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A ketogenic diet rescues the murine succinic semialdehyde dehydrogenase deficient phenotype

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Abstract

Succinic semialdehyde dehydrogenase (SSADH) deficiency is an heritable disorder of GABA degradation characterized by ataxia, psychomotor retardation and seizures. To date, there is no effective treatment for SSADH deficiency. We tested the hypothesis that a ketogenic diet (KD) would improve outcome in an animal model of SSADH deficiency, the SSADH knockout mouse (*Aldh5a1*^{-/-}). Using a 4:1 ratio of fat to combined carbohydrate and protein KD we set out to compare the general phenotype, *in vivo* and *in vitro* electrophysiology and [³⁵S]TBPS binding in both *Aldh5a1*^{-/-} mice and control (*Aldh5a1*^{+/+}) mice. We found that the KD prolonged the lifespan of mutant mice by >300% with normalization of ataxia, weight gain and EEG compared to mutants fed a control diet. *Aldh5a1*^{-/-} mice showed significantly reduced mIPSC frequency in CA1 hippocampal neurons as well as significantly decreased [³⁵S]TBPS binding in all brain areas examined. In KD fed mutants, mIPSC activity normalized and [³⁵S]TBPS binding was restored in the cortex and hippocampus. The KD appears to reverse toward normal the perturbations seen in *Aldh5a1*^{-/-} mice. Our data suggest that the KD may work in this model by restoring GABAergic inhibition. These data demonstrate a successful experimental treatment for murine SSADH deficiency using a KD, giving promise to the idea that the KD may be successful in the clinical treatment of SSADH deficiency.

Keywords

Succinic semialdehyde dehydrogenase deficiency; ketogenic diet; mouse model; GABA; beta-hydroxybutyrate; glucose; electrophysiology; chloride channel

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Introduction

Succinic semialdehyde dehydrogenase (SSADH) deficiency is a rare, autosomal recessive disorder of γ -aminobutyric acid (GABA) biotransformation (Gibson et al., 1983; Gibson and Jakobs, 2001). The absence of SSADH leads to significantly elevated γ -hydroxybutyrate (GHB) and GABA levels throughout the body (Gibson and Jakobs, 2001; Pearl et al., 2003). Elevation of these neuroactive compounds is thought to play a role in the psychomotor retardation, ataxia and epilepsy (Gordon, 2004)—with seizure types ranging from absence seizures to convulsive *status epilepticus*—seen in patients with this disorder (Pearl et al., 2003; Gibson et al., 1998). Currently, there is no effective treatment for human SSADH deficiency (Gropman, 2003; Gibson and Jakobs, 2001).

A murine analog of SSADH deficiency, *Aldh5a1*^{−/−}, was developed to study the pathophysiology and treatment of this disorder (Hogema et al., 2001). *Aldh5a1*^{−/−} mice exhibit developmental delay, ataxia and a seizure disorder that progresses from absence seizures (~post-natal day, P, 15) to lethal *status epilepticus* (~P25) (Cortez et al., 2004). A variety of pharmacological treatments were attempted to rescue *Aldh5a1*^{−/−} mice with limited success (Hogema et al., 2001; Gupta et al., 2002; Cortez et al., 2004). However, it has been reported that pups that continue to suckle beyond weaning age (~P20) live longer than their weanling counterparts, suggesting that the high fat dam's milk may have beneficial effects in this disorder (Hogema et al., 2001). This observation led us to pose the question whether administration of a ketogenic diet (KD)—a high fat, low carbohydrate and adequate protein diet generally used in the treatment of drug-resistant epilepsy and other neurological disorders (Vining, 1999; Thiele, 2003; Kossoff, 2004; Gasior et al., 2006; Hartman and Vining, 2007; Hartman et al., 2007)—might be successful in treating SSADH deficiency.

To test this hypothesis, we examined the effect of the KD on the *Aldh5a1*^{−/−} mouse phenotype. Here we show that the KD normalizes lifespan, ataxia, weight gain and EEG in *Aldh5a1*^{−/−} mice. Previous research has shown a loss of GABA_A receptor-associated chloride channel binding and resultant neural hyperexcitation to be a factor in the SSADH null phenotype (Wu et al., 2006). We therefore examined [³⁵S]TBPS binding and recorded excitatory and inhibitory miniature post synaptic currents in KD and CD fed mutant mice. Our data suggest that the KD may work towards rescuing the SSADH null phenotype by restoring GABAergic activity in the brains of *Aldh5a1*^{−/−} mice.

Methods

Subjects

The SSADH null mice with C57/129Sv background were first generated in the Oregon Health and Science University in Portland (Hogema et al., 2001). Five pairs of heterozygous (*Aldh5a1*^{+/-}) mice were transported to the Hospital for Sick Children in Toronto. The mutant mouse line was maintained by inbreeding. The absence of SSADH was confirmed by two-allele three-primer polymerase chain reaction using tail genomic DNA (Hogema et al., 2001). All animals were maintained in a controlled environment at 12h light-12h dark cycle with lights on at 06:00 h and given ad lib access to food and water. All animal work was approved by the Hospital for Sick Children Division of Laboratory Animal Services and was performed in accordance with the guidelines of the Canadian Council on Animal Care (CCAC).

Diets

In KD treated mutants, the KD replaced normal mouse chow when pups were P12. The young mice fed primarily by suckling until ~P20. The KD was introduced earlier, however, to ensure that any non-suckling feeding was ketogenic in nature. The 4:1 (fat:carbohydrate + protein, by

weight) KD was obtained from Harlan Teklad (Madison, WI; TD.03490) (Likhodii, 2001; Nylen et al., 2005; Nylen et al., 2006). Standard mouse chow served as the CD. Table 1 summarizes the composition of both diets.

Weights and Ataxia

Weights and ataxia scores were determined for all mice 3–5 times per week. Ataxia scores were scored according to Loscher's scale of sedation (Loscher et al., 1987). This scale has 6 levels of ataxia: 1—slight ataxia of hindlegs, 2—dragging hindlegs, 3—ataxia and dragging hindlegs, 4—marked ataxia loss of balance, 5—marked ataxia no balance, and 6—loss of righting reflex with attempts to move forward.

Electrocorticography (ECoG)

Mice (P20–25) were implanted with four epidural monopolar electrodes with their tips placed bilaterally over the frontal and parietal cortices under pentobarbital anesthesia. The electrodes were placed 1mm deep, 2mm anterior to bregma and 2mm lateral from midline. Recordings were made 1, 24 and 48 hours after recovery from anesthesia. Each animal was placed in an individual Plexiglas chamber for a 20-min adaptation period prior to ECoG recordings in order to minimize movement artifact. ECoG recordings were made on paper using a Grass Polysomnograph machine (Grass Instruments, Quincy, MA) (Cortez et al., 2004).

Determination of Serum Analytes

All analytes were measured using methods implemented as *User Defined Assays* using reagents from Randox Laboratories Ltd. (Antrim, United Kingdom). A Vitros Chemistry System 5,1 Fusion Series (Ortho-Clinical Diagnostics, N.J.) was used to analyze all samples. Glucose was determined using the conversion of β -D-glucose gluconate and H_2O_2 via glucose oxidase. The generated hydrogen peroxide oxidizes the 4-aminoantipyrine, 1,7 dihydroxynaphthalene chromogen system in a horseradish peroxidase catalyzed reaction. The density of the resulting dye complex is related to the concentration of glucose in the specimen and is measured by reflectance spectrophotometry at 540nm (User Defined Assay Reference Guide for Vitros 5,1 FS Chemistry System. Ortho-Clinical Diagnostics.). Beta-hydroxybutyrate (β OHB) was measured by the oxidation of β OHB to acetoacetate via β OHB-dehydrogenase. This reaction can be detected by measuring absorbance at 340nm caused by the conversion of NAD^+ to NADH (Randox Ranbut D-3-Hydroxybutyrate Manual, Randox Laboratories Ltd.; User Defined Assay Reference Guide for Vitros 5,1 FS Chemistry System. Ortho-Clinical Diagnostics.). Determination of non-esterified fatty acids (NEFA, or free fatty acids) was performed by the acylation of coenzyme A (CoA) by the fatty acids in the presence of acyl-CoA synthetase. The acyl-CoA produced in the reaction is further oxidized by acyl-CoA oxidase, which generated H_2O_2 as a by-product. Hydrogen peroxide, in the presence of peroxidase permits the oxidative condensation of N-ethyl-N-(2hydroxy-3-sulphopropyl)-m-toluidine with 4-aminoantipyrine (4-AAP) to form a purple coloured adduct. This colour was measured spectrophotometrically at 550nm (Randox NEFA Manual, Randox Laboratories Ltd.; User Defined Assay Reference Guide for Vitros 5,1 FS Chemistry System. Ortho-Clinical Diagnostics.).

Electrophysiology: Brain Slices and Solutions

Brains were taken from P14 to P25 mice under halothane anesthesia. Transverse brain slices (450 μ m) were obtained by a Vibratome (Series 1000; St. Louis, MO) and maintained in artificial cerebrospinal fluid (aCSF) containing (mM) 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4 , 2 $MgSO_4$, 2 $CaCl_2$, 25 $NaHCO_3$, and 10 glucose. aCSF was bubbled with carbogen (95% O_2 , 5% CO_2) and gravity fed to the recording chamber at a rate of 3–4ml/min.

The internal solution for recording mPSCs consisted of (mM) 20 cesium methanesulfonate, 2 Mg-ATP, 10 HEPES, 0.3 GTP, 0.1 EGTA, 130 CsCl₂. Osmolarity was 300±5mOsm and pH was adjusted to 7.2 using cesium hydroxide. For mIPSC recordings, D-2-amino-5-phosphopentanoic acid (D-AP5; 20μM, made of 50mM stock solution in distilled water), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 100μM, made of 50mM stock in dimethylsulfoxide) and tetrodotoxin (TTX; 1μM, made of 1mM stock in dH₂O) were added to the aCSF superfusate. For mEPSC recordings, bicuculline methiodide (BMI; 10μM made of 10mM stock in dH₂O) was added to aCSF superfusate along with TTX. Chemicals were obtained from Sigma and diluted daily.

Electrophysiology: Whole Cell Recordings

Neuronal recordings were obtained from CA1 hippocampal pyramidal neurons using the whole-cell configuration patch-clamp technique. Electrodes had tip resistances between 5 and 8MΩ. Neuronal responses were recorded with the use of a Multiclamp 700B (Molecular Devices, Sunnyvale, CA).

Analysis of mPSCs

mPSCs were analyzed using MiniAnalysis software (Synaptosoft, Decatur, GA).

[³⁵S] Tert-butylbicyclophosphorothionate Autoradiography

Mice were decapitated and their brains were removed and immediately immersed in isopentane at −35°C. Coronal sections were cut from the anterior to posterior boundaries of the cerebral cortex at 20μm at −20°C and thaw-mounted onto gelatin-coated slides that were dried and stored at −80°C until used. The regions analyzed were the frontoparietal cortex, the ventrobasal thalamus, the CA region of the hippocampus and the amygdala (Wu et al., 2006).

Statistical Analysis

Lifespan and weight gain data were analyzed using two-tailed t-tests. Ataxia data were analyzed using a repeated measures two-way analysis of variance (ANOVA). [³⁵S]TBPS binding data and serum analyte were analyzed using a two-way ANOVA with Bonferroni post-hoc tests. All other data were analyzed using a one-way ANOVA with Tukey's post-hoc tests. Significance was considered at p<0.05; numerical values are expressed as means±standard error (s.e.m.).

Results

Lifespan, Ataxia and Weights

Mutant mice weaned onto the CD invariably demonstrated convulsions, which culminated in lethal *status epilepticus* around P25. Although *status epilepticus* was the eventual cause of death in all mice, the onset of *status epilepticus* was significantly delayed in the KD fed mutants, as evidenced by their significantly longer lifespan.

The average lifespan of KD fed mutants was 93.5±20.4 days, almost 4-fold higher than the lifespan of CD fed mutants who had an average lifespan of 24±3.7 days (Fig. 1a; n=9, p<0.0001, two-tailed t-test). All mice were weighed and assessed for ataxia daily. Beyond P12, CD fed mutants exhibited an average daily weight loss of 0.003±0.013g while KD fed mutants gained an average of 0.130±0.010g per day (Fig. 1b; p=0.0002, two-tailed t-test). Ataxia scores were assessed using a 6-point rating scale (Loscher et al., 1987). CD fed *Aldh5a1*^{−/−} mice began to develop ataxia at ~P18 (stage 2 and higher). Onset of ataxia was significantly delayed in KD fed mutants as they only began to develop stage 2 ataxia at ~P70 (Fig. 1c; p=0.0002, repeated-

measures two-way ANOVA, post-hoc analysis revealed significant differences between groups for days P17–40).

In Vivo Electrocorticography

Electrocorticography (ECoG) was performed on freely moving CD and KD fed *Aldh5a1*^{-/-} mice to determine whether the above-mentioned changes corresponded to changes on the electroencephalogram (EEG). The background ECoG baseline in CD fed *Aldh5a1*^{-/-} mice (n=4) consisted of 35 to 60uV cortical activity at 4 to 7 Hz activity with intermingled 3–5 Hz, often associated with fast frequency oscillations at the frequency of 28 Hz during restful waking conditions. There were numerous interruptions of the background activity by intermittent high amplitude 250–300uV bursts of spontaneous, recurrent spike and wave discharges (SWD), whose onset/offset was time-locked with absence-like ictal behavior which consisted of frozen immobility, facial myoclonus, and vibrissal twitching. In contrast, the baseline of the KD fed *Aldh5a1*^{-/-} mice showed remarkable normalization with a fairly well regulated low amplitude 35 to 50uV electrical fields at 4 Hz, with complete absence either slower frequencies in the delta range or fast frequency oscillations within the frontal and parietal cortices recorded with simultaneous standard paper EEG and digital systems (Grass Instruments, Quincy, MA) recorded at 6, 15 and 30 mm/sec (Fig. 2a). We also recoded the number of seizures with corresponding convulsions. KD fed mutants experienced significantly fewer convulsions compared to CD fed mutants (Fig 2b; p=0.031, t-test).

Serum Glucose, Beta-hydroxybutyrate, and Non-esterified Fatty Acids

To determine the biochemical correlates of KD induced changes, we measured serum levels of glucose, beta-hydroxybutyrate (βOHB) and non-esterified fatty acid (NEFA) concentrations (see Table 2). For this experiment we added a KD fed *Aldh5a1*^{+/+} group. Analysis of serum glucose, βOHB and NEFAs was performed given that these analytes are among the most studied blood analytes in the literature investigating the potential mechanism of the KD.

Serum glucose levels were significantly different between groups (p=0.006, one-way ANOVA). Post hoc tests revealed that serum glucose was significantly elevated in KD fed mice when compared to CD fed *Aldh5a1*^{-/-} mice (p<0.05). Serum glucose levels were only significantly different between CD fed *Aldh5a1*^{-/-} and KD fed mice— no groups differed significantly from CD fed *Aldh5a1*^{+/+} control mice.

Examination of βOHB levels demonstrated significant differences between groups (p=0.0004, one-way ANOVA). CD fed *Aldh5a1*^{-/-} mice had significantly elevated βOHB when compared to CD fed *Aldh5a1*^{+/+} control mice (p<0.05, post-hoc). βOHB levels were significantly elevated in KD fed wildtype mice when compared to CD fed wildtype mice, which was expected due to the ketogenic nature of the diet (p<0.01). βOHB levels were highest in KD fed *Aldh5a1*^{-/-} mice and they differed significantly from CD fed wildtype mice (p<0.001).

We measured serum NEFAs in all mice. There were no statistically significant differences between groups.

In Vitro Miniature Post-Synaptic Current Recordings

Whole cell voltage clamp studies were performed to record miniature inhibitory (mIPSC) and excitatory (mEPSC) postsynaptic currents from CA1 pyramidal cells of hippocampal slices. A one-way ANOVA showed that mIPSC activity in slices from *Aldh5a1*^{-/-} mice was significantly diminished compared to wildtype mice (Fig. 3a–b; p=0.04, ANOVA; p<0.05, post-hoc test). This decrease represented a >2.5 fold decrease in mIPSC frequency. There were no statistically significant differences between groups in terms of mEPSC activity (Fig. 3c–d; p=0.46, one-way ANOVA). The decrease in mIPSC activity observed in the *Aldh5a1*^{-/-} was

completely restored in KD fed mutants when compared to wildtype controls (Fig. 3a–b; $p>0.05$, post-hoc test).

[³⁵S]tert-butylbicyclophosphorothionate Binding

[³⁵S]Tert-butylbicyclophosphorothionate ([³⁵S]TBPS) binding in *Aldh5a1*^{−/−} mice has been shown to be significantly reduced in an age-dependent manner when compared with wildtype mice (Wu et al., 2006). The [³⁵S]TBPS binding becomes increasingly diminished until the third postnatal week of life in the mutant animals, reaching a nadir prior to the onset of generalized convulsive seizures (Wu et al., 2006). Here we confirm that [³⁵S]TBPS binding is significantly reduced in *Aldh5a1*^{−/−} mice compared to wildtype controls (Fig. 3d; $p=0.002$, two-way ANOVA). Significantly decreased [³⁵S]TBPS binding in *Aldh5a1*^{−/−} mice was observed in all brain regions studied (hippocampus, cortex, amygdala and thalamus; all regions $p<0.001$, post-hoc test). Post-hoc analysis demonstrated that [³⁵S]TBPS binding is restored fully in hippocampus and frontoparietal cortex of KD fed *Aldh5a1*^{−/−} mice when compared to CD fed *Aldh5a1*^{+/+} mice ($p>0.05$). The KD did not fully restore [³⁵S]TBPS binding in amygdala and thalamus of *Aldh5a1*^{−/−} mice when compared to CD fed wildtype mice ($p>0.05$, both cases). The reduction of [³⁵S]TBPS binding seen in amygdala and thalamus of KD fed mutants was less severe than the reduction seen in CD fed mutants, suggesting that the KD induced partial restoration of [³⁵S]TBPS binding in these areas (Fig. 2d).

Discussion

Our data show that the KD eliminates the premature lethality seen in *Aldh5a1*^{−/−} mutants and increases the lifespan by >300%. KD fed mutants also showed significantly less ataxia, weight loss and fewer abnormalities on the ECoG. These effects were paralleled by a significant restoration of GABAergic spontaneous synaptic activity and region-specific restoration of GABA_A receptor-associated chloride channel binding.

Lifespan

The cause of death in *Aldh5a1*^{−/−} mice is *status epilepticus* (Cortez et al., 2004). Around P18–22, untreated mutants experience tonic-clonic convulsions followed by *status epilepticus* around P25. KD fed mutants did not experience *status epilepticus* at this age, but began to demonstrate susceptibility to auditory evoked convulsions around P70. These convulsions involved a tonic-clonic component with jumping and running fits. Eventually, KD fed *Aldh5a1*^{−/−} mice succumbed to *status epilepticus*, albeit significantly later in life than their untreated counterparts.

To our knowledge, our experiments represent the longest any mouse has been maintained on a KD. The longest-living KD fed mutant was maintained on the KD for 130 days. The long-term effects of a high-fat diet in mice have not been thoroughly investigated. In humans, the KD has been associated with increased risk of coronary heart disease (Best et al., 2000; Bergqvist et al., 2003; Dashti et al., 2003; Kwiterovich et al., 2003) among other adverse effects. It is unclear whether such adverse events played a role in the eventual death of KD fed *Aldh5a1*^{−/−} mice.

Ataxia and Weights

The KD significantly delayed the onset of ataxia in *Aldh5a1*^{−/−} mice. CD fed *Aldh5a1*^{−/−} mice developed marked ataxia by P15–17, as was evidenced by significant dragging of the hind limbs (stage 2 ataxia). Ataxia in these animals quickly progressed to involve complete immobility for prolonged periods with unsuccessful attempts to walk. By P25, untreated mutants had little control over their locomotion. KD fed mutants, however, took significantly longer to develop ataxia. Stage 2 ataxia did not occur until ~P70.

Weights were assessed in KD and CD fed *Aldh5a1*^{-/-} as well as CD fed wildtype mice. We did not measure weight gain in KD fed *Aldh5a1*^{+/+} mice. There are several studies, both clinical and experimental, that report KD fed subjects being lighter than normal diet fed subjects (Rho et al, 1999; Bough & Eagles, 2001; Vining et al, 2002; Zhao et al, 2004; Nylen et al, 2005). Despite having significantly stunted growth, rodents fed a KD do not experience anywhere near the stunting seen in SSADH mutants fed either a KD or a CD.

KD fed mutants showed significantly less stunting of growth compared to CD fed mutants. This was evidenced by significantly higher daily weight gain in the KD fed mutants. Clinically, the KD is associated with significantly attenuated growth compared to humans consuming a non-KD (Vining et al., 2002; Liu et al., 2003; Papandreou et al., 2006). This has also been shown in rats fed a KD (Zhao et al, 2004; Nylen et al, 2005; Nylen et al, 2006). This held true in the KD fed mutants as they showed significant stunting of growth when compared to CD fed wildtype mice. When compared to CD fed *Aldh5a1*^{-/-} mice, however, KD fed *Aldh5a1*^{-/-} mice gained significantly more weight.

It is possible that the cause of this weight gain is simply because the KD fed mutants consumed more food. Previous research from our group, however, has measured food intake in rats on the KD and found that animals regulate their caloric intake (Likhodii et al., 2000). Because the KD is almost twice as calorie-dense as the control diet, this means that KD fed animals tend to eat about half as much food.

We believe significant weight loss in untreated *Aldh5a1*^{-/-} mice may be caused by impaired glucose metabolism (Chowdhury et al., in press) and subsequent metabolism of fat and protein stores. Consumption of a high fat KD provides an alternative energy source for *Aldh5a1*^{-/-} mice, allowing some growth to take place.

Serum Analytes

We examined serum levels of glucose, β OHB and NEFAs. Glucose levels only differed significantly between CD fed *Aldh5a1*^{-/-} mice and KD fed mice. None of the groups differed significantly from CD fed wildtype controls, however.

β OHB levels were significantly elevated in CD fed *Aldh5a1*^{-/-} mice when compared to CD fed wildtype animals. This supports a previous finding showing that impaired glucose oxidation in SSADH mutants leads to a significant elevation of β OHB levels (Chowdhury et al., in press). If *Aldh5a1*^{-/-} mice are unable to oxidize dietary carbohydrate effectively then they will begin to oxidize their fat stores for energy, causing ketosis. This has important implications for the mechanism of the KD in this model. It is possible that giving the mice a high-fat diet simply gives them an energy source that they wouldn't otherwise be able to get from carbohydrate rich mouse chow.

As expected, serum β OHB levels were significantly elevated in KD fed animals due to the ketogenic nature of the diet. β OHB levels in KD fed mutants were very high, possibly a compounded effect of elevated ketosis due to impaired glucose oxidation coupled with the ketogenic nature of the diet.

An unexpected finding was an absence of increased blood NEFA during KD intervention. Similar findings have been reported by Klepper and colleagues (2004), however, using the KD to treat human glucose transporter deficiency. Whereas β OHB rose significantly in their patients, there was no corresponding increase in absolute plasma NEFA. In our study, we assume no differences between genotypes for lipoprotein lipase activity or its ability to activate in response to diet. One explanation for the absence of increased plasma NEFA may reside in the capacity of *Aldh5a1*^{-/-} mice to more effectively convert NEFA to β OHB. The ratios of

mean NEFA to β OHB (Table 2) were: *Aldh5a1*^{+/+} (CD), 1.20; *Aldh5a1*^{-/-} (CD), 0.73; *Aldh5a1*^{+/+} (KD), 1.04; *Aldh5a1*^{-/-} (KD), 0.46. While the ratio for *Aldh5a1*^{+/+} mice was comparable despite diet, the same value in *Aldh5a1*^{-/-} mice was lower on CD and decreased and additional 40% with KD intervention, suggesting enhanced β -oxidation of fatty acids in the *Aldh5a1*^{-/-} mice.

ECoG

KD fed mice show remarkable normalization of the ECoG. This normalization might be due to the KD's ability to elevate ketone bodies. In our model, we show that the KD significantly elevates β OHB (acetone and acetoacetate have not yet been measured). The ketone bodies acetone, β OHB and acetoacetate—all elevated by the KD—have all been shown to have anticonvulsant effects in animal seizure models (Rho et al., 2002; Likhodii et al., 2003; Gasior et al., 2007). Elevation of β OHB may, in part, explain why the ECoG of KD fed *Aldh5a1*^{-/-} mice show less seizure activity, and why the mice have fewer convulsions. Another consideration, discussed below, is that the KD restored spontaneous GABAergic synaptic activity and GABA_A receptor-associated chloride channel binding.

[³⁵S]TBPS Binding

We confirmed that CD fed *Aldh5a1*^{-/-} mice exhibit significantly reduced [³⁵S]TBPS binding compared to wildtype controls. This reduction was seen in all brain areas studied, with the largest decrease (36%) seen in the thalamus. [³⁵S]TBPS binding was completely restored in hippocampus and cortex and partially restored in amygdala and thalamus of KD fed mutants. [³⁵S]TBPS is a specific ligand for the GABA_A receptor-gated chloride channel (Behrends, 2000). These data likely reflect a reduction—and KD induced rescue—of GABA_A receptor binding and/or Cl⁻ channel activity. Interestingly, the KD had no effect on [³⁵S]TBPS binding in wildtype mice.

mPSC Recordings

Here we report a significant decrease in mIPSC frequency in *Aldh5a1*^{-/-} mice. Reductions in mIPSC activity have been implicated as a potential mechanism of epileptogenesis (Hirsch et al., 1999; Shao et al., 2005). mIPSCs are known to play an important role in synaptic maintenance and function (Hartman et al., 2006). A decrease in mIPSC frequency with a corresponding decrease in mIPSC amplitude can suggest postsynaptic changes to GABA_A receptors, however, mIPSC amplitudes were not reduced in KD fed *Aldh5a1*^{-/-} mice (data not shown). The decrease in mIPSC frequency might therefore reflect a decrease in the number of presynaptic inhibitory interneuron terminals in the stratum radiatum. These interneurons synapse on the CA1 pyramidal cells from which we recorded. Decreased input from these interneurons might explain the neural hyperactivity observed in CD fed mutants (Wu et al., 2006). It is possible that the KD may be acting to preserve or repair the interneurons. There exists considerable evidence for the neuro-protective effects of the KD and ketone bodies (Massieu et al., 2003; Noh et al., 2003; Gasior et al., 2006; Noh et al., 2006; Maalouf et al., 2007). This hypothesis requires further studies to determine the exact cause for the mIPSC reduction in CD fed *Aldh5a1*^{-/-} mice.

Role of GABA and GHB in KD's Mechanism

We propose that the KD plays a role in restoring hippocampal mIPSC activity and the hippocampal [³⁵S]TBPS binding in parallel. Although we have not shown the relationship between these events to be causal, there is some evidence to suggest that changes in GABA_A receptor binding could underlie changes in hippocampal mIPSC frequency (Hartman et al., 2006; Swanwick et al., 2006). In untreated mutants, both of these GABAergic components were significantly compromised when compared to wildtype control mice. KD stimulated

restoration of GABAergic activity may have played a role in forstalling the evolution of lethal *status epilepticus* in these mutant animals, thus significantly prolonging their lifespan.

GHB exerts its pharmacological activity via the GABA_B receptor (Snead & Gibson, 2005). In mammalian brain GHB levels approximate 1–4 μ M (Doherty et al., 1978; Snead, 1991), while in *Aldh5a1*^{−/−} mice this level rises to ~250 μ M (Hogema et al., 2001). This concentration of GHB is still well below that which is believed to activate GABA_B receptors (~5 mM; Lingenhoebl et al., 1999; Wu et al., 2004). On the other hand, some of this GHB may interconvert to GABA, as the enzymes responsible for this are still operative (Rumigny et al., 1981), hence elevating the excess GABA due to the block in *Aldh5a1* even more (Snead & Gibson, 2005). The resultant elevated GABA would be predicted to down-regulate both GABA_A (Wu et al., 2006) and GABA_B (Buzzi et al., 2006) receptors. Whether elevated GABA preferentially modulates pre-/postsynaptic GABA_B receptors is unknown, although selected studies using baclofen in cultured hippocampal neurons indicate a preferential desensitization of postsynaptic GABA_B receptors (Wetherington & Lambert, 2002). There is some evidence that GHB may preferentially act on presynaptic GABA_BR (Banerjee et al., 1995; Snead, 1996).

Conclusion

To our knowledge, this is the first evidence that a KD is effective in the treatment of murine SSADH deficiency. Our data provide support for the potential usefulness of the KD in the treatment of human SSADH deficiency. We speculate that detailed characterization of KD mechanisms in SSADH null mice will provide novel insight into the utility of this diet in the treatment of refractory epilepsy.

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Figure 1. Phenotype data (lifespan, ataxia, weights)

This figure outlines the KD's effects on behavior in SSADH deficient mice. a) CD fed mutants lived an average of 23.78 ± 2.70 days ($n=9$, range: 18–43 days) whereas KD fed mutants lived 93.50 ± 13.39 days ($n=9$, range: 58–165 days). Not only did the KD fed mutant mice live longer, but they appeared much healthier— as evidenced by improved weight gain and reduced ataxia. b) All mice were weighed daily from P12 onward. CD fed mutants lost an average of 0.003 ± 0.013 g per day ($n=9$) while KD fed mutants gained 0.13 ± 0.01 g per day ($n=9$). CD fed wildtype mice showed normal weight gain for a mouse, which was significantly higher than both KD and CD fed mutants. The KD is generally associated with stunted growth (both in animals and humans), however, in *Aldh5a1*^{-/-} mice it allows for significantly more weight gain than CD. c) Ataxia was assessed daily. CD fed mutants progressed very quickly to high levels of ataxia ($n=9$). KD fed mutants eventually reached similar levels of ataxia, but took significantly longer to arrive at that stage ($n=9$). *** $p < 0.0001$, d=days, g=grams.

Figure 2. ECoG Recordings in CD and KD fed *Aldh5a1*^{-/-} Mice

a) The baseline ECoG in CD fed *Aldh5a1*^{-/-} (n=4) consisted of 35 to 60uV cortical activity at 4 to 7 Hz activity with intermingled 3–5 Hz often interrupted by intermittent 250–300uV bursts of spontaneous, recurrent spike and wave discharges (SWD), which onset/offset was time-locked with absence-like ictal behavior which consisted of frozen immobility, facial myoclonus, and vibrissal twitching. There were also runs of SWD associated with ictal forelimb clonic movements. The baseline of the KD fed *Aldh5a1*^{-/-} mice showed a fairly well regulated 35 to 50uV electrical fields at 4 Hz, with decreased interictal or ictal bursts of SWD, recorded at 6, 15 and 30 mm/sec. Time / voltage scale indicate 50uV/ 1 second. Sensitivity= 30uV/mm; LFF= 1 Hz; HHF= 100 Hz; Notched Filter (60 Hz) on; LF= Left Frontal; RF= Right frontal; LP= Left Parietal; RP= Right Parietal. b) The number of convulsions per hour were calculated in CD and KD fed mutants. A convulsion was defined as ictal activity with corresponding clonus, tonus, running or jumping.

Figure 3. Electrophysiology and [³⁵S]TBPS data

a) mIPSCs were recorded in the presence of the voltage gated sodium channel blocker TTX (1 μ M) and the glutamatergic blockers CNQX (100 μ M) and APV (20 μ M). The presence of cesium in the patching electrode blocked GABA_B receptor associated potassium channel conductance. mIPSC activity is dramatically reduced in CD fed *Aldh5a1*^{-/-} mice (n=6) compared to CD fed wildtype controls (n=8). KD fed mutants (n=6) had completely restored mIPSC activity. N's indicate the number of animals—recordings were collected from at least two cells from each animal. b) Representative mIPSC traces. c) Representative mEPSC traces. d) mEPSCs were recorded in the presence of the voltage-gated sodium channel blocker TTX (1 μ M) and the GABAergic blocker BMI (10 μ M). A similar, albeit non-significant, trend is apparent here as seen with the mIPSCs. mEPSC activity is reduced non-significantly in CD fed mutant mice (n=8). mEPSC frequency is similar in KD fed mutant mice (n=9) and CD fed wildtype mice (n=8). e) For the [³⁵S]TBPS binding we added a KD fed *Aldh5a1*^{+/+} group. Each binding study involved 4 slices per mouse, with n mice per group. The binding experiment was performed in duplicate, meaning (n*4)*2 slices were analyzed. Consistent with previous studies, [³⁵S]TBPS binding was significantly reduced in CD fed *Aldh5a1*^{-/-} mice (n=3) compared to CD fed *Aldh5a1*^{+/+} mice (n=5) in all regions. [³⁵S]TBPS binding was completely restored in hippocampus and cortex of KD fed *Aldh5a1*^{-/-} mice (n=3). [³⁵S]TBPS binding was partially restored in amygdala and thalamus of KD fed mutants. KD fed *Aldh5a1*^{+/+} (n=4) did not differ from CD fed controls. All n's indicate the number of animals per group. HPC= hippocampus AMY= amygdala THAL= thalamus, *p<0.05 ***p<0.001.

Table 1

Composition of Experimental Diets.

Nutrient	KD (g)	Diet	CD (g)
Protein	146.97		234
Carbohydrate	30		490
Fat	707.83		100
Vitamins	4.6		54
Minerals	44.47		69
Fibre	66.09		53

4:1 KD. The AIN-93G vitamin mix used in the 4:1 KD contains no carbohydrates. Soybean oil came from the vitamin mix where it serves as a carrier for fat-soluble vitamins. The type of fat (i.e. butter or lard) can be varied. Adapted from Likhodii, 2001, modified by reversing quantities of lard and butter. CD. The ash content of the CD is derived from formula AIN-76 mineral mix #F8505. The vitamin mix is derived from formula AIN-76 vitamin mix #F8000. Adapted from Bough et al., 1999. g: grams; KD: ketogenic diet; CD: control diet.

Table 2
Average Group Serum Glucose, Beta-hydroxybutyrate and Non-esterified Fatty Acid Levels.

		Glucose	β OHB	NEFA
<i>Aldh5a1</i> ^{+/+}	CD (n=6)	9.03±2.55	0.59±0.38	0.73±0.17
<i>Aldh5a1</i> ^{-/-}	CD (n=5)	7.03±0.68 [^]	1.11±0.29 [*]	0.81±0.34
<i>Aldh5a1</i> ^{+/+}	KD (n=5)	9.73±0.37	1.12±0.36 [*]	1.16±0.46
<i>Aldh5a1</i> ^{-/-}	KD (n=7)	10.40±0.34	2.06±0.48 [¶]	0.95±0.27

*
p<0.05

[^]
p<0.01

[¶]
p<0.001

one-way ANOVA; β OHB beta-hydroxybutyrate. In terms of glucose, CD fed *Aldh5a1*^{-/-} mice only had significantly lower serum glucose levels than KD fed animals. They did not differ significantly from wildtype controls. CD fed *Aldh5a1*^{-/-} mice and KD fed *Aldh5a1*^{+/+} mice had significantly higher β OHB levels than wildtype controls. KD fed *Aldh5a1*^{-/-} mice had significantly higher β OHB than all other groups. NEFAs did not differ significantly between groups.