Dietary beta-hydroxybutyrate is superior to a ketogenic diet to improve behavior and abnormal myelination in a mouse model of NMDA receptor deficiency

Yuanye Yan  
University of Toronto

Tatiana Lipina  
University of Toronto

Laura Pepera  
University of Toronto

Wendy Horsfall  
University of Toronto

Ali Salahpour  
University of Toronto

Amy J. Ramsey (✉ a.ramsey@utoronto.ca)  
University of Toronto

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Abstract

Background

Dysfunction of N-methyl-D-aspartate receptors (NMDAR) is associated with idiopathic autism and a syndromic form of autism called *GRIN* disorder. Ketogenic therapy is used to treat seizures in *GRIN* disorder, but it is unknown whether it improves other aspects of the disorder. We asked whether a ketogenic diet or exogenous ketone bodies, beta-hydroxybutyrate (BHB), could improve autism-like behaviours in *Grin1* knockdown mice (Grin1KD). Since BHB has been reported to affect myelination, we asked whether improvements in behavior were correlated with changes in myelination.

Methods

WT and Grin1KD mice were randomly assigned to receive control, ketogenic diet (6:1 fat to proteins and carbohydrates ratio), or normal chow with BHB supplementation (6mg/ml in drinking water) starting at postnatal week 3-4. Blood ketones were monitored one-week and nine-week after treatment. Following this, behavioural tests were conducted, and subsequently the myelin integrity of the corpus callosum was studied with transmission electron microscopy.

Results

Ketogenic diet was not well-tolerated by juvenile Grin1KD mice in contrast to BHB supplementation. Both dietary manipulations elevated blood ketone levels after one week of treatment, but these elevations diminished over time. Both treatments reduced hyperactivity of Grin1KD mice. However, only BHB improved sensorimotor gating in Grin1KD mice. Social motivation and spatial working memory were not improved by either treatment. We report, for the first time, a reduced percentage of myelinated axons in the corpus callosum of adult Grin1KD mice, which was ameliorated by long-term BHB supplementation. Surprisingly, mice receiving a ketogenic diet showed increased number of abnormal myelinations, especially decompaction.

Limitations

Our findings are limited to the specific ketogenic regimens. Although findings in Grin1KD mice have significant implications in ASD and GRIN disorder, mice and humans have fundamental differences in their dietary and metabolic requirements. Future studies are required to understand the mechanism by which ketone bodies improve myelination.

Conclusions

We demonstrate that sub-chronic administration of exogenous BHB from early-life is beneficial to some domains of ASD-linked behaviours in Grin1KD mice. One potential mechanism is by improving myelination in the corpus callosum of Grin1KD mice. Our data supports exogenous BHB supplementation as potential treatment for ASD and GRIN disorder.
Background

N-methyl-D-aspartate receptors (NMDAR) play crucial role in neurodevelopment, synaptic plasticity, learning and memory. Impairment of NMDAR function has been associated with a variety of neurological and psychiatric disorders, including neurodevelopmental disorders such as autism spectrum disorder (ASD) (1). Rare pathogenic variants in genes encoding NMDAR subunits, GRIN genes, cause GRIN disorder, a neurodevelopmental disorder with symptoms of intellectual disability, epilepsy, movement disorders, and features of ASD (2). There are no effective treatments for most of the disabling symptoms of GRIN disorder, but ketogenic diet has been reported to improve cases of treatment-resistant seizures in GRIN patients. A possible explanation for the therapeutic benefits of dietary ketosis is the availability of ketones as an energy source instead of glucose. Recent studies point to the presence of bioenergetic dysfunction in patients with ASD, which may lead to novel therapeutic approaches (3, 4).

Bioenergetic changes due to NMDAR dysfunction can be studied in mouse models such as the hypomorphic Grin1 knockdown mouse (Grin1KD). The Grin1KD mouse is homozygous for an intronic insertion mutation that results in a 90% reduction in Grin1 mRNA, GluN1 protein, and NMDAR function (5). Cognitive deficits, reduced social motivation, impaired sensorimotor gating and seizures have been extensively reported in Grin1KD mouse, making it a promising model for GRIN disorder and ASD (5–7).

Several studies suggest the presence of bioenergetic deficits in the Grin1KD mouse. Wesseling et al. reported overexpressed pyruvate kinase and aspartate aminotransferase in the frontal cortex and hippocampus tissue of Grin1KD mice, coupled with the decreased expression of IGF-1 and elevated level of glucagon in serum, indicative of increased energy demands (8). This is also supported by the excessive firing of neurons in the prefrontal cortex (9) and other brain regions of Grin1KD mice (10, 11). However, other studies have shown reduced expression of genes encoding glucose and monocarboxylate transporters GLUT1, GLUT3 and MCT4 and decreased 2-deoxy-glucose (2DG) uptake in Grin1KD mice (12, 13). This suggests deficient glucose uptake to meet the high energy demand.

Although glucose is the major energy source in the brain, other bioenergetic substrates such as ketone bodies are elevated and utilized for oxidative phosphorylation during glucose deprived conditions. Ketone bodies are mainly produced from fatty acid metabolism in the liver. In addition to impaired glucose uptake, Grin1KD mice also show altered serum and brain polyunsaturated fatty acids composition and are vulnerable to omega-3 deprivation (14). Reduced leptin and elevated apolipoprotein-A were also reported in the serum of Grin1KD mice (8). These observations suggest that fatty acid metabolism might play an essential role in Grin1KD mice bioenergetics and survival. Therefore, we asked whether ketone bodies could serve as an alternative energy source that compensates for the deficient glucose utilization in Grin1KD mice.

Ketogenic diets are high-fat, low carbohydrate, and adequate-protein diets that improve treatment-refractory epilepsy (15) and metabolic disorders like GLUT1 deficiency (16) in the clinic. Beyond that, a ketogenic diet showed a neuroprotective effect on cognition (17) and social behaviours (18) in rodents. In particular, ketogenic diet was able to correct (19) and prevent (20) schizophrenia-related phenotypes in
MK-801-induced pharmacological model of acute NMDAR hypofunction in mice. These studies suggest that ketogenic diet may have beneficial effects for NMDAR deficiency and GRIN disorder. One downside of ketogenic diet therapies is the requirement of strict and continuous monitor and oversight. In the absence of such oversight, adverse effect of ketogenic diet therapies were reported in a large percentage of children and often results in treatment discontinuation (21). Thus, alternatives to ketogenic diet are desirable.

An emerging hypothesis suggests that elevation in beta-hydroxybutyrate (BHB) is key to the therapeutic effect of the ketogenic diet. BHB is the principal circulating ketone body synthesized from fatty acid oxidation in liver mitochondria. BHB increases mitochondrial respiration and reduces reactive oxygen species (ROS) production (22). Furthermore, BHB inhibits histone deacetylases (HDAC), which improves memory function and synaptic plasticity in mice (23). BHB also exerts an anti-inflammatory effect specifically on NLRP3 inflammasome (24). Furthermore, in vitro study demonstrated protective actions of BHB against neurotoxicity induced by glucose deprivation, partially mediated by ATP restoration, modulation of reactive oxygen species (ROS) production, or autophagy (25). BHB powder is commercially available as a beverage supplement for human consumption, making it an attractive alternative to a ketogenic diet from a translational perspective.

In this study we compared the effect of ketogenic diet or BHB supplementation to control diets in Grin1KD mice and their wildtype littermates. We examined behaviors that are relevant to ASD symptoms including activity in a novel environment, social approach behavior, sensorimotor gating, and tests of memory and executive function. We investigated the impact of dietary manipulations on myelin integrity in the corpus callosum. Our studies investigated the impact of ketones on non-seizure phenotypes and explored one of the proposed mechanisms by which ketogenic diet exerts its effects in GRIN disorder.

Materials and Methods

Animals

Grin1KD mice were generated through targeted insertion of a neomycin cassette into the Grin1 gene (5). As previously described (7) and recommended (26), congenic C57Bl6/J Grin1+/neo and 129X1/SvJ Grin1+/neo mice were intercrossed to produce the experimental mice: Grin1+/+ (WT) and Grin1neo/neo (Grin1KD) mice. Both sexes were represented in this study. All experimental mice were group housed on a 12h light-dark cycle with ad libitum access to water and food. All experiments were conducted in accordance with the University of Toronto Temerty Faculty of Medicine and Pharmacy Animal Care Committee and the Canadian Council on Animal Care.

Ketogenic diet and BHB-supplemented water

Starting at postnatal week 4, WT and Grin1KD mice were randomly assigned to receive a ketogenic diet with 6:1 fat to proteins and carbohydrates ratio (TD.07797, Envigo Canada) or control diet that provides a similar ratio of Crisco to corn oil as the ketogenic diet (TD.150300, Envigo Canada). For the BHB-
supplementation study, experimental mice of both genotypes received standard rodent diet (Irradiated Teklad Global 18% rodent diet; T.2918.15; Envigo, Canada) and water bottles with or without BHB supplementation starting at postnatal week 3 (NPN 80085843; Natural Health Product, USA; 6mg/ml). Leak-proof stainless steel spouts were used on all water bottles so that liquid consumption can be monitored by weighing the water bottles. Consumption of water or BHB solution per week per mouse was calculated. Ketogenic diet and BHB-supplemented water bottles were replaced twice a week. Control diet and regular water bottles were changed once a week. The regimen of ketogenic diet and BHB dosage were based on previous literature (19, 27).

**Blood ketone measurement**

For better accuracy, experimental animals were fasted 18 hours (6p.m. – 12a.m.) before assessment. Fasting blood ketone level was measured from a drop of tail vein blood using Nova Max Plus ketone monitor (Nova Biomedical Corporation, USA). Measurements were done 1 week and 9 weeks after treatment initiation to assess short-term and chronic blood ketone change, respectively. We assumed 0.05mmol/L for all measurements with blood ketone levels under detection threshold (< 0.1mmol/L).

**Behavioral testing**

Behavioral tests were performed between postnatal week 12–14 for all experimental animals. Both male and female mice were represented in each group. All behavioral tests were performed as previously published (10, 14, 28) and briefly described below:

**Ambulation in novel environment**

Locomotor activity was measured in Plexiglas chambers (20 × 20 × 45cm³) equipped with Versamax activity monitor, which uses infrared light beams to track the mouse' movements (Omnitech Electronics, Columbus, OH, USA). Total distance traveled was collected in 5-min bins for 2 hours.

**Sensorimotor gating**: A paradigm of pre-pulse inhibition of the acoustic startle response (PPI/ASR) was measured using sound-attenuated chambers (SR-LAB ABS System, San Diego Instruments Inc). After 5-min habituation to background white noise (65dB), five startle pulse alone (100dB above background) was conducted, followed by 80 randomized trials of: pulse alone, prepulse alone (4, 8, 16dB above background), pulse + prepulse (100ms delay between prepulse and pulse), no pulse. Five pulse alone trials were performed again at the end. % PPI = (reduction in startle amplitude following prepulse)/(startle amplitude with pulse alone)×100%.

**Y-maze**

The Y-maze paradigm consists of three equivalent arms disposed at 120° from each other. The experimental mouse was initially placed in the center and allowed to explore all three arms (labelled as A, B, C). Sequence and number of entries to each arm were recorded for 5 min. Spontaneous alteration refers to visiting of all three arms in sequence (i.e. ABC or CAB but not CBC). % alteration was calculated as the number of alterations (triplets) / (total number of arm entries − 2) × 100%.
**Spatial Object Recognition:** Spatial object recognition task was performed in a white Plexiglas box (62 × 40.5 × 23 cm$^3$). The procedure consisted of two sessions: 1) habituation towards two identical beakers (10min); 2) object spatial recognition by displacing one beaker to the corner of the box (5min). A two-minute time interval was applied between sessions where the experimental animal was returned to its home cage. Relative discrimination ratio (= (time around displaced object - time around non-displaced object)/(total time in both zones)*100) was calculated indicating preference of one object over the other.

**Social Motivation**

Mice were habituated to a white Plexiglas box (62 × 40.5 × 23 cm$^3$) containing two empty wire cups for 5 minutes. A stranger wildtype mouse of the same age and sex was then placed in one of the cups. The time around each cup and the number of visits to each cup were tracked over a 10-minute period to assess social motivation. To quantify the duration of social interaction, time per visit (= total time around each zone/number of visits to the zone) was calculated.

**Electron microscopy**

Experimental mice were euthanized by cervical dislocation. Coronal brain sections (2 mm thick) containing the corpus callosum (~ 1.5 cm from the olfactory bulbs) were fixed in the phosphate buffer containing 1% glutaraldehyde and 4% paraformaldehyde for 1 hour at room temperature. Next, the fixative was replaced by fresh solution and brains were fixed overnight at 4°C. Fixed brain samples were rinsed with 0.1M phosphate buffer, pH 7.0 and post-fixed in 1% osmium tetroxide in 0.1 M phosphate buffer, pH 7.0. Then brain samples were dehydrated in an ethanol series and infiltrated with Embed 812/Araldite. The samples were trimmed into the blocks, which were cut into the thick section containing the region of interest and stained with toluidine blue. Lastly, the blocks were ultramicrotomed (Leica Reichert Ultracut E) coronally into thin sections (90 nm), mounted on the copper grid, stained with uranyl acetate and lead citrate, and then, imaged using Transmission Electron Microscopy (Thermo Scientific Talos L120C). Ten nonoverlapping images were taken per section at 5300x and 11000x magnifications and analyzed using semi-automated MyelTracer software (v 1.13) as described (29) Assessor was blinded to treatment and genotype. To quantify the thickness of the myelin sheath, G-ratio was calculated, which is defined as the axon diameter divided by the fiber diameter (diameter including myelin sheath). Axon diameter was measured at the narrowest point of the axon. Axons were considered myelinated when at least one complete layer of compacted myelin was observed.

**Statistical analysis**

All data were analyzed using GraphPad Prism 10.0.0 software. Average values are presented as mean ± SEM. Two- or tree-way ANOVA were used, followed by Tukey post hoc analysis for multiple comparisons, as indicated in figure legends. Statistical significance was set at $p < 0.05$.

**Results**
1. BHB supplementation but not ketogenic diet was well tolerated by Grin1KD mice

Although we ensured >10g body weight before initiating ketogenic diet (Keto), 8 out of 16 Grin1KD mice receiving ketogenic diet were found dead or met endpoint (>20% weight loss with or without lesions on face, limbs, or chest) before completing the study. This was not observed in WT or Grin1KD mice receiving control diet (Figure 1A). All animals receiving BHB-supplemented water showed tolerance to the treatment (no health concerns). The weekly consumption of BHB-supplemented water was significantly more than that of control water (Figure 1B, p<0.01), especially after two and three weeks of treatment (p<0.05). The effect of genotype on BHB preference was not tested since mice of both genotypes were housed together.

Consistent with our previous observations, Grin1KD mice had significantly lower body weights than WT mice. (Figure S1, p<0.05). In studies with BHB, sex, age, and treatment were all significant factors on body weight. BHB improved Grin1KD body weights in both male and female mice (Figure S1C-D, males: p=0.0548, female: p=0.0104). A trend of increased body weight in male Grin1KD mice was also observed with ketogenic diet in comparison to control diet (Figure S1A, p=0.0804). However, Grin1KD body weight was still significantly lower than wildtype mice in adulthood (p's<0.05, data not shown).

2. Fasting blood ketone was elevated after short-term ketogenic diet or BHB treatment but not after chronic exposure

As expected, increased blood ketone was observed in WT (2.08±0.26mmol/L) and Grin1KD (3.73±0.24mmol/L) mice receiving ketogenic diet for one week, in comparison with those on control diet (WT control: 1.10±0.13mmol/L, Grin1KD control: 1.91±0.23mmol/L, p's<0.05, Figure 2A). However, after nine weeks of ketogenic diet treatment, elevated blood ketone level was only observed in Grin1KD mice (2.43±0.20mmol/L in Keto-treated vs. 1.00±0.09mmol/L in control, p<0.05) but not in WT mice (1.11±0.17mmol/L in Keto-treated vs. 0.85±0.69mmol/L in control, p>0.05, Figure 2B).

After one week of BHB treatment, we found significantly increased blood ketone levels in both WT (0.57±0.06mmol/L) and Grin1KD (0.98±0.10mmol/L) mice compared to untreated mice of the same genotype (WT: 0.18±0.04mmol/L, p<0.01; Grin1KD: 0.11±0.03mmol/L, p<0.0001, Figure 2C). As with the ketogenic diet, the elevation in blood ketone level was no longer detected with nine weeks of BHB treatment in both genotypes (untreated WT: 0.15±0.03mmol/L, BHB-treated WT: 0.14±0.03mmol/L; untreated Grin1KD: 0.36±0.09mmol/L, BHB-treated Grin1KD: 0.14±0.03mmol/L; Figure 2D). Post hoc comparisons revealed increased blood ketones in untreated Grin1KD mice compared to WT (p<0.01), which was “normalized” after BHB treatment (Grin1KD non-treated vs. treated: p<0.01, BHB-treated WT vs. Grin1KD: ns).

3. BHB supplementation and ketogenic diet showed modest behavioral benefits on Grin1KD mice

As expected, hyperactivity was observed in untreated Grin1KD mice in comparison with WT. Hyperlocomotion was reduced in both Keto-treated (Figure 3A-B) and BHB-treated (Figure 3C-D) Grin1KD
mice in comparison with untreated Grin1KD mice. Neither ketogenic diet nor BHB supplementation affected the locomotor activity of WT mice (Figure 3A-D).

Sensorimotor gating was assessed by measuring the prepulse inhibition of acoustic startle response (PPI). Consistent with previous studies, reduced PPI was observed in Grin1KD mice at baseline. BHB supplementation but not ketogenic diet showed a modestly ameliorating effect on PPI impairment in Grin1KD mice (Figure 3E-F). Analysis of the acoustic startle response (ASR) detected the elevated ASR in Grin1KD treated by control diets but both Keto and BHB had no effect on ASR (Figure S2).

Social behavior was measured with a modified “three-chamber” test, where the study was conducted with a single arena. Video tracking software measured mouse interactions with novel mice constrained to a wire cage in the arena, or with an empty cage providing a non-social stimulus. Given that Grin1KD mice are hyperactive, average duration of time per visit was analyzed and reported for both genotypes. WT mice showed the expected preference for socially unfamiliar mouse over non-social neutral object, spending more time per visit near the stranger mouse (Figure 4A, p’s<0.05). In contrast, Grin1KD mice spent a similar amount of time per visit exploring the stranger mouse and the empty cup. The time per visit near the stranger mouse was significantly reduced in Grin1KD mice in comparison to WT mice (Figure 4B, p<0.001). There was no significant effect of the ketogenic diet on sociability in either genotype (Figure 4A-B, p’s>0.05). BHB treatment did not improve social motivation in Grin1KD mice; both treated and untreated Grin1KD mice spent significantly less time per visit around the stranger mouse in comparison to WT mice (Figure 4D, p<0.05). Interestingly, BHB treatment reduced social motivation in WT mice. BHB treated WT mice showed no preference for social over non-social interactions, spending a reduced amount time investigating the stranger mouse in comparison to non-treated WT mice (Figure 4C-D). In conclusion, BHB supplementation had no beneficial effect on the social motivation of Grin1KD mice but did impair the social motivation of WT mice.

To address potential effects ketogenic diet and BHB on cognition, we tested working memory in a Y-maze assay and short-term spatial novelty detection in a displaced object recognition test. In the Y-maze assay, the percentage of spontaneous alternations was reduced in Grin1KD mice in comparison to WT mice (Figure 5B, D, p’s<0.0001), indicating impaired working memory. Neither the ketogenic diet nor BHB supplementation had a significant effect on working memory in Grin1KD or WT mice (Figure 5) regardless of ketogenic diet’s effect on hyperactivity of Grin1KD mice in Y-maze (Supplementary Figure S3).

Similar results were observed in the test of the short-term spatial memory, assessed by the displaced-object recognition paradigm. In this task, WT mice displayed a preference to explore the displaced object over the non-displaced object while the Grin1KD mice did not (Figure 5A, Genotype: F (1,36) = 8.396, p=0.0064). This observed deficit in spatial recognition was not improved by ketogenic diet. In the cohorts used to study BHB supplementation, similar results were observed in the untreated WT and Grin1KD mice (Figure 5C, Genotype: F (1,35) = 6.885, p=0.0128). BHB supplementation did not improve spatial recognition.
4. **BHB but not ketogenic diet improved myelination in corpus callosum of Grin1KD mice.**

Our previous study showed volumetric deficits in white matter of Grin1KD mice (30), but the reason for the deficits was not understood. Since BHB has been reported to improve myelination in a mouse model of multiple sclerosis (31), we explored the possibility that the benefits of BHB diet on Grin1KD mice might be related to changes in white matter.

Myelin integrity was assessed by electron microscopy of the corpus callosum (Figure 6A). We measured the percentage of axons that were myelinated, the thickness of the myelin sheath (G-ratio), and the incidence of abnormal myelination (decompaction, fragmentation, separation, hypermyelination, aberrant outfolding and invagination). In the ketogenic diet study, we observed no difference in the percentage of myelinated axons or the thickness of myelin sheath between Grin1KD and WT mice. Furthermore, there was no effect of the ketogenic diet in either genotype (Figure 6B; Figure S4A-B). Surprisingly, the ketogenic diet significantly increased the percentage of abnormal myelination in both genotypes (Figure 6C, Keto effect: F (1,14) = 4.790, p=0.0461). Decompaction was the primary form of myelin abnormality observed in ketogenic diet-treated mice (Figure 6D, A).

In the cohort of mice used for the BHB study, two-way ANOVA detected a main effect of genotype: F (1,13) = 4.452, p=0.0548 and genotype´ BHB diet interaction: F (1,13) = 4.031, p=0.0659] on the percentage of myelinated axons. The control Grin1KD mice had a significant reduction in the percentage of axons that were myelinated as compared to control WT mice (Figure 7A-B, p<0.05). BHB supplementation showed a modest trend towards increased the percentage of myelinated axons in Grin1KD mice (Figure 7B). Similarly, no difference in myelination thickness was observed between WT and Grin1KD mice and BHB had no effect in either genotype (Figure S4C-D). Importantly, unlike the ketogenic diet, BHB supplementation did not increase the percentage of abnormally myelinated axons in mice of both genotypes (Figure 7C-D).

**Discussion**

To explore the potential therapeutic effect of a ketogenic diet for GRIN disorder, we initiated a long-term ketogenic diet in juvenile Grin1KD mice. We found that half of the Grin1KD mice developed severe health concerns (weight loss > 20% and skin lesions) and failed to complete the study. Health issues with the ketogenic diet were limited to Grin1KD mice and were not seen in wildtype mice receiving the same diet. There are various options to achieve a ketogenic diet. The choice of ketogenic diet and control (control diet versus regular chow) may contribute to the variation observed in different studies and the low tolerance in Grin1KD mice. Here we used a 6:1 ratio ketogenic diet consists of mainly Crisco (vegetable shortening, hydrogenated, table S1). The hydrogenation process produces trans-fatty acids (if partially hydrogenated) and converts unsaturated fats to saturated fats (if fully hydrogenated). Although the process helps the vegetable shortening to remain solid at room temperature, it makes them highly processed and less nutritious. Given that Grin1KD mice showed vulnerability to omega-3 deficient diet (14), it is possible that the overall lack of unsaturated fat or the omega-6 to omega-3 fatty acid ratio in the
current ketogenic diet is not suitable for Grin1KD mice to tolerate. Moreover, both the fat to protein and carbohydrates ratio and the composition of the lipids used in the ketogenic diet may play an important role in diet effectiveness.

The significant low body weight observed in Grin1KD mice regardless of the dietary interventions agrees with our current findings. It might indicate difficulty in food intake, which is regulated by NMDAR activity at multiple levels such as hunger and satiety sensing and hedonic motivation (32). Several pharmacological studies have reported reduced food intake after chronic antagonism of NMDAR by memantine (33, 34). Therefore, genetically induced NMDAR hypofunction in Grin1KD mice might reduce their food intake at baseline, which might be exaggerated by the ketogenic diet and leads to life-threatening health concerns. However, keto-treated Grin1KD mice who were able to complete the current study gradually increased their body weights and the ketogenic diet did not further exaggerate any behavioral phenotypes in them. Nevertheless, monitoring food intake coupled with indirect calorimetry in Grin1KD mice is necessary in future studies to ensure sufficient energy intake and successful dietary intervention.

To avoid the adverse effect observed in Grin1KD mice receiving ketogenic diet, we provided BHB-supplemented water with regular chow as an alternative. The regular chow contains balanced composition of fats, including soybean and corn oils, which are essential source of unsaturated fatty acids (35). As expected, BHB supplementation did not affect survival and well-being of Grin1KD and WT mice.

Efficient feeding was indicated by elevated blood ketone level one week after dietary initiation (of both ketogenic diet and BHB) in both WT and Grin1KD mice. More profound elevation in blood ketone was observed in mice receiving ketogenic diet compared to BHB supplementation. This is expected since ketogenic diet achieves ketosis and elevated blood ketone level by modifying protein activities in the liver to increase ketone production, while BHB alone does not. Importantly, Grin1KD mice showed greater elevation in blood ketone compared to WT mice receiving the same diet. Elevation in blood ketone bodies has been commonly used in previous studies as an indicator of metabolic ketosis (19, 36), hence suggesting more active use of fatty acids as a source of energy by Grin1KD mice compared to WT. There was no difference in blood ketone levels in WT animals after chronic treatment with either ketogenic diet or BHB for 9 weeks, suggesting metabolic adaptation to both treatments. Weekly monitoring of blood ketone during treatment with ketogenic or/and BHB diets would reveal saturation of cellular metabolism in future studies. For instance, a recent study detected slight reduction of BHB level in serum after 4 weeks of treatment with ketogenic diet in mice (37). Interestingly, blood ketone levels in the Grin1KD mice receiving ketogenic diet remained elevated (but to a lesser extend) after being subjected to a ketogenic diet for a prolonged period, which further supports their increased reliance on fats as an energy source. Similar to a ketogenic diet, chronic BHB supplementation also diminished elevation in blood ketone level but in mice of both genotypes, suggesting metabolic adaptation to BHB in Grin1KD mice. Further investigation into glucose transporters, BHB transporters, hepatic enzymes, and other markers are
necessary to understand how adaptation to chronic BHB supplementation occurs in WT and Grin1KD mice.

In the behavioural phenotype analysis, we found that both ketogenic diet and BHB supplementation reduced hyperactivity in Grin1KD mice. This is consistent to previous studies by Kraeuter et al., where ketogenic diet (19) and BHB injection (38) suppressed the hyperlocomotion effects of acute NMDAR antagonism with MK-801. The prolong exposure to BHB supplementation was efficient to also ameliorate PPI impairment in Grin1KD mice, in agreement with the MK-801 induced NMDAR hypofunction model (38). No rescue effects on PPI deficits were seen in Grin1KD mice receiving ketogenic diet, although previous study observed preventive effect of ketogenic diet on pharmacologically induced PPI impairment (20). Chronic treatment of Grin1KD mice by either ketogenic diet or BHB supplementation was not able to correct socio-cognitive deficiencies, in contrast to pharmacological model of NMDAR deficit (38). Multiple studies documented beneficial effects of the ketogenic diet to ameliorate phenotypes in a range of genetic mouse lines, related to seizures e.g. in Kcna1-null (39), or autism-related behaviors in Shank3 (40), Fmr1KO line (41) or BTBR inbred mice (42). The choice of ketogenic diet or/and regime of treatments may contribute to this discrepancy observed in our study in comparison with others. For instance, although Fmr1KO mice were treated by ketogenic diet with 6:1 ratio of fat to carbohydrate like in our study, a sub-chronic regime (4 weeks) was applied instead of a chronic treatment (9 weeks) used in our study. Besides the lack of ketogenic and BHB efficacies on behaviors of Grin1 deficient mice, we also found that BHB supplementation impaired social motivation in healthy, wildtype animals. Similarly, impairment of the contextual fear memory was found in wildtype mice treated with ketogenic diet (41). Despite the ketogenic diet has been used to alleviate treatment-resistant epilepsy since 1920s, its role in fundamental brain processes and mental disorders is still unclear (43). Hence, these dietary interventions need to be used with caution in healthy human population.

Taken together, even though BHB supplementation does not achieve an elevated blood ketone level that is similar to ketogenic diet, the beneficial effect of BHB supplementation on cognitive behaviour is comparable to that of ketogenic diet with better diet tolerance in Grin1KD mice. However, the comparison is limited to the specific regimens used in this study. Moreover, both interventions showed overall limited benefits on improving cognitive behaviours in Grin1KD mice. Refining BHB supplementation regimen and ketogenic diet with lipid compositions suitable for GRIN disorder are potential strategies to improve treatment effectiveness.

Although structural (30) and molecular (8) evidence points to potential deficits in myelin integrity in Grin1KD mice, the underlying myelination change has not been studied. We reported here, for the first time, a reduction in the percentage of myelinated axons in the corpus callosum of adult Grin1KD mice. Accumulative evidence indicates that NMDAR exists on oligodendrocytes (44) and plays an important role in neuron-glia communication (45). Patients with anti-NMDAR encephalitis develop clinical and radiological evidence of demyelinating disorders (46, 47), linking NMDAR hypofunction to impairment in myelination integrity. Chronic BHB supplementation improved the percentage of myelination in Grin1KD mice, supporting protective effect of BHB on myelination in vivo. Similar to our findings, Sun et al.
reported that one-month of BHB treatment improved demyelination in a mouse model of multiple sclerosis (31). Surprisingly, we observed an increased number of abnormal myelin sheath (especially decompaction) in both WT and Grin1KD mice receiving ketogenic diet. Decompaction is observed in proteolipid protein (PLP)-deficient mouse model (48) and reduces the conduction velocity of action potentials in the optic nerves of these mice (49). Decompaction and myelination swelling is frequently associated with neurodegenerative diseases, especially inflammation-driven neurodegeneration (50). Our result is contradictory to the well-established protective effect of ketogenic diet on neurodegenerative diseases (51). Reasons for this observation needs to be clarified in a future study. Once again, the choice of regimen and the exact lipid composition of the ketogenic diet could be one of the contributors to the conflicting observations.

Taken together, long-term BHB treatment of Grin1KD mice elicited beneficial effects limited to hyperactivity and sensorimotor gaiting coupled with amelioration of abnormal myelination. The prolong usage of the ketogenic diet increased mortality, decompaction of myelination and had no effect on studied behavior, except its modest effect on ambulation in Grin1KD mice. Refining BHB supplementation regimen and ketogenic diet with lipid compositions suitable for GRIN disorder are potential strategies to improve treatment effectiveness. However, BHB diet reduced social motivation in WT animals, hence, precise caution must be bear in mind before usage of ketogenic or/and BHB diets by healthy population in humans.

Limitations

Our findings are limited to the specific regimen used in this study, which may not be generalized to ketogenic diet or BHB supplementation of other regimens. Findings of ketogenic diet on Grin1KD mice are biased to those who tolerated the diet and completed the study.

Although we investigated myelination morphology after dietary intervention, the comprehensive mechanism underlying ketogenic diet and BHB supplementation are unknown. Accumulative evidence suggests that BHB improves mitochondrial respiration (52, 53), has anti-oxidative stress (54), anti-inflammatory (55–57), and neuroprotective effect (58). Based on our hypothesis that bioenergetic defects are present in Grin1KD mice, it is worth investigating the mitochondrial respiration and mROS level in Grin1KD mice. Furthermore, future studies should investigate whether bioenergetic deficits in Grin1KD mice could be improved by BHB, which serves as an alternative energy source and antioxidant.

Conclusions

In conclusion, we examined the effect of long-term exposure to ketone bodies from early-life in Grin1KD mice through two regimens: ketogenic diet or exogenous BHB-supplementation. Ketogenic diet was not well tolerated by Grin1KD mice, even though those who tolerate the diet do show improvements in hyperactivity. We urge close monitoring of food intake and diet tolerance when considering ketogenic diet in the clinic for patients with ASD or GRIN disorder. In contrast, our data demonstrates that exogeneous
BHB supplementation is well tolerated by Grin1KD mice and is sufficient to improve domains of ASD-linked behavioural phenotypes, including hyperactivity and impaired sensory processing. Moreover, BHB supplementation improved myelination in Grin1KD mice, which could be a potential mechanism underlying its beneficial effects. Taken together, exogenous BHB supplementation may be a beneficial treatment for ASD and GRIN disorder.

**List Of Abbreviations**

NMDAR  
N-Methyl-D-aspartate receptor  
ASD  
autism spectrum disorder  
BHB  
beta-hydroxybutyrate

**Declarations**

**Availability of data and materials**

The datasets used and/or analyzed in the current study are available from the corresponding author on reasonable request.

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**Author information**

Yuanye Yan and Tatiana Lipina contributed equally to this work.

**Contributions**
T.L., Y.Y., A.J.R., and A.S. designed the experiments. Y.Y. and T.L. conducted all experiments. L.P. helped conduct behavioural experiments. W.H. bred and maintained all experimental mice. Y.Y. generated all figures. Y.Y. and T.L. wrote the original manuscript draft. The draft was mainly edited by A.J.R. with all other authors comments and approval.

**Corresponding author**

Correspondence to a.ramsey@utoronto.ca

**Ethnics approval and consent to participate**

All experiments were conducted in accordance with the University of Toronto Temerty Faculty of Medicine and Pharmacy Animal Care Committee and the Canadian Council on Animal Care.

**Competing interests**

As a member of the scientific advisory board, AJR received financial renumeration from CureGRIN Foundation.

**References**


20. Kraeuter AK, van den Buuse M, Sarnyai Z. Ketogenic diet prevents impaired prepulse inhibition of startle in an acute NMDA receptor hypofunction model of schizophrenia. Schizophr Res. 2019;206.


47. Sinani A, Al MS, Al, Alshekaili J, Kindi M, Al RK, Al, Khabouri J, Al et al. Overlapping demyelinating syndrome (Neuromyelitis optica spectrum disorders NMOSD with anti-NMDA receptor encephalitis); A case report. Mult Scler Relat Disord. 2020;42.


Figures

Figure 1

Ketogenic diet tolerance and consumption of BHB-supplementation by WT and Grin1KD mice. (A) Kaplan–Meier survival curve for WT and Grin1KD mice on ketogenic diet. (B) Fluid consumption of water with or without BHB-supplementation. BHB-supplemented bottles (BHB; n=7 cages) were replaced at least twice a week whereas control water bottles (H2O; n=8 cages) were replaced once a week. Bottle weights were measured at replacement. Weekly fluid consumption was calculated and normalized to the number of mice in the corresponding cage. Effect of BHB: F (1,13) = 6.151, p=0.0276. Two-way ANOVA. Bonferroni post hoc. Mean ± SEM. *p<0.05.
Figure 2

Fasting blood ketone of WT and Grin1KD mice receiving ketogenic diet or BHB-supplemented water. All animals were fasted for 18 hr (6p.m. – 12p.m.) before assessing tail vein blood ketone level with Nova Max Plus ketone meter. Low values (<0.1mmol/L) were assumed to be 0.05mmol/L to be included for analysis. (A-B) Fasting blood ketone level of mice treated with ketogenic diet or control diet for 1 week (A) or 9 weeks (B). (A) Both WT and Grin1KD mice showed elevated blood ketone. Keto: F (1,44) = 36.68,
(B) Elevated blood ketone was only maintained in Grin1KD mice after prolonged keto treatment. Keto: F (1,38) = 38.01, p<0.0001, Genotype: F (1,38) = 29.46, p<0.0001, Genotype ´ Keto: F (1,38) = 18.57, p=0.0001. (C-D) Fasting blood ketone level of mice treated with H₂O or BHB for 1 week (C) or 9 weeks (D). (C) Elevated blood ketone was observed in both genotypes 1 week after BHB treatment. BHB: F (1,22) = 91.91, p<0.0001, Genotype: F (1,22) = 7.151, p=0.0139, Genotype ´ BHB: F (1,22) = 13.91, p=0.0012. (D) Elevation of blood ketone diminished after chronic BHB treatment in both genotypes. BHB: F (1,39) = 7.130, p=0.0110, Genotype: F (1,39) = 6.059, p=0.0184, Genotype ´ BHB: F (1,39) = 6.188, p=0.0172. Two-way ANOVA. Tukey post hoc. Mean±SEM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Figure 3

Effect of ketogenic diet and BHB-supplemented water on locomotor activity and sensorimotor gating. (A-D) Both ketogenic diet (A-B) and BHB-supplemented water (C-D) reduced the hyperlocomotion of Grin1KD mice. Distance travelled during 2-hour period was tracked in open-field test. (A) Distance travelled plotted over time of mice on ketogenic diet. Genotype: F (1, 37) = 352.2, p<0.0001; Keto: F (1,37) = 14.35, p=0.0005. Genotype ´ Keto: F (1,37) = 17.08, p=0.0002. (B) Total distance travelled during the 2-hour period of mice on ketogenic diet. (C)Distance travelled over time of mice on BHB-supplemented water. Genotype: F (1,31) = 348.2, p<0.0001; BHB: F (1,31) = 6.480, p=0.0161; Genotype ´ BHB: F (1,31) = 7.261,
p=0.0113. **(D)** Total distance travelled during the 2-hour period of mice on BHB-supplemented water. **(E-F)** Effect of ketogenic diet (E) and BHB (F) on prepulse inhibition (PPI) of the acoustic startle response. 

**(E)** Ketogenic diet had no effect on PPI. Genotype: F (1,34) = 7.136, p=0.0115; Keto and Genotype´Keto: ns. **(F)** BHB modestly ameliorated PPI impairment in Grin1KD mice. Genotype´BHB: F (1, 32) = 6.411, p = 0.0164. **(A, C, E-F)** Three-way ANOVA; **(B, D)** Two-way ANOVA. Tukey post hoc. Mean±SEM. *p<0.05, **p<0.01, ****p<0.0001.

**Figure 4**
**Effect of ketogenic diet and BHB-supplemented water on social motivation.** All experimental mice were habituated to the paradigm for 5min followed by a 10min social motivation test at adult age (12-14wks). Time per visit = (total zone time)/(number of visits). E = empty; S = Stranger mouse. **(A-B)** Ketogenic diet had no effect on sociability (A) or social duration (B) of either genotype. **(A)** Three-way ANOVA. Stranger: F (1,36) = 33.90, p<0.0001; Genotype: F (1,36) = 87.85, p<0.0001. Keto: ns. **(B)** Two-way ANOVA. Genotype: F (1,36) = 43.75, p<0.0001. **(C-D)** BHB-supplemented water had no effect on sociability (C) and duration (D) in Grin1KD mice but impaired sociability in WT mice. **(C)** Three-way ANOVA. Stranger: F (1,28) = 33.58, p<0.0001; Genotype: F (1,28) = 23.99, p<0.0001; Stranger ´ BHB: F (1,28) = 7.719, p=0.0096. **(D)** Two-way ANOVA. Genotype: F (1,28) = 10.75, p=0.0028; BHB: F (1,28) = 5.885, p=0.0220. Mean±SEM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns = not significant.
**Figure 5**

**Effect of ketogenic diet and BHB-supplemented water on spatial working memory.** Neither dietary treatment showed an effect on spatial working memory in both genotypes. (A, C) Spatial object recognition test: Discrimination ratio = (time around displaced object – time around non-displaced object)/(total time in both zones)*100. (B, D) Y-maze: % alteration = (number of alterations)/(total arm entries – 2)*100. (A) Genotype: F (1,36) = 8.396, p=0.0064. (B) Genotype: F (1,36) = 82.79, p<0.0001. (C)
Genotype: F (1,35) = 6.885, p=0.0128; BHB: F (1,35) = 3.084, p=0.0878. **(D) Genotype: F (1,53) = 99.36, p<0.00001; BHB: F (1,53) = 3.492, p=0.0672. Two-way ANOVA. Mean±SEM. *p<0.05, ****p<0.0001, ns = not significant.

**Figure 6**

**Effect of ketogenic diet on myelination in the corpus callosum of WT and Grin1KD mice. (A)** Representative TEM images in the corpus callosum at 5300x. **(B)** No difference in % myelinated axons were detected between Grin1KD and WT mice, which was not affected by ketogenic diet in either genotype. % myelination = number of myelinated axons/total number of axons*100. **(C)** Ketogenic diet
increased abnormal myelination in both genotypes. \( \% \) abnormal myelination = number of abnormally myelinated axons/number of myelinated axons*100. Keto: \( F (1,14) = 4.790, p=0.0461 \). (D) \% of different types of abnormal myelination illustrated by pie chart. Decompaction identified as the major type of myelin abnormality correlated to keto treatment. Two-way ANOVA. Mean±SEM.

![Figure 7](image)

**Figure 7**

**Effect of BHB-supplemented water on myelination in the corpus callosum of WT and Grin1KD mice.** (A) Representative TEM images in the corpus callosum at 5300x. (B) Grin1KD mice showed reduced \% myelination compared to WT, which was improved by BHB. \% myelination = number of myelinated
axons/total number of axons*100. Genotype: F (1,13) = 4.452, p=0.0548; Genotype ´ BHB: F (1,13) = 4.031, p=0.0659. (C) BHB did not increase abnormal myelination in either genotype. % abnormal myelination = number of abnormally myelinated axons/number of myelinated axons*100. BHB: ns. (D) % of different types of abnormal myelination illustrated by pie chart. Two-way ANOVA. Tukey post hoc. Mean±SEM. *p<0.05.

Supplementary Files

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- Supplementarymaterials.docx