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Preclinical testing of the ketogenic diet in fragile X mice

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ABSTRACT

The ketogenic diet is highly effective at attenuating seizures in refractory epilepsy, and accumulating evidence in the literature suggests that it may be beneficial in autism. To our knowledge, no one has studied the ketogenic diet in any fragile X syndrome (FXS) model. FXS is the leading known genetic cause of autism. Herein, we tested the effects of chronic ketogenic diet treatment on seizures, body weight, ketone and glucose levels, diurnal activity levels, learning and memory, and anxiety behaviors in *Fmr1*^{KO} and littermate control mice as a function of age. The ketogenic diet selectively attenuates seizures in male but not female *Fmr1*^{KO} mice and differentially affects weight gain and diurnal activity levels dependent on *Fmr1* genotype, sex and age.

1. Introduction

Fragile X syndrome (FXS) is a neurodevelopmental disorder clinically characterized by intellectual disability (overall IQ < 70), autistic-like behaviors and seizures (Hagerman and Hagerman, 2002). FXS results from a mutation in a single gene on the X chromosome, *FMR1*, that is associated with transcriptional silencing of the *FMR1* promoter and loss of expression of fragile X mental retardation protein (FMRP) (Verkerk et al., 1991). FMRP expression is absent or greatly reduced in FXS and many FXS phenotypes are manifested in *Fmr1*^{KO} mice, which lack expression of FMRP (Dutch-Belgian Fragile X Consortium, 1994). *Fmr1*^{KO} mice exhibit many of the physical and behavioral characteristics of humans with FXS and are thus the most widely employed, non-human model system available for testing interventions.

Herein, we conducted preclinical efficacy testing of the ketogenic diet (KD) in *Fmr1*^{KO} mice. The KD, which is clinically used to treat intractable epilepsy, is high in fat with moderate levels of protein and low carbohydrate. Altering diet to treat epilepsy dates back to circa 400 BC when starvation was used to reduce seizures. The classic KD was introduced in 1921 to replace starvation, and forces the body to burn fat for energy, i.e. “ketosis”. Glucose is normally the sole energy source for the human brain, but during ketosis, ketones are produced and used for energy. In addition to intractable epilepsy, ketone- rather than glucose-based metabolism may benefit other conditions. For example, the KD is studied for the treatment of a wide range of disorders and conditions including Alzheimer's disease, amyotrophic lateral sclerosis (ALS), anxiety, attention-deficit hyperactivity disorder (ADHD), autism spectrum disorders (ASD), bipolar disorder, cancer, depression, diabetes, obesity, pain, Parkinson's disease, schizophrenia, stroke and traumatic brain injury (Balietti et al., 2010; Bostock et al., 2017; Cheng et al., 2017; Evangeliou et al., 2003; Frye et al., 2011; Garcia-Penas, 2016; Herbert and Buckley, 2013; Jozwiak et al., 2011; Masino et al., 2009; Napoli et al., 2014; Spilioti et al., 2013; Stafstrom and Rho, 2012; Tai et al., 2008; Verrotti et al., 2017). To our knowledge, no one has studied the ketogenic diet in FXS, albeit there is growing interest in employing the KD for the treatment of autism. FXS is the leading known genetic cause of autism and is highly comorbid with epilepsy (Berry-Kravis et al., 2010; Kaufmann et al., 2017).

Autism is a cluster of complex neurobiological disorders with core features of repetitive stereotyped behavior and impaired social interaction and communication. ASD is highly comorbid with epilepsy, and it has been proposed that epilepsy drives the development of autism (Amiet et al., 2008; Hagerman, 2013; Hartley-McAndrew and Weinstock, 2010; van Eeghen et al., 2013). Thus, treatments that reduce seizure incidence have the potential to prevent the development of ASD or decrease the severity of symptoms. Recent studies in autism rodent models indicate that the KD improves core behavioral symptoms, albeit there were some sex and genotype-specific differences (Ahn et al., 2014; Castro et al., 2017; Dai et al., 2017; Kasprowska-Liskiewicz et al., 2017; Mantis et al., 2009; Ruskin et al., 2013, 2017a,b; Smith et al., 2016; Verpeut et al., 2016). Preliminary studies in humans also indicate improvement in autistic behaviors in response to the KD

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Abbreviations

ALS	amyotrophic lateral sclerosis
ANOVA	analysis of variance
ADHD	attention-deficit hyperactivity disorder
AGS	audiogenic-induced seizures
ASD	autism spectrum disorders
DAG	diacylglycerol
DGKκ	diacylglycerol kinase kappa
FC	fear conditioned
FMRP	fragile X mental retardation protein
FXS	fragile X syndrome
GABA	gamma-aminobutyric acid
IACUC	Institutional Animal Care and Use Committee
KD	ketogenic diet
LFD	low-fat diet
MCFA	medium-chain fatty acids
$mGluR_5$	metabotropic glutamate receptor 5
O-GlnNAc	O-linked-β-N-acetyl glucosamine
PA	phosphatidic acid
pP70S6K	phosphorylated protein 70 S6 kinase
PUFA	polyunsaturated fatty acids
P70SK6	protein 70 S6 kinase
S6K1	ribosomal S6 kinase 1
SEM	standard error of the mean
TST	tail suspension test
WR	wild running
WT	wild type

(Bostock et al., 2017; El-Rashidy et al., 2017; Evangeliou et al., 2003; Frye et al., 2011; Herbert and Buckley, 2013; Lee et al., 2018; Spilioti et al., 2013). Despite these successes, the mechanism underlying the success of the KD and ketosis is not understood, but most likely involves the restoration of aberrant energy metabolism. Possible effectors include adenosine, ketones, lactate dehydrogenase, medium-chain fatty acids (MCFA), neurotrophic factors, O-linked-β-N-acetyl glucosamine (O-GlnNAc), and polyunsaturated fatty acids (PUFA); and affected processes include epigenetic and gene expression mechanisms, the gamma-aminobutyric acid (GABA)ergic and cholinergic systems, inflammatory pathways, mitochondrial dynamics, oxidative stress, synaptic transmission and the gut microbiome (Boison, 2017; Cheng et al., 2017; Freche et al., 2012; Kossoff et al., 2009; Masino et al., 2009; Mychasiuk and Rho, 2017; Napoli et al., 2014; Newell et al., 2016a,b; Newell et al., 2016a,b; Newell et al., 2017; Stafstrom and Rho, 2012; D. C. Wallace et al., 2010; Lutas and Yellon, 2013; Yellon, 2008). Overall, the consensus is that the animal studies are promising, the mechanism of action is not understood, and the evidence in humans is insufficient to form an opinion as to the efficacy or lack thereof of the KD intervention for the treatment of autism. Herein, we tested the effects of chronic KD treatment on audiogenic-induced seizures (AGS), body weight, ketone and glucose levels, diurnal activity levels, fear conditioning and/or anxiety behaviors in *Fmr1*^{KO} mice and littermate controls as a function of age.

2. Materials and methods

Mouse Husbandry: The *Fmr1*^{KO} mice were originally developed by the Dutch-Belgian FXS Consortium and backcrossed > 11 times to FVB mice (Dutch-Belgian Fragile X Consortium, 1994). They were backcrossed into the C57BL/6 background by Dr. Bill Greenough's laboratory (University of Illinois at Urbana-Champaign) and distributed to other laboratories. We have maintained the *Fmr1*^{KO} mice in the C57BL/6 background at the University of Wisconsin-Madison for over 15 years with occasional backcrossing with C57BL/6J mice from Jackson Laboratories to avoid genetic drift. Breeding pairs for these experiments were housed in static microisolator cages on a 6 a.m.-6 p.m. light cycle with *ad libitum* access to food (Teklad2019) and water. Mouse ages and treatments for specific experiments are defined in the figure legends. The bedding (Shepherd's Cob + Plus, ¼ inch cob) contained nesting material as the only source of environmental enrichment. All animal husbandry and euthanasia procedures were performed under NIH and an approved University of Wisconsin-Madison animal care protocol administered through the Research Animal Resources Center with oversight from the Institutional Animal Care and Use Committee (IACUC). *Fmr1* genotypes were determined by PCR analysis of DNA extracted from tail biopsies with HotStarTaq polymerase (Qiagen Inc, Germantown, MD, USA; catalog #203205) and Jackson Laboratories' (Bar Harbor, ME, USA) primer sequences oIMR2060 [mutant forward; 5′-CAC GAG ACT AGT GAG ACG TG-3'], oIMR6734 [wild type (WT) forward; 5′-TGT GAT AGA ATA TGC AGC ATG TGA-3'], and oIMR6735 [common reverse; 5′-CTT CTG GCA CCT CCA GCT T-3'], which produces PCR products of 400 base pairs (*Fmr1*^{KO}) and 131 base pairs (WT). Heterozygote females exhibit both the 400 and 131 base pair bands.

Diets: The test diets included: Teklad2019 (Envigo, Fitchburg, WI, USA), AIN-76A (Bio-Serv catalog number F1515, Flemington, NJ, USA) and KD (Bio-Serv catalog number F3666, Flemington, NJ, USA]. Tekald2019 is a fixed formula diet with a nutritional profile of 19.0% protein, 9.0% fat, 2.6% fiber, 12.1% neutral detergent fiber, 5.0% ash, and 44.9% carbohydrate. The ingredients are ground wheat, ground corn, corn gluten meal, wheat middlings, soybean oil, calcium carbonate, dicalcium phosphate, brewers dried yeast, L-lysine, iodized salt, magnesium oxide, choline chloride, DL-methionine, calcium propionate, L-tryptophan, vitamin E acetate, menadione sodium bisulfite complex (source of vitamin K activity), manganous oxide, ferrous sulfate, zinc oxide, niacin, calcium pantothenate, copper sulfate, pyridoxine hydrochloride, riboflavin, thiamin mononitrate, vitamin A acetate, calcium iodate, vitamin B12 supplement, folic acid, biotin, vitamin D3 supplement, cobalt carbonate. The energy density is 3.3 kcal/kg. AIN-76A is a purified ingredient diet with a nutritional profile of 18.1% protein, 5.1% fat, 4.8% fiber, 2.9% ash, and 65.2% carbohydrate. The ingredients are sucrose, casein, corn starch, corn oil, cellulose, mineral mix, vitamin mix, DL-methionine, and choline bitartrate. The energy density is 3.79 kcal/g. The KD is a modified AIN-76A high fat paste with a ratio of fat to carbohydrate of 6:1 and a nutritional profile of 8.6% protein, 75.1% fat, 4.8% fiber, 3.0% ash, and 3.2% carbohydrate. The ingredients are lard, butter, corn oil, casein, cellulose, mineral mix, vitamin mix, and dextrose, and the caloric profile is 7.24 kcal/g.

Audiogenic-Induced Seizures: Mice were randomly assigned to treatment groups at weaning (postnatal day 18; P18), treated for 3 days with one of three diets (Teklad2019, AIN-76A, KD), and tested for susceptibility to AGS at P21, which is the age of peak sensitivity to AGS (Yan et al., 2004). Mice were transferred to a Plexiglas box (13″L X 8″W X 7″H) and exposed to a high-pitched siren (110 dB) from a personal body alarm (LOUD KEY™). The number of mice exhibiting wild running (WR), tonic seizures (AGS) and death were scored. Treatment groups were compared by the Fisher exact test.

Actigraphy: Rest-activity rhythms were assessed under standard lighting conditions in home-made Plexiglas® chambers containing passive infrared sensors mounted on the underside of the lids (Fenoglio-Simeone et al., 2009; E. Wallace et al., 2015). The dimensions of the transparent, cylindrical, Plexiglas® chambers were 6″ diameter X 10″ height. Mice were individually housed during actigraphy with access to food and water. Each gross movement of the animal was recorded as an activity count with VitalView acquisition software (Mini Mitter Inc., Bend, OR, USA). Activity counts were binned into 60-s epochs and

scored on an activity scale (0–50) over a 5-9-day period. Data were analyzed with ACTIVIEW Biological Rhythm Analysis software (Mini Mitter Company, Inc., Bend, OR, USA). One-minute activity epochs were averaged for the full days of the recording period (excluding the first and last partial days) to calculate total activity. One-minute activity epochs were averaged for the full recording days and binned into light and dark 6-h quadrants (first and last 6 h of lights ON, first and last 6 h of lights OFF) for the diurnal activity profiles. A Chi-square periodogram method was used to determine the diurnal rest-activity period. To determine habituation to the novel activity chambers, activity count data from the first 2 h of the first day in the chamber were analyzed. Average data was plotted ± the standard error of the mean (SEM) and statistical significance determined by Student's t-test.

Marble Burying: Corncob bedding was added to clean, empty cages and sixteen emerald-colored glass gems per cage were arranged in 4 × 4 grids over 2/3 of the cage. The cage was photographed before a mouse was added to the 1/3 of the cage without glass gems. After 30 min, the mouse was returned to its home cage, the cage was photographed, and the number of gems that were more than half buried were counted. Average data was plotted ± SEM.

Tail Suspension: The tail suspension test was performed as previously described (Steru et al., 1985). Briefly, the mouse was equilibrated to the test room for at least 30 min before testing. White bench paper was placed on the backside of and below a laboratory bench shelf to mask visual clues. A 1 mL pipette tip or straw was cut and placed on the mouse's tail to prevent the mouse from grabbing its tail or the shelf during the test. The mouse was suspended from the edge of the shelf approximately 60 cm above the benchtop using lab tape that was placed 1 cm from the tip of the tail. The mouse's abdomen faced outward toward a camera and the mouse's eyes faced the white bench paper. The 5-min test was recorded with a video camera and the footage was scored for the latency time to immobility as well the number and length of immobility bouts. Immobility was defined as lack of motion but included head movements to look around and sustained foot grabs as long as the mouse was not actively trying to escape. Average data was plotted ± SEM and statistical significance determined by analysis of variance (ANOVA) and post-hoc Bonferroni multiple comparison test.

Fear Conditioning: Delayed fear conditioning was tested in a Panlab Startle and Fear Combined System that included a LE1188 stimuli interface unit, LE111 load cell amplifier, and LE10026 shock generator with scrambler (Harvard Apparatus, Holliston, MA, USA). The chamber was prepared by turning the light on and setting the white noise to 70 dB. The load cell amplifier was set to 0.5 Hz with a gain of 5,000, and the shock generator was set to 0.6 mA. The mouse was equilibrated to the testing room for approximately 2 min before placement into the novel chamber. For the delayed fear conditioning paradigm (training day), the mice were: (1) acclimated or allowed to explore the chamber for 2 min; (2) exposed to a 30 s, 90 dB, 4,000 Hz sound with the last 2 s of sound exposure co-terminant with a 0.6 mA shock; (3) allowed to explore for 2 min; (4) re-exposed to a 30 s, 90 dB, 4000 Hz sound with the last 2 s of sound exposure co-terminant with a 0.6 mA shock; (5) allowed to explore for 1 min; and (6) removed from the chamber and returned to home cage. Percent freezing was analyzed with PACKWIN software (Harvard Apparatus, Holliston, MA, USA). After 24 h, the mice were tested for context learning. The context of the chamber was the same as the training day. The mice were allowed to explore the chamber for 6 min without sound or shock, and the percent freezing was determined. After a minimum of 30 min, the mice were tested for cued learning in which the shock grid was replaced with a round, flat-floored chamber and vanilla scent. The mice were presented with the same timing/sound paradigm as the training day, but no shocks. Percent of freezing was measured. For data analysis, the duration of freezing value was set to 1 s such that if the mouse did not freeze for at least 1 s, the time was not counted as freezing. The threshold was set at 7 based on visual assessment of scans of total freezing behavior throughout the experiment. Average data was plotted ± SEM and statistical significance determined by ANOVA and post-hoc Bonferroni multiple comparison test.

Ketone & Glucose Measurements: Ketone and glucose levels were assessed in urine or blood as indicated in the figure legends using a Precision Xtra blood glucose and ketone monitoring system (Abbott Diabetes Care Inc., Alameda, CA, USA). For urine samples, the mice were gently restrained at the neck and urine collected. It was not always possible to collect urine from live mice. Blood samples were collected from the abdominal artery after euthanization. Low (LO) glucose meter readings were adjusted to 20 md/dL. High (HI) off-scale glucose meter readings were adjusted to 500 mg/dL. High off-scale ketone meter readings were adjusted to 8.0 mmol/L. All samples were collected during the light phase. Fasting status is indicated in the table legends. Average data presented ± SEM and statistical significance determined by Student's t-test or ANOVA with post-hoc Bonferroni multiple comparison test.

PTZ-Induced Seizures: Seizures were induced with PTZ and scored as previously described (C. J. Westmark et al., 2008).

Nest Building Assay: Nest building was tested as previously described (R. M. Deacon, 2006; Moretti et al., 2005; Udagawa et al., 2013). Briefly, corncob bedding and two 2 × 2″ white cotton nestlets (catalog #NES3600; Ancare, Baltimore, NY, USA) were added to clean standard cages with feed and water. A single mouse was transferred to a nestlet cage at 3 pm. At 7 am the following day, the mouse was returned to its home cage. The empty nestlet cage was photographed and the length, width, and height of the nests were measured. Nests were scored based on the scale: (0) nestlet intact, (1) flat nest with partially shredded material, (2) shallow nest with shredded material but lacks fully formed walls, (3) nest with well-developed walls, and (4) nest in shape of cocoon with partial or complete roof. The volume of the nest was calculated by multiplying the length x height x width of the nest. Average data was plotted ± SEM and statistical significance determined by Student's t-test.

Sleep Deprivation: Sleep deprivation was induced by covering home cages with black fitted tarps for 7 days.

3. Results

The KD is associated with decreased seizures in juvenile male *Fmr1*^{KO} mice. We assessed seizures in *Fmr1*^{KO} mice in response to a 3-day treatment with KD (postnatal days 18–21; P18–P21) (Fig. 1). There was a reduction in wild running, seizures and deaths in response to 110 dB audiogenic stimulation in male mice (3% rates for each). The attenuation of wild running and seizures with the KD was statistically significant when compared with both Teklad2019 (chow) and AIN-76A (purified ingredient) control diets. In contrast, the KD did not attenuate seizure phenotypes in female *Fmr1*^{KO} mice indicating a strong sex-specific difference in response to diet (Fig. 1). A 3-day treatment with the KD also significantly reduced body weight by 17% in *Fmr1*^{KO} females, 18% in *Fmr1*^{KO} males, and 27% in WT males ($P \leq 0.004$), but not in *Fmr1*^{HET} females compared to mice maintained on AIN-76A, (Table 1). Urine ketone and glucose levels were elevated in response to KD (Supplementary Tables 1 and 2).

The KD alters diurnal activity levels in young adult WT and *Fmr1*^{KO} mice. In addition to juvenile seizures, the KD affects diurnal rest-activity rhythms in young adult, adult and aged adult *Fmr1*^{KO} mice. We tested two KD treatment paradigms in young adult mice. In the first cohort, WT and *Fmr1*^{KO} male littermate mice were weaned onto AIN-76A versus KD at P18 and maintained on their respective diets throughout testing (Fig. 2A). Actigraphy testing was conducted at 3 months of age (equivalent to 23 human years) after 10 weeks on AIN-76A versus KD. There was a greater than 30% decrease in total activity in both WT and *Fmr1*^{KO} mice in response to KD (Fig. 2B, Supplementary Fig. 1). Habituation to the novel actigraphy chambers was not affected by diet (Fig. 2C). Actigraphy counts were averaged over 7 days and binned into 6-h quadrants ([first (6am-12pm) and second (12pm–6pm)

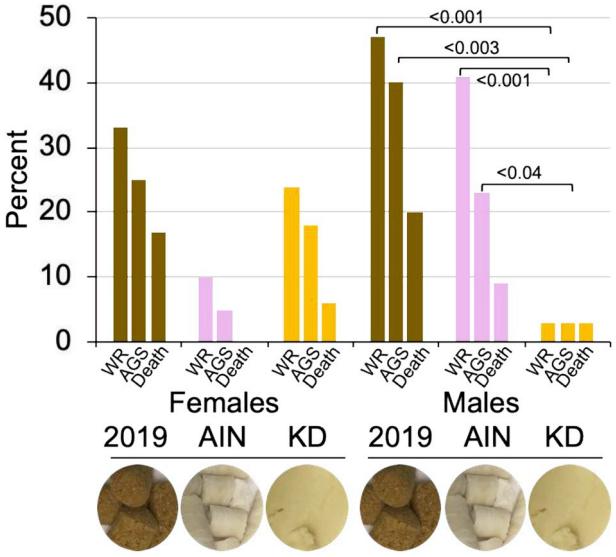

Fig. 1. The KD rescues seizures in *Fmr1KO* mice. Juvenile *Fmr1KO* mice were weaned onto vivarium chow (Teklad2019; brown bars), AIN-76A (control purified ingredient diet, vitamin-matched to KD; pink bars) or KD (Bio-Serv F3666, gold bars) at postnatal day (P18) and tested for AGS susceptibility at P21. Female cohorts contained: Teklad2019 (n = 12), AIN-76A (n = 20) and KD (n = 33). Male cohorts contained: Teklad2019 (n = 15), AIN-76A (n = 22) and KD (n = 32) mice. Data for WT littermates are not shown as they have a low susceptibility to AGS (0% in this experiment, usually < 5%), which did not change in response to the KD (0%). WR was significantly reduced in male *Fmr1KO* mice with KD compared to both Teklad2019 ($P < 0.001$) and AIN-76A ($P < 0.001$) by Fisher exact test. AGS was significantly reduced in male *Fmr1KO* mice with KD compared to both Teklad2019 ($P < 0.003$) and AIN-76A ($P < 0.04$) by Fisher exact test.

halves of the light cycle and first (6pm-12am) and second (12am–6am) halves of the dark cycle]. Activity was significantly reduced during the first half of the light cycle and the first and second halves of the dark cycle in both WT and *Fmr1*^{KO} male mice in response to KD (Fig. 2D). The second half of the light cycle is the lowest activity period for the mice and was unchanged in response to KD. The circadian period length, or the amount of time from an organism's waking to the next day's waking, was unchanged in response to diet or genotype for animals that exhibited measurable circadian periods [WT/AIN-76A 1448 ± 6.4 min, WT/KD 1453 ± 6.0 min, *Fmr1*^{KO}/AIN-76A 1446 ± 4.8 min, and *Fmr1*^{KO}/KD 1440 ± 0 min]; however, 50% of WT mice (n = 3/6) and 78% of *Fmr1*^{KO} mice (n = 7/9) on KD did not exhibit measurable circadian periods compared to 14% (n = 1/7) and 18% (n = 2/11), respectively, on AIN-76.

In addition to actigraphy, these mice were assessed in marble burying, tail suspension, and fear conditioning behavioral tests. Marble burying was tested in the afternoons (second half of light cycle) and was unchanged in response to KD (Fig. 2E, Supplementary Fig. 2). Mice were tested in the tail suspension test in the mornings (first half of the light cycle) and the frequency of immobility, latency time to first immobility, and the total duration of immobility were not significantly different between cohorts in response to KD (Fig. 2F) suggesting that significantly decreased locomotion in the actigraphy assay is not due to a depressed state. Mice were tested in context and cued fear conditioning (Fig. 2G; Supplementary Fig. 3). Our data normalized to baseline by the subtraction method indicate impaired learning during training and in context testing in *Fmr1*^{KO} compared to WT, but no significant changes in cued learning or in response to KD. Data normalization to baseline measures can be a major source of variability in fear conditioned testing (Tipps et al., 2014). The raw data without background correction indicate impaired learning during training and in both context and cued learning in *Fmr1*^{KO} and no significant effect with KD (Supplementary Fig. 4).

Mice were weighed at 3 and 5 months of age and there was a 21–56% reduction in body weight gain in response to KD ($P \leq 0.0003$) (Table 2). Blood ketone levels (nonfasting) were elevated at 5 months of age in both WT and *Fmr1*^{KO} mice on KD, and glucose levels (nonfasting) were significantly reduced in *Fmr1*^{KO} mice (Supplementary Table 3). In terms of adverse reactions, 3 WT mice treated with the KD died before the end of the study, 1 WT mouse on the KD was culled because it was sickly, and 2 *Fmr1*^{KO} mice (1 on KD and 1 runt weaned onto AIN-76A) died before the end of the study.

For the second KD treatment paradigm in young adult mice, female and male mice were transferred to KD versus AIN-76A as adults (2–3 months old) (Fig. 3A). Actigraphy was assessed after 4 weeks of treatment. Although, average total 24-h activity counts in the chambers did not differ as a function of diet or genotype in females or males (Fig. 3B and 3C), the KD increased activity in *Fmr1*^{KO} female mice during both the first (27%) and second (49%) halves of the light cycle ($P \leq 0.01$) (Fig. 3D), and reduced activity in *Fmr1*^{KO} male mice during the first (27%) and second (24%) halves of the dark cycle (Fig. 3E). In addition, there was a significant 38% increase in activity in *Fmr1*^{KO} mice compared to WT during the first half of the light cycle ($P \leq 0.04$) in the mice maintained on AIN-76A. We have repeatedly observed a 40% increase in activity during this time quadrant in *Fmr1*^{KO} mice compared to WT maintained on Teklad2019 (unpublished results). Thus, there are sex-specific differences in the *Fmr1*^{KO} response to the KD for both AGS and actigraphy. After 4 weeks KD treatment, male WT and *Fmr1*^{KO} mice on KD weighed significantly less than littermates on AIN-76A, female *Fmr1*^{HET} and *Fmr*^{KO} mice did not exhibit altered body weight, urine ketones were elevated in *Fmr1*^{KO} females on KD, and urine glucose was highly elevated in male and female *Fmr1*^{KO} on KD (Fig. 3F, Supplementary Table 4, Supplementary Note 1). After 8 weeks treatment, male WT and *Fmr1*^{KO} mice exhibited significantly reduced body weight, female *Fmr1*^{HET} did not exhibit altered body weight, and surprisingly, *Fmr1*^{KO} female mice exhibited increased body weight (10%) on KD compared to AIN-76A (Fig. 3F). Individual weight data for each mouse is graphed (Supplementary Figs. 5 and 6). Blood ketone levels (fasting) were elevated in all cohorts in response to KD. Blood glucose (fasting) was significantly reduced in *Fmr1*^{HET} females and *Fmr1*^{KO} males with trends for reduced glucose in all cohorts in response to KD (Supplementary Table 4). Marble burying, habituation to the actigraphy chambers, and depression as assessed by the tail suspension assay were not different in response to diet (Fig. 3G, 3H, 3I). In terms of

Table 1
Body weight in postnatal mice in response to KD.

Genotype	Teklad2019	AIN-76A	KD	[a]*P*
Fmr1^{HET} Female	ND	7.64 ± 0.51 (n = 10)	7.15 ± 0.25 (n = 9)	0.42
Fmr1^{KO} Female	7.80 ± 0.51 (n = 12)	8.38 ± 0.46 (n = 20)	6.99 ± 0.10 (n = 33)	0.004, [b]AIN vs KD
WT Male	ND	8.63 ± 0.43 (n = 3)	6.29 ± 0.20 (n = 12)	0.0002
Fmr1^{KO} Male	7.75 ± 0.41 (n = 15)	8.69 ± 0.40 (n = 22)	7.13 ± 0.17 (n = 32)	0.001, [b]AIN vs KD

[a] Statistical significance was determined by Student t-test to compare 2 diets and by one-way ANOVA followed by a Bonferroni's multiple comparison test to compare 3 diets.
[b] Statistically significant difference.

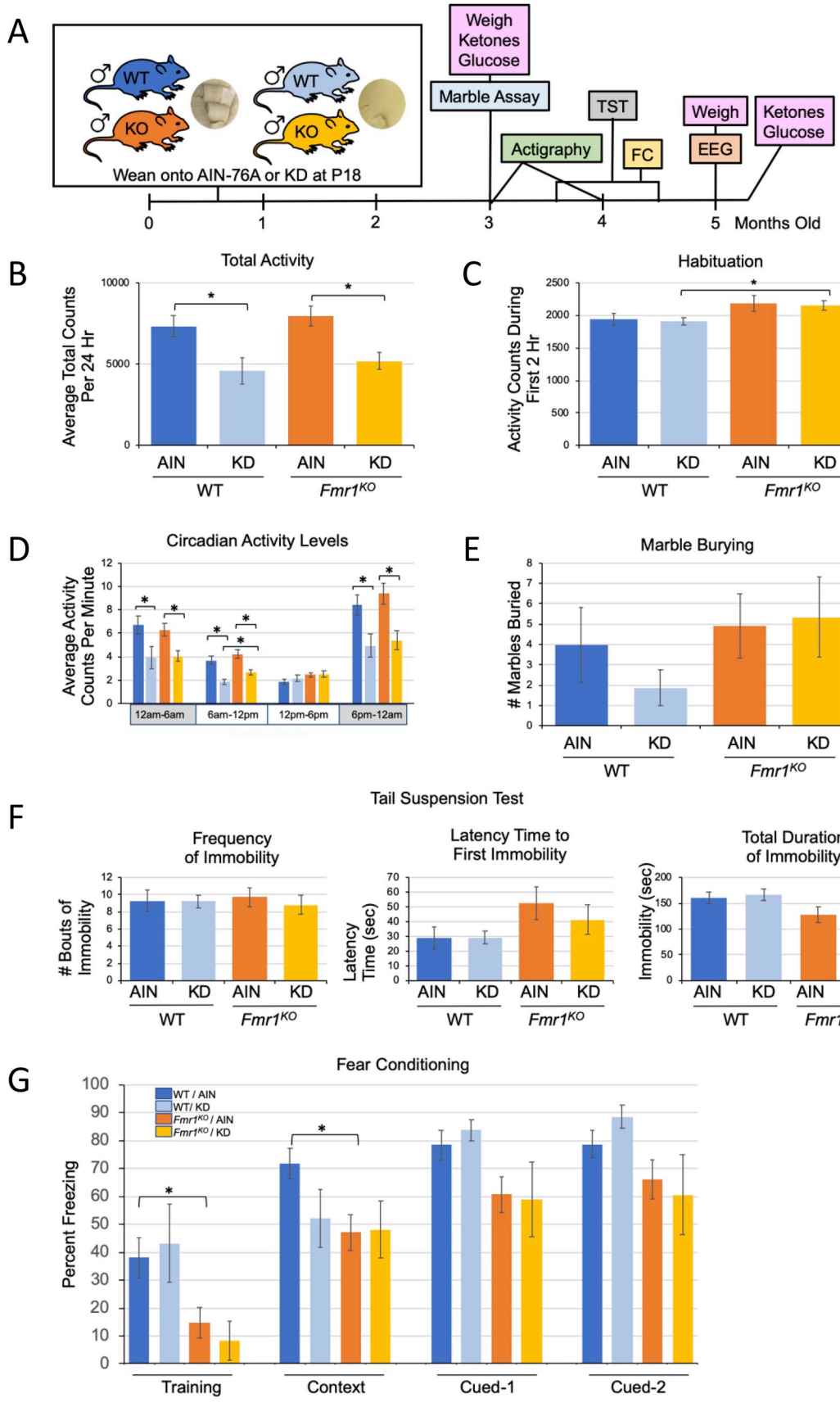

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Fig. 2. The KD affects diurnal rest-activity rhythms in young adult WT and *Fmr1KO* mice (cohort 1). Juvenile WT and *Fmr1KO* littermate male mice were weaned onto AIN-76A versus KD at P18 and maintained on their respective diets throughout testing. (A) Schematic of testing timeline. (B) Activity counts over 24 h in the actigraphy chambers in WT/AIN-76A (n = 7; blue bars), WT/KD (n = 6; light blue bars), *Fmr1KO*/AIN-76A (n = 11; orange bars), and *Fmr1KO*/KD (n = 9; gold bars) were averaged over 7 full days and plotted ± SEM per treatment condition to assess total activity levels, which were decreased with KD treatment in both WT ($P = 0.021$) and *Fmr1KO* ($P = 0.004$) by Student's t-test. (C) Activity counts during the first 2 h in the actigraphy chambers were summed, averaged and plotted ± SEM per treatment condition to assess habituation, which was not altered in response to diet. (D) Diurnal activity levels were assessed over 7 full days in the actigraphy chambers and found to be reduced in WT and *Fmr1KO* mice during both halves of the dark cycle and the first half of the light cycle ($P < 0.05$). (E) Marble burying was not significantly different in response to genotype or diet. (F) Tail suspension test (TST) metrics (frequency of immobility, latency time to the first episode of immobility and total duration time of immobility) were not significantly different in response to genotype or diet. (G) Fear conditioned (FC) learning was impaired in *Fmr1KO*/AIN-76A mice compared to WT/AIN-76A during training and in the context test by Student t-test. Learning was not significantly different in response to KD by ANOVA.

Table 2
Body weight in adult mice in response to KD.

Metric	Genotype	AIN-76A	KD	% Δ	*P*
Body Weight at 3 mo	WT	27.2 ± 0.30 (n = 7)	12.1 ± 1.7 (n = 7)	56	1.7E-6
	$Fmr1^{KO}$	24.7 ± 0.89 (n = 10)	15.8 ± 1.8 (n = 9)	36	0.0003
Body Weight at 5 mo	WT	32.9 ± 0.78 (n = 7)	17.4 ± 1.6 (n = 6)	47	2.1E-6
	$Fmr1^{KO}$	28.2 ± 0.59 (n = 10)	22.2 ± 1.3 (n = 8)	21	0.0004

adverse reactions, at the end of the study when the mice were culled, two $Fmr1^{KO}$ mice on KD exhibited abnormal intestinal pathology.

The KD alters diurnal activity levels in adult WT and $Fmr1^{KO}$ mice. We conducted a crossover study to test behavioral and biochemical measures in adult male WT and $Fmr1^{KO}$ littermate mice (6 months old; the human equivalent of 34 years) in response to KD (Fig. 4A). Adult mice were transferred from Teklad2019 to AIN-76A for 3 weeks and then crossed over to KD. A neuroassessment battery was performed pre- and post-crossover and showed no abnormalities except reduced body weight and dirty coat (fur in contact with greasy feed) with KD (Supplementary Table 5). Reduced body weight was statistically significant for the WT mice after 2 weeks of treatment and for the $Fmr1^{KO}$ mice after 1-week treatment (Fig. 4B). Specifically, WT mice lost 24% body weight on KD ($P = 0.01$) and $Fmr1^{KO}$ lost 11% body weight with KD ($P < 0.01$). Urine ketone levels were significantly higher in $Fmr1^{KO}$ mice compared to WT at both 2 and 3 of weeks KD treatment (Supplementary Table 6). Urine glucose levels were higher in $Fmr1^{KO}$ mice after both 3 weeks of AIN-76A treatment and 3 weeks of KD treatment (Supplementary Table 6).

Actigraphy was performed pre- and post-crossover to KD and demonstrated significantly increased activity in $Fmr1^{KO}$ mice compared to WT during the first half of the light cycle (41%) in the pre-crossover test as well as significantly reduced activity in $Fmr1^{KO}$ mice post-KD in 3 of 4 binned timeframes (12am–6am, 6am-noon and 6pm-midnight) (Fig. 4C). Passive avoidance and PTZ-induced seizures were also conducted post-crossover and demonstrated no statistically significant differences between WT and $Fmr1^{KO}$ mice maintained on KD (Fig. 4D and 4E).

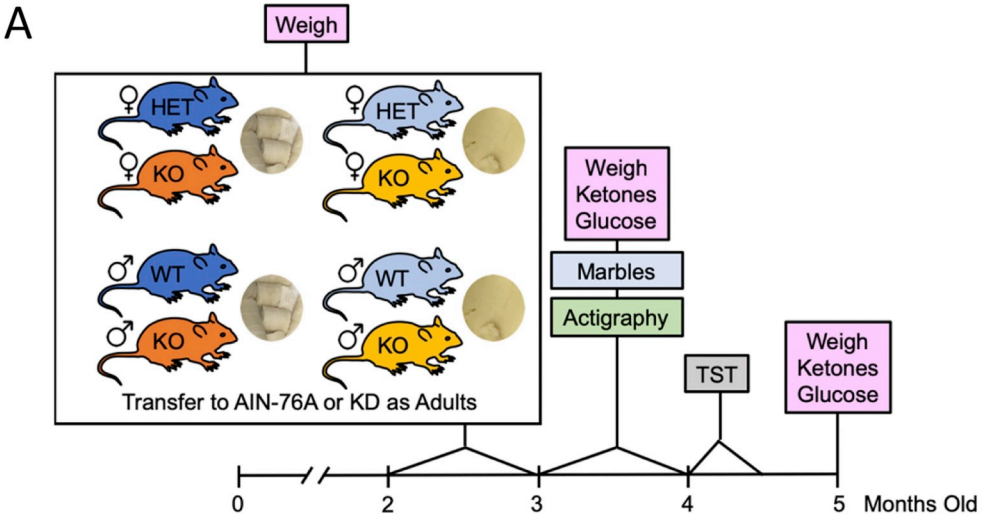
The KD alters diurnal activity levels in aged $Fmr1^{KO}$ mice. Aged $Fmr1^{KO}$ animals were tested in actigraphy, behavioral and biochemistry assays in response to diet. Two experiments were performed, Teklad2019 versus KD and AIN-76A versus KD, with female and male $Fmr1^{KO}$ mice (Fig. 5A). Treatments commenced at 19–21 months of age (human equivalent of 67 years). The pre-treatment neuroassessment battery in the aged mice showed that 4 out of 26 of the females and 1 out of 28 of the male $Fmr1^{KO}$ mice had cataracts and 2 mice had eyelid closure at the start of the experiment (Supplementary Table 7). A spontaneous seizure was observed in 1 mouse. Actigraphy was conducted over 5 days (data averaged for 3 full days) and showed a statistically significant decrease in total activity in the males (43%, $P = 0.005$) (Fig. 5B), decreased activity in females during the second half of the dark cycle (43%, $P = 0.008$), and decreased activity in males during both halves of the dark cycle (49–54%, $P \leq 0.001$) in response to KD compared to Teklad2019 (Fig. 5C). Habituation data from the first 2 h in the chambers were collected for the male mice and showed a significant 29% decrease in activity counts with KD ($P = 0.025$) (Fig. 5D). For the AIN-76A versus KD experiment, KD significantly reduced total activity levels in female mice (29%, $P = 0.04$) (Fig. 5E), and activity in females and males during the first half of the dark cycle (29–43%, $P < 0.03$) (Fig. 5F). Females maintained on AIN-76A were significantly more active than males (36%, $P = 0.04$) (Fig. 5E). $Fmr1^{KO}$ mice treated with KD built poorer nests compared to Teklad2019 (Fig. 5G; Supplementary Table 8; Supplementary Fig. 7).

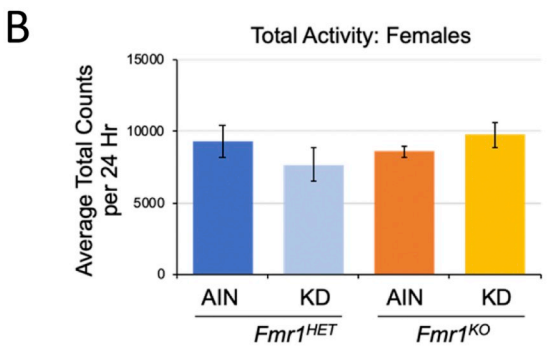
Urine ketone levels were elevated in response to KD in comparison to both Teklad2019 and AIN-76A in males and females (Supplementary Tables 9 and 10). Urine glucose levels were elevated in male $Fmr1^{KO}$ in response to KD versus Teklad2019 and AIN-76A (Supplementary Table 10). A limited number of aged $Fmr1^{HET}$ female and WT male mice fed Teklad2019 were tested for ketone and glucose levels, which appeared equivalent to aged $Fmr1^{KO}$ mice fed Teklad2019 (Supplementary Table 11). In terms of adverse effects in the female $Fmr1^{KO}$ on KD, 1 mouse died 5 days after the start of treatment. Upon completion of the study, one female $Fmr1^{KO}$ mouse on KD had a hard mass in the stomach, one had an enlarged pancreas and liver, and one had a bad sore on the back of the neck. With the AIN-76A, one female $Fmr1^{KO}$ had a huge mass in the abdomen. For the male $Fmr1^{KO}$ on KD, one mouse had an ear wound that was bleeding, one had an enlarged spleen and liver with hemolysis of the blood and shrunken blood vessels, and one had a pale liver and small spleen at the time of tissue collection.

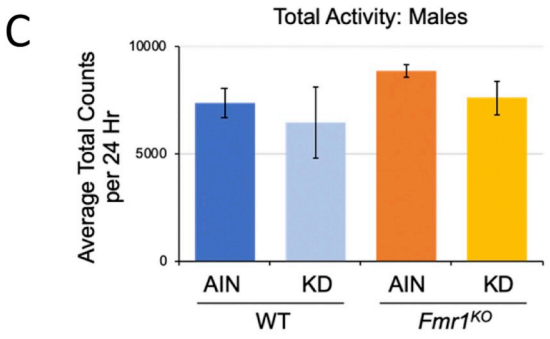
Aged $Fmr1^{KO}$ mice do not exhibit AGS. A separate cohort of aged $Fmr1^{KO}$ mice (17–19 months old) were tested for susceptibility to AGS and glucose levels. AGS are typically tested at P21 because C57BL/6J exhibit age-related hearing loss. The mitochondrial deacetylase Sirt3 prevents age-related hearing loss under caloric restriction in C57BL/6J (Someya et al., 2010), and *Sirt3* is an FMRP binding target (Darnell et al., 2011). There was no incidence of AGS in aged male (n = 8) or female (n = 8) $Fmr1^{KO}$ mice, urine glucose levels were 2.2-fold higher in males [76.88 ± 10.66 (n = 8)] than in females [35.67 ± 6.90 (n = 5), $P = 0.01$]. Dermatitis was present in 63% of aged females and 13% of aged males.

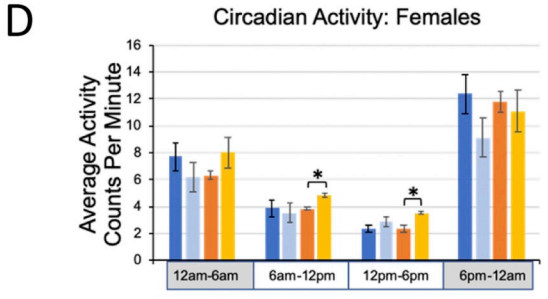
Sleep deprivation increases rest during the light cycle in aged $Fmr1^{KO}$ mice. Sleep deprivation was tested in a third cohort of aged male $Fmr1^{KO}$ mice. Pre-treatment neuroassessments were normal (Supplementary Table 12). Mice were sleep deprived for 7 days by exposure to constant darkness. Actigraphy under standard lighting conditions resulted in a 34% reduction ($P = 0.002$) in activity in the sleep-deprived cohort during the 2nd half of the light cycle (Fig. 5H), which corresponds to improved rest when the mice are supposed to be sleeping. Sleep deprivation did not alter overall activity levels (normal

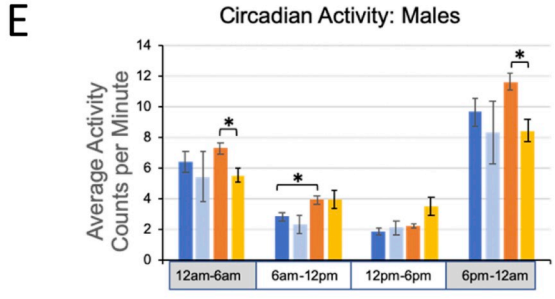
lighting 8,420 ± 664 counts per 24 h; sleep deprived, 8,168 ± 419 counts per 24 h). After completion of actigraphy testing, mice underwent a second sleep deprivation treatment followed by light/dark box transition testing and urine glucose measurements. Sleep deprivation did not alter latency time to enter the dark side of a light/dark shuttle box or baseline urine glucose levels (Supplementary Table 13).











F

Body Weight in Adult Mice in Response to KD

Metric	Genotype	AIN-76A	KD	*P*
Body Weight pre-treatment (grams)	*Fmr1*^{HET} (females)	21.16 ± 0.66 (n=5)	20.85 ± 0.58 (n=5)	0.74
	Fmr1^{KO} (females)	20.11 ± 0.33 (n=4)	20.24 ± 0.53 (n=3)	0.83
	WT (males)	26.18 ± 0.77 (n=6)	25.01 ± 0.61 (n=4)	0.31
	Fmr1^{KO} (males)	25.96 ± 0.86 (n=4)	26.27± 0.61 (n=6)	0.77
Body Weight after 4 weeks treatment (grams)	*Fmr1*^{HET} (females)	21.63 ± 0.69 (n=5)	21.34 ± 0.20 (n=2)	0.69
	Fmr1^{KO} (females)	20.80 ± 0.36 (n=4)	21.87 ± 0.42 (n=3)	0.11
	WT (males)	27.37 ± 0.72 (n=6)	22.21 ± 0.80 (n=4)	0.002
	Fmr1^{KO} (males)	26.80 ± 0.98 (n=4)	23.50 ± 0.79 (n=6)	0.03
Body Weight after 8 weeks treatment (grams)	*Fmr1*^{HET} (females)	23.51 ± 0.52 (n=5)	23.29 ± 0.37 (n=4)	0.75
	Fmr1^{KO} (females)	22.08 ± 0.52 (n=4)	24.25 ± 0.20 (n=3)	0.02
	WT (males)	30.94 ± 0.87 (n=6)	21.62 ± 1.53 (n=4)	0.0004
	Fmr1^{KO} (males)	28.47 ± 0.88 (n=4)	23.50 ± 1.13 (n=6)	0.01

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Fig. 3. The KD affects diurnal rest-activity rhythms in young adult WT, *Fmr1HET*, and *Fmr1KO* mice (cohort 2). Adult littermate female and male mice were weaned onto AIN-76A versus KD at 2–3 months of age and maintained on their respective diets throughout testing. (A) Schematic of testing timeline. (B) Activity counts over 24 h in the actigraphy chambers in female *Fmr1HET*/AIN-76A (n = 5; blue bars), *Fmr1HET*/KD (n = 5; light blue bars), *Fmr1KO*/AIN-76A (n = 4; orange bars) and *Fmr1KO*/KD (n = 3; gold bars) were averaged over 7 full days and plotted ± SEM per treatment condition to assess total activity levels in females, which were unchanged in response to diet. (C) Activity counts over 24 h in the actigraphy chambers in male WT/AIN-76A (n = 6; blue bars), WT/KD (n = 4; light blue bars), *Fmr1KO*/AIN-76A (n = 4; orange bars) and *Fmr1KO*/KD (n = 6; gold bars) were averaged over 7 full days and plotted ± SEM per treatment condition to assess total activity levels in males, which were unchanged in response to diet. (D) Diurnal activity levels were assessed over 7 full days in the actigraphy chambers and found to be elevated in response to KD in female *Fmr1KO* mice during both halves of the light cycle by Student t-test, $P \leq 0.01$. (E) Diurnal activity levels were assessed over 7 full days in the actigraphy chambers and found to be reduced in male *Fmr1KO* during both halves of the dark cycle, $P < 0.03$. In addition, there was a statistically significant increase in activity during the first half of the light cycle comparing WT and *Fmr1KO* maintained on AIN-76A, $P < 0.04$. (F) Body weight was measured after 4- and 8-weeks of KD treatment and was unchanged in *Fmr1HET*, elevated at 8 weeks in *Fmr1KO* fed KD, and reduced in WT and *Fmr1KO* males fed KD at both 4 and 8 weeks by Student t-test, $P \leq 0.03$. (G) Marble burying was not significantly different in response to genotype or diet. (H) Activity counts during the first 2 h in the actigraphy chambers were summed, averaged and plotted ± SEM per treatment condition to assess habituation, which was not altered in response to diet in females or males. (I) Tail suspension metrics (frequency of immobility, latency time to the first episode of immobility and total duration time of immobility) were not significantly different in response to genotype or diet.

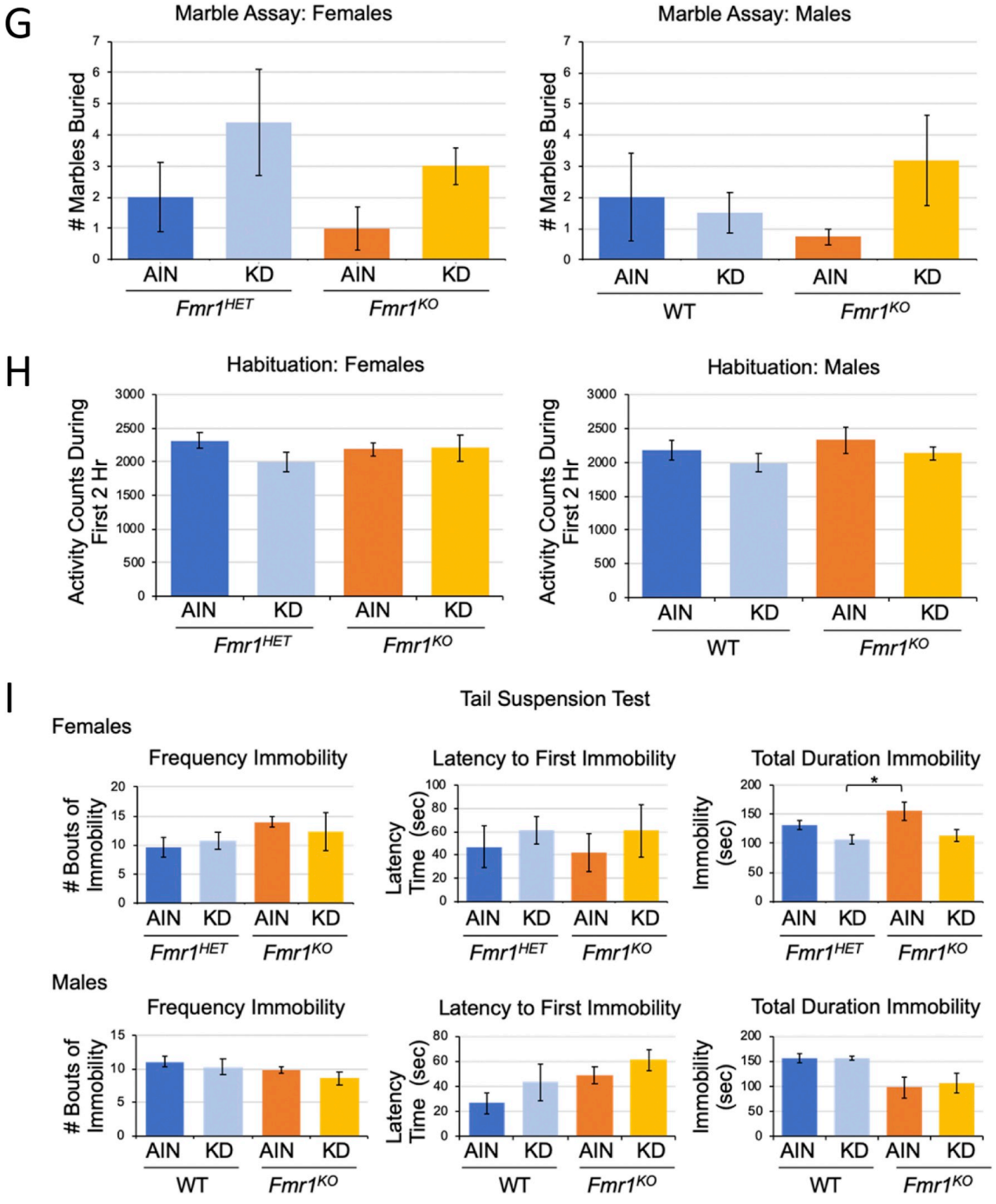


Fig. 3. (*continued*)

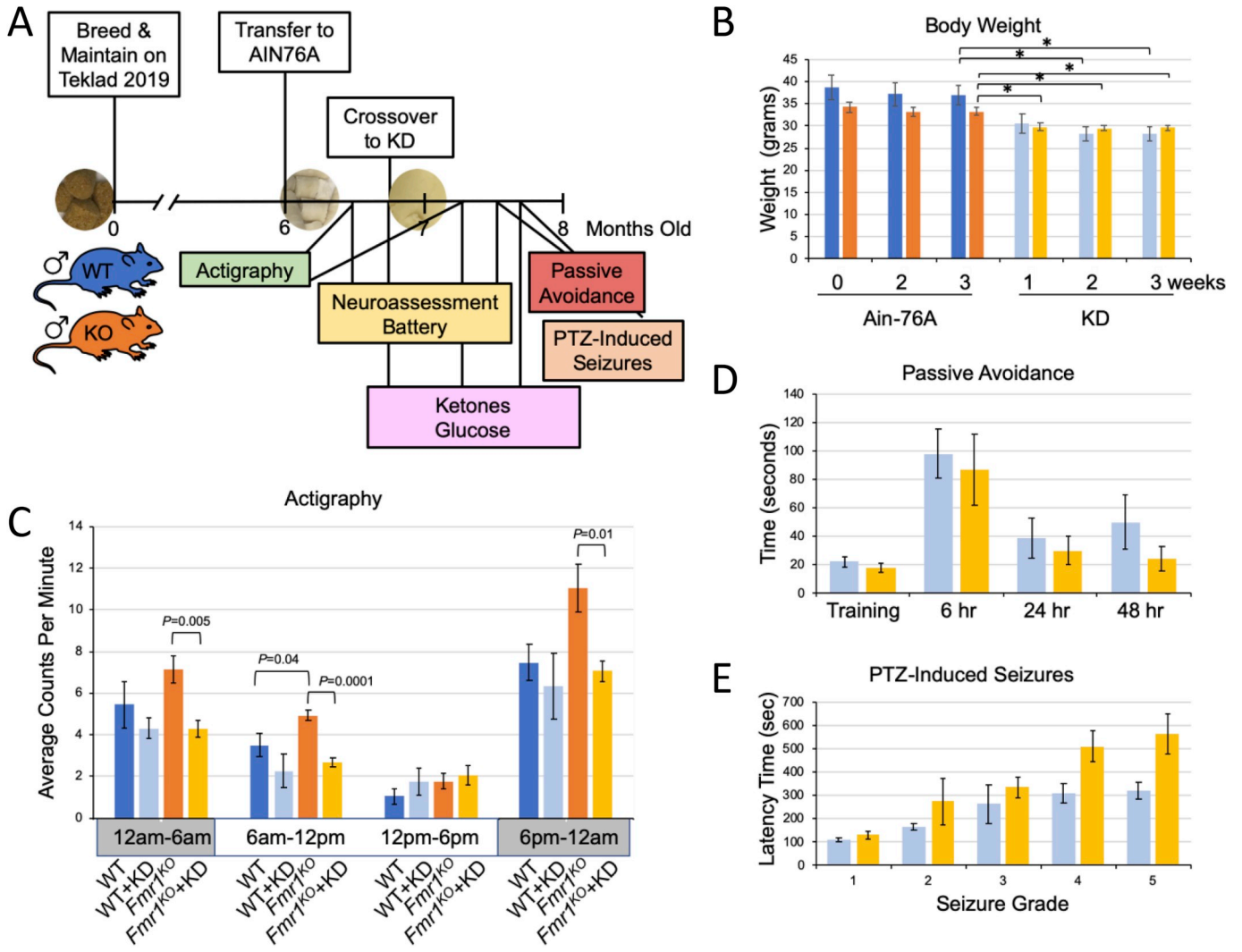
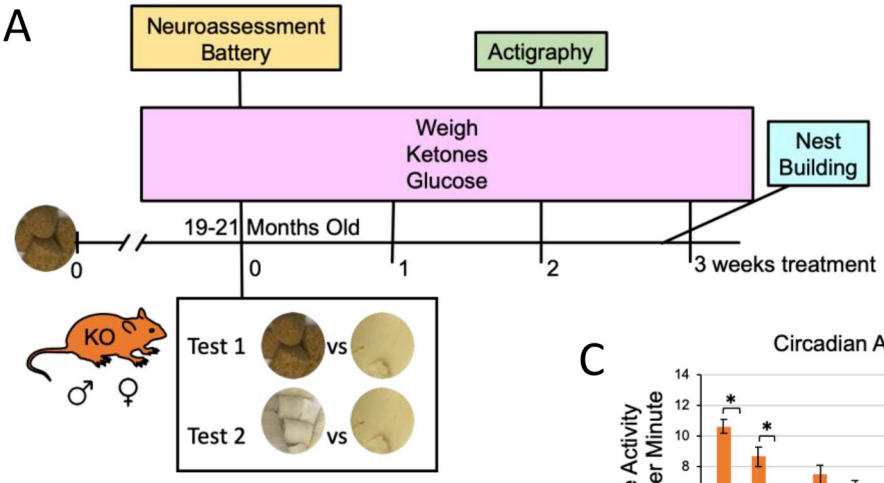
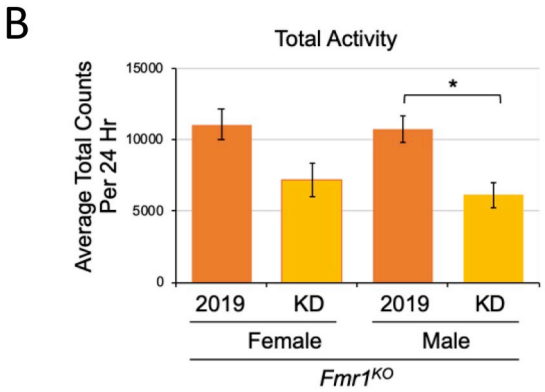


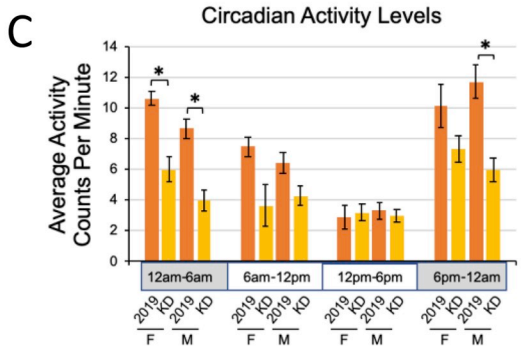
Fig. 4. The KD affects diurnal rest-activity rhythms in adult WT and *Fmr1KO* mice. Adult mice were transferred from Teklad2019 to AIN-76A for 3 weeks and then crossed over to KD. (A) Schematic for behavioral and biochemical testing in adult WT and *Fmr1KO* crossover study with KD. Mice (6 months old) that had been maintained on Teklad2019 were weighed and transferred to the AIN-76A control diet for 2 weeks. After 2 weeks on AIN-76A (Day 1), mice underwent an abbreviated neuroassessment battery and actigraphy for 5 days (Days 2–6) followed by an abbreviated neuroassessment and urine ketone and glucose measurements before transfer to KD (Day 8). After 2 weeks on KD (Day 22), mice underwent an abbreviated neuroassessment and urine ketone and glucose measurements. Actigraphy was conducted for 5 days (Days 23–27) followed by a neuroassessment battery and passive avoidance (Day 30), and ketone/glucose measurements and PTZ-induced seizure testing (Day 32). (B) Body weight was measured weekly and found to be significantly reduced in both WT (n = 5; blue bars AIN-76A and light blue bars KD) and *Fmr1KO* (n = 11; orange bars AIN-76A and gold bars KD) mice by 2 weeks of KD treatment by Student test, $P \leq 0.01$. (C) Diurnal activity levels were quantitated from the first full day in the actigraphy chambers pre- and post-crossover to KD and found to be reduced in response to KD in *Fmr1KO* mice (n = 5) compared to WT (n = 3) during the first half of the light cycle and during both halves of the dark cycle, $P \leq 0.01$. As each mouse was tested twice in the chambers (pre- and post-KD), there could be a small habituation effect with the KD data. *Fmr1KO* mice maintained on AIN-76A during the pre-crossover testing were hyperactive during the first half of the dark cycle compared to WT littermates, $P = 0.04$. (D) Passive avoidance was tested post-crossover and exhibited no differences between WT (n = 5; light blue bars) and *Fmr1KO* (n = 11; gold bars) on KD. (E) Sensitivity to PTZ-induced seizures was tested post-crossover and exhibited no differences between WT (n = 5; light blue bars) and *Fmr1KO* (n = 11; gold bars) on KD.

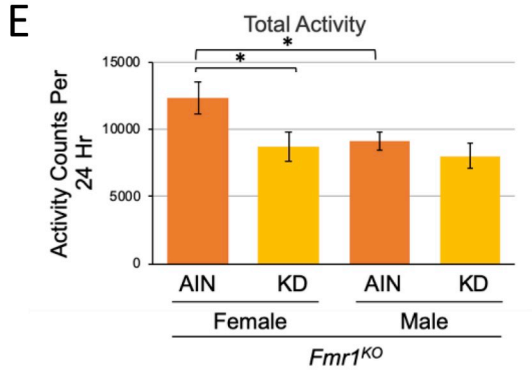
4. Discussion

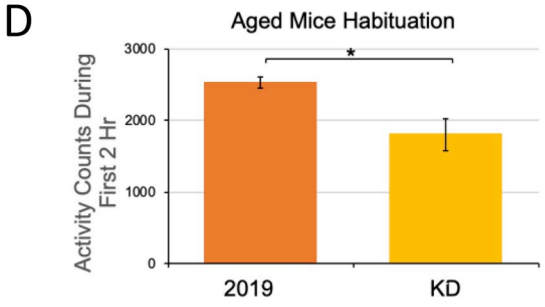
The KD is the new fad diet for weight loss and is purported to improve a wide range of disorders including diabetes, Alzheimer's disease, and ASD. Others have reviewed current nutritional approaches in managing ASD, the fundamental metabolic processes that promote brain health, and case studies testing the KD in autism (Boison et al., 2017; Bostock et al., 2017; Cekici and Sanlier, 2017). In addition, three new case studies successfully employed the KD in the treatment of autism and thus increase the available evidence regarding the safety and efficacy of this treatment for ASD (El-Rashidy et al., 2017; Lee et al., 2018; Zarnowska et al., 2018). At the molecular level, the KD enhances mitochondrial function and affects molecular targets related to the symptoms and comorbidities of ASD (Cheng et al., 2017). The effects of KD on mouse behavior have been studied in several autism models including succinic semialdehyde dehydrogenase (SSADH) deficiency (normalizes EEG activity), Rett syndrome (improves motor behavior and reduced anxiety), BTBR mice (decreases sociability in 3 chamber test, decreases self-directed repetitive behavior, and improves social communication in a food preference assay), El mouse that models progressive spontaneous epilepsy (improves sociability, decreases repetitive behaviors, has more pronounced effects in females), and valproic acid exposure model of autism (improves social behavior in mice) (Cheng et al., 2017). BTBR mice maintained on KD from weaning for 14 days exhibit lower average body weight, lower average brain weight, and an increased level of the ketone beta-hydroxybutyrate (Mychasiuk





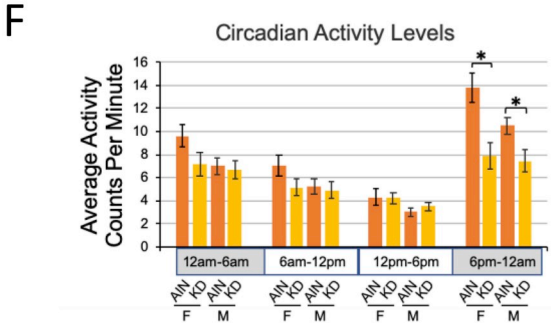


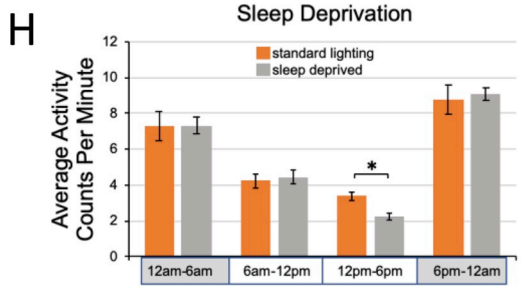




G

Nest Scores in Aged *Fmr1*^{KO} Mice in Response to Diet			
Metric	**2019 (n=8)**	**KD (n=10)**	***P***
Nest Score	1.2 ± 0.42	0.35 ± 0.13	0.05
Volume of Nest	33 ± 9	36 ± 13	0.87
% Shredded Material	44 ± 15	21 ± 11	0.21





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and Rho, 2017). Gene expression analysis indicates a limited number of differentially expressed genes in the brains of KD fed BTBR mice as compared to control diet with the most substantial changes found in mitochondrial bioenergetics, neurotransmitter signaling, and hormonal metabolism (Mychasiuk and Rho, 2017). Long-term KD contributes to glycemic control but promotes lipid accumulation and hepatic steatosis

Fig. 5. The KD affects diurnal rest-activity rhythms in aged *Fmr1KO* mice. Aged *Fmr1KO* female and male mice that had been maintained on Teklad2019 until 19–21 months of age were randomized onto Teklad2019 versus KD or onto AIN-76A versus KD. (A) Schematic of testing timeline. (B) Activity counts over 24 h in the actigraphy chambers in female *Fmr1KO*/Teklad2019 (n = 3; orange bars), female *Fmr1KO*/KD, (n = 3; gold bars), male *Fmr1KO*/Teklad2019 (n = 5; orange bars), and male *Fmr1KO*/KD, (n = 7; gold bars) were averaged over 3 full days and plotted ± SEM per treatment condition to assess total activity levels, which were decreased with KD in male *Fmr1KO* by Student t-test, $P = 0.005$. (C) Diurnal activity levels were assessed over 3 full days in the actigraphy chambers and found to be reduced in female *Fmr1KO* mice during the second half of the dark cycle and during both halves of the dark cycle in *Fmr1KO* male mice, $P \leq 0.002$. (D) Activity counts during the first 2 h in the actigraphy chambers were summed, averaged and plotted ± SEM per treatment condition to assess habituation, which was decreased in response to KD in male mice, $P < 0.03$. (E) Activity counts over 24 h in the actigraphy chambers in female *Fmr1KO*/AIN-76A (n = 8), female *Fmr1KO*/KD, (n = 8), male *Fmr1KO*/AIN-76A (n = 7), and male *Fmr1KO*/KD, (n = 8) were averaged over 3 full days and plotted ± SEM per treatment condition to assess total activity levels, which were decreased with KD in female *Fmr1KO*. $P = 0.04$. (F) Diurnal activity levels were assessed over 3 full days in the actigraphy chambers and found to be reduced in both female and male mice during the first half of the dark cycle in response to KD, $P < 0.03$. (G) Nest building was assessed overnight with cotton nestlets, and aged *Fmr1KO* mice fed KD (n = 10) built poorer nests compared to aged *Fmr1KO* fed Teklad2019 (n = 8) by Student test, $P = 0.05$. (H) Diurnal activity levels were assessed over 3 full days in the actigraphy chambers after ± sleep deprivation (orange bars standard lighting, gray bars sleep deprivation) for 7 days. Actigraphy under standard lighting conditions showed reduced activity in the sleep-deprived cohort during the second half of the light cycle, $P < 0.003$.

in type 2 diabetic mice (X. Zhang et al., 2016), and improves longevity and health span in adult mice (12-months old) (Roberts et al., 2018). Thus, the KD has profound effects on metabolism, behavior and health span.

To our knowledge, no one has tested the KD in an FXS model. *Fmr1*^{KO} mice are a well-established mouse model for preclinical drug testing in FXS and are highly susceptible to AGS (Chen and Toth, 2001; Musumeci et al., 2000). We typically observe an 80% or greater reduction in wild running, seizures and death outcomes in the AGS assay in *Fmr1*^{KO} mice in response to acute treatment with inhibitors of metabotropic glutamate receptor 5 ($mGluR_5$) (C. J. Westmark et al., 2011; P. R. Westmark et al., 2018). Herein, we demonstrate that the KD reduces AGS selectively in male *Fmr1*^{KO} mice as well as alters diurnal activity profiles and body weight in WT and *Fmr1*^{KO} mice in a sex, age and genotype-specific manner. Regarding the attenuation of seizures, we have tested several thousand mice in the AGS assay over the past decade in response to over two dozen pharmaceutical, dietary or genetic interventions. The KD (3-day chronic treatment) was as effective as the $mGluR_5$ inhibitors MPEP (30 mg/kg) and AFQ-056 (3–10 mg/kg) in attenuating seizure phenotypes in the AGS assay and more effective than a lower dose of AFQ-056 (1 mg/kg) in male *Fmr1*^{KO} mice (P. R. Westmark et al., 2018). AGS was not reduced in female *Fmr1*^{KO} mice. This is the first time we have observed a strong sex-specific response to an intervention using the AGS assay. There is contradictory evidence in the literature regarding the role(s) of sex hormones in seizures. Sex-specific differences in response to the KD have not been reported in humans with epilepsy; however, the KD compared to Japanese, Mediterranean, American and standard mouse chow had the largest effect on mouse body composition, particularly for females (Wells et al., 2018). These data suggest that the KD may differentially benefit males rather than females in the presence of FMRP deficiency.

In addition to AGS, the KD differentially affected diurnal activity patterns in mice. Actigraphy is a sensitive, noninvasive, reliable biomarker to measure rest-activity cycles that conveniently records mouse activity levels continuously 24/7. We tested young adult, adult and aged male *Fmr1*^{KO} mice by actigraphy, and in all cases total 24-h activity counts and/or activity during the light or dark cycles were significantly reduced in response to KD. Of note, the KD significantly reduced total activity levels in young adult male *Fmr1*^{KO} mice treated from weaning but not in young adult mice with treatment commencing in adulthood. These data suggest that longer treatment or earlier intervention is required to reduce overall hyperactivity in young adult mice. Nocturnal activity, as well as activity during the first half of the light cycle, were significantly reduced in both WT and *Fmr1*^{KO} young adult male mice fed KD from weaning. Hyperactivity during nocturnal hours was similarly decreased in *Fmr1*^{KO} young adult male mice fed KD commencing as adults. Caregivers report a 32–47% rate of sleep problems in children with FXS with the most frequent problems of trouble falling asleep and frequent nighttime awakenings (Kronk et al, 2009, 2010). Since mice are nocturnal, increased activity during the beginning of the light cycle corresponds to the trouble falling asleep period.

In overnight polysomnography, FXS subjects exhibit a higher percentage of stage 1 non-REM sleep and a lower percentage of REM sleep compared to normal controls indicative of disrupted sleep microstructure (Miano et al., 2008). We hypothesize that actigraphy may be a viable outcome measure that corresponds to altered sleep architecture, responds to pharmaceutical and dietary interventions, and translates between preclinical and clinical FXS studies. Saré and colleagues employed a home-cage monitoring system to assess sleep in the light and dark phases in *Fmr1*^{KO} mice as a function of development (Sare et al., 2017). They define sleep as 40 s of inactivity. They find that juvenile *Fmr1*^{KO} mice (P21) do not differ from WT controls and that reduced sleep, defined as decreased activity, in adult *Fmr1*^{KO} mice (P70 and P180) occurs selectively during the light phase, which we also observed in young adult (Fig. 3E) and adult (Fig. 4C) mice during the first half of the light cycle. Thus, sleep disturbances may emerge during the later stages of brain maturation.

We did not observe KD-responsive effects in several other mouse behavioral testing paradigms (marble burying, tail suspension, fear conditioning). Marble burying assesses repetitive behavior in mice (A. Thomas et al., 2009), and was not significantly altered. The tail suspension test is used to screen antidepressants, and if coupled with a locomoter test, can differentiate locomotor stimulant doses from antidepressant doses (Steru et al., 1985). The KD is a potential metabolic therapy for mood disorders (Brietzke et al., 2018), and rats on a KD are less likely to exhibit behavioral despair (depression) (Murphy et al., 2004). We found no significant effect of KD in WT or *Fmr1*^{KO} mice. There are contradictory reports in the literature regarding fear conditioned learning in *Fmr1*^{KO} mice with reports of no difference compared to WT (Dobkin et al., 2000; Huynh et al., 2015; Spencer et al., 2006; A. M. Thomas et al., 2011; Uutela et al., 2012; Van Dam et al., 2000) as well as reports of decreased learning in the *Fmr1*^{KO} (de Diego-Otero et al., 2009; R. M. Deacon et al., 2015; Mao et al., 2013; Martinez and Tejada-Simon, 2018; Neuwirth et al., 2015; Nolan et al., 2017; Olmos-Serrano et al., 2011; Paradee et al., 1999; Reinhard et al., 2019). We found impaired learning during training and in context testing in *Fmr1*^{KO} compared to WT, but no significant changes in response to KD. Potential confounding issues with this experiment are altered baseline activity levels and pain threshold. *Fmr1*^{KO} mice exhibited increased activity compared to WT, and both WT and *Fmr1*^{KO} mice on the KD exhibited significantly decreased activity levels by actigraphy testing. Decreased freezing (immobility) in the fear conditioning chamber may be due to increased activity levels. Although the KD may reduce pain (Masino and Ruskin, 2013), there is likely not a KD-induced analgesic effect here as all mice exhibited significantly decreased freezing in response to the shock (Supplementary Fig. 4). In contrast, nest building is a paradigm for activities of daily living and well-being in mice (R. Deacon, 2012; Jirkof, 2014), and was reduced in aged *Fmr1*^{KO} mice in response to KD.

The choice of control diet for comparison to the KD can cause profound differences in results. Other laboratories use vivarium chow as their control diet. We chose to test both a typical vivarium chow

Teklad2019 and AIN-76A as control diets because the KD (Bio-Serv, Flemington, NJ, USA) that was used is a high fat paste diet based on the AIN-76A purified ingredient diet formulation, and because we have previously reported reduced seizure phenotypes in *Fmr1*^{KO} mice in response to AIN-76A (C. J. Westmark et al., 2013). A recent publication tested KD in comparison to a low-fat diet (LFD) in young adult male C57BL/6 mice (Genzer et al., 2015). Their KD was based on stearin (fatty acid composition: 0.1% C:12, 0.3% C:14, 14.9% C:16, 11.4% C:18, 25.44% C:18-1, 41.42% C:18-2, and 4.7% C:18-3) and contained 67.4% w/w fat (90.5% of calories) and less than 1% carbohydrate (Genzer et al., 2015). We commenced KD at 10–12 weeks of age and maintained for 8 weeks. They commenced KD at 6 weeks of age and maintained for 8 weeks (Genzer et al., 2015). We both fasted the mice before blood collection on the last day. In both studies, the mice gained weight throughout the experiment; however, we observed significantly reduced weight gain in the KD cohort throughout the study whereas they observed similar final body weights. At the end of the study, we found decreased blood glucose with KD (142 mg/dL) compared to AIN-76A (275 mg/dL) in WT males whereas they found increased blood glucose with KD (150 mg/dL) compared to LFD (95 mg/dL) indicating that the different results are most likely due to the varied control diets. We both found that ketone levels were significantly increased 3-fold in response to KD [0.88 mmol/L AIN-76A, 2.7 mmol/L Bio-Serv KD, 1.25 mmol/L LFD, and 3.5 mmol/L stearin-based KD]. They found that KD leads to an overall increase in basal locomotor activity during the dark and light cycles compared to LFD (Genzer et al., 2015). We also tested locomotor activity 24/7 but found significantly reduced activity during the dark cycle in *Fmr1*^{KO} on KD versus AIN-76A and no significant change in activity in WT male mice between the two diets. Again, these opposite results are likely due to comparison with different control diets as we both observed 6–7 average counts per minute in KD-fed WT mice during the dark phase. However, their WT male mice on LFD exhibited 3.5 average counts per minute during the dark phase whereas our mice on AIN-76A had 8 average counts per minute. Thus, LFD appears to reduce blood glucose and activity levels compared to AIN-76A without increasing ketone levels. These results have important implications regarding which biological effects of the KD result from increased ketones as opposed to increased fat with decreased carbohydrate consumption.

In addition, Genzer and colleagues found that KD increases insulin levels 1.5-fold, causes a 40% downregulation of the phosphorylated protein 70 S6 kinase (pP70S6K)/protein 70 S6 kinase (P70SK6) ratio in the brain, delays the rhythms of clock genes, and downregulates the amplitudes of clock genes (Genzer et al., 2015). These findings are of interest because *Fmr1*^{KO} mice and FXS patients exhibit reduced circulating glucose and insulin (Leboucher et al., 2019), Ribosomal S6 kinase 1 (S6K1) inhibitors are under study to normalize translational homeostasis in FXS (Bhattacharya et al., 2016), and fragile-X related proteins regulate circadian behavioral rhythms (J. Zhang et al., 2008). Indirect calorimetry studies in *Fmr1*^{KO} mice indicate that FMRP deficiency shifts metabolism toward enhanced utilization of lipids as energy substrates (Leboucher et al., 2019). Fasted *Fmr1*^{KO} mice exhibit reduced triglycerides, total cholesterol, carnitine, leptin and insulin, and increased free fatty acid and ketone bodies, specifically acetone and acetoacetate (Leboucher et al., 2019). These variations in metabolic markers in the absence of FMRP underscore the need to study metabolism and dietary effects in FXS models especially since drugs that target insulin signaling such as metformin are prescribed off-label for FXS.

The KD induces a unique metabolic state in C57BL/6J mice associated with increased expression of fatty acid oxidation genes and a reduction in lipid synthesis genes (Kennedy et al., 2007). Of interest, Tabet and colleagues recently demonstrated that FMRP binds to and regulates the expression of diacylglycerol kinase kappa (DGKκ), which is a key enzyme that modulates the switch between diacylglycerol (DAG) and phosphatidic acid (PA) signaling pathways. The knockdown of DGKκ in a WT mouse phenocopied the major symptoms in *Fmr1*^{KO} mice while overexpression of DGKκ rescued phenotypes (Tabet et al., 2016a; Tabet et al., 2016b). The KD increases hepatic DAG content more than 3-fold in male C57BL/6J mice (Jornayvaz et al., 2010). The effects of the KD on gene, protein and metabolite expression in *Fmr1*^{KO} remain to be determined.

Patients and family members are desperate for cures and symptomatic relief for many currently untreatable conditions including FXS. The accumulating evidence showing the success of the KD in autism may prompt patients to try this dietary intervention. KD plans are available on the internet and can be commenced without doctor supervision or laboratory monitoring. It is important to note that there can be adverse side effects associated with continuous maintenance on the KD. Children receiving the KD long-term are at higher risk for growth retardation, gastrointestinal symptoms, carnitine deficiency, kidney stones, hypercholesterolemia, hypertriglyceridemia, cardiac abnormalities due to selenium deficiency, Fanconi renal tubular acidosis, pancreatitis, bone fractures, and micronutrient deficiencies (Kossoff et al., 2009; Kwiterovich et al., 2003). It is unknown if the positive effects associated with the KD in humans can be maintained after discontinuation of the diet. In rats, after one-week cessation of the KD, social activity deficits returned to control levels (Kasprowska-Liskiewicz et al., 2017). Drugs such as minocycline and metformin are prescribed off label for FXS. A prescription is not required for a dietary intervention such as the KD. Thus, it is imperative to understand the biological effects of the KD and provide appropriate advice and warnings. Since carbohydrates and protein are restricted, meals need to be carefully prepared to guarantee adequate nutrition and to avoid negative side effects. An important feature of the KD is that ingestion of even a small amount of carbohydrate by patients who have achieved seizure control on the diet can rapidly reduce the diet's effectiveness and result in seizure recurrence. Thus, medical supervision is necessary. With these caveats, the KD could offer fewer chronic negative side effects than medications given that this diet has been used for over 90 years and serious or systematic negative consequences would likely have surfaced by now. Chronic KD treatment may not be a feasible treatment option for FXS, particularly for older children and adults, as patients already have gastrointestinal issues and the diet is difficult to maintain. However, if early short-term intervention with KD was proven beneficial in FXS, it would be possible to administer KD to infants as a baby formula or to toddlers. Early postnatal exposure to KD significantly alters neonatal brain structure and retards growth (Sussman et al., 2013); thus, more research is required.

In summary, there is a growing body of knowledge from both rodent and human studies demonstrating the rescue of ASD phenotypes in response to the KD. We present the first evidence that the KD rescues FXS phenotypes in a mouse model of the disorder. We also demonstrate sex-specific effects. The KD is a very restrictive diet that is difficult to maintain long-term; thus, elucidation of the mechanism underlying the success of the diet may identify pharmaceutical interventions that mimic the KD and may be beneficial in FXS.

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CRediT authorship contribution statement

Pamela R. Westmark: Methodology, Validation, Investigation, Writing - review & editing, Visualization. **Alejandra Gutierrez:** Investigation, Visualization. **Aaron K. Gholston:** Investigation. **Taralyn M. Wilmer:** Investigation. **Cara J. Westmark:** Conceptualization, Methodology, Formal analysis, Investigation,

Resources, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuint.2020.104687.

References

Ahn, Y., Narous, M., Tobias, R., Rho, J.M., Mychasiuk, R., 2014. The ketogenic diet modifies social and metabolic alterations identified in the prenatal valproic acid model of autism spectrum disorder. Dev. Neurosci. 36 (5), 371–380. https://doi.org/10.1159/000362645. [doi].

Amiet, C., Gourfinkel-An, I., Bouzamondo, A., Tordjman, S., Baulac, M., Lechat, P., et al., 2008. Epilepsy in autism is associated with intellectual disability and gender: evidence from a meta-analysis. Biol. Psychiatry 64 (7), 577–582. https://doi.org/10.1016/j.biopsych.2008.04.030.

Balietti, M., Casoli, T., Di Stefano, G., Giorgetti, B., Aicardi, G., Fattoretti, P., 2010. Ketogenic diets: an historical antiepileptic therapy with promising potentialities for the aging brain. Ageing Res. Rev. 9 (3), 273–279. https://doi.org/10.1016/j.arr.2010.02.003. [doi].

Berry-Kravis, E., Raspa, M., Loggin-Hester, L., Bishop, E., Holiday, D., Bailey, D.B., 2010. Seizures in fragile X syndrome: characteristics and comorbid diagnoses. Am. J. Intellect. Dev. Disabil. 115 (6), 461–472. https://doi.org/10.1352/1944-7558-115.6.461.

Bhattacharya, A., Mamcarz, M., Mullins, C., Choudhury, A., Boyle, R.G., Smith, D.G., et al., 2016. Targeting translation control with p70 S6 kinase 1 inhibitors to reverse phenotypes in fragile X syndrome mice. Neuropsychopharmacology : Off. Publ. Am. Coll. Neuropsychopharmacol. 41 (8), 1991–2000. https://doi.org/10.1038/npp.2015.369. [doi].

Boison, D., 2017. New insights into the mechanisms of the ketogenic diet. Curr. Opin. Neurol. 30 (2), 187–192. https://doi.org/10.1097/WCO.0000000000000432. [doi].

Boison, D., Meier, J.C., Masino, S.A., 2017. Editorial: metabolic control of brain homeostasis. Front. Mol. Neurosci. 10, 184. https://doi.org/10.3389/fnmol.2017.00184. [doi].

Bostock, E.C., Kirkby, K.C., Taylor, B.V., 2017. The current status of the ketogenic diet in psychiatry. Front. Psychiatry 8, 43. https://doi.org/10.3389/fpsyt.2017.00043. [doi].

Brietzke, E., Mansur, R.B., Subramaniapillai, M., Balanza-Martinez, V., Vinberg, M., Gonzalez-Pinto, A., et al., 2018. Ketogenic diet as a metabolic therapy for mood disorders: evidence and developments. Neurosci. Biobehav. Rev. 94, 11–16 S0149-7634(18)30376-2 [pii].

Castro, K., Baronio, D., Perry, I.S., Riesgo, R.D.S., Gottfried, C., 2017. The effect of ketogenic diet in an animal model of autism induced by prenatal exposure to valproic acid. Nutr. Neurosci. 20 (6), 343–350. https://doi.org/10.1080/1028415X.2015.1133029. [doi].

Cekici, H., Sanlier, N., 2017. Current nutritional approaches in managing autism spectrum disorder: a review. Nutr. Neurosci. 1–11. https://doi.org/10.1080/1028415X.2017.1358481. [doi].

Chen, L., Toth, M., 2001. Fragile X mice develop sensory hyperreactivity to auditory stimuli. Neuroscience 103 (4), 1043–1050.

Cheng, N., Rho, J.M., Masino, S.A., 2017. Metabolic dysfunction underlying autism spectrum disorder and potential treatment approaches. Front. Mol. Neurosci. 10, 34. https://doi.org/10.3389/fnmol.2017.00034. [doi].

Dai, Y., Zhao, Y., Tomi, M., Shin, B.C., Thamotharan, S., Mazarati, A., et al., 2017. Sex-specific life course changes in the neuro-metabolic phenotype of Glut3 null heterozygous mice: ketogenic diet ameliorates electroencephalographic seizures and improves sociability. Endocrinology 158 (4), 936–949. https://doi.org/10.1210/en.2016-1816. [doi].

Darnell, J.C., Van Driesche, S.J., Zhang, C., Hung, K.Y., Mele, A., Fraser, C.E., et al., 2011. FMRP stalls ribosomal translocation on mRNAs linked to synaptic function and autism. Cell 146 (2), 247–261. https://doi.org/10.1016/j.cell.2011.06.013.

de Diego-Otero, Y., Romero-Zerbo, Y., el Bekay, R., Decara, J., Sanchez, L., Rodriguez-de Fonseca, F., del Arco-Herrera, I., 2009. Alpha-tocopherol protects against oxidative stress in the fragile X knockout mouse: an experimental therapeutic approach for the Fmr1 deficiency. Neuropsychopharmacology : Off. Publ. Am. Coll. Neuropsychopharmacol. 34 (4), 1011–1026. https://doi.org/10.1038/npp.2008.152. [doi].

Deacon, R., 2012. Assessing burrowing, nest construction, and hoarding in mice. J. Vis. Exp. : J. Vis. Exp.(59), e2607. https://doi.org/10.3791/2607. doi(59), e2607.

Deacon, R.M., 2006. Assessing nest building in mice. Nat. Protoc. 1 (3), 1117–1119 nprot.2006.170 [pii].

Deacon, R.M., Glass, L., Snape, M., Hurley, M.J., Altimiras, F.J., Biekofsky, R.R., Cogram, P., 2015. NNZ-2566, a novel analog of (1-3) IGF-1, as a potential therapeutic agent for fragile X syndrome. NeuroMolecular Med. 17 (1), 71–82. https://doi.org/10.1007/s12017-015-8341-2. [doi].

Dobkin, C., Rabe, A., Dumas, R., El Idrissi, A., Haubenstock, H., Brown, W.T., 2000. Fmr1 knockout mouse has a distinctive strain-specific learning impairment. Neuroscience 100 (2), 423–429 S030645220000292X [pii].

Dutch-Belgian Fragile X Consortium, 1994. Fmr1 knockout mice: a model to study fragile X mental retardation. Cell 78 (1), 23–33.

El-Rashidy, O., El-Baz, F., El-Gendy, Y., Khalaf, R., Reda, D., Saad, K., 2017. Ketogenic diet versus gluten free casein free diet in autistic children: a case-control study. Metab. Brain Dis. 32 (6), 1935–1941. https://doi.org/10.1007/s11011-017-0088-z. [doi].

Evangeliou, A., Vlachonikolis, I., Mihailidou, H., Spilioti, M., Skarpalezou, A., Makaronas, N., et al., 2003. Application of a ketogenic diet in children with autistic behavior: pilot study. J. Child Neurol. 18 (2), 113–118. https://doi.org/10.1177/08830738030180020501. [doi].

Fenoglio-Simeone, K.A., Wilke, J.C., Milligan, H.L., Allen, C.N., Rho, J.M., Maganti, R.K., 2009. Ketogenic diet treatment abolishes seizure periodicity and improves diurnal rhythmicity in epileptic Kcna1-null mice. Epilepsia 50 (9), 2027–2034. https://doi.org/10.1111/j.1528-1167.2009.02163.x.

Freche, D., Lee, C.Y., Rouach, N., Holcman, D., 2012. Synaptic transmission in neurological disorders dissected by a quantitative approach. Commun. Integr. Biol. 5 (5), 448–452. https://doi.org/10.4161/cib.20818. [doi].

Frye, R.E., Sreenivasula, S., Adams, J.B., 2011. Traditional and non-traditional treatments for autism spectrum disorder with seizures: an on-line survey. BMC Pediatr. 11https://doi.org/10.1186/1471-2431-11-37. 37-2431-11-37.

Garcia-Penas, J.J., 2016. Autism spectrum disorder and epilepsy: the role of ketogenic diet. [Trastornos del espectro autista y epilepsia: el papel de la dieta cetogenica]. Rev. Neurol. 62 (Suppl. 1), S73–S78 rn2015525 [pii].

Genzer, Y., Dadon, M., Burg, C., Chapnik, N., Froy, O., 2015. Ketogenic diet delays the phase of circadian rhythms and does not affect AMP-activated protein kinase (AMPK) in mouse liver. Mol. Cell. Endocrinol. 417, 124–130. https://doi.org/10.1016/j.mce.2015.09.012. [doi].

Hagerman, R.J., 2013. Epilepsy drives autism in neurodevelopmental disorders. Dev. Med. Child Neurol. 55 (2), 101–102. https://doi.org/10.1111/dmcn.12071. 10.1111/dmcn.12071.

Hagerman, R.J., Hagerman, P.J., 2002. In: Hagerman, R.J., Cronister, A. (Eds.), Physical and Behavioral Phenotype. John Hopkins University Press, Baltimore.

Hartley-McAndrew, M., Weinstock, A., 2010. Autism spectrum disorder: correlation between aberrant behaviors, EEG abnormalities and seizures. Neurol. Int. 2 (1). https://doi.org/10.4081/ni.2010.e10. e10.

Herbert, M.R., Buckley, J.A., 2013. Autism and dietary therapy: case report and review of the literature. J. Child Neurol. 28 (8), 975–982. https://doi.org/10.1177/0883073813488668. [doi].

Huynh, T.N., Shah, M., Koo, S.Y., Faraud, K.S., Santini, E., Klann, E., 2015. eIF4E/Fmr1 double mutant mice display cognitive impairment in addition to ASD-like behaviors. Neurobiol. Dis. 83, 67–74. https://doi.org/10.1016/j.nbd.2015.08.016. [doi].

Jirkof, P., 2014. Burrowing and nest building behavior as indicators of well-being in mice. J. Neurosci. Methods 234, 139–146. https://doi.org/10.1016/j.jneumeth.2014.02.001. [doi].

Jornayvaz, F.R., Jurczak, M.J., Lee, H.Y., Birkenfeld, A.L., Frederick, D.W., Zhang, D., et al., 2010. A high-fat, ketogenic diet causes hepatic insulin resistance in mice, despite increasing energy expenditure and preventing weight gain. Am. J. Physiol. Endocrinol. Metabol. 299 (5), E808–E815. https://doi.org/10.1152/ajpendo.00361.2010. [doi].

Jozwiak, S., Kossoff, E.H., Kotulska-Jozwiak, K., 2011. Dietary treatment of epilepsy: rebirth of an ancient treatment. Neurol. Neurochir. Pol. 45 (4), 370–378 S0028-3843(14)60108-0 [pii].

Kasprowska-Liskiewicz, D., Liskiewicz, A.D., Nowacka-Chmielewska, M.M., Nowicka, J., Malecki, A., Barski, J.J., 2017. The ketogenic diet affects the social behavior of young male rats. Physiol. Behav. 179, 168–177 S0031-9384(17)30179-8 [pii].

Kaufmann, W.E., Kidd, S.A., Andrews, H.F., Budimirovic, D.B., Esler, A., Haas-Givler, B., et al., 2017. Autism spectrum disorder in fragile X syndrome: cooccurring conditions and current treatment. Pediatrics 139 (Suppl. 3), S194–S206. https://doi.org/10.1542/peds.2016-1159F. [doi].

Kennedy, A.R., Pissios, P., Otu, H., Roberson, R., Xue, B., Asakura, K., et al., 2007. A high-fat, ketogenic diet induces a unique metabolic state in mice. Am. J. Physiol. Endocrinol. Metabol. 292 (6), E1724–E1739 00717.2006 [pii].

Kossoff, E.H., Zupec-Kania, B.A., Rho, J.M., 2009. Ketogenic diets: an update for child neurologists. J. Child Neurol. 24 (8), 979–988. https://doi.org/10.1177/0883073809337162. [doi].

Kronk, R., Bishop, E.E., Raspa, M., Bickel, J.O., Mandel, D.A., Bailey Jr., D.B., 2010. Prevalence, nature, and correlates of sleep problems among children with fragile X syndrome based on a large scale parent survey. Sleep 33 (5), 679–687.

Kronk, R., Dahl, R., Noll, R., 2009. Caregiver reports of sleep problems on a convenience sample of children with fragile X syndrome. Am. J. Intellect. Dev. Disabil. 114 (6), 383–392. https://doi.org/10.1352/1944-7588-114.6.383. [doi].

Kwiterovich Jr., P.O., Vining, E.P., Pyzik, P., Skolasky Jr., R., Freeman, J.M., 2003. Effect of a high-fat ketogenic diet on plasma levels of lipids, lipoproteins, and apolipoproteins in children. Jama 290 (7), 912–920. https://doi.org/10.1001/jama.290.7.912. [doi].

Leboucher, A., Pisani, D.F., Martinez-Gili, L., Chilloux, J., Bermudez-Martin, P., Van

Dijck, A., et al., 2019. The translational regulator FMRP controls lipid and glucose metabolism in mice and humans. Mol. Metabol. 21, 22–35 S2212-8778(18)31159-1 [pii].
Lee, R.W.Y., Corley, M.J., Pang, A., Arakaki, G., Abbott, L., Nishimoto, M., et al., 2018. A modified ketogenic gluten-free diet with MCT improves behavior in children with autism spectrum disorder. Physiol. Behav. 188, 205–211 S0031-9384(18)30050-7 [pii].
Lutas, A., Yellon, G., 2013. The ketogenic diet: metabolic influences on brain excitability and epilepsy. Trends Neurosci. 36 (1), 32–40. https://doi.org/10.1016/j.tins.2012.11.005.
Mantis, J.G., Fritz, C.L., Marsh, J., Heinrichs, S.C., Seyfried, T.N., 2009. Improvement in motor and exploratory behavior in Rett syndrome mice with restricted ketogenic and standard diets. Epilepsy Behav. : E&B 15 (2), 133–141. https://doi.org/10.1016/j.yebeh.2009.02.038. [doi].
Mao, S.C., Chang, C.H., Wu, C.C., Orejarena, M.J., Manzoni, O.J., Gean, P.W., 2013. Inhibition of spontaneous recovery of fear by mGluR5 after prolonged extinction training. PLoS One 8 (3), e59580. https://doi.org/10.1371/journal.pone.0059580. [doi].
Martinez, L.A., Tejada-Simon, M.V., 2018. Increased training intensity induces proper membrane localization of actin remodeling proteins in the hippocampus preventing cognitive deficits: implications for fragile X syndrome. Mol. Neurobiol. 55 (6), 4529–4542. https://doi.org/10.1007/s12035-017-0666-4. [doi].
Masino, S.A., Kawamura, M., Wasser, C.D., Pomeroy, L.T., Ruskin, D.N., 2009. Adenosine, ketogenic diet and epilepsy: the emerging therapeutic relationship between metabolism and brain activity. Curr. Neuropharmacol. 7 (3), 257–268. https://doi.org/10.2174/157015909789152164. [doi].
Masino, S.A., Ruskin, D.N., 2013. Ketogenic diets and pain. J. Child Neurol. 28 (8), 993–1001. https://doi.org/10.1177/0883073813487595. [doi].
Miano, S., Bruni, O., Elia, M., Scifo, L., Smerieri, A., Trovato, A., et al., 2008. Sleep phenotypes of intellectual disability: a polysomnographic evaluation in subjects with down syndrome and fragile-X syndrome. Clin. Neurophysiol. : Off. J. Int. Feder. Clin. Neurophysiol. 119 (6), 1242–1247. https://doi.org/10.1016/j.clinph.2008.03.004. [doi].
Moretti, P., Bouwknecht, J.A., Teague, R., Paylor, R., Zoghbi, H.Y., 2005. Abnormalities of social interactions and home-cage behavior in a mouse model of Rett syndrome. Hum. Mol. Genet. 14 (2), 205–220. https://doi.org/10.1093/hmg/ddi016.
Murphy, P., Likhodii, S., Nylen, K., Burnham, W.M., 2004. The antidepressant properties of the ketogenic diet. Biol. Psychiatry 56 (12), 981–983 S0006-3223(04)01006-6 [pii].
Musumeci, S.A., Bosco, P., Calabrese, G., Bakker, C., De Sarro, G.B., Elia, M., et al., 2000. Audiogenic seizures susceptibility in transgenic mice with fragile X syndrome. Epilepsia 41 (1), 19–23.
Mychasiuk, R., Rho, J.M., 2017. Genetic modifications associated with ketogenic diet treatment in the BTBR(T + Tf/J) mouse model of autism spectrum disorder. Autism Res. : Off. J. Int. Soc. Autism Res. 10 (3), 456–471. https://doi.org/10.1002/aur.1682. [doi].
Napoli, E., Duenas, N., Giulivi, C., 2014. Potential therapeutic use of the ketogenic diet in autism spectrum disorders. Front. Pediatr. 2, 69. https://doi.org/10.3389/fped.2014.00069. [doi].
Neuwirth, L.S., Volpe, N.P., Ng, S., Marsillo, A., Corwin, C., Madan, N., et al., 2015. Taurine recovers mice emotional learning and memory disruptions associated with fragile x syndrome in context fear and auditory cued-conditioning. Adv. Exp. Med. Biol. 803, 425–438. https://doi.org/10.1007/978-3-319-15126-7_33. [doi].
Newell, C., Bomhof, M.R., Reimer, R.A., Hittel, D.S., Rho, J.M., Shearer, J., 2016a. Ketogenic diet modifies the gut microbiota in a murine model of autism spectrum disorder. Mol. Autism. 7 (1). https://doi.org/10.1186/s13229-016-0099-3. 37-3016-0099-3. eCollection 2016.
Newell, C., Johnsen, V.L., Yee, N.C., Xu, W.J., Klein, M.S., Khan, A., et al., 2017. Ketogenic diet leads to O-GlcNAc modification in the BTBR(T + tf/j) mouse model of autism. Biochim. Biophys. Acta 1863 (9), 2274–2281 S0925-4439(17)30150-3 [pii].
Newell, C., Shutt, T.E., Ahn, Y., Hittel, D.S., Khan, A., Rho, J.M., Shearer, J., 2016b. Tissue specific impacts of a ketogenic diet on mitochondrial dynamics in the BTBR (T + tf/j) mouse. Front. Physiol. 7, 654. https://doi.org/10.3389/fphys.2016.00654. [doi].
Nolan, S.O., Reynolds, C.D., Smith, G.D., Holley, A.J., Escobar, B., Chandler, M.A., et al., 2017. Deletion of Fmr1 results in sex-specific changes in behavior. Brain Behav. 7 (10), e00800. https://doi.org/10.1002/brb3.800. [doi].
Olmos-Serrano, J.L., Corbin, J.G., Burns, M.P., 2011. The GABA(A) receptor agonist THIP ameliorates specific behavioral deficits in the mouse model of fragile X syndrome. Dev. Neurosci. 33 (5), 395–403. https://doi.org/10.1159/000332884. [doi].
Paradee, W., Melikian, H.E., Rasmussen, D.L., Kenneson, A., Conn, P.J., Warren, S.T., 1999. Fragile X mouse: strain effects of knockout phenotype and evidence suggesting deficient amygdala function. Neuroscience 94 (1), 185–192.
Reinhard, S.M., Rais, M., Afroz, S., Hanania, Y., Pendi, K., Espinoza, K., Razak, K.A., 2019. Reduced perineuronal net expression in Fmr1 KO mice auditory cortex and amygdala is linked to impaired fear-associated memory. Neurobiol. Learn. Mem. 164, 107042 S1074-7427(19)30109-1 [pii].
Roberts, M.N., Wallace, M.A., Tomilov, A.A., Zhou, Z., Marcotte, G.R., Tran, D., et al., 2018. A ketogenic diet extends longevity and healthspan in adult mice. Cell Metabol. 27 (5), 1156 S1550-4131(18)30247-X [pii].
Ruskin, D.N., Fortin, J.A., Bisnauth, S.N., Masino, S.A., 2017a. Ketogenic diets improve behaviors associated with autism spectrum disorder in a sex-specific manner in the EL mouse. Physiol. Behav. 168, 138–145 S0031-9384(16)30338-9 [pii].
Ruskin, D.N., Murphy, M.I., Slade, S.L., Masino, S.A., 2017b. Ketogenic diet improves behaviors in a maternal immune activation model of autism spectrum disorder. PLoS One 12 (2), e0171643. https://doi.org/10.1371/journal.pone.0171643. [doi].
Ruskin, D.N., Svedova, J., Cote, J.L., Sandau, U., Rho, J.M., Kawamura, M., et al., 2013. Ketogenic diet improves core symptoms of autism in BTBR mice. PLoS One 8 (6), e65021. https://doi.org/10.1371/journal.pone.0065021. [doi].
Sare, R.M., Harkless, L., Levine, M., Torossian, A., Sheeler, C.A., Smith, C.B., 2017. Deficient sleep in mouse models of fragile X syndrome. Front. Mol. Neurosci. 10, 280. https://doi.org/10.3389/fnmol.2017.00280. [doi].
Smith, J., Rho, J.M., Teskey, G.C., 2016. Ketogenic diet restores aberrant cortical motor maps and excitation-to-inhibition imbalance in the BTBR mouse model of autism spectrum disorder. Behav. Brain Res. 304, 67–70. https://doi.org/10.1016/j.bbr.2016.02.015. [doi].
Someya, S., Yu, W., Hallows, W.C., Xu, J., Vann, J.M., Leeuwenburgh, C., et al., 2010. Sirt3 mediates reduction of oxidative damage and prevention of age-related hearing loss under caloric restriction. Cell 143 (5), 802–812. https://doi.org/10.1016/j.cell.2010.10.002. [doi].
Spencer, C.M., Serysheva, E., Yuva-Paylor, L.A., Oostra, B.A., Nelson, D.L., Paylor, R., 2006. Exaggerated behavioral phenotypes in Fmr1/Fxr2 double knockout mice reveal a functional genetic interaction between fragile X-related proteins. Hum. Mol. Genet. 15 (12), 1984–1994 ddl121 [pii].
Spilioti, M., Evangeliou, A.E., Tramma, D., Theodoridou, Z., Metaxas, S., Michailidi, E., et al., 2013. Evidence for treatable inborn errors of metabolism in a cohort of 187 Greek patients with autism spectrum disorder (ASD). Front. Hum. Neurosci. 7, 858. https://doi.org/10.3389/fnhum.2013.00858. [doi].
Stafstrom, C.E., Rho, J.M., 2012. The ketogenic diet as a treatment paradigm for diverse neurological disorders. Front. Pharmacol. 3, 59. https://doi.org/10.3389/fphar.2012.00059. [doi].
Steru, L., Chermat, R., Thierry, B., Simon, P., 1985. The tail suspension test: a new method for screening antidepressants in mice. Psychopharmacology 85 (3), 367–370. https://doi.org/10.1007/bf00428203. [doi].
Sussman, D., Ellegood, J., Henkelman, M., 2013. A gestational ketogenic diet alters maternal metabolic status as well as offspring physiological growth and brain structure in the neonatal mouse. BMC Pregnancy Childbirth 13https://doi.org/10.1186/1471-2393-13-198. 198-2393-13-198.
Tabet, R., Moutin, E., Becker, J.A., Heintz, D., Fouillen, L., Flatter, E., et al., 2016a. Fragile X mental retardation protein (FMRP) controls diacylglycerol kinase activity in neurons. Proc. Natl. Acad. Sci. U. S. A 113 (26), E3619–E3628. https://doi.org/10.1073/pnas.1522631113. [doi].
Tabet, R., Vitale, N., Moine, H., 2016b. Fragile X syndrome: are signaling lipids the missing culprits? Biochimie 130, 188–194 S0300-9084(16)30169-9 [pii].
Tai, K.K., Nguyen, N., Pham, L., Truong, D.D., 2008. Ketogenic diet prevents cardiac arrest-induced cerebral ischemic neurodegeneration. J. Neural Transm. 115 (7), 1011–1017. https://doi.org/10.1007/s00702-008-0050-7. [doi].
Thomas, A., Burant, A., Bui, N., Graham, D., Yuva-Paylor, L.A., Paylor, R., 2009. Marble burying reflects a repetitive and perseverative behavior more than novelty-induced anxiety. Psychopharmacology 204 (2), 361–373. https://doi.org/10.1007/s00213-009-1466-y.
Thomas, A.M., Bui, N., Graham, D., Perkins, J.R., Yuva-Paylor, L.A., Paylor, R., 2011. Genetic reduction of group 1 metabotropic glutamate receptors alters select behaviors in a mouse model for fragile X syndrome. Behav. Brain Res. 223 (2), 310–321. https://doi.org/10.1016/j.bbr.2011.04.049. [doi].
Tipps, M.E., Raybuck, J.D., Buck, K.J., Lattal, K.M., 2014. Delay and trace fear conditioning in C57BL/6 and DBA/2 mice: issues of measurement and performance. Learn. Mem. 21 (8), 380–393. https://doi.org/10.1101/lm.035261.114. [doi].
Udagawa, T., Farny, N.G., Jakovcevski, M., Kaphzan, H., Alarcon, J.M., Anilkumar, S., et al., 2013. Genetic and acute CPEB1 depletion ameliorate fragile X pathophysiology. Nat. Med. 19 (11), 1473–1477. https://doi.org/10.1038/nm.3353. [doi].
Uutela, M., Lindholm, J., Louhivuori, V., Wei, H., Louhivuori, L.M., Pertovaara, A., et al., 2012. Reduction of BDNF expression in Fmr1 knockout mice worsens cognitive deficits but improves hyperactivity and sensorimotor deficits. Genes Brain Behav. 11 (5), 513–523. https://doi.org/10.1111/j.1601-183X.2012.00784.x. [doi].
Van Dam, D., D'Hooge, R., Hauben, E., Reyniers, E., Gantois, I., Bakker, C.E., et al., 2000. Spatial learning, contextual fear conditioning and conditioned emotional response in Fmr1 knockout mice. Behav. Brain Res. 117 (1–2), 127–136 S0166432800002965 [pii].
van Eeghen, A.M., Pulsifer, M.B., Merker, V.L., Neumeyer, A.M., van Eeghen, E.E., Thibert, R.L., et al., 2013. Understanding relationships between autism, intelligence, and epilepsy: a cross-disorder approach. Dev. Med. Child Neurol. 55 (2), 146–153. https://doi.org/10.1111/dmcn.12044. 10.1111/dmcn.12044.
Verkerk, A.J., Pieretti, M., Sutcliffe, J.S., Fu, Y.H., Kuhl, D.P., Pizzuti, A., et al., 1991. Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. Cell 65 (5), 905–914.
Verpeut, J.L., DiCicco-Bloom, E., Bello, N.T., 2016. Ketogenic diet exposure during the juvenile period increases social behaviors and forebrain neural activation in adult engrailed 2 null mice. Physiol. Behav. 161, 90–98 S0031-9384(16)30135-4 [pii].
Verrotti, A., Iapadre, G., Pisano, S., Coppola, G., 2017. Ketogenic diet and childhood neurological disorders other than epilepsy: an overview. Expert Rev. Neurother. 17 (5), 461–473. https://doi.org/10.1080/14737175.2017.1260004. [doi].
Wallace, D.C., Fan, W., Procaccio, V., 2010. Mitochondrial energetics and therapeutics. Ann. Rev. Pathol. 5, 297–348. https://doi.org/10.1146/annurev.pathol.4.110807.092314. [doi].
Wallace, E., Kim, D.Y., Kim, K.M., Chen, S., Blair Braden, B., Williams, J., et al., 2015. Differential effects of duration of sleep fragmentation on spatial learning and synaptic plasticity in pubertal mice. Brain Res. 1615, 116–128 S0006-8993(15)00333-9 [pii].
Wells, A., Barrington, W.T., Dearth, S., May, A., Threadgill, D.W., Campagna, S.R., Voy, B.H., 2018. Tissue level diet and sex-by-diet interactions reveal unique metabolite and clustering profiles using untargeted liquid chromatography-mass spectrometry

on adipose, skeletal muscle, and liver tissue in C57BL6/J mice. J. Proteome Res. 17 (3), 1077–1090. https://doi.org/10.1021/acs.jproteome.7b00750. [doi].
Westmark, C.J., Westmark, P., Beard, A., Hildebrandt, S., Malter, J.S., 2008. Seizure susceptibility and mortality in mice that over-express amyloid precursor protein. Int. J. Clin. Exp. Pathol. 1 (2) 157-157-168.
Westmark, C.J., Westmark, P.R., Malter, J.S., 2013. Soy-based diet exacerbates seizures in mouse models of neurological disease. J. Alzheimer's Dis. : JAD 33 (3), 797–805. https://doi.org/10.3233/JAD-2012-121426. 10.3233/JAD-2012-121426.
Westmark, C.J., Westmark, P.R., O'Riordan, K.J., Ray, B.C., Hervey, C.M., Salamat, M.S., et al., 2011. Reversal of fragile X phenotypes by manipulation of AbetaPP/Abeta levels in Fmr1 mice. PLoS One 6 (10), e26549. https://doi.org/10.1371/journal.pone.0026549.
Westmark, P.R., Dekundy, A., Gravius, A., Danysz, W., Westmark, C.J., 2018. Rescue of Fmr1(KO) phenotypes with mGluR5 inhibitors: MRZ-8456 versus AFQ-056. Neurobiol. Dis. 119, 190–198 S0969-9961(18)30440-6 [pii].
Yan, Q.J., Asafo-Adjei, P.K., Arnold, H.M., Brown, R.E., Bauchwitz, R.P., 2004. A phenotypic and molecular characterization of the fmr1-tm1Cgr fragile X mouse. Genes Brain Behav. 3 (6), 337–359. https://doi.org/10.1111/j.1601-183X.2004.00087.x.
Yellon, G., 2008. Ketone bodies, glycolysis, and K_{ATP} channels in the mechanism of the ketogenc diet. Epilepsia 49, 80–82. https://doi.org/10.1111/j.1528-1167.2008.01843.x.
Zarnowska, I., Chrapko, B., Gwizda, G., Nocun, A., Mitosek-Szewczyk, K., Gasior, M., 2018. Therapeutic use of carbohydrate-restricted diets in an autistic child; a case report of clinical and 18FDG PET findings. Metab. Brain Dis. https://doi.org/10.1007/s11011-018-0219-1. [doi].
Zhang, J., Fang, Z., Jud, C., Vansteensel, M.J., Kaasik, K., Lee, C.C., et al., 2008. Fragile X-related proteins regulate mammalian circadian behavioral rhythms. Am. J. Hum. Genet. 83 (1), 43–52. https://doi.org/10.1016/j.ajhg.2008.06.003. [doi].
Zhang, X., Qin, J., Zhao, Y., Shi, J., Lan, R., Gan, Y., et al., 2016. Long-term ketogenic diet contributes to glycemic control but promotes lipid accumulation and hepatic steatosis in type 2 diabetic mice. Nutr. Res. 36 (4), 349–358 S0271-5317(15)00301-2 [pii].