β-Hydroxybutyrate Fuels Synaptic Function during Development

Histological and Physiological Evidence in Rat Hippocampal Slices

Yukitoshi Izumi,* Kazumoto Ishii,§ Hiroshi Katsuki,* Ann M. Benz,* and Charles F. Zorumski*‡

**Department of Psychiatry and ‡Department of Neurobiology, Washington University School of Medicine, St. Louis, Missouri 63110; and §Department of Pediatrics, Jonan Hospital, Mito City, Japan*

Abstract

To determine whether ketone bodies sustain neuronal function as energy substrates, we examined the effects of β-hydroxybutyrate (βHB) on synaptic transmission and morphological integrity during glucose deprivation in rat hippocampal slices. After the depression of excitatory postsynaptic potentials (EPSPs) by 60 min of glucose deprivation, administration of 0.5–10 mM D-βHB restored EPSPs in slices from postnatal day (PND) 15 rats but not in slices from PND 30 or 120 rats. At PND 15, adding D-βHB to the media allowed robust long-term potentiation of EPSPs triggered by high frequency stimulation, and prevented the EPSP-spike facilitation that suggests hyperexcitability of neurons. Even after PND 15, D-βHB blocked morphological changes produced by either glucose deprivation or glycolytic inhibition. These results indicate that D-βHB is not only able to substitute for glucose as an energy substrate but is also able to preserve neuronal integrity and stability, particularly during early development. (*J. Clin. Invest.* 1998. 101:1121–1132.) Key words: suckling • weaning • ketone bodies • synaptic plasticity • hypoglycemia

Introduction

Because very little glycogen is stored in the brain, the high energy requirements of the central nervous system must be met by external sources of glucose. Thus, the brain is highly vulnerable to hypoglycemia and may suffer irreversible damage after hypoglycemic events (1). However, the favorable prognosis of primary ketotic hypoglycemia (2) suggests that ketone bodies function as alternative energy substrates in the brain during hypoglycemia. The brain is particularly at risk when hypoglycemia is associated with hypoketonemia (3, 4). Because of their high permeability across the blood-brain barrier (BBB)[1] (5, 6), ketone bodies may be an essential link between fatty acid stores in the body and energy production in the brain.

Although several studies have demonstrated the utilization of ketone bodies in the brain (7–9), there is no direct evidence that ketone bodies can sustain neuronal function or preserve histological integrity in the absence of glucose. In hippocampal slices from adult guinea pigs, a prior study found that D-β-hydroxybutyrate (D-βHB), a major ketone body in the circulation (10–13), preserved high energy phosphate levels but failed to maintain synaptic function (14). This observation suggests that D-βHB itself does not work as an energy substrate to sustain brain function. Because D-βHB provides only respiratory energy whereas glucose supplies both glycolytic and respiratory energy, it could be hypothesized that energy substrates providing only respiratory energy are not sufficient to sustain synaptic function. However, lactate and pyruvate, other monocarboxylates that provide only respiratory energy, sustain synaptic function in hippocampal slices (15, 16). Therefore, the failure of D-βHB to preserve synaptic function must be explained by a different mechanism.

An alternative possibility concerns developmental changes in D-βHB utilization. In brain slices from neonatal rats, ketone bodies are more efficient sources of acetyl-CoA than glucose (17). The rate of D-βHB incorporation into rat brain is high during the suckling period but rapidly decreases after weaning (9, 18). The substantial energy production from ketone bodies, accounting for at least 30% of the total energy balance at early developmental periods (18), is consistent with the nutritional supply of the suckling period. Maternal milk, the primary food source during the suckling period, contains as much as 12–15% fat but only 3% carbohydrates (19, 20). Based on these observations, it appears likely that the ability of ketone bodies to serve as an energy source is developmentally regulated. To test this, we assessed the effects of D-βHB on the preservation of neuronal integrity in hippocampal slices prepared from nonprecocial animals at several developmental stages. We chose the hippocampus for these studies because it is a brain region that is extremely sensitive to hypoglycemia.

Methods

Determination of glucose and D-βHB. Albino male rats (postnatal day [PND] 15±1, PND 30±1, PND 120±2), which were not food restricted, were anesthetized with halothane. In some animals, cerebrospinal fluid (CSF) from the cisterna magna and cardiac arterial blood were sampled before slice preparation to analyze glucose and D-βHB. Glucose concentrations from CSF and whole blood were determined immediately with an Advantage Monitor (Boehringer Mannheim, Indianapolis, IN) after standardization. Serum and CSF were frozen at −20°C for weeks until they were used for D-βHB determination with the 3-hydroxybutyrate kit from Sigma Chemical Co. (St. Louis, MO). Briefly, a 25-μl sample and a 750-μl reagent containing 4.6 mM nicotinamide adenine dinucleotide were mixed with 375 μg/750 μl D-βHB dehydrogenase for 20 min at 37°C and NADH was quantified with a spectrometer at 340 nm.

Address correspondence to Yukitoshi Izumi, M.D., Ph.D., Department of Psychiatry, Washington University School of Medicine, 4940 Children's Place, St. Louis, MO 63110. Phone: 314-362-8659; FAX: 314-362-8649; E-mail: izumiy@psychiatry.wustl.edu

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1. *Abbreviations used in this paper:* aCSF, artificial cerebrospinal fluid; BBB, blood-brain barrier; CSF, cerebrospinal fluid; EPSP, excitatory postsynaptic potentials; βHB, β-hydroxybutyrate; HFS, high frequency stimulation; IA, iodoacetate; LTP, long-term potentiation; PND, postnatal day; PS, population spike.

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Table I. Levels of Glucose in Blood and CSF during Development

	Blood	CSF	Ratio	*n*
	mM	*mM*	%	
PND 15±1	9.0±1.3	3.3±0.3	39±4	5
PND 30±1	7.8±0.6	4.7±0.3*	61±7*	5
PND 120±2	8.2±0.9	5.2±1.1‡	63±5*	4

‡$P < 0.05$, *$P < 0.01$ by *t* test compared to PND 15.

Table II. Levels of D-βHB in Plasma and CSF during Development

	Plasma	CSF	*n*
	mM	*mM*	
PND 15±1	1.12±0.06	1.01±0.13	9
PND 30±1	0.36±0.07*	0.01±0.01*	6
PND 120±2	0.41±0.05*	0.03±0.02*	4

*$P < 0.01$ by U test compared to PND 15.

Slice preparation. Slices were prepared from the septal half of the hippocampus using standard techniques (21). The hippocampi were rapidly dissected at 4–6°C and cut into 500-μm-thick slices using a WPI vibroslicer. Slices were then kept in artificial CSF (aCSF) containing (mM): 124 NaCl, 5 KCl, 2 $MgSO_4$, 2 $CaCl_2$, 1.25 NaH_2PO_4, 22 $NaHCO_3$, 10 glucose, bubbled with 95% O_2/5% CO_2 in an incubation chamber for at least 60 min at 30°C. In certain experiments designed to test the need for continuous exposure to D-βHB, slices were incubated in aCSF with 1 mM D-βHB in addition to 10 mM glucose starting immediately after dissection.

Slice physiology. For physiological experiments, slices were transferred to a submerged recording chamber where they were continuously perfused with aCSF (2 ml/min) at 30°C. Extracellular recordings were obtained from the dendritic region of CA1 using 5–10 MΩ glass electrodes filled with 2 M NaCl. During an experiment, the Schaffer collateral-commissural fibers were stimulated once per minute in stratum radiatum with bipolar electrodes and 0.1–0.2 ms constant current pulses at an intensity sufficient to evoke 60–70% maximal excitatory postsynaptic potentials (EPSPs) based on the baseline stimulus–response curve. Long-term potentiation (LTP) was induced using a high frequency stimulus that consisted of 100 pulses at 100 Hz using the same stimulus intensity.

Slice histology. For histological experiments, slices from the same animal were incubated in parallel in individual 10-ml beakers. Each hippocampus provided six to eight slices. At completion of an experiment, slices were fixed in 1% paraformaldehyde and 1.5% glutaraldehyde overnight at 4°C. Slices were then rinsed in 0.1 M pyrophosphate buffer, placed in 1% buffered osmium tetroxide for 60 min, and dehydrated with ethanol and toluene. The fixed slices were embedded in araldite, cut into 1-μm sections, stained with methylene blue and azure II, and evaluated by light microscopy. Damage in the CA1 region was rated on a 0 (completely intact) to 4 (severe morphological alteration) scale by a rater who was unaware of the experimental conditions. Using this system, control slices from PND 30 rats that were incubated for 120 min in the standard aCSF were rated as 0.2±0.1 (n = 34, mean±standard error). Slices treated with 100 μM *N*-methyl-D-aspartate for 20 min followed by 90 min of postincubation in standard aCSF exhibited damage scores of 3.7±0.1 (n = 34). Photomicrographs were developed on Kodak F3 black and white paper.

Chemicals and statistics. All chemicals were obtained from Sigma Chemical Co. Values of each age group were compared with Jandel Scientific Software (San Rafael, CA) Sigma Stat 2.0 by means of Mann-Whitney U test unless Student's *t* test was applicable. Data are expressed as mean±standard error.

Results

Concentrations of glucose and D-βHB in CSF. To our knowledge, there are no studies describing glucose and D-βHB levels in CSF from developing rats. To determine physiological levels of both substrates during development, we sampled CSF from the cisterna magna. Although blood glucose levels were similar among albino rats of different ages, glucose levels in CSF were significantly lower at PND 15 than at PND 30 and PND 120. At PND 15, substantial levels of D-βHB were detected in both blood and CSF, although there was considerable animal-to-animal variation. D-βHB levels at PND 30 and PND 120 were significantly lower than those at PND 15 (Tables I and II).

Electrophysiological assessment during glucose deprivation. To assess the functional effects of D-βHB in the absence

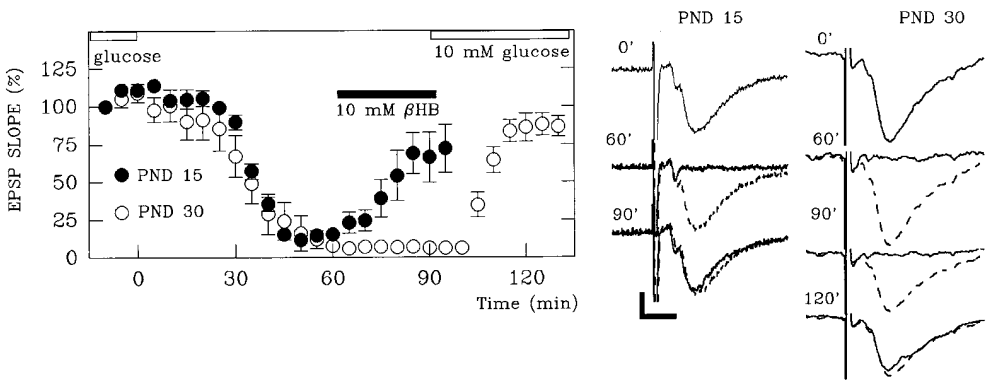

Figure 1. Functional effects of 10 mM D,L-βHB during glucose deprivation in slices from PND 15 and PND 30 rats. The graph shows the changes in EPSP slope in the CA1 region of hippocampal slices from PND 15 (*filled circles*) and PND 30 rats (*open circles*). Glucose (*left open bar*) was removed from the solution at time 0. EPSPs that were depressed by 60 min of glucose deprivation were restored by administration of 10 mM D,L-βHB (*filled bar*) in slices from PND 15 rats. Although EPSPs were not restored by D,L-βHB in slices from PND 30 rats, subsequent administration of 10 mM glucose (*right open bar*) restored the EPSPs. The numbers on each representative EPSP trace depict the time when the responses were sampled. Dotted traces are the control EPSPs obtained before glucose deprivation. Calibration bar: 1 mV, 5 ms.

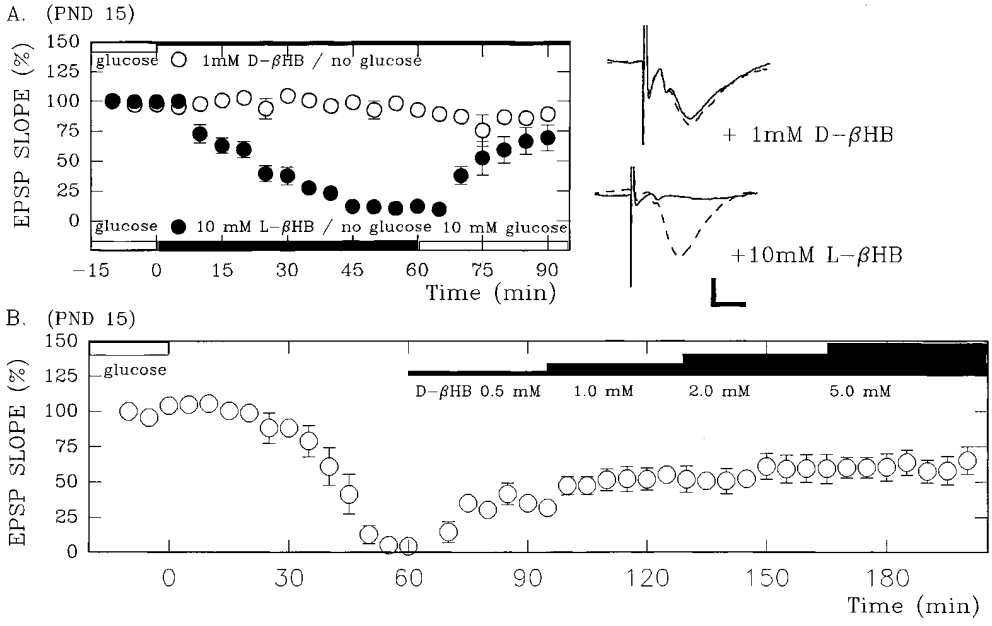

Figure 2. Substitution of glucose with stereoisomers of βHB in slices from PND 15 rats. (*A*). The graph shows the changes in EPSP slope in slices treated with 1 mM D-βHB in a glucose-free solution for 90 min (*open circles*) and changes in slices treated with 10 mM L-βHB in a glucose-free solution for 60 min (*filled circles*) followed by readministration of 10 mM glucose. At time 0, 10 mM glucose in the aCSF (*left open bar*) was switched to a stereoisomer of βHB (*filled bar*). EPSPs were maintained by 1 mM D-βHB (*open circles*) but not by 10 mM L-βHB (*filled circles*) in the absence of glucose. The traces show EPSPs obtained during the initial perfusion of glucose (*dotted traces*), 60 min after glucose depletion (*solid traces*) in the presence of 1 mM D-βHB (*upper*) and 10 mM L-βHB (*lower*). Calibration bar: 1 mV, 5 ms. (*B*) The graph shows the changes in EPSP slope in slices treated with 0.5, 1.0, 2.0, and 5.0 mM D-βHB successively beginning 60 min after glucose deprivation.

of glucose, field EPSPs were monitored in the CA1 region of hippocampal slices. In slices from PND 30 rats, we have shown previously that EPSPs that are depressed by 60 min of glucose deprivation are restored by the subsequent administration of 10 mM pyruvate, lactate, or glucose (16).

Consistent with a previous report using adult guinea pig hippocampal slices (14), administration of 10 mM D,L-βHB for 30 min after 60 min of glucose deprivation did not restore EPSPs in slices from PND 30 rats (Fig. 1, *open circles*, $n = 5$). The failure to restore EPSPs did not result from irreversible deterioration during the 60 min of glucose deprivation because EPSPs were restored by subsequent administration of 10 mM glucose for 30 min (EPSP slope compared with initial values: 88±4%, $n = 5$). In contrast, 10 mM D,L-βHB readily restored EPSPs after glucose depletion in slices from PND 15 rats (Fig. 1, *filled circles*, $n = 5$).

The effects of βHB in slices from PND 15 rats were stereospecific. Whereas EPSPs were suppressed within 60 min after replacement of glucose in aCSF with 10 mM L-βHB (Fig. 2 *A*, *filled circles*, $n = 5$), EPSPs were sustained by 1 mM D-βHB for 90 min in the absence of glucose (Fig. 2 *A*, *open circles*, $n = 5$). Similarly, EPSPs that were depressed during glucose deprivation were restored by D-βHB at concentrations as low as 0.5 mM (Fig. 2 *B*), suggesting that physiological levels of D-βHB are sufficient to sustain synaptic transmission in the absence of glucose during suckling periods. The ability to restore synaptic function was not observed when 10 mM L-βHB was administered 60 min after glucose deprivation ($n = 3$, data not shown).

The ability of D-βHB to restore synaptic transmission during glucose deprivation was age dependent. Administration of 10 mM D-βHB restored EPSPs only at PND 15, but not at PND 30 or PND 120 (Fig. 3, *filled circles*), while 3.3 mM glucose, a level found physiologically in CSF at PND 15 (Table I), restored EPSPs at all ages examined (Fig. 3, *open circles*).

Electrophysiological assessment during iodoacetate (IA) treatment. We next tested whether D-βHB is used as a functional energy substrate when glycolysis is blocked by IA, an inhibitor of glyceraldehyde-3-phosphate dehydrogenase. It has been shown that the suppression of synaptic activity by IA is prevented by addition of pyruvate or lactate to aCSF (15, 16).

At all ages tested (PND 15, 30, 120), 60 min of administration of 200 μM IA depressed EPSPs (Fig. 4, *A–C*, *open circles*). Because D-βHB did not support synaptic transmission in slices from PND 30 or 120 rats, 10 mM D-βHB was simply added to the aCSF containing 10 mM glucose, then 200 μM IA was administered. In slices from PND 15 rats, EPSPs supported by both glucose and D-βHB were temporarily depressed and then fully restored during IA administration (Fig. 4 *A*, *filled circles*). In slices from PND 120 rats, however, the presence of D-βHB did not alter the time course of IA-mediated synaptic depression, indicating that D-βHB does not substitute for glucose in the adult brain (Fig. 4 *C*, *filled circles*). Although D-βHB did not restore EPSPs during glucose deprivation at PND 30 (Fig. 1), the depression of EPSPs after IA administration was followed by a partial recovery in the presence of D-βHB (Fig. 4 *B*, *filled circles*). EPSPs were also restored partially after administration of IA and D-βHB in the absence of glucose at PND 30 (Fig. 4 *D*). Thus, the ability of D-βHB to restore EPSPs after IA administration was not dependent on the presence of glucose.

D-βHB attenuates EPSP-spike dissociation and allows more robust LTP in immature hippocampus. LTP, a lasting enhancement of synaptic efficacy induced by high frequency stimulation (HFS), is a useful model for understanding mechanisms of synaptic plasticity and possibly memory formation. In the CA1 hippocampal region, LTP primarily reflects enhanced synaptic transmission as measured by population EPSPs but may include increased neuronal excitability as measured by

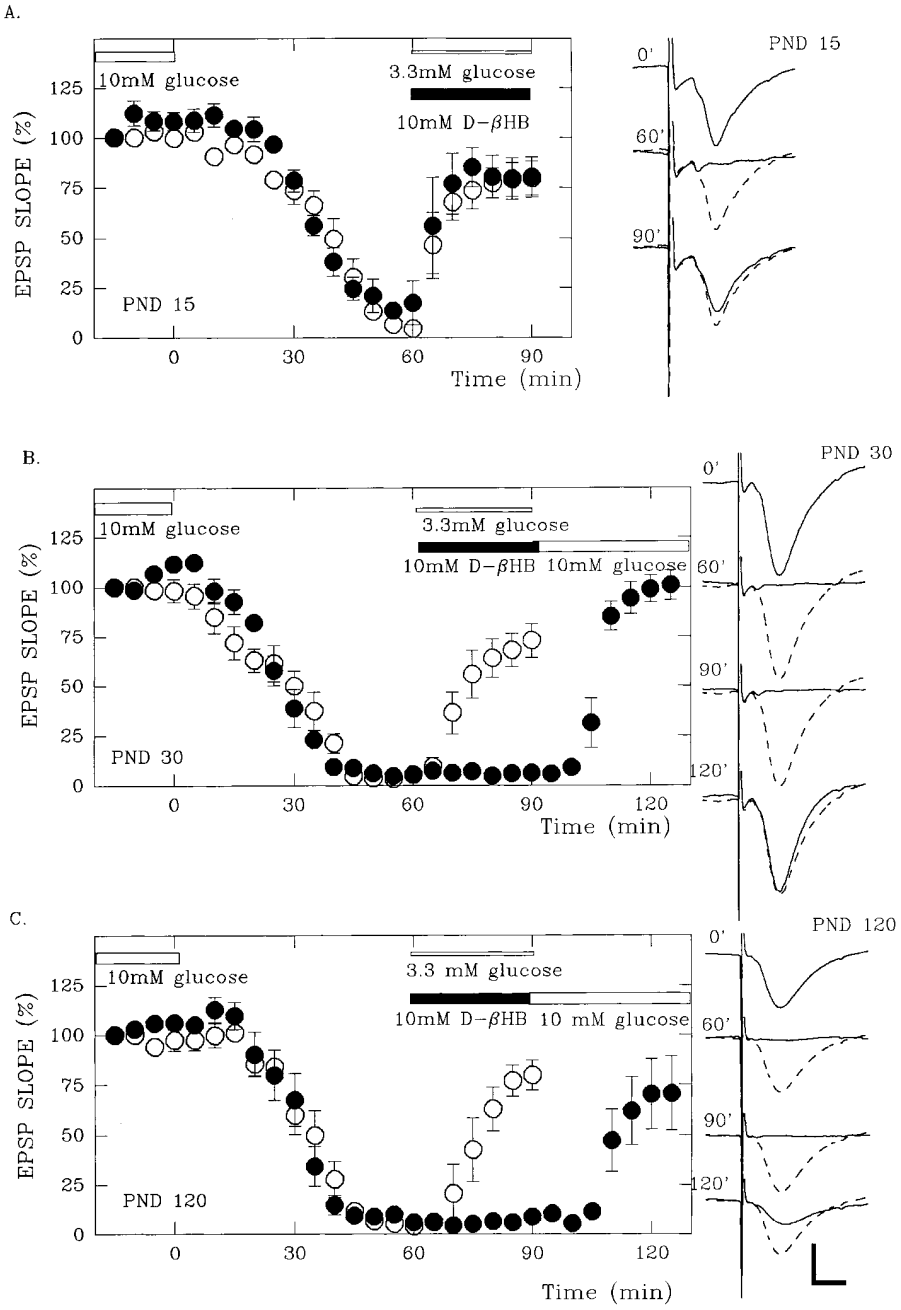

Figure 3. Functional effects of 10 mM D-βHB after glucose deprivation in slices from PND 15, 30, and 120 rats. Administration of 10 mM D-βHB (*right filled bar*) after 60 min of glucose deprivation restored EPSPs in hippocampal slices from PND 15 rats (*A*, *filled circles*), but did not restore EPSPs in slices from PND 30 and 120 rats (*filled circles*, *B* and *C*, respectively). Subsequent administration of 10 mM glucose restored EPSPs in slices from PND 30 and 120 rats. Administration of 3.3 mM glucose 60 min after glucose deprivation restored EPSPs at all ages examined (*A–C*, *open circles*). The traces are representative EPSPs recorded before glucose deprivation (0′), 60 min after glucose deprivation (60′), 30 min after administration of D-βHB (90′), and 30 min after successive administration of 10 mM glucose (120′). For comparison, the control EPSPs recorded before glucose deprivation are shown as dotted traces. Calibration bar: 1 mV, 5 ms.

population spikes (PS). Under most conditions, changes in PS can be explained by changes in EPSPs. However, there are conditions in which neuronal hyperexcitability occurs out of proportion to changes in synaptic input, a condition known as EPSP-spike dissociation (22–24). As reported previously (25), we found that the peak degree of LTP in PS occurs at PND 15 but there is significant EPSP-spike dissociation at this age (26). We have replicated this result and found that, in the presence of 10 mM glucose, the augmentation of PS in the CA1 hippocampal region measured 60 min after HFS cannot be explained simply by changes in synaptic input, reflecting EPSP-spike facilitation (Fig. 5). When 1 mM D-βHB was added to 10 mM glucose, the degree of enhancement of PS amplitude was accurately predicted by changes in EPSPs (Table III). Although the overall degree of increase in PS amplitude was similar to that observed in the presence of 10 mM glucose alone, the change in PS directly reflected potentiation of EPSPs (Fig. 5, *B* and *C*). This suggests that D-βHB facilitates the induction of synaptic (EPSP) LTP while preventing a disproportionate augmentation of neuronal excitability. However, LTP could not be induced in slices treated with 10 mM D-βHB in the absence of glucose (EPSP changes 60 min after tetanic stimulation; $-11.8\pm5.5\%$, $n = 4$), indicating a requirement for glucose as well.

As described above, glucose levels in the CSF of PND 15 rats are < 10 mM, a concentration that has been used typically in studies of LTP in vitro. In slices from PND 30 rats, we have shown that LTP can be induced in the presence of 5–10 mM

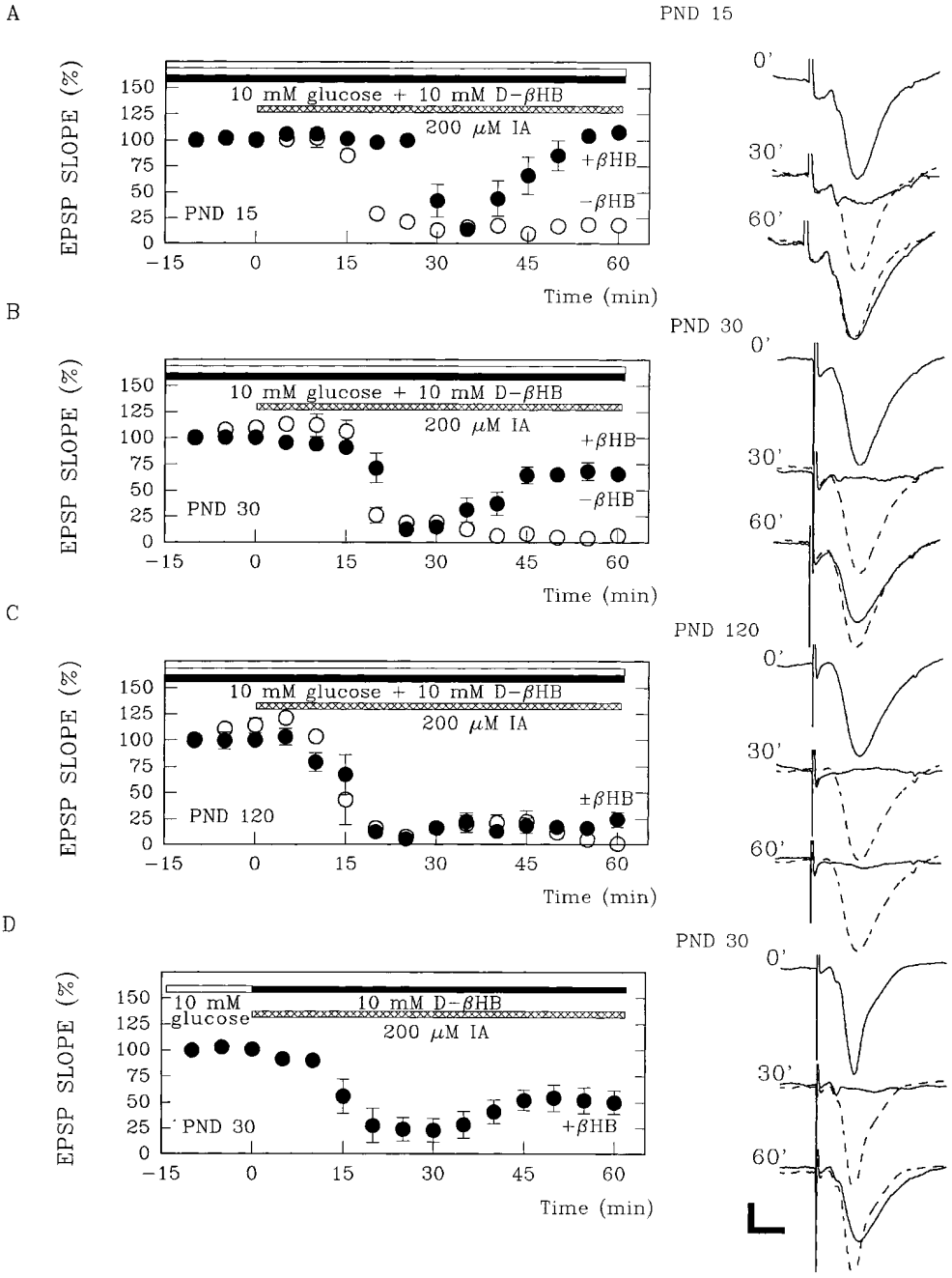

Figure 4. Effects of 10 mM D-βHB on synaptic depression by IA. The upper three graphs show the changes in EPSP slope in slices from PND 15 (*A*), 30 (*B*), and 120 (*C*) rats by administration of 200 μM IA for 60 min (*hatched bar*) in the presence of 10 mM glucose (*open bar*) with or without 10 mM D-βHB (*filled bar*). At all ages tested, IA suppressed EPSPs supported by 10 mM glucose alone (*open circles*). In the presence of D-βHB, the depression of EPSPs by IA was only temporary and was followed by a full recovery in slices from PND 15 rats (*A*, *filled circles*). A partial but substantial recovery was also observed at PND 30 (*B*, *filled circles*) but not at PND 120 (*C*, *filled circles*). The partial recovery produced by D-βHB at PND 30 was also observed in the absence of glucose (*D*). The traces to the right of each graph depict representative EPSPs obtained before (0′), 30 min after (30′), and 60 min after (60′) IA administration. Calibration bar: 1 mV, 5 ms.

glucose but not in the presence of 2–3.3 mM glucose (27). The failure of low glucose to support LTP also occurs in slices from PND 15 rats (Fig. 6, *open circles*). Although LTP could not be induced in the presence of 3.3 mM glucose alone, LTP was successfully generated in the presence of 1 mM D-βHB plus 3.3 mM glucose (Fig. 6 and Table III). This LTP was not accompanied by EPSP-spike dissociation. D-βHB (1 mM) failed to allow LTP in the presence of 3.3 mM glucose in slices from PND 30 rats (EPSP changes 60 min after tetanic stimulation; $+7.5\pm5.4\%$, $n = 7$).

Morphological assessment during glucose deprivation. We also evaluated the effects of D-βHB on the morphological integrity of slices during glucose deprivation. Glucose deprivation for 180 min produces histological damage to CA1 pyramidal neurons at PND 30 that is characterized by the appearance of dark cells with relative sparing of the dendritic fields (16). The dark cell appearance is accompanied by somatic shrinkage and results in a widening of intercellular spaces ($n = 10$, Fig. 7 *B*). In control slices incubated with 10 mM glucose for 240 min, neuronal nuclei took on a pale appearance, but dark cell changes were not observed ($n = 10$, Fig. 7 *A*). Similarly, in slices from PND 120 rats, 180 min of glucose deprivation produced dark cell changes, making it difficult to distinguish nuclei from cytoplasm ($n = 5$, Fig. 7 *E*). Such changes were not typically observed in control slices at PND 120 ($n = 8$, Fig. 7 *D*).

To examine whether D-βHB preserves neuronal morphology during glucose deprivation, slices were treated with 10 mM D-βHB in the absence of glucose for 180 min. In slices from

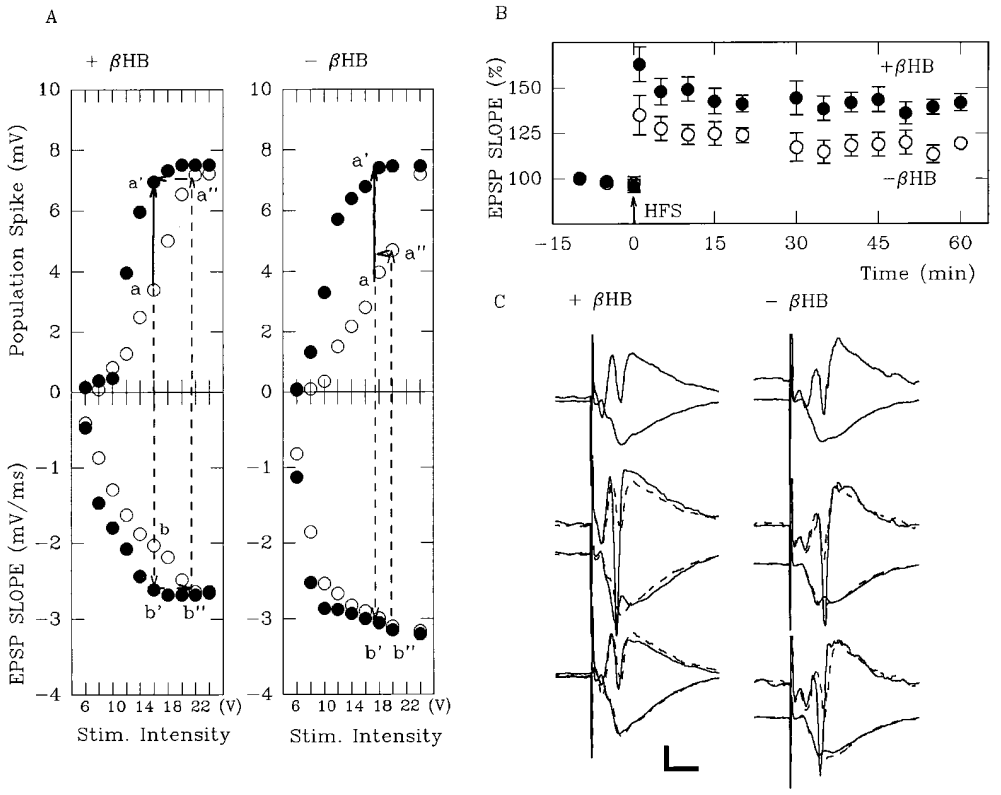

Figure 5. Suppression of EPSP-spike facilitation by D-βHB at PND 15. (*A*) The graphs show typical input–output curves for PS height (*top*) and dendritic EPSP slope (*bottom*) from a PND 15 slice in the presence of 10 mM glucose with 1 mM D-βHB (*left*) and without D-βHB (*right*). The curves were obtained 10 min before (*open circles*) and 60 min after (*filled circles*) the delivery of HFS. In the presence of D-βHB (*left*), the stimulus intensity needed to evoke a 50% maximal PS in the control period (*a*) produced ~70% maximal EPSP slope (*b*). Both values were observed to be augmented 60 min after HFS ($a \rightarrow a'$, $b \rightarrow b'$). Based on the input–output curves it would be necessary to increase the stimulus intensity to the value shown as b'' during the control period to elicit an EPSP of the same magnitude as b'. The stronger stimulus would evoke a PS (a'') of comparable size to that observed (a'). Under the assumption that the change in PS was entirely accounted for by the change in dendritic EPSP change ($b \rightarrow b'$), the expected PS amplitude after HFS was calculated from the input–output curve obtained before HFS ($b'' \rightarrow a''$). In Table III, the actual PS change is presented as $a'/a \times 100\%$, whereas the expected PS change is calculated as $a''/a \times 100\%$. In the slice supported by glucose alone (*right*), the calculated PS value (a''), based on corresponding changes in EPSP slope does not accurately reflect the observed change in PS after HFS (a'), whereas the prediction is accurate in the slice treated with 1 mM D-βHB. (*B*) The graph shows the time course of change in dendritic EPSP slope as a percentage of control (±standard error) produced by a single 100 Hz × 1 s HFS (*arrow*). Slices prepared from PND 15 rats were fueled by 10 mM glucose alone (*open circles*) or 10 mM glucose plus 1 mM D-βHB beginning immediately after dissection (*filled circles*). (*C*) Traces are representative EPSPs from a slice treated with 1 mM D-βHB (*left*) and from a slice untreated with D-βHB (*right*). The upper and middle traces depict EPSPs obtained before and 60 min after HFS, respectively. Dotted traces are the initial EPSPs obtained before HFS. Although PS augmentation was apparent in both slices, potentiation in EPSPs in the untreated slice is smaller. 60 min after HFS, the stimulus intensity was reduced so that EPSPs overlapped the initial EPSPs (*lower traces*). In the untreated slice, the PS amplitude is still facilitated (EPSP-spike facilitation at the lower stimulus intensity). Calibration bar: 1 mV, 5 ms.

PND 30 rats, 10 mM D-βHB protected slices from the dark cell appearance produced by glucose deprivation ($n = 5$, Fig. 7 *C*). In contrast, D-βHB was ineffective in slices from PND 120 rats ($n = 5$, Fig. 7 *F*).

In control slices from PND 15 rats, incubation with 10 mM glucose for 240 min revealed dark cell changes and widened intercellular spaces ($n = 12$, Fig. 8 *A*). These changes occurred over time during incubation and slices fixed immediately after dissection failed to exhibit dark cells ($n = 6$, Fig. 8 *B*). These observations suggest that control slices from PND 15 rats may suffer from energy deficiency despite the presence of 10 mM glucose. Because physiological studies indicated that D-βHB helps to maintain synaptic function in slices from PND 15 rats, we incubated slices with D-βHB plus glucose starting immediately after dissection. Slices treated with 10 mM glucose plus 1 mM D-βHB showed evidence of dark cell changes over 240 min of incubation ($n = 5$, Fig. 8 *C*). Thus, in subsequent experiments at PND 15, slices were preincubated with 1 mM D-βHB plus 10 mM glucose until the beginning of experiments. Deprivation of both glucose and D-βHB for 180 min produced dark cell appearances similar to that of the conventional control slices ($n = 5$, Fig. 8 *D*). The dark cell changes were not observed after glucose deprivation in the presence 10 mM D-βHB ($n = 5$, Fig. 8 *E*). Results of these experiments are summarized in Table IV.

Table III. LTP at PND 15 with/without 1 mM D-βHB

Changes (%)	10 mM glucose	+1 mM D-βHB	3.3 mM glucose	+1 mM D-βHB
EPSP slope	+18.8±4.7	+40.2±4.7*	+3.0±7.9	+26.8±3.3*
PS (expected)	+20.7±14.7	+84.5±16.0*	+5.4±4.4	+64.9±3.0*
PS (observed)	+95.0±23.0‡	+99.4±12.4	+32.6±25.3	+92.4±23.1

*$P < 0.05$ compared to glucose alone in each group by *t* test. ‡$P < 0.05$ compared to expected changes by paired *t* test. Five slices were examined in each group. Expected PS values were calculated using methods described in the legend for Fig. 5.

Morphological assessment during glycolytic inhibition. To confirm the neuroprotective actions of D-βHB during energy deprivation, we examined the effects of D-βHB on neuronal damage produced by IA. Administration of 200 μM IA for 90

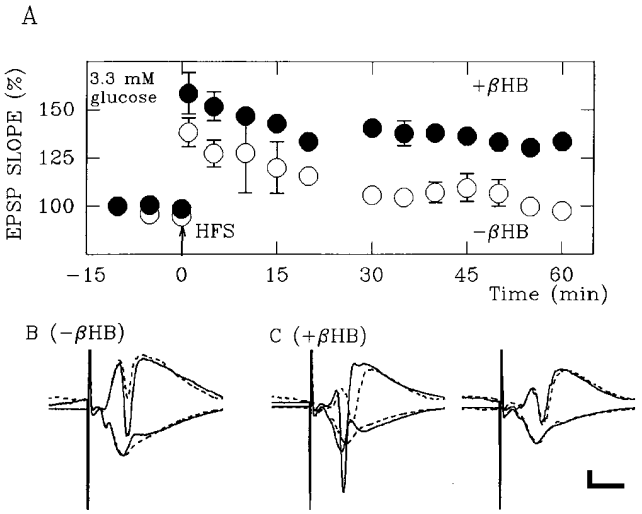

Figure 6. Effects of D-βHB on LTP inhibition by low glucose at PND 15. (*A*) The graph shows the time course of change in dendritic EPSP slope produced by a single 100 Hz × 1 s HFS (*arrow*) in the presence of 3.3 mM glucose in slices from PND 15 rats. LTP was not induced in the presence of 3.3 mM glucose alone (*open circles*) but was induced in the presence of 1 mM D-βHB plus 3.3 mM glucose (*filled circles*). (*B*) The traces depict representative EPSPs and PS recorded before (*dotted traces*) and 60 min after HFS (*solid traces*) in the presence of 3.3 mM glucose without D-βHB. Although there is no significant change in EPSP slope, the PS is facilitated. (*C*) The traces on the left depict EPSPs and PS recorded before (*dotted traces*) and 60 min after (*solid traces*) HFS in the presence of 3.3 mM glucose plus 1 mM D-βHB. The traces on the right are EPSPs and PS recorded with the stimulus intensity reduced so that the test EPSP overlaps the control EPSP (*lower dotted traces*). With the reduced stimulus, the PS height returns to the initial level (*upper dotted trace*). Calibration bar: 1 mV, 5 ms.

min caused severe damage in hippocampal slices at all ages examined (Fig. 9, *A–C*). The damage was characterized by swelling of neuronal cell bodies and dendritic processes (16). Although 10 mM D-βHB failed to preserve morphological integrity during glucose deprivation in slices from PND 120 rats, administration of 10 mM D-βHB prevented the neuronal deterioration by IA at all ages studied (Fig. 9, *D–F*, and Table V).

Taken together, these results suggest that although D-βHB substitutes for glucose in sustaining synaptic transmission only during suckling periods, its neuroprotective action during energy deprivation can be seen even after animals have been weaned. These results are summarized in Table VI.

Discussion

This study presents the first direct evidence that D-βHB can preserve neuronal functional and morphological integrity during the suckling period. Ketone bodies may be essential links between fatty acids in milk and energy production in the brain because the brain cannot use fatty acids directly as an energy source (6). The rate of incorporation of D-βHB into brain is greater than that of glucose at PND 14 but declines after weaning (PND 21–25), while glucose utilization increases continuously after birth (9). The developmental changes in D-βHB utilization may be determined by changes in circulating levels,

Table IV. Histological Damage Scores during Glucose Deprivation with/without 10 mM D-βHD

	Damage score (mean±SE)	*n*
PND 15		
Control	1.8±0.2	6
+ 1 mM D-βHB	0.3±0.3*	4
No glucose	2.5±0.2‡	6
No glucose/10 mM D-βHB	0.4±0.2§‖	5
PND 30		
Control	0.6±0.3	6
No glucose	2.7±0.3§	6
No glucose/10 mM D-βHB	0.3±0.2‖	6
PND 120		
Control	0.9±0.3	11
No glucose	2.6±0.2§	6
No glucose/10 mM D-βHB	2.1±0.2§	10

*$P < 0.05$, §$P < 0.01$ by U test compared to control in each group. ‖$P <$ 0.01 compared to no glucose in each group by U test. ‡$P < 0.01$ compared to 1 mM D-βHB by U test.

transport across the BBB, and metabolizing enzyme activities (28). Plasma levels of D-βHB are low at birth (29) but remain at high levels until weaning. Circulating levels of D-βHB are as high as 1–2 mM in PND 14–16 rats. These concentrations are two- to fourfold higher than those of acetoacetate and the levels of both D-βHB and acetoacetate decline after weaning (10–13). A similar pattern of changes in plasma D-βHB levels was observed in this study (Table II), although low levels of D-βHB were still detected in plasma even after weaning. The transport rate for D-βHB across the BBB increases during the suckling period then fades after weaning (30). Consistent with this, substantial levels of D-βHB were found in CSF from PND 15 rats and the average levels of D-βHB detected in CSF were high enough to restore synaptic transmission in hippocampal slices during glucose deprivation (Fig. 2). This indicates that during the suckling period D-βHB is an important substrate not only for synthesis of cerebral lipids and neurotransmitters (9, 31, 32) but also as an energy source to sustain neuronal function.

Table V. Histological Damage Scores during Glycolytic Inhibition by IA for 90 min

	Damage score (mean±SE)	*n*
PND 15		
IA	2.8±0.2	6
IA + D-βHB	1.4±0.2*	6
PND 30		
IA	4.0±0.0	5
IA + D-βHB	1.5±0.2‡	4
PND 120		
IA	3.5±0.2	4
IA + D-βHB	1.3±0.5‡	4

200 μM IA was administered with/without 10 mM D-βHB. ‡$P < 0.05$, *$P < 0.01$ by U test compared by IA alone in each group.

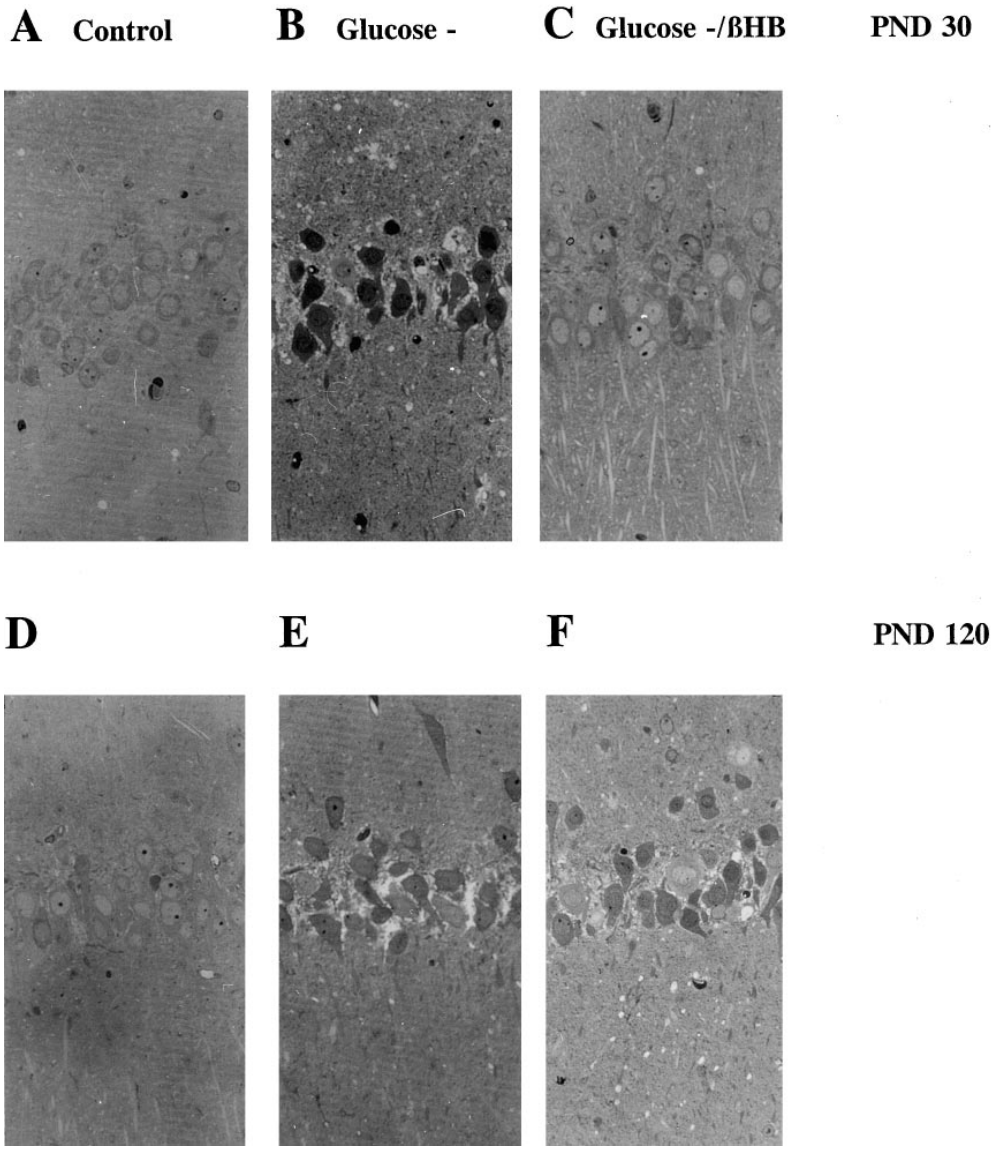

Figure 7. Morphological assessment during glucose deprivation at PND 30 and 120. Effects of 10 mM D-βHB on the dark cell appearance produced by glucose deprivation in slices from PND 30 rats (*upper panels*) and PND 120 rats (*lower panels*). The left (control) slices were incubated with a conventional aCSF containing 10 mM glucose for 240 min after dissection (*A* and *D*). The middle panels depict the effects of 180 min of glucose deprivation after 60 min of preincubation with 10 mM glucose, revealing dark cell appearance and widened intercellular spacing in the CA1 pyramidal cell layer (*B* and *E*). 10 mM D-βHB prevented the dark cell appearance by 180 min of glucose deprivation in slices from PND 30 rats (*C*) but not in slices from PND 120 rats (*F*). ×300.

In contrast to the high concentrations of D-βHB at PND 15, significant levels were not detected in CSF from PND 30 rats, suggesting that D-βHB does not serve as a physiological energy source after weaning. However, it has been reported that D-βHB levels in CSF reach 30% of plasma levels within 180 min after intravenous infusion of D-βHB (33), implying that the low transport rate across the BBB may not be a critical factor for D-βHB utilization if circulating levels are maintained. Indeed, the effects of D-βHB in restoring EPSPs in hippocampal slices were not observed after the suckling period, suggesting that in the mature brain, the failure to use D-βHB as an energy source is not simply explained by a decrease in circulating levels or a reduction in transport of D-βHB across the BBB. It appears likely that the transport rate across neuronal membranes is an important determinant for the use of D-βHB as a functional energy substrate, because the amount of D-βHB that accumulates in dissociated brain cells from PND 28 rats is only half of that in cells from PND 15 animals (34). Further studies should clarify the properties of the monocarboxylate transporters responsible for D-βHB transport into the brain and neurons. The activities of ketone body metabolizing enzymes, which increase during the suckling period, are also important determinants of D-βHB use. In contrast to the developmental changes in D-βHB levels, transport rate, and

Table VI. Functional and Morphological Effects of 10 mM D-βHB in Various Ages

	PND 15	PND 30	PND 120
Synaptic transmission without glucose	+	−	−
Synaptic transmission during glycolytic inhibition	+	+	−
Dark cell appearance without glucose	+	+	−
Neurodegeneration during glycolytic inhibition	+	+	+

+, effective to preserve neuronal integrity.

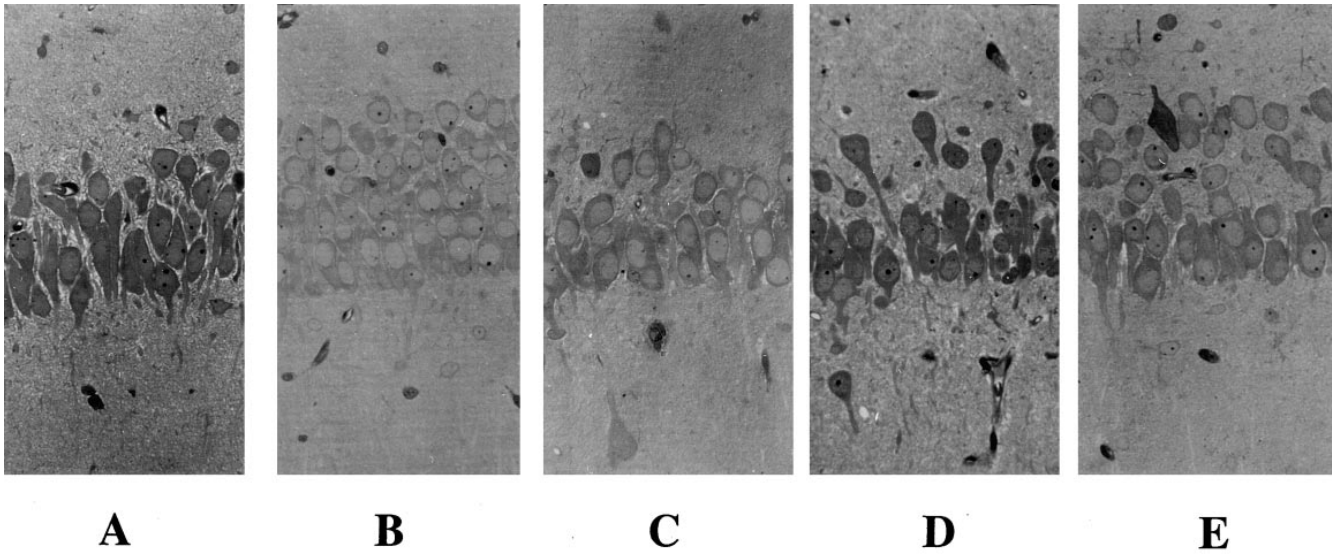

Figure 8. Morphological assessment during glucose and D-βHB deprivation at PND 15. The slice on the left was incubated with aCSF containing 10 mM glucose for 240 min after dissection and showed dark cell changes and increased intercellular spacing in the CA1 pyramidal cell layer (*A*). Such an appearance was not observed in a slice that was fixed immediately after dissection (*B*) or in a slice that was incubated with 1 mM D-βHB plus 10 mM glucose for 240 min starting immediately after dissection (*C*). Removal of both glucose and D-βHB for 180 min after 60 min of preincubation with both agents (*D*) produced dark cell appearances similar to that observed in the control slice (*A*). In a slice treated with 1 mM D-βHB in the absence of glucose for 180 min after 60 min of preincubation with both agents, dark cell changes were rare. In the slice shown, only a single dark cell is apparent (*E*). ×300.

utilization in the brain, the activities of ketone body metabolizing enzymes peak between PND 20 and PND 30, then decline gradually (12, 35, 36). The persisting activities of these enzymes suggest that D-βHB may be used to a lesser extent for a period of time after weaning. Indeed, D-βHB prevented morphological changes produced by glucose deprivation even in PND 30 slices, although D-βHB did not maintain synaptic transmission at this age. Although D-βHB did not overcome LTP inhibition by low glucose in slices from PND 30 rats, it has been shown that βHB administration to mice with insulin-induced hypoglycemia promotes recovery from stupor (37). Similarly, D-βHB ameliorates cognitive impairment in humans during insulin-induced hypoglycemia by increasing the threshold for neuroglycopenic symptoms (38), suggesting a role for D-βHB even after early development. The L isomer of βHB is not a substrate for ketone body metabolizing enzymes but may play some metabolic roles (28). In this study, however, L-βHB was not effective in sustaining synaptic responses even at PND 15, suggesting that the L isomer is unlikely to serve as an energy substrate.

In contrast to D-βHB utilization, glucose utilization in the brain remains low during the suckling period (9). Because of high circulating levels in the blood (11–13) (Table I), the low utilization of glucose during the suckling period cannot be explained by the composition of maternal milk. Glucose is transported into the brain through the GLUT1 transporter that is concentrated in endothelial cells of the BBB and then into neurons mainly via the specific transporter, GLUT3. Expression of GLUT1 in the BBB remains low until the mid-suckling period and does not attain adult levels until after PND 30 (39). Consistent with this, the glucose transport rate across the BBB is lower in suckling rats than in adults (40). As is often observed in human infants, we found that the CSF/blood ratio of glucose was significantly lower at PND 15 than in mature animals (Table I). Developmental changes in GLUT3 expression are even more robust. GLUT3 is very low in the immature rat hippocampus and reaches adult levels after PND 14 (41). A similar developmental regulation of GLUT3 is found in human cortex where the expression is two to three times higher in adults compared with neonates (42). The slow maturation of glucose transporters during development suggests that during the suckling period D-βHB may be an indispensable substitute for glucose.

In the presence of 10 mM glucose, the peak degree of PS LTP recorded in the CA1 pyramidal cell layer occurs at PND 15 (25). However, PS LTP can reflect increases in neuronal excitability in addition to potentiation of synaptic transmission. Previously, we reported that the peak of synaptic LTP, as measured by changes in dendritic EPSPs, occurs at a later age and that the remarkable facilitation in PS at PND 15 does not fully correspond to the degree of synaptic enhancement (26). This discrepancy, called EPSP-spike facilitation, reflects an increase in excitability of the neuronal soma. Although adding 1 mM D-βHB did not alter the degree of PS enhancement during LTP (Table III), the change in PS amplitude directly mirrored changes in EPSPs. The prevention of EPSP-spike facilitation by D-βHB could be involved in the anticonvulsant effects of ketogenic diets, because EPSP-spike facilitation is believed to be a cellular correlate of enhanced neuronal excitability (26). In mice, it has been shown that ketogenic diets increase the electroconvulsive threshold (43) and ketogenic diets have been used in the treatment of epilepsy and convulsive encephalopathies since the 1920s (44–48). Other disorders in which D-βHB may have therapeutic potential include Leigh syndrome in which there is a deficiency of the pyruvate dehydrogenase complex (48), as well as a syndrome due to glucose transport

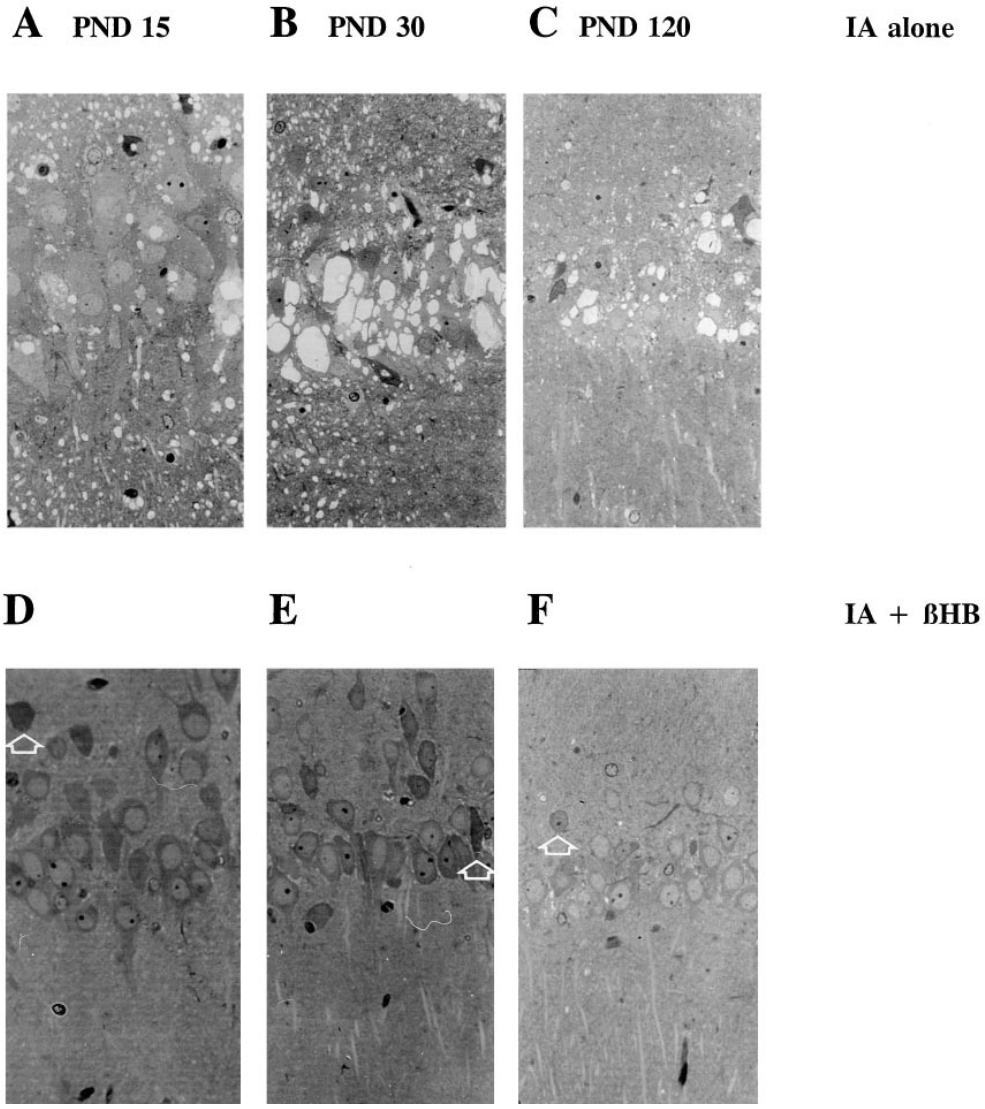

Figure 9. Morphological assessment during glycolytic inhibition. Effects of D-βHB on IA-mediated neuronal damage in slices from PND 15 rats (*left panels*), PND 30 rats (*middle panels*), and PND 120 rats (*right panels*). The upper panels depict the effects of 200 μM IA for 90 min (*A–C*). 10 mM D-βHB prevented the damage at each age examined (*D–F*), although some neurons exhibited dark cell changes (*arrows*). ×300.

protein deficiency (49). In both of these disorders there are defects in glucose metabolism in the brain. As is true of memory disorders occurring during hypoglycemia, LTP induction is influenced by extracellular levels of glucose. In hippocampal slices from PND 30 rats, LTP is readily induced in the presence of 5–10 mM glucose but not in the presence of 3.3 mM glucose (27). In this study, 3.3 mM glucose also failed to allow LTP in slices from PND 15 rats, even though 3.3 mM is near the physiological glucose level in CSF at this age. The observation that addition of 1 mM D-βHB allowed LTP induction suggests a potentially important role for D-βHB in synaptic plasticity in immature neurons.

The importance of ketone bodies during the suckling period is also supported by the observation that D-βHB maintained the morphological integrity of hippocampal slices from immature rats, an effect not mimicked by glucose alone (Fig. 8). In the presence of glucose alone, pyramidal neurons in slices from PND 15 rats revealed dark cell changes (Fig. 7), resembling the effects of glucose deprivation in slices from PND 30–120 rats (16). These changes were not observed in slices incubated with 1 mM D-βHB plus glucose, suggesting that glucose is unable to fulfill the energy demands required for preservation of morphology in immature neurons.

Although D-βHB failed to restore EPSPs during glucose deprivation at PND 30, an unexpected observation was the recovery of EPSPs after IA administration in the presence of D-βHB. This strongly suggests that the change in energy utilization produced by IA is not identical to glucose deprivation. We initially speculated that the presence of glucose, even during glycolytic inhibition, would facilitate the use of D-βHB at this age because it has been shown that glucose facilitates ketone body use in adult rat brain (17, 50–52). It is also plausible that in the presence of both IA and glucose, nicotinamide adenine dinucleotide accumulates and results in acceleration of the conversion of D-βHB to acetoacetate. These possibilities seem unlikely because D-βHB attenuated IA-mediated suppression of EPSPs even in the absence of glucose (Fig. 4 *D*). Another possibility is that IA, an inhibitor of glyceraldehyde-3-phosphate dehydrogenase, may also modify the affinity of transporters for D-βHB. It has been reported that one of the monocarboxylate transporters is sensitive to thiol reagents such as IA (53). It should also be noted that even at PND 120,

D-βHB ameliorated the acute damage produced by IA, although D-βHB did not provide neuronal protection during glucose deprivation. The pattern of damage produced by IA, including the extreme swelling of neuronal cell bodies, is apparently different from the damage induced by glucose deprivation, but is similar to the damage produced by *N*-methyl-D-aspartate or simulated ischemia (oxygen and glucose deprivation) (54, 55). 1,3-Butanediol, a precursor of D-βHB, has neuroprotective effects in models of stroke (34, 56–58). The neuroprotection offered by D-βHB at PND 120 suggests that, during neurodegenerative conditions, D-βHB may substitute for glucose or that D-βHB may play additional neuroprotective roles despite the fact that it is only weakly transported under normal conditions.

Taken together, these studies indicate that D-βHB is an important substrate that not only substitutes for glucose in maintaining synaptic function but also helps to preserve neuronal integrity during development. Further studies should address the role of ketone bodies in memory impairment and seizure disorders during development. Additionally, the observation that D-βHB has neuroprotective properties even after the suckling period makes it important to characterize pathological conditions in which D-βHB can be used.

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