A ketogenic diet reduces long-term potentiation in the dentate gyrus of freely-behaving rats.

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Running title: Ketogenic diet reduces long-term potentiation in rats
Abstract:

Ketogenic diets are very low in carbohydrates and can reduce epileptic seizures significantly. This dietary therapy is particularly effective in pediatric and drug-resistant epilepsy. Hypothesized anticonvulsant mechanisms of ketogenic diets focus on increased inhibition and/or decreased excitability/excitation. Either of these consequences might not only reduce seizures but could also affect normal brain function and synaptic plasticity. Here we characterized effects of a ketogenic diet on hippocampal long-term potentiation, a widely studied form of synaptic plasticity. Adult male rats were placed on a control or ketogenic diet for three weeks prior to recording. To maintain the most physiological conditions possible we assessed synaptic transmission and plasticity using chronic in vivo recordings in freely behaving animals. Rats underwent stereotaxic surgery to chronically-implant a recording electrode in the hippocampal dentate gyrus and a stimulating electrode in the perforant path; they recovered for one week. After habituation and stable baseline recording, 5 Hz theta-burst stimulation was delivered to induce long-term potentiation. All animals showed successful plasticity, demonstrating that potentiation was not blocked by the ketogenic diet. Compared to rats fed a control diet, rats fed a ketogenic diet demonstrated significantly diminished long-term potentiation. This decreased potentiation lasted for at least 48h. Reduced potentiation in ketogenic diet-fed rats is consistent with a general increase in neuronal inhibition (or decrease in excitability) and decreased seizure susceptibility. A better understanding of the effects of ketogenic diets on synaptic plasticity and learning is important, as diet-based therapy is often prescribed to children with epilepsy.

Keywords
Hippocampus; epilepsy; metabolic therapy; paired-pulse; perforant path; plasticity; theta-burst
A ketogenic diet (KD) is a high fat, low carbohydrate diet used most often to treat epilepsy in young children unresponsive to anticonvulsant medications. The low availability of carbohydrates (and thus limited glucose) creates a state of ketosis in which ketone bodies become the primary source of energy for the nervous system. Introduced in the 1920s, a KD reduces seizure occurrence significantly in a majority of epileptic children, and eliminates seizures in a sizable minority (Kossoff and Rho 2009; Neal et al. 2009), often with fewer cognitive side effects than anticonvulsant drugs. These benefits are seen across gender and seizure etiology, but can reverse quickly with the reintroduction of dietary carbohydrates. A KD is also effective in reducing seizures in adults (Klein et al. 2010; Sirven et al. 1999), and there is growing interest in and evidence for the application of a KD for clinical benefits in other conditions, such as pain (Ruskin et al. 2009) and brain injury (Appelberg et al. 2009; Hu et al. 2009).

Despite the clinical efficacy of the KD in reducing seizures, researchers and clinicians remain uncertain as to the precise mechanism by which it raises seizure threshold to control epilepsy. Proposed mechanisms include (but are not limited to) increases in inhibitory neuromodulators, activated potassium channels, enhanced inhibitory neurotransmission, or diminished excitatory neurotransmission (Hartman et al. 2007; Juge et al. 2010; Ma et al. 2007; Masino and Geiger 2008; Nylen et al. 2009; Kawamura et al. 2010; Masino et al. 2011). Because it is effective in drug-resistant epilepsy, the diet is likely to activate mechanisms other than those targeted by any one type of antiepileptic drug,

The anticonvulsant effects of a KD might also affect normal synaptic functions. Thus far, some electrophysiological evidence has been reported for such effects in hippocampus and neocortex (Bough et al. 2003; Cantello et al. 2007), although not all published work using KD or
diet-related manipulations are consistent (Stafstrom 2004; Thio et al. 2010). Changes in synaptic function might, in turn, lead to diverse cognitive and behavioral side-effects such as changes in learning and memory. Some studies have found improved cognitive function (Appelberg et al. 2009; Silva et al. 2005; Thio et al. 2010; Todorova et al. 2000); other rodent studies have found impaired spatial learning in water maze paradigms (Su et al. 2000; Zhao et al. 2004).

To date, no research has characterized the impact of a KD on synaptic transmission in vivo without the use of anesthetics, which necessarily alter central inhibition and/or excitability (Cain et al. 1992; MacIver et al. 1989). To achieve the most physiologically-relevant conditions possible, the current study uses chronic electrophysiological recordings in freely-behaving rats to evaluate the effects of a KD on transmission in the perforant pathway-hippocampal dentate gyrus (DG) synapse, a well-established model of rodent synaptic plasticity in vivo (Blaise and Bronzino 2003; Blaise et al. 2008). After feeding a KD to adult rats, we assessed both short-term plasticity via recording of paired-pulse responses and long term plasticity via induction of long-term potentiation (LTP) using theta-burst stimulation (TBS). Results indicate long-term synaptic plasticity was reliably induced in all animals. KD significantly reduced the magnitude of TBS-induced potentiation at time points up to 48 hours after induction. However, we found no significant effects of KD on baseline synaptic parameters or paired pulse ratios,
MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats were assigned diets starting at an age range of 51-73 d. Each cage of animals was randomly assigned to be fed ad libitum either a KD (F3666, Bio-Serv, Frenchtown, NJ; with a >6:1 ratio of fat:(carbohydrate + protein)) or a regular control diet (CD; Purina 5001, W.F. Fisher, Somerville, NJ). Animals were maintained on diets for 14 d before surgery. We previously showed that this KD fed to adult male rats ad libitum had no significant effect on body weight over a three week period, but did elevate plasma ketone bodies significantly (Ruskin et al. 2009) and we replicated this effect in a separate group of animals (data not shown). All surgical and experimental protocols were approved by the Trinity College Animal Care and Use Committee and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Chronic Implantation Surgery. Details of our stereotaxic surgical procedures have been described in detail elsewhere (Blaise and Bronzino 2003). Briefly, anesthetized (IP, 50 mg/kg, sodium pentobarbital) male rats were chronically implanted with a concentric bipolar stimulating electrode positioned in the angular bundle to activate the medial perforant path, and with a monopolar single-strand flat-point tungsten recording electrode positioned in the ipsilateral DG. Ground and reference electrodes consisting of stainless steel machine screws were positioned contralaterally on the cortical surface of the parietal cortex. Dorsal-ventral positioning of the recording and stimulating electrodes was optimized by maximizing the amplitude of the evoked field potential on a digital oscilloscope (Nicolet Instruments, Madison, WI). The electrode wires were then led to a contact pin headstage assembly which was fixed to the skull with fast-drying dental acrylic (Lang Dental Manufacturing, Wheeling, IL). Throughout surgery, body temperature was maintained at 37°C with heat lamps, and animals were allowed a 7 d recovery
prior to testing. During recovery, animals were housed individually and maintained on their respective diets.

Electrophysiology. On the day of recording, rats were placed in a sound-attenuating recording chamber and attached to the recording apparatus via low-noise cabling through a counter-weighted commutator assembly, which allowed free movement of the animal about the chamber. Animals were allowed 1 hr to habituate to the recording environment and recordings were acquired only when animals were in a vigilance state of quiet waking (characterized behaviorally by animals posturally relaxed with eyes open, and electrographically by desynchronized activity in the DG with occasional low amplitude spindles and delta waves) to minimize evoked response variability (Blaise and Bronzino 2003; Hargreaves et al. 1990). Details of our stimulation parameters and data acquisition/analysis systems are available elsewhere (Blaise and Bronzino 2003; Blaise et al. 2008).

Fig. 1A illustrates the methodology for measuring the amplitude of the population spike (PS). After verifying an appropriate evoked response from the chronic implant, an input/output curve was constructed for each animal by averaging 10 PSs induced by single-pulse current stimuli at current intensities from 200 - 1500 μA. From this input/output curve, the stimulation intensity required to evoke 50% of the maximum recorded PS amplitude was determined for each individual animal; this current intensity was then used for both paired-pulse analysis and LTP induction. With these parameters, the excitatory post-synaptic potential is contaminated by the PS; thus we limited our analysis to PS amplitude. Paired-pulse ratios were also determined for a varying range of interstimulus intervals (ranging from 20 to 500 ms) to assess local circuit short-term plasticity. Following paired-pulse analysis, a pre-tetanus baseline of evoked responses to single pulse stimuli was established. To induce LTP, a train consisting of a 100-
pulse, 5 Hz TBS (10 bursts of 10 pulses; 400 Hz within-burst frequency) was delivered to the perforant path at the same 50% stimulus intensity. All animals were monitored closely for signs of seizure during and immediately after tetanus using behavioral and electrographic criteria; this never occurred. Five evoked DG PSs were recorded and averaged for each minute for the first 15 minutes. The average of 10 responses within one minute was again recorded at 30 min, 60 min, 120 min, 180 min, 24 hr and 48 hr after tetanization. The mean percent change in PS amplitude relative to baseline was determined at each timepoint for each animal in each treatment group. Data from 5 out of 19 animals were removed from analysis due to input/output curves that were non-monotonic or did not reach a clear maximal asymptote, or due to signal loss during recording. Significance was determined by analysis of variance with Newman-Keuls post hoc comparisons. Data are shown as mean ± standard error.
RESULTS

We recorded input/output curves, paired-pulse ratios, and synaptic plasticity in 14 animals (CD: n = 7; KD: n = 7). Treatment with a KD did not affect the input/output relationship significantly (data not shown; diet: F = 2.8, ns; diet/stimulation current interaction: F = 0.3, ns). Paired-pulse analysis demonstrated the characteristic depression / facilitation / depression pattern with increasing inter-pulse intervals (from 20-500 ms) at the perforant path-DG synapse (Bekenstein and Lothman 1991; Blaise and Bronzino 2000), regardless of diet (Fig. 2). Although there was a trend for the paired-pulse ratios to be reduced by KD treatment at the 70 ms (facilitation) and 150 ms (late depression) intervals, and a trend towards a reduced latency to the maximal paired-pulse ratio (50 ms vs. 70 ms), these effects were not significant (Fig 2).

TBS tetanization successfully potentiated PS amplitude in all animals (representative PSs at baseline vs. 1 hr post-TBS shown in CD- and KD-fed rats; Fig. 1B, C, respectively). Synaptic potentiation was significantly less in KD-treated rats at nearly all time points (Fig. 3). At the later time points qualifying as LTP, KD treatment reduced PS potentiation consistently, including the longest time points recorded (24 and 48 h; Fig. 3). No animals from either treatment group showed behavioral or electrographic seizures during tetanization, and observed behavior throughout the recording process did not differ between CD- and KD-fed animals.
DISCUSSION

Feeding with a KD decreased synaptic potentiation but had no significant effects on baseline synaptic transmission or paired-pulse ratios at the perforant path-DG synapse in freely-behaving rats. TBS-induced potentiation, including stable and significant LTP, was induced successfully in all animals in all diet groups, underscoring that the KD reduced the magnitude of but did not block synaptic plasticity. Decreased potentiation in KD-fed rats is consistent with increased seizure threshold and thus reduced seizure susceptibility: these two measures are modified in parallel by a number of treatments and conditions (Casasola et al. 2004; Johnston 1996; Lopez de Armentia et al. 2007; Mazarati et al. 2000). Here we used normal, non-epileptic animals; to our knowledge the relationship between a KD and synaptic plasticity in in vivo animal models of epilepsy remains unknown.

Our findings contrast somewhat with those of a recent paper using the identical KD. Thio et al. (2010) reported no significant effect of 2-3 wk KD feeding on LTP in the DG in vivo recorded out to ~90 min post-induction. This difference might arise from the use of halothane-anesthetized rats by Thio et al. and of awake freely behaving rats in our study: a putative moderate inhibition and/or moderately-reduced excitability of the KD impacting LTP might be overwhelmed by anesthetic actions. Interestingly, a parallel finding appears in the literature for anticonvulsant drugs which inhibit LTP induction in awake animals but have no effect on LTP in anesthetized animals (Kubota et al. 1994; Stringer 2000; Xiong and Stringer 1997). This pattern suggests that anesthetized subjects may be of limited value in electrophysiological studies of the effects of KD or other ketogenic treatments on synaptic plasticity. Other methodological differences between Thio et al. and our study include subject age (Thio et al. used juvenile rats: PD > 21, we used adult rats: PD > 73), acute vs. chronic recording, and type of electrical stimulation: the theta-burst patterned stimulation used presently might be more physiologically
relevant than the sustained high-frequency tetanus used by Thio et al., and thus more sensitive to moderate experimental treatments (Jedlicka et al. 2009; Morozov et al. 2003; Staubli et al. 1999).

Nevertheless, both we and Thio et al. (2010) found no significant effect of KD feeding on paired-pulse depression or facilitation in the dentate gyrus. A lack of effect was also reported in an in vitro study of another hippocampal region, CA1 (Stafstrom et al. 1999). In contrast, Bough et al. (2003) found that a KD enhanced early-phase paired-pulse depression in urethane-anesthetized rats. This effect might be attributable to caloric restriction, as only a restricted KD was tested and the same effect occurred in animals on a restricted standard pellet diet. A similar explanation might underlie shifted input/output relationships in Bough et al. (2003), whereas such an effect was not found in CA1 (Stafstrom et al. 1999) or DG (present study) with ad libitum KD treatment; more research is needed to resolve these issues.

Although a major effect of any KD is to increase circulating ketones, ketones might not be directly responsible for the present effect. Ketones applied in vitro do not appear to affect inhibitory synaptic transmission in the hippocampus (Thio et al. 2000) and actually prevent oxidative-stress induced impairment of hippocampal LTP (Maalouf and Rho 2008); there are conflicting data on ketones affecting hippocampal excitatory synaptic transmission (Juge et al. 2010; Thio et al. 2000). KD-induced reduction of LTP thus might be secondary to ketonemia (or mild hypoglycemia). Because all diets were provided ad libitum, and CD- and KD-fed groups did not differ in weight, these findings are unlikely to be due to caloric restriction. At this time we cannot completely rule out that altered intake of vitamins and minerals may have influenced the results; the high caloric density of the KD leads animals to eat less (by weight) (Al-Khalifa et al. 2009).

Animal studies report a variety of cognitive outcomes with KD treatment in normal animals of varying ages and in multiple models of epilepsy, ranging from impairment (Su et al.
2000; Zhao et al. 2004) to no negative effect (Appelberg et al. 2009; Silva et al. 2005; Thio et al. 2010; Todorova et al. 2000). Clinical studies of pediatric epileptic patients on the KD have reported improvements in general cognitive/behavioral measures such as alertness, attention, and social functioning (Kinsman et al. 1992; Pulsifer et al. 2001; Svedova et al. 2010). Any negative cognitive effects of the KD in pediatric patients, however, might be overpowered by positive effects due to seizure control or reduction, or elimination or reduction of antiepileptic drugs. Whereas explicit effects of a KD on cognition in non-epileptic children have not been examined, some studies have been performed in non-epileptic adults. One study found a transient, moderate impairment in one cognitive task (out of three), present at one week of diet treatment but not at later time points (Wing et al. 1995). Two studies examining chronic KD treatments reported improved processing speed and working memory lasting up to one year (Brinkworth et al. 2009; Halyburton et al. 2007). Thus, negative cognitive effects of KD feeding might be minimal and/or transient, at least in adults.

It is important to recognize that our diet formulation was stricter than that typically applied clinically; even so, all animals did display significant synaptic plasticity. Furthermore, there are no established guidelines and limited scientific discussions regarding “how much” plasticity is best, and mechanisms underlying synaptic plasticity are thought to overlap those associated with epileptogenesis (Gall and Lynch 2004; Golarai and Sutula 1996). It is clear that more in vivo electrophysiological characterization of KD effects needs to be performed in unanesthetized animals, with clinically-relevant diet formulations. This work should include juvenile animals, epilepsy models, and parallel cognitive/behavioral analyses. In particular, additional work in juveniles will help characterize the effects of a KD during development, and maximize the clinical relevance to the mainly pediatric target of current KD treatments.
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REFERENCES


FIGURE LEGENDS

Figure 1. Representative examples of field post-synaptic potentials and superimposed PSs in the DG, recorded during quiet waking. A: Illustration of PS measurement. The gray line tangents the beginning and ending of the PS; the dashed line rising from the spike’s nadir to the tangent line is the measured amplitude. B: Traces from baseline and 60 minutes after TBS to induce LTP in a rat fed the CD. Baseline stimulation was calibrated to produce 50% of maximal PS amplitude, and, using this same stimulation intensity, robust potentiation was produced by TBS. All traces are averages of five single traces. C: As in B, except from a rat fed the KD. TBS produces significant potentiation, but of lesser magnitude on average (Fig. 3) than in a CD-fed animals.

Figure 2. Paired-pulse analysis in the DG of CD- vs. KD-fed awake adult rats. The paired-pulse ratio was calculated across a 20-500 ms interpulse interval. All rats demonstrated the expected paired-pulse facilitation (50-70 ms interstimulus interval) and early- and late-phase depression as published previously. Feeding with a KD had no significant effect on this pattern or on the amplitude of the paired-pulse ratio at any interstimulus interval. Diet F = 2.1, n.s. (p = 0.15); Current-x-Diet F = 1.0, n.s.; n = 7 for each group.

Figure 3. Synaptic potentiation induced by TBS of the perforant path - DG pathway of CD- vs. KD-fed awake adult rats. KD-fed rats show a significantly smaller degree of short-term potentiation (defined as less than 30 min after TBS, delivered at time zero) and a significantly smaller degree of LTP (defined as more than 30 min after TBS) than CD-fed rats. This increased potentiation persisted in CD- vs. KD-fed rats for at least 48 hours. Diet F = 7.1, p<0.05, Time-x-Diet F = 3.0, p<0.001. * p<0.05, compared to CD; n = 7 for each group.
Fig. 1

[Graphs showing voltage (mV) over time (ms) for baseline and 60 minutes after induction in A and B, and another graph in C with similar data.]
Fig. 2

![Graph showing paired pulse ratio against inter-pulse interval (ms) for two diets, control diet and ketogenic diet. The graph illustrates the difference in response between the two diets at various intervals.]
Fig. 3

Time (min)
-15 -10 -5 0 5 10 15 30 60 120 180 24h 48h

% change in population spike amplitude
0 100 200 300 400

- control diet
- ketogenic diet

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