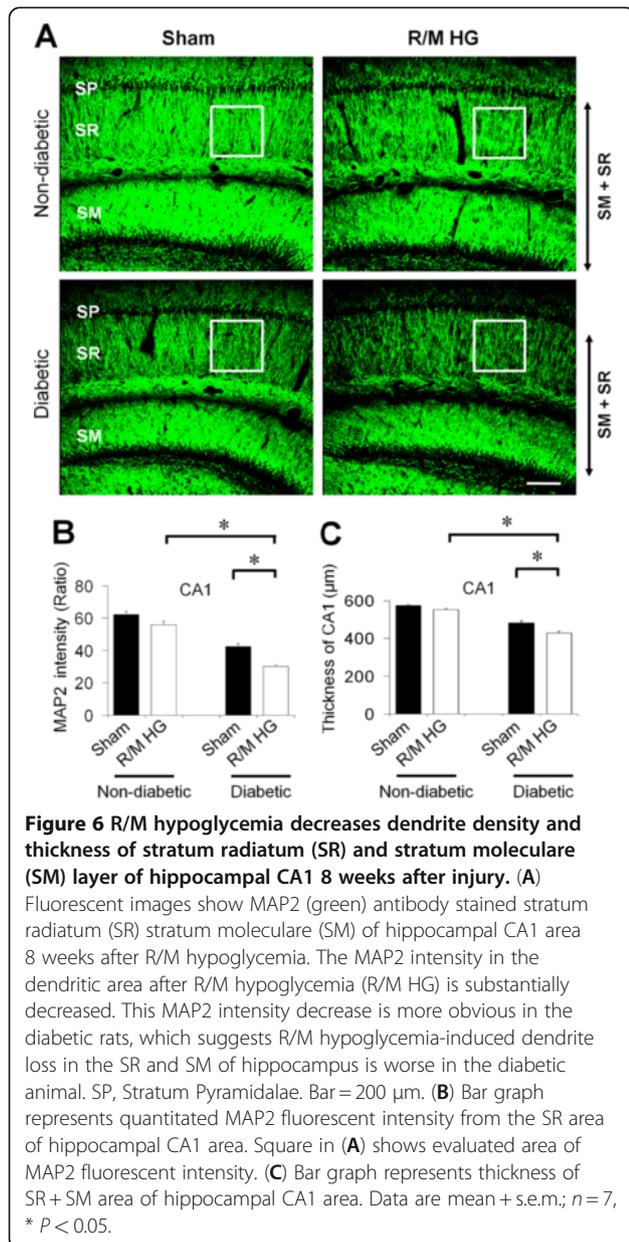
Figure 5 R/M hypoglycemia induces cognitive impairment in diabetic rats. STZ induced impairment in spatial learning and memory by increasing the path length traveled in the BM test (*: sham/saline vs. sham/STZ: $P < 0.05$; #: R/M-HG/saline vs. R/M-HG/STZ: $P < 0.01$). R/M hypoglycemia (R/M-HG) induced spatial learning and memory impairment only in the diabetic (*: R/M-HG/STZ vs. sham/STZ: $P < 0.05$), but not in non-diabetic rats (R/M-HG/saline vs. sham/saline: $P = 0.65$) (**A**). Diabetes but not R/M-HG reduced overall locomotor activity as evidenced by reduced total distance traveled (**B**), total active time (**C**) and vertical activity in rearing (**D**). Similarly, diabetes but not R/M-HG altered exploratory behavior by reducing activity in the center zone (Z2) of an open field (**E, F**) (D1, D2, and D3 = Day1, Day2, and Day3). *Post-hoc* analysis suggests that there are significant differences between R/M-HG/saline and R/M-HG/STZ in total distance (labeled as & in B), total active time (labeled as * in C), rearing events (labeled as & in D), Z2 distance (labeled as % in E) and Z2 total time (labeled as * in F). There were also significant differences between sham/saline and sham/STZ in total distance (# in B), rearing events (& in D), Z2 distance (# in E) and Z2 total time (* in F). *: $P < 0.05$, #: $P < 0.01$, %: $P < 0.005$, and &: $P < 0.001$.

impairment. However, moderate hypoglycemia produces neuronal death that is restricted to the cerebral cortex [34,49], It is not clear why this variation occurs.

To investigate whether differences in neuronal death depend on a pre-existing diabetic condition before insult, we rendered rats diabetic by STZ injection. The present study showed cortical but not hippocampal neuronal death, similar to what was seen in a previous

study [46]. Thus, the pattern of neuronal death in the cerebral cortex or in the hippocampus in the diabetic rats after R/M hypoglycemia is almost identical with other studies, which used non-diabetic rats [34,35,49] .

Whether R/M hypoglycemia causes cognitive impairment remains an important question. Repetitive hypoglycemia in the developing brain causes selective impairment of synaptic plasticity, in the absence of
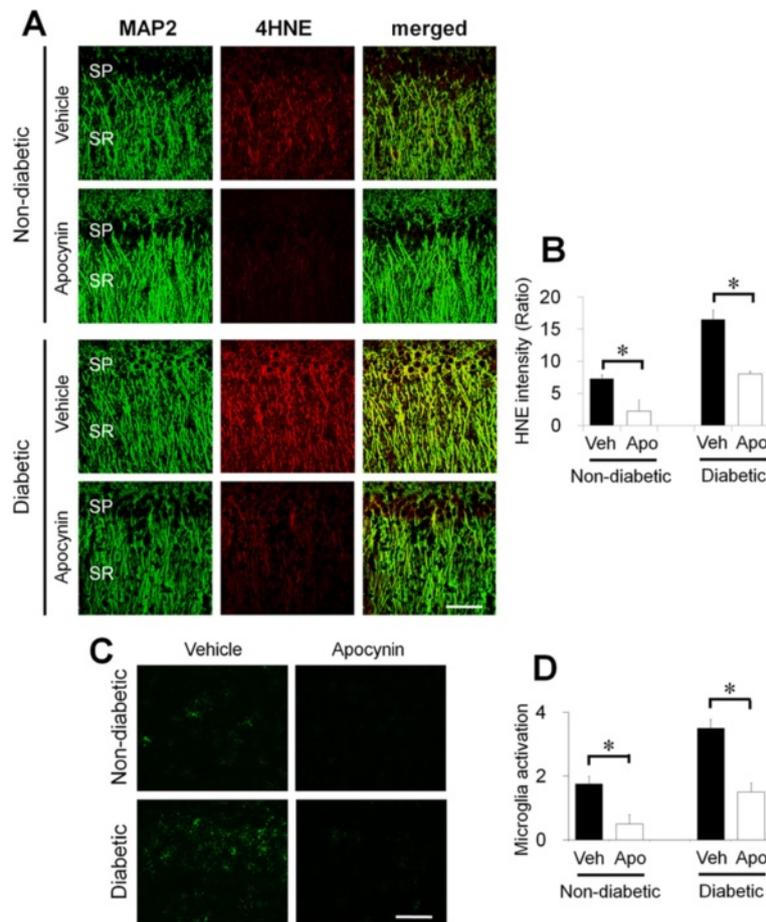
Figure 6 R/M hypoglycemia decreases dendrite density and thickness of stratum radiatum (SR) and stratum moleculare (SM) layer of hippocampal CA1 8 weeks after injury. (**A**) Fluorescent images show MAP2 (green) antibody stained stratum radiatum (SR) stratum moleculare (SM) of hippocampal CA1 area 8 weeks after R/M hypoglycemia. The MAP2 intensity in the dendritic area after R/M hypoglycemia (R/M HG) is substantially decreased. This MAP2 intensity decrease is more obvious in the diabetic rats, which suggests R/M hypoglycemia-induced dendrite loss in the SR and SM of hippocampus is worse in the diabetic animal. SP, Stratum Pyramidalae. Bar = 200 μm. (**B**) Bar graph represents quantitated MAP2 fluorescent intensity from the SR area of hippocampal CA1 area. Square in (**A**) shows evaluated area of MAP2 fluorescent intensity. (**C**) Bar graph represents thickness of SR + SM area of hippocampal CA1 area. Data are mean + s.e.m.; *n* = 7, * *P* < 0.05.

be induced after only a single episode of moderate hypoglycemia. Neither diabetic nor non-diabetic rats showed oxidative injury after only one episode, suggesting that repetitive moderate hypoglycemia events are required to induce oxidative injury in the hippocampal dendritic area. Our results also confirm previous findings that hypoglycemia induces lipid peroxidation in discrete regions of the forebrain [34,50].

Previously, McNay and Sherwin published the intriguing result that R/M hypoglycemia actually led to cognitive improvement in rats [51]. In the study, they performed a spatial working memory test shortly (20 min) after R/M hypoglycemia (3 h daily for 3 days). This improvement was equal in diabetic and non-diabetic rats, in contrast to the present study. However, a major difference between these studies is that behavioral testing in the present study was conducted 6 weeks after R/M hypoglycemia. Another study showed that R/M hypoglycemia prevented subsequent severe hypoglycemia-induced neuronal death and cognitive impairment [38]. This acute preconditioning effect is not relevant to the chronic functional impairment described here.

Microglial activation is also induced by severe hypoglycemia, and can contribute to neuronal injury [52], by releasing several neurotoxic substances, including superoxide, nitric oxide, and metalloproteinase [43,53-56] In the present study, we found that R/M hypoglycemia also induced microglial activation in the hippocampus, as seen in severe hypoglycemia [52], and diabetic rats showed higher microglial activation after R/M hypoglycemia. Thus, this confirms that the inflammatory response was also induced after R/M hypoglycemia, but whether this inflammatory response contributes to the oxidative stress or later cognitive impairment is not established.

Debate continues over the role of hypoglycemia and/or hyperglycemia in producing cognitive impairment in type 1 diabetes. In the present study, non-diabetic rats showed no obvious cognitive impairments after R/M hypoglycemia, but diabetic rats showed statistically significant cognitive impairment. Why was cognitive impairment only seen in the diabetic rats after R/M hypoglycemia? The present study does not provide a clear answer for this; however, we can speculate that less severe oxidative injury in the non-diabetic rats or impaired recovery in the diabetic rats influenced this. In support of these possibilities, we found that R/M hypoglycemia reduced MAP2 intensity and thickness in the stratum radiatum area of hippocampal CA1, more so in diabetic than in non-diabetic rats. Synaptic density in the hippocampus plays a crucial role in memory [57]. For example, synaptic density in the CA1 stratum radiatum is regulated by estrogen, leading to modulation of long-term depression (LTD) and long-term potentiation

hippocampal neuronal death, suggesting that impaired synaptic plasticity in the hippocampus caused by repetitive hypoglycemia could underlie memory and cognitive deficits observed in diabetic children [35]. In the present study, we tested the hypothesis that R/M hypoglycemia may cause oxidative injury in hippocampal dendrites [16,45]. To do this, we subjected young diabetic and healthy rats to R/M hypoglycemia and evaluated by immunostaining for 4HNE, an α,β-unsaturated hydroxyalkenal produced by lipid peroxidation in cells. 4HNE immunoreactivity in the hippocampal dendritic area was substantially increased after sequential hypoglycemic events, and was higher in diabetic rats compared to non-diabetic rats. Next we asked if oxidative injury could

Figure 7 R/M hypoglycemia-induced dendritic oxidative injury and microglial activation was reduced by the NADPH oxidase inhibitor, apocynin. (**A**) Fluorescent images show MAP2 (green) and 4HNE (red) antibody stained stratum radiatum (SR) of hippocampal CA1 area. The 4HNE intensity in the dendritic area after R/M hypoglycemia (R/M HG) is substantially increased in diabetic and non-diabetic rats. The NADPH oxidase inhibitor, apocynin (Apo), reduced 4HNE intensity is much higher in the diabetic and in the non-diabetic rats, which suggests R/M hypoglycemia-induced oxidative injury in the dendritic area of hippocampus is mediated by NADPH oxides activation. SP, Stratum pyramidalae. Bar = 100 μm. (**B**) Bar graph represents quantitated 4HNE fluorescent intensity from the SR area of hippocampal CA1 area. Data are mean + s.e.m.; $n = 7$, * $P < 0.05$. (**C**) Brain sections were harvested at 1 day after the last episode of moderate hypoglycemia and immunostained with CD11b. R/M hypoglycemia-induced microglial activation in the hippocampus is apparent in both non-diabetic and diabetic rats. Apocynin (Apo) treatment reduced microglia activation in the diabetic or in the non-diabetic rats. Scale bar = 100 μm. (**D**) Quantification of microglial activation was performed from hippocampal CA1 pyramidal area. Microglial activation is quantified based on morphological change and intensity of CD11b immunostaining. Data are mean + s.e.m.; $n = 5$, * $P < 0.05$.

(LTP) [58]. Evidence suggests that diabetes, stress, and aging negatively affect synaptic plasticity in brain regions including the hippocampus and the cortex, which can lead to persistent inhibition of LTP and facilitation of LTD and in turn might lead to activity-dependent synapse weakening and contribute to cognitive impairments [59].

We found that R/M hypoglycemia-induced oxidative damage in hippocampal dendrites and microglial activation was reduced by the NADPH oxidase inhibitor, apocynin, suggesting a mechanism by which R/M hypoglycemia may promote oxidative stress in the hippocampus during reperfusion. Glucose reperfusion-induced dendritic injury results in part from the activation by superoxide

produced through NADPH oxidase. NADPH oxidase generates superoxide through a process that requires glucose as a substrate for NADPH production, such that glucose availability can be the rate-limiting factor in superoxide production by this process [42,60,61].

Extrapolating these results to clinical settings has some limitations. The experimental paradigm of hypoglycemia for 5 consecutive days is intended to model recurrent hypoglycemia, but the effects over time of widely spaced hypoglycemic intervals may differ from the tightly spaced recurrences used here. Similarly, the diabetic rats were subjected to much greater variations in glucose than would typically occur in clinical settings. They also

were subjected to much greater changes in plasma glucose than the non-diabetic rats, a factor which may have contributed to the differences observed between the diabetic and non-diabetic rats. Last, rodent brains may differ from human brains in their responses to R/M hypoglycemia. Further research, particularly from the translational and clinical perspective is needed to resolve these questions.

Additional files

Additional file 1: Figure S1. Experimental protocol for R/M hypoglycemia or A/S hypoglycemia in the diabetic rats. Type 1 diabetes rats were induced by 2 consecutive days of intraperitoneal streptozotocin (50 mg/kg) injection. One week after STZ injection, diabetic rats were subjected to either moderate hypoglycemia for 5 consecutive days (**A**) or severe hypoglycemia once (**B**). For moderate hypoglycemia, rats were unanesthetized during entire experiment. One group of rats was sacrificed at 24 h after the last moderate hypoglycemia episode for histological evaluation for neuron death (FJB), oxidative damage (4HNE), and microglial activation (CD11b). The other group of rats was subjected to cognitive function evaluation with the Barnes maze behavioral test 6 weeks after R/M hypoglycemia and then sacrificed for MAP2 staining. For severe hypoglycemia, rats were anesthetized by isoflurane during entire hypoglycemia surgery. Rats were sacrificed 1 week after hypoglycemia for assessment of neuron death (FJB), oxidative damage (4HNE), and microglial activation (CD11b).

Additional file 2: Figure S2. EEG and blood glucose changes during hypoglycemia in diabetic rats. Insulin injection increases amplitude and reduces frequency of cortical EEG. Iso-EEG (flat line, e) was induced 4 h after 30 U/kg insulin injections. Initial fasting blood glucose concentration from diabetic rats in this experiment was above 15 mM. This blood glucose concentration was quickly decreased immediately after insulin injection. During the iso-EEG, blood glucose concentration was below 0.5 mM. After glucose reperfusion (G/R), blood glucose concentration was increased above 9 mM.

Abbreviations
ANOVA: Analysis of variance; A/S hypoglycemia: Acute/severe (A/S) hypoglycemia; EEG: Electrocardiogram; CA1: Cornu Ammonis area 1; CD11b: Cluster of differentiation molecule 11b; DMSO: Dimethylsulphoxide; FJB: Fluoro-Jade B; H&E: Haematoxylin/eosin; 4HNE: 4-hydroxy-2-nonenal; i.p: Intraperitoneal; LTD: Long-term depression; LTP: Long term potentiation; MAP2: Microtubule-associated protein 2; NADPH: Nicotinamide-adenine dinucleotide phosphate; RANOVA: Repeated measure of analysis of variance; R/M hypoglycemia: Recurrent/moderate hypoglycemia; SR: Stratum radiatum; STZ: Streptozotocin.

Competing interests
The authors declare no conflict of interest.

Author contributions
SW, BY, TK, BC, JK, BJ, ML, and JL researched data. MS performed the data analysis; SW, MS, JL, RS, and SS reviewed and edited the manuscript. RS and SS contributed to the discussion. SS wrote the manuscript. All authors read and approved the final manuscript.

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