Published in final edited form as:

Brain Res. 2007 August 24; 1165: 30–39.

Effects of Relative Hypoglycemia on LTP and NADH Imaging in Rat Hippocampal Slices

Matthew P. Sadgrove, Christopher J. Beaver, and Dennis A. Turner

Neurosurgery and Neurobiology, Duke University Medical Center Research and Surgery Services, Durham VAMC, Durham, NC 27710

Abstract

Cognitive and neuronal impairment in diabetes may be associated with iatrogenic hypoglycemia, particularly at low serum glucose levels (< 3 mM). To evaluate cellular impairment, we assessed acute hippocampal slice functioning during decreased ambient glucose, by monitoring evoked field excitatory post-synaptic potentials (fEPSP), and slice nicotinamide adenine dinucleotide (NADH) fluorescence. The effects of lowered glucose levels (60 min) were analyzed by examining the induction and maintenance of long-term potentiation (LTP), and NADH metabolic imaging in the CA1 region. The basal fEPSP response was reduced by lowered ambient glucose, an effect that was reversible in 2.5 mM glucose, partially reversible in 1.25 mM glucose and irreversible in 0 mM glucose, after 25 min recovery. LTP induction and maintenance declined during glucose restriction, demonstrating reversibly failed maintenance in 5 mM and 2.5 mM ambient glucose, and absent induction in 1.25 mM glucose. Peak NADH levels observed during train-induced biphasic transients were significantly reduced during 1.25 mM and 2.5 mM glucose. Significant functional compromise in our slice model occurred at 2.5 mM ambient glucose, equivalent to < 1 mM tissue glucose in the slice center, due to diffusion limitations and active glucose utilization. This tissue glucose level correlates with human observations of a serum threshold of < 3 mM for cognitive impairment, since brain tissue glucose is approximately one third of serum levels. The physiological effects of hypoglycemia in our slice model, assessed through fEPSP, LTP, and NADH responses, replicate closely the *in vivo* situation, confirming the usefulness of this model in assessing consequences of relative hypoglycemia.

Keywords

Metabolism; CNS; Diabetes; Memory

1. Introduction

Aggressive treatment of diabetes requires close adherence to normal blood sugar values, which may be complicated by intermittent episodes of iatrogenic hypoglycemia. These hypoglycemic episodes can produce both acute and chronic cognitive dysfunction [2,3,9,35]. The threshold for the appearance of cognitive impairment is ~ 3 mM serum glucose [3,22,29]. Although normal serum and brain tissue glucose levels (6.9 mM and 2.2 mM, respectively, approximately a 3:1 ratio) have been reported in humans using microdialysis [27], the symptomatic brain tissue glucose level during hypoglycemia is unknown. However, the use of brain slices

Correspondence to: Matthew P. Sadgrove, PhD, Box 3807, Neurosurgery, DUMC, Durham, NC 27710, Phone 919-684-6706, FAX 919-681-8068, Email Matthew.Sadgrove@duke.edu

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

facilitates the manipulation of media glucose levels. Although tissue levels and concentration gradients of glucose within brain slices are not precisely known in this model, they can be mathematically estimated [43]. This *in vitro* approach also separates systemic protective factors (such as autonomic and hepatic responses) from those of direct relevance to the brain. Although impairment of cognitive function cannot be directly measured in an *in vitro* preparation various surrogate physiological measures may (as described below) be used to estimate neuronal function.

At a basic level tissue viability and physiological function can be assessed using evoked field potentials (fEPSPs), an averaging of post-synaptic responses from multiple neurons. However, even tissue that retains a robust fEPSP may show impairment of more complex physiological function, such as long-term potentiation (LTP). LTP is a long lasting increase in synaptic strength, which is well documented both *in vivo* and *in vitro* [8]. Although the precise function of LTP, if any, is controversial it has long been linked with learning and memory through a contribution to synaptic plasticity [30], and at least one group has directly linked LTP with spatial memory [38,42].

Experimentally LTP can be induced by repeated electrical stimulation at high frequency (reviewed in [33]). Induction requires increased cytoplasmic calcium concentration to activate multiple signal transduction pathways, which increase synaptic strength. This leads to a sustained increase of the fEPSP (at least 30 mins; *maintenance*), the duration of which depends on the method of induction [33,34]. LTP induction can readily be inhibited, for example by Ca^{2+} chelation or NMDA receptor inhibition [31]. LTP maintenance is also prone to disruption [37] or loss, especially during the initial 30 mins after induction [26], which is detected as a failure of potentiation. Cognitive dysfunction arising from iatrogenic hypoglycemia in humans, which includes impairment of learning and memory [46], may be parallel to failure of one or more of these aspects of LTP [23]. For example, in young rats repeated insulin-induced hypoglycemia impairs LTP [47].

NADH fluorescence imaging can be used to assess metabolic responses of tissue, and is complementary to physiological assessment. NADH imaging can reveal relative changes in tissue NADH levels and particularly metabolic NADH turnover, but has rarely been combined with studies of hypoglycemia. It is known that NADH concentrations measured at static time points differ considerably with hypoglycemia [16], hence we used NADH fluorescence imaging as a tool to measure relative NADH changes in real time.

Our hypothesis is that reduced ambient glucose, leading to decreased tissue glucose, significantly affects slice function, and that the slice model accurately reflects appropriate *in vivo* critical glucose levels. Tissue oxygen tension gradients have been reported in the slice preparation [12], and a model for predicting glucose gradients within slices has been proposed [43]. Thus, we used a range of ambient glucose concentrations to investigate our hypothesis, to identify the *in vitro* equivalent of critical serum glucose (3 mM). These experiments examine the tolerance of brain slices to relevant levels of glucose, including physiological measures of neuronal function, as well as the ability to induce and maintain LTP and functional recovery.

2. Results

The experiments were carried out in three groups (see also experimental procedures). Firstly we will discuss fEPSP amplitude and recovery with different levels of glucose (Fig 1A). Since recovery is a static measure of slice health we then used LTP as a dynamic measure of slice function and explored both induction and maintenance (Fig 1A +B). Additionally under these conditions we considered the effects of glucose levels on metabolic function and measured NADH responses to synaptic train stimuli. The final group of experiments formed an internal

control to exclude any effect of the NADH stimulus train on LTP induction (Fig 1B+C). Together these results map out clinically relevant functional changes associated with relative hypoglycemia.

Hypoglycemia and fEPSP amplitude

All slices were kept in the recording chamber for 30 min prior to the start of the experiment and then followed for 2.5 hours to monitor fEPSP amplitude and slope, at a temperature of 36°C. In experiments some spontaneous upwards drift of the fEPSP levels was noted, possibly due to the length of recording and the normothermic conditions (a relatively high temperature for slices, but appropriate for metabolism studies). We measured this drift in the control group (i.e., 10 mM glucose for the entire experiment), and then compensated for the drift by estimating the slope for each individual group. The fEPSP increased similarly in the control and treatment groups (over a baseline of 100% at the start of the experiment) by $42 \pm 10\%$ after an additional 22.5 min. All other glucose levels showed similar increases in fEPSP amplitude (5 mM $26 \pm 10\%$, 2.5 mM $39 \pm 13\%$, 1.25 mM $38 \pm 31\%$, 0 mM $53 \pm 17\%$; all n.s. vs. the 10 mM glucose group). The baseline fEPSP amplitude in the control group also continued to trend upward for the duration of the experiment, but at the same relative slopes. Therefore, comparisons were made to time matched controls (exposed to 10 mM glucose) of raw data.

A glucose concentration of 5 mM was adequate to maintain intermittent synaptic stimulation; the fEPSP amplitude was comparable to those of slices maintained in 10 mM glucose (Fig. 2). fEPSP amplitude was increased over baseline by $34 \pm 19\%$ after 25 min in 5 mM glucose, $56 \pm 37\%$ after 60 min in 5 mM glucose, and $102 \pm 53\%$ after the 25 min recovery ($n = 4$; all n.s. vs. corresponding 10 mM glucose; Fig 2 *dark gray bars*).

In contrast the fEPSP amplitude slowly declined in 2.5 mM glucose ($n = 5$): to $11 \pm 10\%$ below baseline after 25 min (n.s. vs. 10 mM glucose), and $90 \pm 6\%$ below baseline after 60 min ($P < 0.001$ vs. 10 mM glucose). The fEPSP amplitude partially recovered when returned to normal glucose, although it remained significantly decreased compared to time matched control slices ($19 \pm 39\%$ above baseline after 25 min recovery; $P < 0.01$ vs. 10 mM glucose; Fig 2).

Slices in 1.25 mM glucose showed a rapid decline in fEPSP amplitude ($93 \pm 4\%$, and $97 \pm 2\%$ below baseline after 25 and 60 min respectively; both $P < 0.001$ vs. corresponding 10 mM control; $n = 5$). On return to 10 mM glucose some recovery of synaptic activity was evident although the fEPSP amplitude remained significantly depressed, at $56 \pm 28\%$ below baseline after 25 min ($P < 0.001$ vs. 10 mM control). The same rapid loss of fEPSP amplitude occurred in slices perfused in the absence of glucose ($92 \pm 5\%$, and $93 \pm 5\%$ below baseline after 25 and 60 min respectively; both $P < 0.001$ vs. corresponding 10 mM control; $n = 4$). However, after aglycemia no recovery of function was observed (fEPSP amplitude $96 \pm 4\%$ below baseline; $P < 0.001$ vs. 10 mM glucose; $n = 4$; Fig 2).

In summary these experiments showed that the 2.5 mM ambient glucose level demonstrated a critical decrease in function, compared to 5 mM and 10 mM, which demonstrated near normal recovery, whereas there was irreversible loss of function at levels below 2.5 mM.

Effects of hypoglycemia on LTP induction and maintenance

LTP is consistently induced in control conditions (10 mM glucose)—A single LTP induction protocol applied either in the middle (at 52.5 min, Fig 1B) or late (at 112.5 min, Fig 1A) in the recording induced an early fEPSP slope increase ($100.3 \pm 23.3\%$, $P < 0.01$, $n = 8$, and $127.9 \pm 51.7\%$, $P < 0.05$, $n = 5$ respectively at 5 min post tetanus; One-way ANOVA with repeated measures and Bonferroni post test). This increase persisted for at least 25 min (190.4

$\pm 34.9\%$, $P < 0.001$, $n = 8$, and $160.8 \pm 45.9\%$, $P < 0.05$, $n = 5$ respectively at 25 min post tetanus; One-way ANOVA with repeated measures and Bonferroni post test). These observations persist after correction for baseline drift was applied to the fEPSP slope data (Fig 3), indicating the significant increases in fEPSP slope are not due to the baseline drift but to the applied LTP tetanus protocol. After drift correction, the significant fEPSP slope potentiation observed in the raw data was still rapidly induced ($82.0 \pm 21.8\%$, $P < 0.01$, $n = 8$, and $125.9 \pm 53.0\%$, $P < 0.05$, $n = 5$ respectively at 5 min post tetanus; One-way ANOVA with repeated measures and Bonferroni post test; Fig 3) and persisted for at least 25 min ($153.5 \pm 34.0\%$, $P < 0.001$, $n = 8$, and $125.6 \pm 40.9\%$, $P < 0.05$, $n = 5$ respectively at 25 min post tetanus; One-way ANOVA with repeated measures and Bonferroni post test; Fig 3). The LTP induction protocol we used was also sufficient to induce maximal potentiation. Application of a second LTP protocol after 60 min interval (Fig 1B), did not elicit a significant further increase in fEPSP slope ($60.9 \pm 34.7\%$ increase at 5 mins, and $62.3 \pm 32.3\%$ increase at 25 mins post tetanus; $n = 8$; both n.s. vs. pre tetanus, One-way ANOVA with repeated measures and Bonferroni post test; Fig 3). These control experiments in 10 mM glucose confirm that our LTP induction protocol is sufficient to saturate LTP under control conditions.

LTP maintenance is interrupted by mild + moderate hypoglycemia (5 or 2.5 mM glucose)—10 mM glucose was required for persistent maintenance (>20 min) of induced fEPSP slope potentiation. However, 5 and 2.5 mM glucose were sufficient conditions for LTP induction. In contrast to slices maintained in 10 mM glucose, which demonstrate persistent potentiation ($155 \pm 30\%$ increase in slope from 5 min before to 25 min after induction; Fig 4A), fEPSP slopes in slices maintained in 5 mM and 2.5 mM glucose showed a $39 \pm 47\%$ decrease ($n = 7$) and a $4 \pm 55\%$ increase ($n = 5$), respectively, over the same time. Stimulation of slices in 5 mM glucose did elicit a transient increase in fEPSP slope following the LTP induction; however, this increase was not persistent, lasting less than 25 min (Fig 4B).

The absence of significant LTP under these moderate hypoglycemic (5 or 2.5 mM) conditions appears to be due to a failure of LTP maintenance, rather than a lack of LTP induction, since the restoration of 10 mM glucose to slices led significant increases, above baseline, in the fEPSP slope during recovery. The fEPSP slope increased by $272 \pm 104\%$ 25 min after return to 10 mM glucose, compared with the slope in 5 mM glucose before LTP induction, and by $253 \pm 104\%$ compared with the slope in 2.5 mM glucose before LTP induction (both $P < 0.05$; $n = 7$ and $n = 5$ respectively; Fig. 4B,C).

Differences between the efficacy of LTP induction in 5 mM and 2.5 mM glucose became more apparent when a second LTP induction protocol was performed, as the slice recovered in 10 mM glucose, after the low glucose episode. During the recovery period, following treatment in 5 mM glucose, the slice fEPSP slope responses ($75 \pm 46\%$ decrease in slope from 5 min before to 25 min after second LTP induction) were similar to the responses of slices maintained in 10 mM glucose throughout ($81 \pm 56\%$ increase in slope from 5 min before to 25 min after second LTP protocol; Fig. 4B). In contrast, slices recovering after treatment with 2.5 mM glucose produced a further increase in fEPSP slope ($122 \pm 51\%$ increase from 5 min before to 25 min after second LTP protocol; Fig. 4C). Although this is not statistically significant, the stepwise shape of this further potentiation in recovery after 2.5 mM glucose suggests a real increase in LTP. The initial induction in 2.5 mM glucose may thus not have been maximal, with further induction possible on return to 10 mM glucose.

In our experiments hypoglycemic treatment did not reduce the maximal level of LTP induction as a consequence of either mild (5 mM) or moderate (2.5 mM) glucose restriction (for the 60 min exposure). fEPSP slopes at the end of the experiment were comparable in these cases to 10 mM controls (10 mM glucose, $433 \pm 121\%$; 5 mM glucose, $217 \pm 104\%$; and 2.5 mM glucose, $353 \pm 72\%$ above baseline; Fig 4A,B,C).

After prolonged severe hypoglycemia or aglycemia (1.25 or 0 mM glucose) slice health is insufficient to support LTP—fEPSP slope continued to decline after attempted

LTP induction in either 1.25 mM or 0 mM glucose, decreasing by $28 \pm 15\%$ and $46 \pm 22\%$ from 5 min before LTP to 25 min after, respectively (Fig. 4D,E). Consistent with our fEPSP amplitude measurements (Fig 2), fEPSP slope measurements in slices exposed to 1.25 mM glucose exhibited only partial recovery of synaptic function ($76 \pm 25\%$ of initial slope after 25 min in 10 mM glucose). LTP induction during the 1.25 mM glucose condition appears to have failed, with the fEPSP slope below baseline after 25 min of recovery, and significantly lower compared to time matched controls ($P < 0.001$ vs. 10 mM; Fig 4D). A gradual recovery of fEPSP slope continued during the recovery in 10 mM glucose, after exposure to 1.25 mM glucose. However, the second LTP induction protocol failed to significantly potentiate the fEPSP slope (increase of $57 \pm 23\%$ from 5 min before to 25 min after stimuli), which remained significantly lower than 10 mM controls ($P < 0.001$; Fig 4D). Synaptic activity became negligible during aglycemia, and failed to recover on return to 10 mM glucose ($9 \pm 9\%$ after 25 min in 10 mM glucose; Fig. 4E). Also, there was no subsequent response to the second LTP stimulus ($8 \pm 8\%$ of baseline 25 min after LTP 2). Thus, severe hypoglycemia prevented LTP induction both during and after the insult, as a consequence of the severe, irreversible loss of neuronal function.

NADH imaging of metabolic function

NADH imaging was performed for two reasons: 1) to monitor gradual changes in slice metabolic turnover during restricted glucose by recording fluorescence intensity throughout the experiments; and 2) to assess metabolic capacity by examining the biphasic response produced by an intense stimulus train (250 pulses at 10 Hz).

Baseline measurements

Slices imaged in control conditions (10 mM glucose) showed a steady decline in baseline NADH fluorescence [12,14]. The background fluorescence in the stratum radiatum decreased by $7.2 \pm 1.5\%$ during the experiment (approx. 2.5 hrs; Fig. 5A). The application of the two stimulation protocols, i.e., high frequency LTP inducing trains (200 pulses at 100 Hz) and low frequency stimulus trains (250 pulses at 10 Hz), both produced brief biphasic responses ($\sim 1\%$ and $\sim 6\%$ total NADH change, respectively), but did not appear to alter the long term NADH profile (Fig 5A). In the glucose restriction experiments no differences in relative NADH fluorescence intensity were evident at the start of the hypoglycemic period, when compared to time-matched controls ($P = 0.30$, one-way ANOVA; Fig 5).

Decreased glucose levels produced a further decline in NADH fluorescence, which became clearly apparent when glucose concentration was lowered to 2.5 mM for 60 min (Fig. 5C). Mild hypoglycemia (5 mM glucose) exposure for 55 min did not significantly alter basal NADH fluorescence (5 mM, $-9.1 \pm 2.1\%$ below initial fluorescence intensity; $n = 6$; compared to 10 mM, $-6.9 \pm 1.0\%$ below initial fluorescence intensity; $n = 7$; n.s.; Fig 5A,B). However, moderate glucose restriction (2.5 mM glucose) led to a significant decrease in baseline NADH fluorescence by 55 min ($-13.9 \pm 1.7\%$ below initial fluorescence intensity; $P < 0.01$, $n = 5$; Fig 5C). Full recovery of NADH fluorescence intensity to control levels occurred within 25 min of return to 10 mM glucose (after 5 mM, $-8.2 \pm 2.0\%$ below initial fluorescence intensity, $n = 6$; and after 2.5 mM, $-8.7 \pm 1.5\%$ below initial fluorescence intensity, $n = 5$; vs. 10 mM, $-7.9 \pm 1.1\%$ below initial fluorescence intensity, $n = 7$; both n.s.; Fig 5A,B,C). Moderate hypoglycemia does not appear to cause irreversible damage to the slice metabolic state, since the NADH fluorescence response is reversible.

The decrease in NADH fluorescence was accelerated by severe hypoglycemia/aglycemia (1.25 or 0 mM glucose) and became irreversible after 60 min deprivation (Fig. 5D,E). NADH fluorescence intensity became significantly attenuated within 25 min in both 1.25 mM and 0

mM glucose and continued to decline further: at 55 min, 1.25 mM glucose was $-22.9 \pm 1.8\%$; and 0 mM glucose was $-37.7 \pm 0.6\%$; both $P < 0.001$ vs. 10 mM glucose, $-6.9 \pm 1.0\%$; Fig 5A,D,E). As with milder hypoglycemia, recovery of slices in 10 mM glucose ACSF led to a relative increase in NADH fluorescence intensity. 25 min after return to 10 mM glucose NADH remained significantly lower than in time-matched controls (1.25 mM glucose, $-16.6 \pm 1.5\%$, $n = 6$; and 0 mM glucose, $-30.0 \pm 1.2\%$, $n = 3$; both $P < 0.001$ vs. 10 mM glucose; Fig 5). This change in baseline NADH fluorescence was permanent, as no further recovery occurred during the remainder of the experiment.

Transient NADH responses

Application of a low frequency stimulus train (250 pulses at 10 Hz) to slices under control conditions induced a transient (< 5 min), reproducible, biphasic NADH response [12,39]. This response consisted of an initial small oxidation, followed by a larger, more prolonged reduction (values shown in Table 1). Prior to glucose restriction there was no significant difference between experimental groups in the magnitudes of either phase of the biphasic response assessed by one-way ANOVA (Table 1). Mild (5 mM) glucose restriction did not affect the reduction phase, when compared to slices in 10 mM glucose. However, during moderate (2.5 mM) and severe (1.25 mM) glucose restriction the prolonged increase in NADH fluorescence (reduction phase) was significantly attenuated compared with the 10 mM glucose controls (train 2: 10 mM, $3.9 \pm 1.1\%$, $n = 7$; vs. 2.5 mM, $1.8 \pm 0.9\%$; $p < 0.05$; and vs. 1.25 mM, $1.1 \pm 1.5\%$, $p < 0.01$, $n = 5$, and 6 respectively). Subsequent responses to a stimulus train during recovery in 10 mM glucose were partially, but not significantly, attenuated (train 3: after 2.5 mM, $2.3 \pm 1.1\%$, $n = 5$; and after 1.25 mM, $2.1 \pm 1.3\%$, $n = 6$, compared to 10 mM control, $4.1 \pm 1.9\%$, $n = 7$). However, zero glucose irreversibly abolished the transient NADH response to stimulation (0 mM: train 2, $0.1 \pm 0.2\%$; train 3, $0.3 \pm 0.3\%$; both $P < 0.01$ vs. equivalent 10 mM train, $n = 3$).

The low frequency synaptic train does not interfere with LTP induction

The low frequency trains (for the NADH biphasic response measurement) could potentially interfere with or enhance LTP induction. Therefore, additional controls were performed at 10 mM and 2.5 mM glucose, omitting the 10 Hz synaptic trains but retaining the LTP protocols (Fig 1C), these were compared with slices receiving both NADH trains and LTP protocols (Fig 1B). These control experiments demonstrated that the low frequency stimulus train protocols (250 pulses at 10 Hz) did not significantly alter subsequent electrophysiological responses to LTP induction, in either control conditions (10 mM glucose) or under moderate hypoglycemia (2.5 mM glucose for 60 min). In both conditions a Bonferroni *post-hoc* test found no significant difference at 2.5, 17.5, 47.5, 77.5, 107.5, or 137.5 min (*data not shown*). Thus, there were no significant interactions between the low frequency trains and LTP induction.

3. Discussion

The primary conclusions of our *in vitro* slice experiments are that: 1) lowered ambient glucose, particularly at 2.5 mM and below, reduces the metabolic capacity of the tissue in response to neuronal activation; 2) LTP induction and maintenance are altered below 5 mM glucose, which may correspond to cognitive disturbances experienced *in vivo* at similar levels. These results confirm the usefulness of this *in vitro* slice model of hypoglycemia, particularly the physiological consequences of the various glucose levels and their equivalents *in vivo*, as well as potentially to study treatment effects.

Comparison of *in vitro* glucose ambient levels and *in vivo* clinical relevance

In humans, a 'normal' physiological plasma glucose concentration (5–7 mM) correlates with a brain extracellular glucose concentration of approximately 2 mM, by invasive microdialysis

studies [1,17,27], resulting in a linear 3:1 ratio between plasma and tissue glucose concentrations [27]. Plasma glucose concentrations of <3 mM lead to impaired cognitive function [3,4], and limitations of glycolysis [17,18].

The interface chamber used in our *in vitro* experiments supplies unlimited glucose directly to the slice tissue surface, but leads to a large diffusion gradient between the slice surface and interior, as compared to *in vivo* brain. This gradient can be modeled [43], and the glucose at a given slice depth can be estimated. This gradient is similar to oxygen diffusion and demand within slice tissue [12]. By adapting the calculations given by Tekkok [43], the interior glucose concentration at a depth of 200µm in the slice can be estimated for various ambient ACSF glucose concentrations: 10 mM in ACSF decreases to 7.24 mM; 5 mM ACSF to 2.24 mM; and 2.5 mM ACSF would lead to almost zero tissue glucose, provided the rate of glucose utilization remains constant. The observed decline in fEPSP amplitude in slices exposed to 2.5 mM ACSF glucose in the present study suggest a significant reduction in utilization. Thus 2.5 mM ambient glucose *in vitro* would likely result in <1 mM glucose in the tissue interior, comparable with 2–3 mM plasma glucose *in vivo* [27]. Therefore our slice model provides experimental glucose tissue levels parallel to that expected during iatrogenic hypoglycemia.

The observed decline in fEPSP amplitude following reductions in glucose concentrations to 2.5 mM or lower was gradual [20,36]. Clear cognitive sequelae appear to occur in the presence of low serum glucose after only minutes, suggesting that brain energy reserves (ie, glycogen) are very limited. This difference in the latency of functional changes in response to hypoglycemia between clinical and *in vitro* studies may be due to changed glycogen reserves induced by slice preparation and recovery [11], or differences in basal tissue activity, since tissue slices are effectively quiescent whereas the *in situ* tissue of the clinical brain is constantly stimulated. The rate of fEPSP loss during hypoglycemia in past *in vitro* studies varies with the temperature used to maintain the slices (< 30 min at 35–36°C and ≈ 60 min at 30°C), since metabolic demands of tissue during hypoglycemia are highly temperature dependent [6,41]. The reversible loss of fEPSP induced by 30 min of aglycemia at 35°C [36] becomes irreversible in our experiments (also at 35°C) by 60 min. Other studies using submerged slices demonstrate low glucose can induce decreased fEPSP slope at a variety of temperatures [10,36,45].

Effects of Hypoglycemia on LTP

LTP responses may be more susceptible to moderate hypoglycemia than fEPSP amplitude alone, since it is a more complex process and may require a higher degree of metabolic support. LTP is a functional measure of *in vitro* retention of patterned inputs, which closely parallels short-term, post-distraction memory *in vivo*. Many reviews have assessed the specificity and validity of this model [28,40]. LTP induction in the CA1 region of the hippocampus requires bursts of postsynaptic action potentials, coincident with incoming pre-synaptic activity [32, 44]. Thus failure of LTP induction in 1.25 mM and zero glucose conditions in our experiments can be attributed to failure of synaptic transmission.

Our data clearly show that LTP maintenance is significantly altered, even in 5 mM ambient glucose, and severely compromised at lower glucose levels. These slice media levels parallel observed *in vivo* levels, where 2.5 mM serum glucose commonly leads to severe, temporary cognitive sequelae, particularly loss of memory and altered response to the environment. Our data, however, may also give insight to processes leading to LTP maintenance, since we added a second LTP after recovery to assess whether full LTP was initially induced (and occluded or not). Though full maintenance of LTP has been known for some time to require protein synthesis [19], the required degree of metabolic integrity has not been fully delineated. Thus, in our intermediate glucose levels (5 or 2.5 mM) LTP is temporarily not maintained, and may not be either fully induced or maintained, so that the second LTP stimulus adds further to the initial tetanic stimulus. This could be due either to incomplete induction, or inadequate

maintenance, after full induction. We have determined that LTP maintenance fails in an ambient glucose concentration of between 5 and 10 mM, and is likely close to 5 mM, since at this concentration induction occurs but maintenance is transient. The return of significant fEPSP slope potentiation on recovery in 10 mM glucose with no other intervention demonstrates continued induction of LTP, but with reversible inhibition of maintenance. This effect appears to last for at least 30 min, even when synaptic transmission is not sustained. The failure of maintenance appears due to a lack of synaptic transmission although a block of the postsynaptic modifications that occur in response to LTP induction cannot be excluded. These observations suggest that metabolic manipulation may provide a novel approach for investigating the various processes underlying LTP *in vitro*.

While the observation of successful LTP induction but failed maintenance during hypoglycemia is novel to this study, previous studies [21,24] have examined the effect of brief hypoglycemia on LTP induction with varied results. Kamal et al. also demonstrated that LTP could be induced by high frequency stimulation after a brief (20 minute) exposure to 0 mM glucose, although importantly in this study normal glucose (10 mM) was returned immediately after tetanus [24]. This observation is similar to the response elicited in our experiments using 2.5 mM glucose, where LTP could be induced but not maintained in the hypoglycemic condition; again differences in the severity of glucose deprivation required to produce the effect are likely due to the temperatures used (30°C and 35–36°C respectively). Critically the fEPSP was still present if attenuated in both these studies.

The observations of Izumi and Zorumski are markedly different; they reported no alteration of baseline synaptic transmission when extracellular glucose was reduced to 2 mM glucose, but found that a brief exposure to 2 mM glucose blocked LTP induction [21]. This discrepancy is partially attributable to the widely divergent metabolic conditions; our study used interface slices at 35–36°C, whereas the Izumi study used submerged slices at 30°C. Glucose gradients in these two slice preparations are very different and are predicted to be considerably smaller in the submerged slice, since the glucose media completely surrounds the slice [43]; metabolic demand is also significantly lower due to temperature effects. The lack of LTP induction observed by Izumi and Zorumski may also be due to lower oxygen tension in the submerged slices, leading to a hypoxic/ischemic insult as much as a hypoglycemic insult. Foster et al. have measured tissue oxygen tension as a function of depth in the interface slice used in our experiments, reporting a nadir at ~265 μm depth (pO_2 326 ± 101 mm Hg)[12]. However, the oxygen profile for submerged slices starts at a much lower pO_2 and falls to 12 ± 23 mm Hg at a depth of 254 μm ($n = 9$, unpublished observation, personal communication, Dr. Kelley Foster). Thus, the radically different conclusions offered by Izumi regarding LTP competency likely arises from the differences in metabolism of the various slice conditions.

NADH measurements

By recording both slice electrophysiology and NADH fluorescence intensity we were able to observe the energetic rundown that accompanies loss of synaptic function. NADH fluorescence is sensitive to ambient glucose concentration, declining gradually in both 2.5 and 1.25 mM glucose, and corresponding with the loss of synaptic transmission and irreversible neuronal damage. These changes show, in real time, significant reductions in the available energy pool within cells. This method also has the advantage of being non-invasive, in contrast with most studies directly measuring ATP, which use rapid freezing, homogenization and subsequent optical quantification [13], and thus require destruction of the tissue.

Levels of ATP in tissue slices may be critical in determining the efficacy of synaptic transmission. It has been shown that adenosine release is linked to synaptic transmission loss in hypoglycemia [7,15,48]. The adenosine is presumed to derive from ATP, which is decreased by hypoglycemia. The decrease in basal NADH fluorescence during hypoglycemia reflects a

reduction in the potential for ATP regeneration but in the presence of normal oxygen levels, in effect forcing most of the NADH to NAD. Brennan et al. demonstrated that an adenosine A1 receptor antagonist could alleviate 2-deoxyglucose (chemical hypoglycemia) induced attenuation of the biphasic NADH response to a stimulus train [5]. We also found that glucose concentrations modulate the second phase of the NADH biphasic response, which may reflect impaired recovery of mitochondrial energy intermediates in the lowered glucose media [25, 39].

4. Experimental Procedures

Tissue slice preparation

Hippocampal tissue slices were prepared from male Fischer 344 rats (5–6 weeks; Harlan, Indianapolis, IN, USA). The Institutional Animal Care Committees at both Duke University and Durham VAMC approved all experimental protocols. Experiments conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals used and their suffering. The rats were anesthetized with halothane (Abbott Laboratories, North Chicago, IL, USA) in an anesthesia induction chamber until respirations ceased, and decapitated. The brain was rapidly removed from the skull and placed in chilled artificial cerebrospinal fluid (ACSF). Both hippocampi were isolated, and the center third of each cut into 400 μm thick transverse slices using a tissue chopper. Slices were permitted to recover for 2 hours in oxygenated ACSF at 22°C before use. For all experiments, slices were transferred to an interface recording chamber, where they were perfused with oxygenated ACSF (1.67 ml/min), continuously aerated with 95% O₂-5% CO₂ humidified gas mixture and maintained at 36°C for a further 30 min. Each animal provided up to three slices for recording, with only one slice used per experimental condition.

Solutions

ACSF used to maintain tissue during slice preparation, recovery, and initially after transfer to the recording chamber (normal ACSF) consisted (in mM): glucose 10, NaCl 130, NaH₂PO₄ 1.25, NaHCO₃ 24, KCl 3.5, CaCl₂ 1.2, MgSO₄ 1.2 (Sigma Aldrich, St. Louis, MO, USA). ACSF used during glucose restriction (test ACSF) retained the same basic formulation as normal ACSF, with osmotic changes due to reduced glucose concentrations (5, 2.5, 1.25 and 0 mM) compensated for by modifying NaCl concentrations.

Synaptic stimulation

A bipolar stimulating electrode made of insulated stainless steel wire was placed in the stratum radiatum to stimulate Schaffer collaterals in the CA1 region. fEPSPs were recorded using glass microelectrodes filled with 1M NaCl, placed in the stratum radiatum, at a depth at which fEPSP amplitude response was optimal, within the middle third of each slice. After a 20-minute stabilization period, stimulus-response curves were used to establish a stimulation voltage that would induce a 60–70% maximal fEPSP amplitude response.

Slope and amplitude measurement

To determine fEPSP amplitude, an average of the peak value of the first deviation (excluding the fiber volley) from baseline after the stimulus artifact and the values 0.1 ms before and after, were used to calculate the maximum observed potential. This average was then subtracted from the baseline potential taken 5 ms before each stimulus. Least squares linear regression was applied to 10 points (1 ms) prior to the maximal EPSP response to obtain a value for EPSP slope, on the initial, falling phase of the field EPSP response. Additionally, all data were normalized by dividing each observation by an average of observations from the first 150 s of each experiment and expressing this as a percentage.

Experimental paradigm

For all slices, 22.5 min of baseline was recorded in normal ACSF (10 mM glucose); the perfusate was switched to the test ACSF (containing 10, 5, 2.5, or 1.25 mM glucose) for 60 min, and then returned to normal ACSF for an hour recovery before the experiment was terminated. The experiments were divided into 3 groups based on the stimulation protocols they received (Fig. 1). Two groups were subjected to a low frequency stimulus train (250 pulses at 10 Hz) during the baseline-recording period (Figs 1A, B). This stimulus train induces a reproducible NADH biphasic response and was used to establish an NADH pre-treatment response and confirm slice health. Further low frequency stimulus trains, followed after 5 min by high frequency LTP inducing trains (200 pulses at 100 Hz, repeated after 1 min), were applied; either during the recovery following altered glucose only (Fig 1A), or during both altered glucose and recovery (Fig 1B). The third group only received 2 high frequency LTP inducing trains and did not receive any of the low frequency stimulus trains (Fig 1C). A single LTP induction protocol included two trains (each 200 pulses at 100 Hz) separated by 60 s.

NADH imaging

For fluorescence imaging, slices were epi-illuminated (excitation 330 – 370 nm) with an incident angle of 45°, using a Lambda DG-4 (Sutter Instrument Company, Novato, CA, USA) light source equipped with a stabilized xenon arc lamp. Slices were imaged (emission above 400 nm) through a Nikon upright microscope (UM-2) with a compound lens (4x, N.A. 0.13), using a linear, cooled 12-bit CCD camera (Cooke Instruments Sensicam, Auburn Hills, MI) with 640x480 digital spatial resolution. Images were taken every 5 s, with 1–1.5 s exposure for each image. To compensate for low fluorescence emission, NADH images were acquired as 8x8 binned images, which reduces the effective spatial resolution to 80x60 pixels but increases sensitivity; the area of a single pixel becomes approx. 400µm². Control experiments were performed to assess the maximum oxidation and reduction levels of NADH by exposing slices to 0 mM glucose for 60 min, or hypoxia for 30 min which resulted in a ~40 % decrease and ~45 % increase in fluorescence intensity, respectively. These responses indicate that the resting NADH level in the slice is juxtaposed fairly equally between oxidation and reduction states, at the 95% oxygenation level used [12]. The imaging analysis consisted of creating a region of interest (ROI; approx area 15000–25000 µm²) within the stratum radiatum, between the stimulating and recording electrodes, and then averaging the imaging values across the ROI. ROI values were calculated for each image in the series using initial ROI values as a baseline for each further image in the series: $\Delta I/I = (ROI_i - ROI_{\text{control}})/ROI_{\text{control}}$.

Data analysis

For each stimulus, the maximal fEPSP amplitude and slope were obtained and reported as percentage changes from baseline (average of the first 5 responses obtained). Data were analyzed by one-way ANOVA with Bonferroni's post hoc test (GraphPad Prism; version 4.0) to assess statistically significant deviation from control (10 mM glucose) experiments, or by one-way ANOVA with repeated measures, and Bonferroni post hoc test (GraphPad Prism; version 4.0) to assess slope potentiation within the control group. Data are shown as mean ± standard error of mean, with the number of independent slices (n) given.

Acknowledgments

We thank Dr. Kelley Foster for her help and effort with the experimental preparation and advice on imaging. This research was supported by NIH grants R21 NS45304 and RO1 NS051856, and a VAMC Merit Review Award.

This research was supported by NIH grants R21 NS45304 and RO1 NS051856, and a VAMC Merit Review Award.

References

1. Abi-Saab WM, Maggs DG, Jones T, Jacob R, Srihari V, Thompson J, Kerr D, Leone P, Krystal JH, Spencer DD, During MJ, Sherwin RS. Striking differences in glucose and lactate levels between brain extracellular fluid and plasma in conscious human subjects: effects of hyperglycemia and hypoglycemia. *J Cereb Blood Flow Metab* 2002;22:271–279. [PubMed: 11891432]
2. Auer RN. Hypoglycemic brain damage. *Metab Brain Dis* 2004;19:169–175. [PubMed: 15554413]
3. Blackman JD, Towle VL, Lewis GF, Spire JP, Polonsky KS. Hypoglycemic thresholds for cognitive dysfunction in humans. *Diabetes* 1990;39:828–835. [PubMed: 2191886]
4. Boyle PJ. Alteration in brain glucose metabolism induced by hypoglycaemia in man. *Diabetologia* 1997;40:S69–S74. [PubMed: 9248704]
5. Brennan AM, Connor JA, Shuttleworth CW. NAD(P)H fluorescence transients after synaptic activity in brain slices: predominant role of mitochondrial function. *J Cereb Blood Flow Metab*. 2006
6. Buldakova S, Dutova E, Ivlev S, Weiss M. Temperature change-induced potentiation: a comparative study of facilitatory mechanisms in aged and young rat hippocampal slices. *Neuroscience* 1995;68:395–397. [PubMed: 7477949]
7. Calabresi P, Centonze D, Pisani A, Bernardi G. Endogenous adenosine mediates the presynaptic inhibition induced by aglycemia at corticostriatal synapses. *J Neurosci* 1997;17:4509–4516. [PubMed: 9169511]
8. Cooke SF, Bliss TV. Plasticity in the human central nervous system. *Brain* 2006;129:1659–1673. [PubMed: 16672292]
9. Cryer PE. Hypoglycaemia: the limiting factor in the glycaemic management of Type I and Type II diabetes. *Diabetologia* 2002;45:937–948. [PubMed: 12136392]
10. Fan P, O'Regan PA, Szerb JC. Effect of low glucose concentration on synaptic transmission in the rat hippocampal slice. *Brain Res Bull* 1988;21:741–747. [PubMed: 2851374]
11. Fiala JC, Kirov SA, Feinberg MD, Petrak LJ, George P, Goddard CA, Harris KM. Timing of neuronal and glial ultrastructure disruption during brain slice preparation and recovery in vitro. *J Comp Neurol* 2003;465:90–103. [PubMed: 12926018]
12. Foster KA, Beaver CJ, Turner DA. Interaction between tissue oxygen tension and NADH imaging during synaptic stimulation and hypoxia in rat hippocampal slices. *Neuroscience* 2005;132:645–657. [PubMed: 15837126]
13. Foster KA, Galeffi F, Gerich FJ, Turner DA, Muller M. Optical and pharmacological tools to investigate the role of mitochondria during oxidative stress and neurodegeneration. *Prog Neurobiol* 2006;79:136–171. [PubMed: 16920246]
14. Foster KA, Margraf RR, Turner DA. NADH hyperoxidation correlates with enhanced susceptibility of aged rats to hypoxia. *Neurobiol Aging*. 2006
15. Fowler JC. Glucose deprivation results in a lactate preventable increase in adenosine and depression of synaptic transmission in rat hippocampal slices. *J Neurochem* 1993;60:572–576. [PubMed: 8380436]
16. Garofalo O, Cox DW, Bachelard HS. Brain levels of NADH and NAD⁺ under hypoxic and hypoglycaemic conditions in vitro. *J Neurochem* 1988;51:172–176. [PubMed: 3379400]
17. Gruetter R, Novotny EJ, Boulware SD, Rothman DL, Mason GF, Shulman GI, Shulman RG, Tamborlane WV. Direct measurement of brain glucose concentrations in humans by ¹³C NMR spectroscopy. *Proc Natl Acad Sci U S A* 1992;89:1109–1112. [PubMed: 1736294]
18. Gruetter R, Ugurbil K, Seaquist ER. Steady-state cerebral glucose concentrations and transport in the human brain. *J Neurochem* 1998;70:397–408. [PubMed: 9422387]
19. Huang YY, Pittenger C, Kandel ER. A form of long-lasting, learning-related synaptic plasticity in the hippocampus induced by heterosynaptic low-frequency pairing. *Proc Natl Acad Sci U S A* 2004;101:859–864. [PubMed: 14711997]
20. Izumi Y, Benz AM, Katsuki H, Zorumski CF. Endogenous monocarboxylates sustain hippocampal synaptic function and morphological integrity during energy deprivation. *J Neurosci* 1997;17:9448–9457. [PubMed: 9391000]
21. Izumi Y, Zorumski CF. Involvement of nitric oxide in low glucose-mediated inhibition of hippocampal long-term potentiation. *Synapse* 1997;25:258–262. [PubMed: 9068123]

22. Jones TW, McCarthy G, Tamborlane WV, Caprio S, Roessler E, Kraemer D, Starick-Zych K, Allison T, Boulware SD, Sherwin RS. Mild hypoglycemia and impairment of brain stem and cortical evoked potentials in healthy subjects. *Diabetes* 1990;39:1550–1555. [PubMed: 2245879]
23. Kamal A, Biessels GJ, Urban IJ, Gispen WH. Hippocampal synaptic plasticity in streptozotocin-diabetic rats: impairment of long-term potentiation and facilitation of long-term depression. *Neuroscience* 1999;90:737–745. [PubMed: 10218775]
24. Kamal A, Spoelstra K, Biessels GJ, Urban IJ, Gispen WH. Effects of changes in glucose concentration on synaptic plasticity in hippocampal slices. *Brain Res* 1999;824:238–242. [PubMed: 10196454]
25. Kasischke KA, Vishwasrao HD, Fisher PJ, Zipfel WR, Webb WW. Neural activity triggers neuronal oxidative metabolism followed by astrocytic glycolysis. *Science* 2004;305:99–103. [PubMed: 15232110]
26. Lynch G, Rex CS, Gall CM. LTP consolidation: substrates, explanatory power, and functional significance. *Neuropharmacology* 2007;52:12–23. [PubMed: 16949110]
27. Magnoni S, Ghisoni L, Locatelli M, Caimi M, Colombo A, Valeriani V, Stocchetti N. Lack of improvement in cerebral metabolism after hyperoxia in severe head injury: a microdialysis study. *J Neurosurg* 2003;98:952–958. [PubMed: 12744353]
28. Martin SJ, Grimwood PD, Morris RG. Synaptic plasticity and memory: an evaluation of the hypothesis. *Annu Rev Neurosci* 2000;23:649–711. [PubMed: 10845078]
29. McCall AL. Cerebral glucose metabolism in diabetes mellitus. *Eur J Pharmacol* 2004;490:147–158. [PubMed: 15094081]
30. McNaughton BL. The mechanism of expression of long-term enhancement of hippocampal synapses: current issues and theoretical implications. *Annu Rev Physiol* 1993;55:375–396. [PubMed: 8466180]
31. Miyamoto E. Molecular mechanism of neuronal plasticity: induction and maintenance of long-term potentiation in the hippocampus. *J Pharmacol Sci* 2006;100:433–442. [PubMed: 16799259]
32. Pike FG, Meredith RM, Olding AW, Paulsen O. Rapid report: postsynaptic bursting is essential for 'Hebbian' induction of associative long-term potentiation at excitatory synapses in rat hippocampus. *J Physiol* 1999;518(Pt 2):571–576. [PubMed: 10381601]
33. Raymond CR. LTP forms 1, 2 and 3: different mechanisms for the "long" in long-term potentiation. *Trends Neurosci* 2007;30:167–175. [PubMed: 17292975]
34. Reymann KG, Frey JU. The late maintenance of hippocampal LTP: requirements, phases, 'synaptic tagging', 'late-associativity' and implications. *Neuropharmacology* 2007;52:24–40. [PubMed: 16919684]
35. Rosenthal JM, Amiel SA, Yaguez L, Bullmore E, Hopkins D, Evans M, Pernet A, Reid H, Giampietro V, Andrew CM, Suckling J, Simmons A, Williams SC. The effect of acute hypoglycemia on brain function and activation: a functional magnetic resonance imaging study. *Diabetes* 2001;50:1618–1626. [PubMed: 11423484]
36. Sakurai T, Yang B, Takata T, Yokono K. Synaptic adaptation to repeated hypoglycemia depends on the utilization of monocarboxylates in Guinea pig hippocampal slices. *Diabetes* 2002;51:430–438. [PubMed: 11812751]
37. Sanna PP, Cammalleri M, Berton F, Simpson C, Lutfjens R, Bloom FE, Francesconi W. Phosphatidylinositol 3-kinase is required for the expression but not for the induction or the maintenance of long-term potentiation in the hippocampal CA1 region. *J Neurosci* 2002;22:3359–3365. [PubMed: 11978812]
38. Shimizu E, Tang YP, Rampon C, Tsien JZ. NMDA receptor-dependent synaptic reinforcement as a crucial process for memory consolidation. *Science* 2000;290:1170–1174. [PubMed: 11073458]
39. Shuttleworth CW, Brennan AM, Connor JA. NAD(P)H fluorescence imaging of postsynaptic neuronal activation in murine hippocampal slices. *J Neurosci* 2003;23:3196–3208. [PubMed: 12716927]
40. Stevens CF. A million dollar question: does LTP = memory? *Neuron* 1998;20:1–2. [PubMed: 9459434]
41. Takata T, Sakurai T, Yang B, Yokono K, Okada Y. Effect of lactate on the synaptic potential, energy metabolism, calcium homeostasis and extracellular glutamate concentration in the dentate gyrus of the hippocampus from guinea-pig. *Neuroscience* 2001;104:371–378. [PubMed: 11377841]

42. Tang YP, Shimizu E, Dube GR, Rampon C, Kerchner GA, Zhuo M, Liu G, Tsien JZ. Genetic enhancement of learning and memory in mice. *Nature* 1999;401:63–69. [PubMed: 10485705]
43. Tekkok SB, Godfraind JM, Krnjevic K. Moderate hypoglycemia aggravates effects of hypoxia in hippocampal slices from diabetic rats. *Neuroscience* 2002;113:11–21. [PubMed: 12123680]
44. Thomas MJ, Watabe AM, Moody TD, Makhinson M, O'Dell TJ. Postsynaptic complex spike bursting enables the induction of LTP by theta frequency synaptic stimulation. *J Neurosci* 1998;18:7118–7126. [PubMed: 9736635]
45. Wada H, Okada Y, Uzuo T, Nakamura H. The effects of glucose, mannose, fructose and lactate on the preservation of neural activity in the hippocampal slices from the guinea pig. *Brain Res* 1998;788:144–150. [PubMed: 9554988]
46. Warren RE, Zammitt NN, Deary IJ, Frier BM. The effects of acute hypoglycaemia on memory acquisition and recall and prospective memory in type 1 diabetes. *Diabetologia* 2007;50:178–185. [PubMed: 17143604]
47. Yamada KA, Rensing N, Izumi Y, De Erausquin GA, Gazit V, Dorsey DA, Herrera DG. Repetitive hypoglycemia in young rats impairs hippocampal long-term potentiation. *Pediatr Res* 2004;55:372–379. [PubMed: 14681492]
48. Zhu PJ, Krnjevic K. Adenosine release is a major cause of failure of synaptic transmission during hypoglycaemia in rat hippocampal slices. *Neurosci Lett* 1993;155:128–131. [PubMed: 8397349]

Abbreviations

fEPSP, field excitatory post-synaptic potential; NADH, nicotinamide adenine dinucleotide; LTP, long-term potentiation; ACSF, artificial cerebrospinal fluid; SD, spreading depression; CNS, central nervous system.

Fig 1.

Time line showing electrical stimulation protocols, and ACSF alterations applied during experiments. Two types of stimulation protocol were used, low frequency stimulus trains (Train; 250 pulses at 10 Hz) and high frequency LTP inducing trains (LTP; 200 pulses at 100 Hz, repeated after 60 s). Where glucose concentration is reduced NaCl concentrations were altered to maintain osmotic balance. **A)** Slice competence during and after hypoglycemia; **B)** Slice competence for LTP induction and metabolic activation during and after hypoglycemia; and **C)** slice competence for LTP induction at control and critical (2.5 mM glucose) level of hypoglycemia.

Fig 2.

Changes induced in fEPSP amplitude during glucose restriction and subsequent 10 mM recovery. Bars represent % changes in EPSP amplitude from an initial 100% baseline (average from 0 – 2.5 min in 10mM glucose ACSF), during perfusion (25 and 60 min) in altered glucose ACSF: 10 mM (black bars, n = 5), 5 mM (dark gray, n = 4), 2.5 mM (gray, n = 5), 1.25 mM (light gray, n = 5) or 0 mM (white, n = 4), and after 25 min recovery in 10 mM glucose ACSF. Data are mean $\pm$ SEM, analyzed by one-way ANOVA with Bonferroni's post hoc test, ** P < 0.01, and *** P < 0.001 relative to 10 mM glucose at corresponding times.

Fig 3.

Averaged traces of baseline drift corrected fEPSP slope in control (10mM glucose) conditions. A single LTP induction protocol (200 pulses at 100 Hz, repeated after 60 s) after 112.5 mins (**dotted arrow**) induces LTP (**dotted line, n = 5**). Two LTP induction protocols separated by 60 minutes (**solid arrows**) demonstrate LTP induction is maximal (**solid line, n = 8**). Data are mean $\pm$ SEM, with significant changes determined by One-way ANOVA with repeated measures and Bonferroni post-hoc test, * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Fig 4.

Averaged traces of fEPSP slope (*left*), with typical physiological traces (*right*). The average traces show responses to a high frequency LTP induction protocol (200 pulses at 100 Hz, repeated after 60 s) applied during altered glucose, and repeated during 10 mM glucose recovery (**arrows**). During altered glucose (between dotted lines) ACSF concentrations were: **A)** 10 mM, **n** = 8, **B)** 5 mM, **n** = 6, **C)** 2.5 mM, **n** = 5, **D)** 1.25 mM, **n** = 6 or **E)** 0 mM, **n** = 3. Data are mean $\pm$ SEM, analyzed by one-way ANOVA with Bonferroni's post hoc test, * P < 0.05, and *** P < 0.001 relative to 10 mM glucose at corresponding times. The typical physiological traces shown were obtained: before glucose alteration (**a** – solid line), 30 mins

after first LTP induction protocol (**b – dashed line**), and 30 mins after second LTP induction (**c – dotted line**).

Fig 5.

Averaged traces showing changes (%) in NADH fluorescence induced by altered ACSF glucose concentration and subsequent recovery in 10mM glucose. *Insets*; show changes in the magnitude of biphasic responses to reversible stimulus trains (10 Hz for 25 s) applied before (*left trace*), during (*center trace*), and after (*right trace*) glucose restriction. During altered glucose (between dotted lines) ACSF concentrations were: **A)** 10 mM, n = 8, **B)** 5 mM, n = 6, **C)** 2.5 mM, n = 5, **D)** 1.25 mM, n = 6 or **E)** 0 mM, n = 3. Data are mean $\pm$ SEM, analyzed by one-way ANOVA with Bonferroni's post hoc test, ** P < 0.01, and *** P < 0.001 relative to 10 mM glucose at corresponding times.

The biphasic NADH fluorescence response to a stimulus train (250 pulses at 10 Hz) under different glucose concentration conditions
Maximum oxidative (phase 1; Fig 5) and reduction (phase 2; Fig 5) changes in the biphasic NADH fluorescence response to a stimulus train (250 pulses at 10 Hz) under different glucose concentration conditions. The stimulus train was repeated 3 times, before, during and after altered glucose ACSF. Data are mean SEM, analyzed by one-way ANOVA with Bonferroni's post hoc test, * P <0.05, ** P <0.01, *** P <0.001 relative to 10 mM glucose at corresponding times. Numbers of slices are given in parentheses.

Phase	Stimulus	[Glucose] during hypoglycemia				
		10mM (8)	5mM (6)	2.5mM (5)	1.25mM (6)	0mM (3)
1 Oxidation	A (pre)	-1.60 ± 0.21	-1.57 ± 0.42	-1.92 ± 1.02	-1.17 ± 0.19	-1.98 ± 0.74
	B (during)	-1.95 ± 0.27	-1.55 ± 0.32	-1.74 ± 0.94	-0.77 ± 0.17	-0.71 ± 0.12
	C (post)	-1.83 ± 0.41	-2.03 ± 0.33	-1.53 ± 0.44	-0.64 ± 0.35	-0.24 ± 0.13
2 Reduction	A (pre)	3.66 ± 0.37	3.44 ± 0.69	2.98 ± 0.51	3.22 ± 0.31	2.54 ± 0.87
	B (during)	3.90 ± 0.38	4.33 ± 0.80	1.77 ± 0.50 *	1.10 ± 0.67 **	0.13 ± 0.16 ***
	C (post)	4.10 ± 0.67	3.43 ± 0.82	2.30 ± 0.55	2.14 ± 0.57	0.32 ± 0.21 **