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# Ketogenic diet exposure during the juvenile period increases social behaviors and forebrain neural activation in adult *Engrailed 2* null mice.

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**Abstract**

Prolonged consumption of ketogenic diets (KD) has reported neuroprotective benefits. Several studies suggest KD interventions could be useful in the management of neurological and developmental disorders. Alterations in the *Engrailed* (*En*) genes, specifically *Engrailed 2* (*En2*), have neurodevelopmental consequences and produce autism-related behaviors. The following studies used *En2* knockout (KO; *En2* $^{-/-}$*),* and wild-type (WT; *En2*$^{+/+}$*),* male mice fed either KD (80% fat, 0.1% carbohydrates) or control diet (CD; 10% fat, 70% carbohydrates). The objective was to determine whether a KD fed from weaning at postnatal day (PND) 21 to adulthood (PND 60) would alter brain monoamines concentrations, previously found dysregulated, and improves social outcomes. In WT animals, there was an increase in hypothalamic norepinephrine content in the KD-fed group. However, regional monoamines were not altered in KO mice in KD- fed compared with CD-fed group. In order to determine the effects of juvenile exposure to KD in mice with normal blood ketone levels, separate experiments were conducted in mice removed from the KD or CD and fed standard chow for 2 days (PND 62). In a three-chamber social test with a novel mouse, KO mice previously exposed to the KD displayed similar social behavior and self-grooming behaviors compared with the WT group. Groups previously exposed to a KD, regardless of genotype, had more c-Fos-positive cells in the cingulate cortex, lateral septal nuclei, and anterior bed nucleus of the stria terminalis. In the novel object condition, KO mice previously exposed to KD had similar behavioral responses and pattern of c-Fos immunoreactivity compared with the WT group. Thus, juvenile exposure to a KD resulted in short-term consequences of improving social interactions and appropriate exploratory behaviors in a mouse model that displays autism-related behaviors. Such findings further our understanding of metabolic-based therapies for neurological and developmental disorders.

**Key words**: Low-carbohydrate diet, no-carbohydrate diet, β-hydroxybutyrate, serotonin, dopamine, nutrition therapy, adolescence

## 1. Introduction

Nutritional diets that are high in fat and low in carbohydrates, but sufficient in protein, increase fatty acid oxidation leading to a metabolic condition characterized by an elevation inketone bodies (β-hydroxybutyrate, acetoacetate, and acetone)[1]. Prolonged consumption of such ketogenic diets (KD) have been used to effectively control seizures in children with drug-resistant epilepsy [2-5]. Despite their clinical use for many decades, the mechanisms for the neuroprotective effects of KD have not been fully elucidated [6], but there is evidence that the KD may protect neurons against excitotoxicity, neuroinflammation, and reactive oxygen species (ROS), as well as improvements in mitochondrial function [7-10]. Emerging evidence suggests that KD or modified KD could have clinical utility for other neurological and developmental disorders [11-15]. Several developmental disorders, such as autism spectrum disorder (ASD), could realize benefits from KD because there are few effective treatment options and some behaviors are thought to involve neurometabolic impairments [16, 17]. Indeed, there is limited clinical evidence to suggest mild to moderate improvements in autism-related behaviors in ASD subjects undergoing a 6-month intermittent KD intervention [18]. Several difficulties are inherent in investigating developmental disorders since they often have complex heterogeneous symptomatology [19]. Despite their unknown etiologies, developmental disorders, such as ASD and related behaviors, are suspected to be influenced by heritable factors. Indeed, twin studies have found concordance rates for ASD as high as 80% for monozygotic twins and 13.6% for dizygotic twins [20]. In the same dataset, the heritability for social impairment in ASD was estimated to be 60.9% [20]. Thus, understanding the role of these genes in ASD may be critical for providing future treatments. One set of genes that have been found to be associated with neurodevelopmental impairments are the *Engrailed* (*En*) *genes* [21-24].

*The En* genes are important homeobox transcription factors for neurodevelopmental events, such as mid-hindbrain regionalization, cerebellar development, and neural growth and

maturation [25-31]. During early embryogenesis, *En* genes are expressed in the mid-brain and hindbrain border and regulate gene expression by binding to AT-rich DNA cis-sequences [32-36]. Mutations in the *En* genes affect the ventral mid-hindbrain nuclei, the locus coeruleus (LC) and the raphe nuclei (RN), ultimately resulting in abnormal levels of norepinephrine (NE) and serotonin (5HT) in forebrain and hindbrain areas during development [37-39]. Mice with a deletion of the *En2* gene from birth ($En2^{-/-}$) demonstrate severe cerebellar hypoplasia, reduced Purkinje cell numbers, disruptions in cerebellar patterning and foliation, reduced hippocampal weight, increased dentate gyrus cell turnover, and an anterior shift in the position of the amygdala nuclei [24, 33, 40-48]. From a behavioral standpoint, juvenile $En2^{-/-}$ mice, compared with wild-type (WT, $En2^{+/+}$) mice, display impaired social interaction, memory deficits, improper sensory-motor gating, decreased play, reduced social sniffing, reduced aggressiveness, and depression-related behaviors [24, 33, 41, 43-47]. The behavioral impairments demonstrated by the $En2^{-/-}$ mice parallel the reduced social interactions, abnormal communication skills, and restricted or repetitive behaviors, which are core behaviors demonstrated in individuals diagnosed with neurodevelopmental disorders, such as autism [44, 49-51]. In particular, intronic SNP of *EN2*, *rs1861972* (A/G) *and rs1861973 (*C/T), are transmitted as an ASD-associated haplotype [24, 42, 52]. Postmortem analysis of cerebellar tissue of ASD subjects (26 case and control samples) has also revealed hypermethylation of the promoter region of the *En2* DNA [53]. However, not all populations of individuals with ASD have revealed associations with *EN2* polymorphisms [54]. Hence, the exact contribution of the *En2* gene product in developmental events related to autistic behaviors is still not known.

In the following study, we used KO ($En2^{-/-}$) and WT ($En2^{+/+}$) male mice fed the KD or control diet (CD) from postnatal day (PND) 21 to 60 to determine if a KD intervention could improve the neural and behavioral deficits associated with $En2^{-/-}$ mice. Prolonged exposure to diets high in fat has resulted in altered brain monoamines [55]. Therefore, it was hypothesized

that *En2*$^{-/-}$ mice would display altered monoamine content, specifically regarding dopamine (DA), 5-HT, NE in forebrain regions, which would be improved by exposure to the KD. Either because of compliance issues or developmental neuroplasticity, several human studies suggest that KD exposure for treatment of epilepsy during childhood development is more effective than during adulthood [1, 13, 56], therefore, KD exposure in this study was limited to the juvenile period (PND 21-60). We further hypothesized that social behaviors, analyzed by a three-chamber social test, would be improved by the KD and accompanied by increased immunoreactivity of c-Fos, an immediate early gene, in forebrain structures critical to social behavior. In this study, c-Fos immunoreactivity was determined in limbic and hypothalamic brain regions of mice following exposure to a novel animal in the 3-chambered social test. These studies are the first to investigate whether KD intervention during the juvenile period alters monoamines and whether this exposure impacts social behavior and neural activation of related brain areas.

## 2. Materials and Methods

### *2.1. Animals*

*En2*$^{tm1Alj/tm1Alj}$ mice were originally purchased from The Jackson Laboratory (Bar Harbor, ME, USA). *En2*$^{tm1Alj/tm1Alj}$ mice were generated on a 129S2/SvPas background as previously described [57]. Offspring *En2* heterozygous (*En2*$^{+/-}$) breeding pairs were used for the following study and placed on a 12:12 h light:dark cycle with lights off at 1800 h. Heterozygous breeding pairs were used for all studies. Every ten generations, *En2*$^{+/-}$ mice were crossed to B6129SF2/J mice for creation of new *En2*$^{+/-}$ breeding pairs. Mice were fed standard chow (Purina Mouse Diet 5015, 25.34% fat, 19.81% protein, 54.86 CHO, 3.7 Kcal/g) and water was available at all times, unless otherwise noted. Pups were kept with the dam until weaning at PND 21. After weaning, male mice were group-housed, with at least 2 different litters per cage and with equal KO to WT genotype ratios, and placed on experimental diets. All procedures were approved by the

Institutional Animal Care and Use Committee of Rutgers University and were in accordance with NIH guidelines.

*2.2. Genotyping*

Animals were genotyped by PCR analysis of ear tissue DNA using standard polymerase chain reaction (PCR) methods. Ear snips (2 mm) were digested using Promega ReliaPrep gDNA Tissue Miniprep System (Promega, Madison, WI, USA). The following primers were used for PCR: GTTCACAGTCCTGTGAAATGCAGC, common to both *En2*$^{+/+}$ and *En2*$^{-/-}$ mice; ACCAACAGGTACCTGACAGAGC, specific for the *En2*$^{+/+}$ homeobox; and CTTGGGTGGAAGGGCTATTC, a sequence in the neomycin gene specific for the *En2*$^{-/-}$ mutation. These primers amplify a 600-bp band in *En2*$^{+/+}$ mice, a 950-bp band in *En2*$^{-/-}$ mice, and 600-bp and 950-bp band in *En2*$^{+/-}$ mice [33, 58].

*2.3. Experimental diet*

To determine the effects of the KD on monoamines, KO and WT mice were *ab libitum* fed either a lard-based ketogenic diet (KD; 80% fat, 20% protein, 0.1% CHO, 6.14 Kcal/g; D03022101; Research Diets, New Brunswick, NJ, USA) or a protein-matched control diet (CD; 10% fat, 20% protein, 70% CHO, 3.85 Kcal/g; D12450K; Research Diets) from PND 21 to 60, which encompasses the juvenile developmental period; see supplemental Table 1 for diet breakdown. Notably, neither diet contained sucrose.

*2.4. KD exposure on forebrain and cerebellar monoamines*

At PND 60, KO-KD (n = 19), KO-CD (n=19), WT-KD (n=20), WT-CD (n=20) mice were fasted for 5 hours (0800h-1300h), then sacrificed by decapitation for analysis of monoamines and metabolites in regional brain tissue. Trunk blood β-hydroxybutyrate levels were measured by a ketone meter (Precision Plus Ketone Meter, Abbott Laboratories, North Chicago, Il, USA).

Brains were dissected to isolate the frontal cortex (+2.46 to +1.98 mm from Bregma), medial hypothalamus (-1.06 to -2.06 mm from Bregma), hippocampus (-1.06 to -2.06 mm from Bregma), and cerebellum (-5.40 to -8.24 mm from Bregma) [59]. For the forebrain regions, a brain matrix was used for standardized dissection. For the cerebellum, the entire cerebellum was removed by blunt dissection. Monoamines for each individual brain section were extracted and analyzed as previously described [60] by reverse-phase high-performance liquid chromatography (HPLC) (Dionex Ultimate 3000, Thermo Fisher Scientific, Sunnyvale, CA, USA) with electrochemical detection (Coulochem III, Thermo Fisher Scientific). An acetonitrile-based phosphate buffer mobile phase (MD-TM; Thermo Fisher Scientific) was used for all experiments. The internal standard, 3,4-dihydroxybenzylamine (DHBA), was added to all samples prior to extraction. Quantification of NE, DA, 5-hydroxyindoleacetic acid (5-HIAA), homovanillic acid (HVA), and 5-HT was determined by Chromeleon 7.1 software (Thermo Fisher Scientific). Values were expressed as picograms (pg) of monoamine relative to milligrams (mg) of wet tissue weight per sample. Body weights (to the nearest 0.01 g) were also measured at the time of sacrifice.

*2.5. Persistence of KD alterations in blood β-hydroxybutyrate levels*

A group of mice (n=8) were fed the KD from PND 21 to PND 60 and then placed on a standard chow diet (25.34% fat, 19.81% protein, 54.86 CHO, 3.7 Kcal/g; Purina Mouse Diet 5015) for 2 days (until PND 62) to determine if changes in ketone body concentrations were restored to baseline. Tail nick blood β-hydroxybutyrate levels were measured by a ketone meter.

*2.6. Assessment of social behaviors*

At PND 62, KO-KD (n = 14), KO-CD (n = 13), WT-KD (n = 12), WT-CD (n = 13) were subjected to a three-chamber social test (Stoelting Co, Wood Dale, Illinois, USA) to determine effects of a KD on social behavior, as previously described [43]. During three 10-min recorded phases the

following were quantified: time spent in each chamber, frontal contact (time spent between experimental and novel mice), and self-grooming. In phase 1, all mice had 10 min to explore and acclimate to all three chambers. In phase 2, a novel mouse (adult male *En2*$^{+/-}$ non-litter mate) was placed in a wire cage in chamber 1 and the experimental mouse could choose between social interaction or continual exploration of the chambers and empty wire cage in chamber 3, for 10 min. In phase 3, the novel mouse was now familiar and a second novel mouse was placed in chamber 3 in a wire cage. For 10 min, the experimental mouse could choose to spend time with mice in chamber 1 or 3 or be alone in the middle chamber 2. It is important to note that chambers 1 and 3 were reversed for half the animals, to avoid confounding side preference. All behaviors were recorded and analyzed using a time-sampling computer program, Hindsight (version 1.3), and each mouse was scored by 3 observers blind to the treatment conditions and genotypes. To avoid the acute effect of the experimental diets, all animals were tested on standard chow. Testing occurred following 2 days on standard chow.

*2.7. Novel object exploratory behaviors in En2 mice with diet exposures.*

At PND 62, a separate set of *En2*$^{-/-}$ (KO, n = 8) and *En2*$^{+/+}$ (WT, n = 8) previous fed either KD or CD, but maintained on standard chow were placed in a three-chamber social test (Stoelting, Wood Dale, Illinois, USA) to expose mice to a novel object prior to analyzing for c-Fos immunoreactivity. This experiment acted as a control group for c-Fos immunoreactivity studies for mice exposed to a novel mouse. During two 10-min recorded phases the following was quantified: time spent in each chamber, time spent with the novel object, and self-grooming. In phase 1, all mice had ten minutes to explore and acclimate to all three chambers. In phase 2, a novel object (wooden block that was previously housed with novel male mice) was placed in a wire cage in one of the side chambers. The free mouse could choose to spend time with the novel object or explore the chamber for 10 min. All behaviors were recorded and analyzed using

a time-sampling computer program, Hindsight (version 1.3), and each mouse was scored by three observers blind to the experimental groups.

*2.8. Neural activation in forebrain regions in En2 mice with diet exposures*

After the 3-chamber test for sociability using a novel mouse or object, all mice were returned to home cages for 90 min [61]. Mice were deeply anesthetized with 0.1% Euthasol (pentobarbital sodium and phenytoin sodium) solution intraperitoneal (IP), exsanguinated with 0.9% phosphate buffered saline (PBS), and perfused with 4% paraformaldehyde in PBS. Brains were extracted and post-fixed for 24 h in 4% paraformaldehyde in PBS, then switched to 20% sucrose in 4% paraformaldehyde until sectioning. Free-floating sections (40 µm) of the forebrain were obtained by using a Leica cryostat (Leica Microsystems, Rijswijk, The Netherlands). Sections were stored in cryoprotectant until immunohistochemistry was performed. Sections were washed 3 x 10 min in PBS (10 mM phosphate, 150 mM NaCl, pH 7.5). Endogenous peroxidases were neutralized with 0.3% $H_2O_2$ in $H_2O$. After a 3 x 10 min PBS wash, sections were incubated in normal goat serum (PK-4001, Vectastain ABC kit, Vector Laboratories, Burlingame, CA) with 0.3% Triton-X-100 in PBS for 30 min. c-Fos immunolabeling was performed with a polyclonal rabbit IgG anti-human c-Fos (sc-52, Santa Cruz Biotechnology, Santa Cruz, CA), diluted 1:1 in antibody diluent (Dako, An Agilent Technologies Company, Carpinteria, CA), then diluted 1:000 in PBS. Tissue incubated overnight (~20 h). Sections were transferred to a new clean plate, washed in three 10 min in 0.1% Triton X-100 in PBS, then incubated for 30 min in biotinylated secondary antibody (goat IgG anti-rabbit, PK-4001, Vectastain ABC kit, Vector Laboratories) with 0.3% Triton X-100 in PBS. After three 10 min wash in PBS, sections were incubated in an avidin-peroxidase complex (PK-4001, Vectastain ABC kit, Vector Laboratories) for 45 min. Again sections were washed in three 10 min in PBS. Staining was performed using Nickel Diaminobenzidine Tetrahydrochloride (Ni-DAB) Chromagen (SK-4100, DAB Peroxidase Substrate Kit, 3,3'-diaminobenzidine, Vector Laboratories) for approximately 30 sec to stain Fos-like products

black. PBS was added immediately after desired stain was reached and sections were washed in three 10 min in PBS to halt the Ni-DAB reaction. Sections were mounted on gelatin coated slides (Fisherbrand Double Frosted Microscope Slides, Thermo Fisher Scientific Inc, Bridgewater, NJ) and dehydrated with ethanol and xylenes prior to coverslip with permount [62].

*2.9. Imaging and quantification of c-Fos positive nuclei*

Imaging was performed using an Olympus FSX-BSW imaging scope and FSX100 software (Olympus videoscope, Tokyo, Japan). Quantification was performed by identifying c-Fos positive black nuclei using Image J software system (NIH, Bethesda, MD) image analysis software [62]. Three anatomically matched tissue slices of each region (unilateral) of each mouse was used in data analysis. Cells were counted by two observers blinded to the experimental conditions.

Several forebrain regions were examined, which included the dorsal prefrontal cortex (PFC; +1.98 mm from Bregma), cingulate cortex (area 1 and 2; Cing Cortex; + 0.86 mm from Bregma), piriform cortex (+1.78 mm from Bregma), lateral septal nuclei (LSN; +0.98 mm from Bregma), nucleus accumbens core (NAc core; +0.98 mm from Bregma), anterior bed nucleus of the stria terminalis (aBNST;+0.38 mm from Bregma), dentate gyrus (-1.22 mm from Bregma), anterior hypothalamus area (AH; -0.58 mm from Bregma), and paraventricular hypothalamic nucleus (PVH;-0.94 mm from Bregma) [59]. These regions were selected based on previous investigations with En2 KO mice [63] and pattern of c-Fos immunoreactivity.

*2.10. Statistical analysis*

Regional brain mass, monoamines, and β-hydroxybutyrate levels were analyzed with a factorial analysis of variance (ANOVA). To determine changes in β-hydroxybutyrate from PND 60-62, a dependent t-test was used. Measurements in the three-chamber test were analyzed by a multivariate ANOVA (MANOVA). Cell counts for c-Fos-like immunoreactivity were analyzed with

MANOVA. Post-hoc comparisons were made, when justified, with Newman-Keuls tests. All statistical analyses were performed using Statistica 7.1 software (StatSoft, Tulsa, OK, USA) and significance was set at $\alpha = 0.05$.

## 3. Results

### *3.1. Effects of the KD on monoamines and metabolites in brain regions of En2 null mice*

As illustrated in Table 1, brain regions (frontal cortex, hippocampus, medial hypothalamus, and cerebellum) were assessed for monoamines and metabolites in knockout (KO; *En2*$^{-/-}$) and WT mice. In the frontal cortex there were no significant differences in genotype, diet, or genotype X diet for any monoamine and metabolites measurements. In the hippocampus there was a genotype effect [$F(1, 69) = 4.3$, $p < 0.05$] for NE. Levels of NE were approximately 30% lower in KO than WT genotype ($p < 0.05$). Also in the hippocampus there was also genotype effect [$F(1, 53) = 5.0$, $p < 0.05$] for 5HT. Levels of 5HT were approximately 35% lower in KO than WT genotype ($p < 0.05$). There were no significant differences in DA or 5-HIAA concentrations in the hippocampus of KO or WT mice fed the KD or CD. In the medial hypothalamus there was a diet x genotype [$F(1, 61) = 4.1$, $p < 0.05$] for NE. Levels of NE were approximately 86% higher in the WT-KD compared with WT-CD group ($p < 0.05$). Also in the medial hypothalamus there was also a genotype effect [$F(1, 62) = 4.3$, $p < 0.05$] for DA. Levels of DA were approximately 32% lower in KO than WT groups ($p < 0.05$). There was also a genotype effect for 5-HT [$F(1, 59) = 4.8$, $p < 0.05$] in the medial hypothalamus, whereby 5-HT levels were 28% lower in KO compared with WT genotype ($p < 0.05$). There were no significant differences in 5-HIAA or HVA in the medial hypothalamus with respect to genotype or diet. In cerebellum for NE, there was a genotype effect [$F(1, 62) = 4.3$, $p < 0.05$] with NE levels being approximately 20% higher in the KO compared with the WT genotype ($p < 0.05$). For 5-HT in the cerebellum, there was also genotype effect [$F(1, 52) = 11.8$, $p < 0.01$], whereby KO mice had approximately 60% higher 5HT levels compared with WT groups ($p < 0.05$), regardless of diet. There were no significant

differences in 5-HIAA or HVA in the cerebellum with respect to genotype or diet. Terminal body weights were 26.2 ± 0.6 g for KO-KD, 23.4 ± 0.5 g for KO-CD, 27.2 ± 0.5 g for WT-KD, and 23.5 ± 0.3 g for WT-CD. There was an effect for diet [$F(1, 74) = 39.6$, $p < 0.001$] with KD-fed animals having an approximate 15% greater body weight compared with CD-fed animals ($p < 0.001$).

*3.2. Ketone body (β-hydroxybutyrate) alterations as a result of the KD in En2 KO mice.*

At PND 60, there was an effect for blood β-hydroxybutyrate [$F(1, 59) = 26.9$, $p < 0.0001$]. Levels of β-hydroxybutyrate were elevated by approximately 67% in the KD-fed groups compared with CD group ($p< 0.001$). β-hydroxybutyrate levels were 1.1 ± 0.1 mmol/L for KO-KD, 0.68 ± 0.1 mmol/L for KO-CD, 1.13 ± 0.1 mmol/L for WT-KD, and 0.74 ± 0.1 mmol/L for WT-CD.

*3.3. Persistence of β-hydroxybutyrate levels following standard diet re-feeding*

Compared to active experimental diet feeding, blood β-hydroxybutyrate levels were decreased by approximately 55% in mice fed a KD from PND 21 to 60 and then switched to a standard diet for 2 days ( $t = 4.29$, $p<0.005$). At PND 60, β-hydroxybutyrate levels were 0.92 ± 0.06 mmol/L. After two days of standard chow feeding, β-hydroxybutyrate levels were 0.41 ± 0.1 mmol/L.

*3.4. Social behaviors in En2$^{-/-}$ mice were similar to WT as a result of exposure to the KD.*

In the three-chamber social test there was a significant phase effect [$F(1,141) = 19.5$, $p < 0.0001$)], diet effect [$F(1,141) = 4.09$, $p < 0.05$)], and a genotype x diet effect [$F(1,141) = 20.3$, $p < 0.0001$)] for frontal contact. Post-hoc analysis revealed that KO previously exposed to KD had increased social contact over all three phases compared with KO with previous exposure to CD ($p < 0.01$); see Fig. 1A. There was a phase effect [$F(2, 134) = 17.1$, $p < 0.0001$], diet effect [$F(1, 134) = 5.8$, $p < 0.05$], phase x diet effect [$F(2, 134) = 4.0$, $p < 0.05$], and genotype x diet effect [$F(1, 134) = 4.9$, $p < 0.05$] for self-grooming repetitive behaviors. Post-hoc analysis revealed that

KO previously exposed to KD had reduced self-grooming behaviors compared with KO with previously exposure to CD ($p < 0.05$); see Fig. 1B. There was a phase effect [$F(2,144) = 15.0$, $p < 0.001$)], genotype x diet effect [$F(1,144) = 5.4$, $p < 0.05$)], and phase x genotype x diet effect [$F(2,144) = 5.4$, $p < 0.01$)] for chamber 1. Despite these overall effects, post-hoc testing failed to reveal any differences between groups (data not shown).

*3.5. En2$^{-/-}$ mice spent significantly more time with a novel object*

For a control group, two days after the switch to standard chow, at PND 62, mice were placed in a three-chamber test and exposed to a novel object (n = 4/group) prior to analysis for c-Fos positive nuclei. There was a diet effect [$F(1, 12) = 11.4$, $p < 0.01$], whereby KO previously exposed to KD spent little time with the novel object, similar to WT mice, but KO previously exposed to CD spent significantly more time with the novel object compared with all other groups ($p < 0.05$); see Fig 2A. For the chamber that was empty in phase 1, but contained a novel object in phase 2, there was a phase effect [$F(1, 24) = 29.2$, $p<0.0001$]. KO previously exposed to CD spent more time in this chamber compared with all other groups ($p < 0.05$); see Fig 2B.

*3.6. Neural activation in forebrain regions of En2$^{-/-}$ mice in response to novel mouse.*

Immunohistochemistry for exposure to novel mouse (n = 6/group), counts of immunopositive cells indicated a diet effect [$F (1, 179) = 65.8$, $p < 0.0001$], region effect [$F (8, 179) = 13.6$, $p < 0.001$] and diet X region effect [$F (8, 179) = 4.1$, $p < 0.0005$]. Post-hoc testing revealed mice previously exposed to KD displayed more c-Fos counts in the cingulate cortex ($p < 0.005$), lateral septal nuclei ($p < 0.005$) and anterior region of the BNST ($p < 0.05$), see Fig 3. Representative immunohistochemistry micrographs for cingulate cortex, lateral septal region, and anterior BNST are illustrated in Fig 4.

*3.7. Neural activation in forebrain regions of En2$^{-/-}$ mice in response to novel object.*

Immunohistochemistry for exposure to novel object (n = 4/group), counts of immunopositive cells indicated a genotype effect [$F(1, 108) = 6.8$, $p < 0.05$], diet effect [$F(1, 108)=4.4$, $p < 0.05$], genotype X diet [$F(1, 108)=21.5$, $p<0.005$], and region effect [$F(8, 108) = 24.2$, $p < 0.005$]. In the cingulate cortex, post-hoc testing revealed KO previously exposed to CD had higher c-Fos counts than the KO mice previously exposed to KD ($p<0.05$), see Fig. 5). In the lateral septal nuclei and anterior region of BNST, KO mice previously exposed to CD had higher c-Fos counts than WT mice previously exposed to CD ($p < 0.05$ for both regions). In the PVH, KO previously exposed to the CD had higher c-Fos counts than WT previously exposed to the CD and KO previously exposed to the KD ($p < 0.05$ for both). Representative immunohistochemistry images for the cingulate cortex, lateral septal region, anterior BNST, and PVH are illustrated in Fig 6.

## 4. Discussion

Diets high in fat and low in carbohydrate content, which elevate blood ketone bodies, have been used as nutritional therapy for the management for a variety of neurological disorders [13]. This was the first study to investigate how a ketogenic diet influences the neural circuitry modulated by the *En2* gene during juvenile periods of brain development. As such, this study examined the neural and behavioral outcomes following exposure to a ketogenic diet in *En2* knockout (KO) and wild-type (WT) male mice. Mice were fed a ketogenic or control diet during the juvenile period (PND 21-60). The rationale for the timing of the diet intervention is that the juvenile developmental window represents a period of brain reorganization and maturation influenced by dietary events [64, 65].

Our findings indicated an elevation in blood levels of β-hydroxybutyrate with exposure to a diet high in fat (80%) and low in carbohydrate (0.1%) content compared with a protein-

matched control diet (10% fat; 70% carbohydrate). Notably, both diets contained no sucrose. The ketogenic diet produced an approximate 15% elevation in body weight in both the *En2* KO and WT groups. In order to examine the effects of exposure to the ketogenic diet during the juvenile period, the mice were removed from their respective diets (i.e., ketogenic and control) and placed on standard chow (~56% carbohydrate, ~25% fat) for 2 days. One finding of our study was that the ketogenic diet-induced effect on blood β-hydroxybutyrate levels was reversed. Similar rapid effects on blood ketone levels following high-fat, low-carbohydrate diet cessation have been previously reported [57]. Experiments using 16-day-old mice fed a ketogenic diet for 10 days demonstrated an anticonvulsive effect for bicuculline-induced seizures. However, when mice were returned to the standard diet for 3.5 h, blood ketone bodies returned to baseline levels and the anticonvulsant benefits of the diet were also lost [58]. Therefore, in order to determine whether the KD exposure improved social interactions and related behaviors *independent* of blood ketone levels, KD mice were tested following 2 days of standard chow exposure.

Initially, this study found novel effects of the *En2* gene on brain and behavior. In the hippocampus, levels of norepinephrine (NE) and serotonin (5-HT) were found to be 30% and 35% lower, respectively, in *En2* KO compared to WT genotype. Also, in the medial hypothalamus, levels of dopamine (DA) and 5-HT were 32% and 28% lower, respectively, in *En2* KO compared with WT groups. In the cerebellum, NE and 5-HT levels were 20% and 60% higher, respectively, in *En2* KO than WT genotype. Reductions in regional monoamine content in adult (PND 60) *En2* KO mice compared with WT have been reported by Genestine and colleagues and were found to be more prominent at PND 14 and PND 21 than at PND 60 [63]. Reductions in NE content, in particular the hippocampus, have been associated abnormal neurogenesis resulting from a lack of En2 [63]. In addition to changes in monoamine concentration observed in the present study, *En2* KO mice spent more time with the novel

object than WT mice. Previous studies with *En2* KO mice have demonstrated deficits in novel object recognition. In that, *En2* KO mice spent equal time investigating a novel or familiar objects, whereas WT mice spent more time investigating a novel object [43]. The increased time with novel object in our study was accompanied by increased number of cFos positive cells in the lateral septal nuclei, anterior region of BST, and PVH compared with WT mice following novel object exposure. The increase time spent with novel object along with the increased neural activation following novel object exposure could reflect a deficit in sensory processing. This notion, however, needs further experiments to support.

This study also found ketogenic diet-induced effects. Within the brain, there was elevated norepinephrine (NE) content observed in the medial hypothalamus. While the NE signaling pathways are thought to be critical for the anticonvulsive effect of ketogenic diets, and prolonged exposure to a ketogenic diet increases hippocampal NE levels [66, 67]. This is the first study to indicate that NE is increased in the medial hypothalamus. The increase in NE content was only observed in the WT mice fed the ketogenic diet compared with WT fed the control diet. In fact, the *En2* KO mice did not have altered monoamines in response to the diet intervention. Such data suggest that the ketogenic diet does not alter the monoamine content in *En2* KO mice in the regions examined. One notable limitation of our study is that we used tissue homogenates to measure monoamine concentration. Another limitation to this study is the analysis of the cerebellum as a whole instead of distinct regions, since individual regions of cerebellum have been hypothesized to contribute to the pathology of ASD [68]. We also observed ketogenic diet-induced effects in the neural response to novel mouse exposure. Our c-Fos immunohistochemistry findings indicated more neural activation of forebrain areas, such as the cingulate cortex, lateral septal region, and anterior bed nucleus of the stria terminalis, with previous exposure to the ketogenic diet. Because mice fed the ketogenic diet were heavier than those animals fed the control diet, one contributing factor to these observed differences

could be the metabolic alterations caused by weight gain. Future studies will use a more targeted *in vivo* approach to examine the role of ketogenic diet-induced increases in monoamines in specific hypothalamic regions.

Our findings indicated an interaction between *En2* gene status and diet was observed for some outcomes. That is, previous exposure to the ketogenic diet increased frontal social contact and reduced grooming behavior in the *En2* KO mice. These behavioral results support previous findings by Ruskin and colleagues in the BTBR T +tf/J (BTBR) mouse model, in which BTBR mice that were fed and maintained on a ketogenic diet demonstrated increased sociability compared to control diet-fed BTBR counterparts [69]. In contrast, our findings in the WT mice, which showed a similar behavioral response in the three-chamber regardless of diet, indicate that exposure to a ketogenic diet per se does not result in a generalized increase in social interaction with a novel mouse. In addition, it appears that ketogenic diet-induced improvement is likely a result of the brain changes that accompany elevation in ketonemia since recent findings by Zilkha and colleagues (2016) demonstrated maintenance of a high fat diet (60% fat, 20% carbohydrates, 20% protein) compared with maintenance of a control diet (10% fat, 70% carbohydrates, 20% protein) reduced social interaction of BTBR mice [70]. In the study by Zilkha and colleagues, separately run (and analyzed) control mice (C57) maintained on the high fat diet did not show social impairments compared with control mice maintained on the control diet [70]. It is worth noting in our study that we only observed a ketogenic diet-induced increase in frontal contact time and a decrease in grooming behavior in the *En2* KO mice. That is, we did not observe differences in frontal contact time between the WT mice previously fed the control diet and *En2* KO mice previously fed the control diet. Our c-Fos immunohistochemistry findings indicate that neural activation of forebrain areas by previous exposure to the ketogenic diet could have differential behavioral effects in *En2* KO mice to improve social interaction with a novel mouse. Brielmaier and colleagues [43] previously reported deficits in sociability and

related behaviors in a three chamber test in adult (8-10 weeks old) *En2* KO mice compared with WT (*En 2*$^{+/+}$) and heterozygous (*En 2* $^{+/-}$) controls. These mice were fed standard chow throughout the study and test periods [43]. Taken together with our study, it is plausible that the switch in diet two days prior to social testing could have influence our ability to replicate the social impairments in *En2* KO compared with WT mice previous fed the control diet. Therefore, a limitation of our studies was that behavioral testing and neural activation were performed after a 2 day wash-out of control and ketogenic diets. This diet switch does not allow us to determine whether there is a persistent effect of the diets fed during the juvenile period or whether the result were caused by metabolic changes due to the diet switch. Future studies will be needed to determine whether our reported effects are consistent with *En2* KO mice maintained on ketogenic and control diets, as well as the long term (> 2 days) effects of juvenile ketogenic diet exposure on adult neural and behavioral outcomes. Notwithstanding, our findings demonstrate that exposure to ketogenic diet during the juvenile period increases social performance in a mouse model with autistic-like behaviors.

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**Figure 1. Social behaviors in a three-chamber social interaction test with a novel mouse at PND 62.** Two days after switching from experimental diets (KD or CD) to standard chow, mice were exposed to a three-chamber social test. Groups were designated KO-KD (n = 14), KO-CD (n = 13), WT-KD (n = 12), and WT-CD (n = 13) based on diet exposure during the juvenile period (PND 21-60). Mice were placed in the three-chamber test for a total of 30 min with three 10-min phases with an adult male *En2*$^{+/-}$ non-litter mate (novel mouse). Average times are mean ± SE. **A:** Average total time (30 min) engaging in frontal contact with the novel mouse. **B.** Average total time (30 min) engaging in self-grooming. * indicates differences ($p < 0.05$) from KO-CD.

**Figure 2. Behavior in a three-chamber apparatus when exposed with a novel object at PND 62.** Two days after switching from experimental diets (KD or CD) to standard chow, mice were exposed to a novel object in the three-chamber test. KO-KD, KO-CD, WT-KD, and WT-CD mice (n = 4/group) were placed in the three-chamber test for a total of 20 min with two 10-min phases with or without a novel object previously housed in a cage of novel male mice. Average times are mean ± SE. **A.** Average total time (20 min) engaging in contact with a novel object. ** indicates difference from all other groups ($p < 0.05$). **B.** Average total time (10 min per phase) spent in chamber that has a novel object in phase 2. ** indicates difference from all other groups ($p < 0.05$).

**Figure 3. Average immunoreactive c-Fos counts in the forebrain regions in mice with exposure to a novel mouse.** Mice were euthanized 90 minutes after novel mouse exposure (see Figure 1; n = 6/group). Counts are mean ± SE. Mice previously exposed to KD had higher counts in the cingulate cortex, lateral septal nuclei, and anterior region of the BNST. ##

indicates difference from CD exposed groups ($p < 0.005$), # indicates difference from CD exposed groups ($p < 0.05$). Abbreviations; PFC (prefrontal cortex), Cing Cortex (Cingulate cortex), LSN (lateral septal nuclei), NAc core (Nucleus accumbens core region), aBNST (anterior bed nucleus of the stria terminalis), AH (anterior hypothalamus), PVH (paraventricular hypothalamic nucleus).

**Figure 4. Representative coronal micrographs of c-Fos staining of forebrain regions in mice with exposure to a novel mouse.** Images are **A:** cingulate cortex (area 1 and 2) from WT mouse with previous exposure to KD. **B:** cingulate cortex (area 1 and 2) from WT mouse with previous exposure to CD. **C:** Lateral septal nuclei from WT mouse with previous exposure to KD. **D:** Lateral septal nuclei from WT mouse with previous exposure to CD. **E:** Anterior region of the bed nucleus of the stria terminalis from WT mouse with previous exposure to KD. **F:** Anterior region of the bed nucleus of the stria terminalis from WT mouse with previous exposure to CD. Scale bars are 153 μm. Abbreviations: CC: corpus callosum; cg: cingulum; LV: lateral ventricle; MS: medial septal region; aca: anterior commissure; ic: internal capsule.

**Figure 5. Average immunoreactive c-Fos counts in the forebrain regions in mice with exposure to novel object.** Mice were euthanized 90 minutes after novel object exposure (see Figure 2, n = 4/group). Counts are mean ± SE. KO mice previously exposed to CD had higher counts in the cingulate cortex, lateral septal nuclei, and anterior region of the BNST, and PVH. # indicates difference ($p<0.05$) from KO- KD exposed groups, $ indicates difference ($p< 0.05$) from WT-CD. Abbreviations; PFC (prefrontal cortex), Cing Cortex (Cingulate cortex), LSN (lateral septal nuclei), NAc core (Nucleus accumbens core region), aBNST (anterior bed nucleus of the stria terminalis), AH (anterior hypothalamus), PVH (paraventricular hypothalamic nucleus).

**Figure 6. Representative coronal micrographs of c-Fos staining of forebrain regions in mice with exposure to a novel object**. Images are **A:** cingulate cortex (area 1 and 2) from KO mouse with previous exposure to CD. **B:** cingulate cortex (area 1 and 2) from KO mouse with previous exposure to KD. **C:** Lateral septal nuclei from KO mouse with previous exposure to CD. D: Lateral septal nuclei from WT mouse with previous exposure to CD. **E:** Anterior region of the bed nucleus of the stria terminalis from KO mouse with previous exposure to CD. **F:** Anterior region of the bed nucleus of the stria terminalis from WT mouse with previous exposure to CD. **G:** Paraventricular hypothalamic nucleus from KO mouse with previous exposure to CD. **H:** Paraventricular hypothalamic nucleus from WT mouse with previous exposure to CD. Scale bars are 153 μm. Abbreviations: CC: corpus callosum; cg: cingulum; LV: lateral ventricle; MS: medial septal region; aca: anterior commissure; ic: internal capsule; 3V: 3$^{rd}$ ventricle; f: fornix.

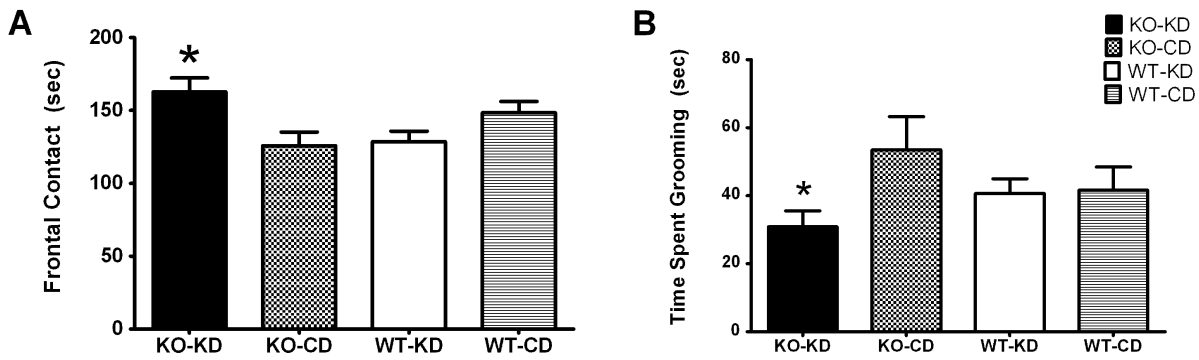


Figure 1

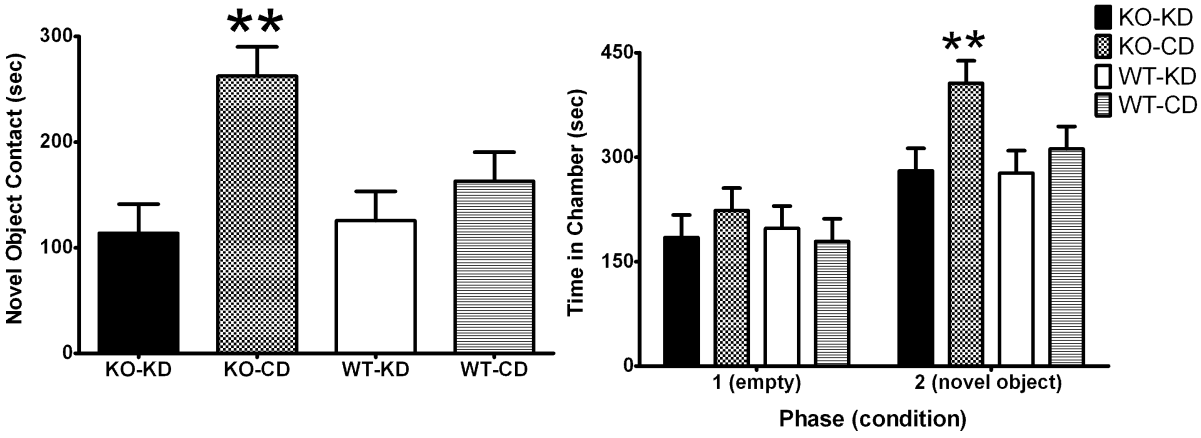


Figure 2

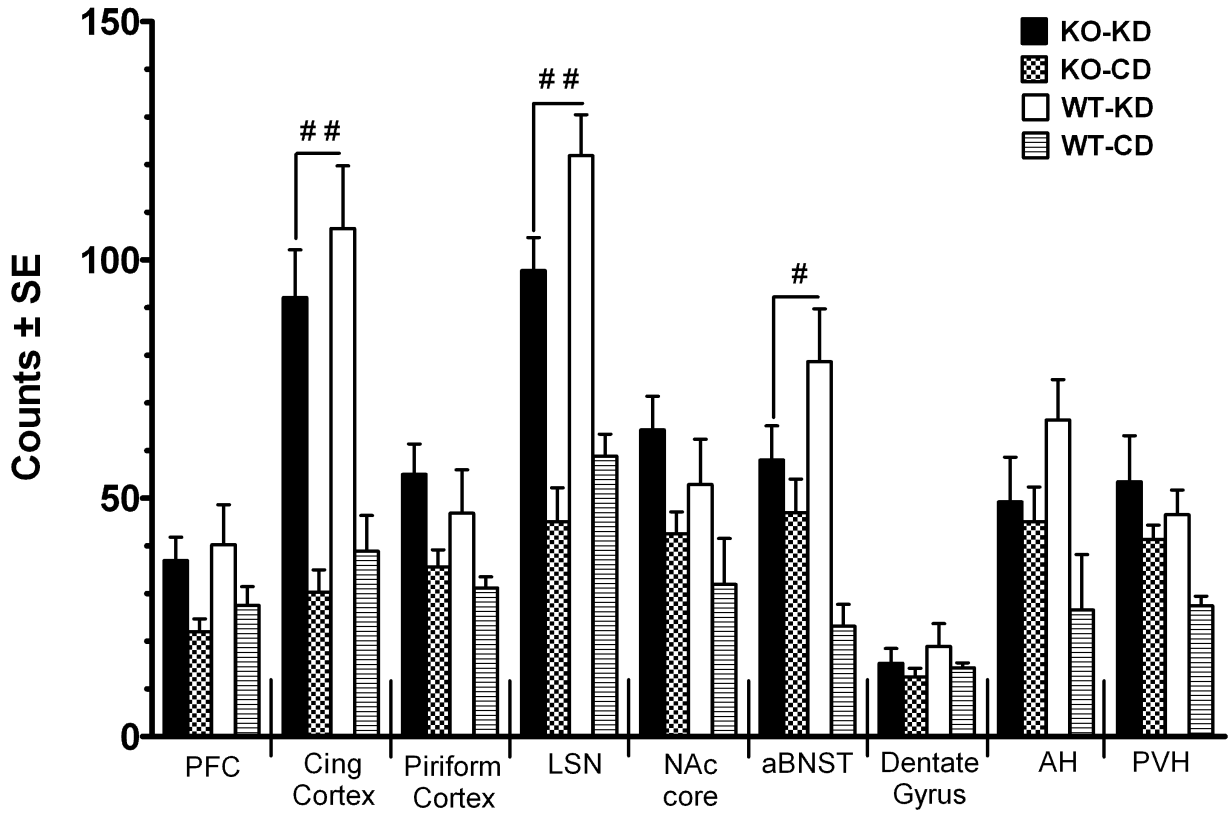


Figure 3

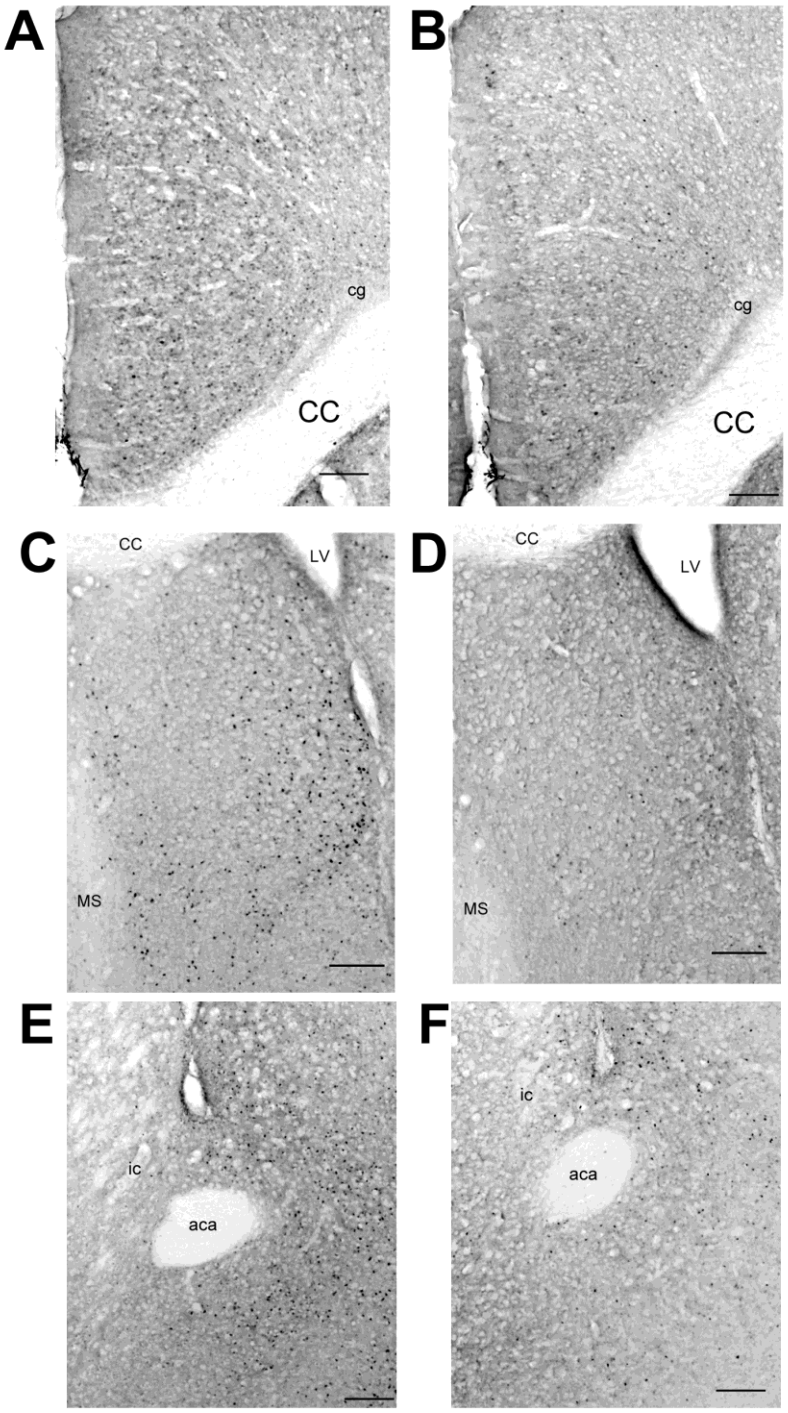


Figure 4

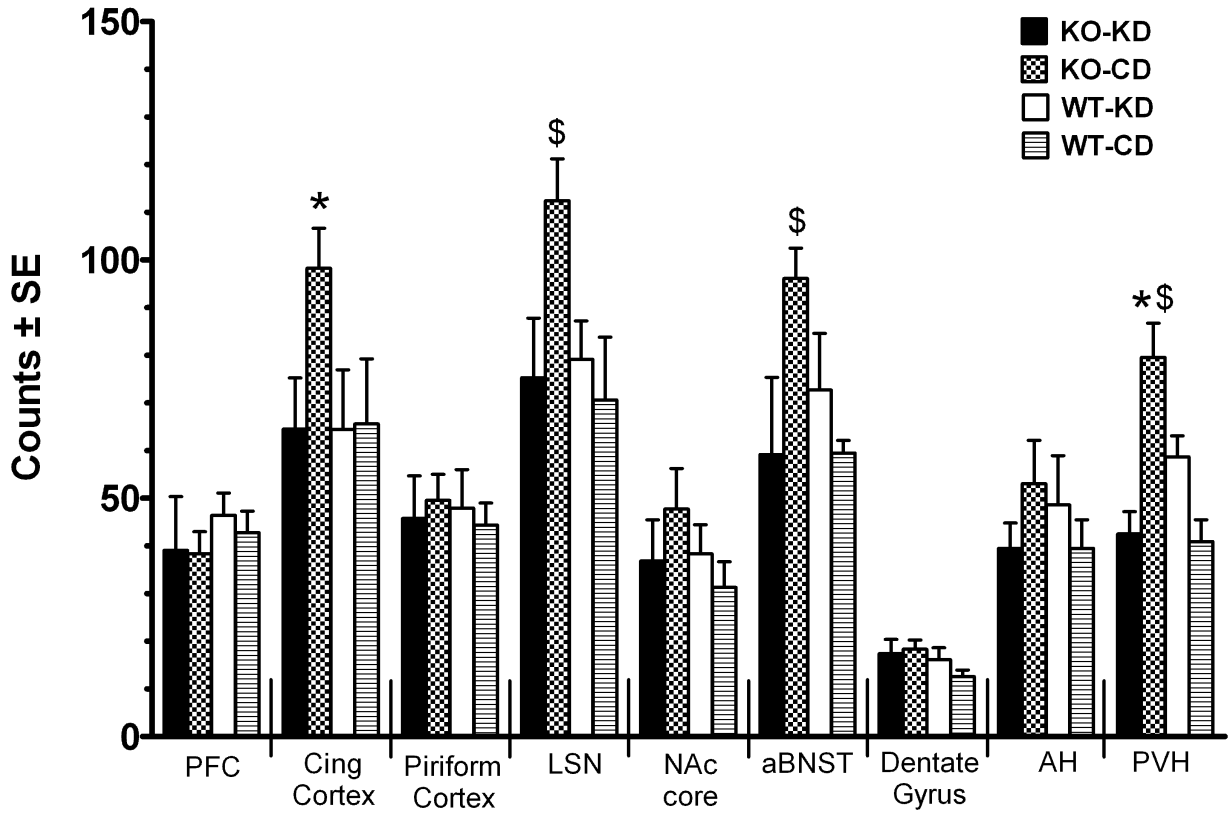


Figure 5

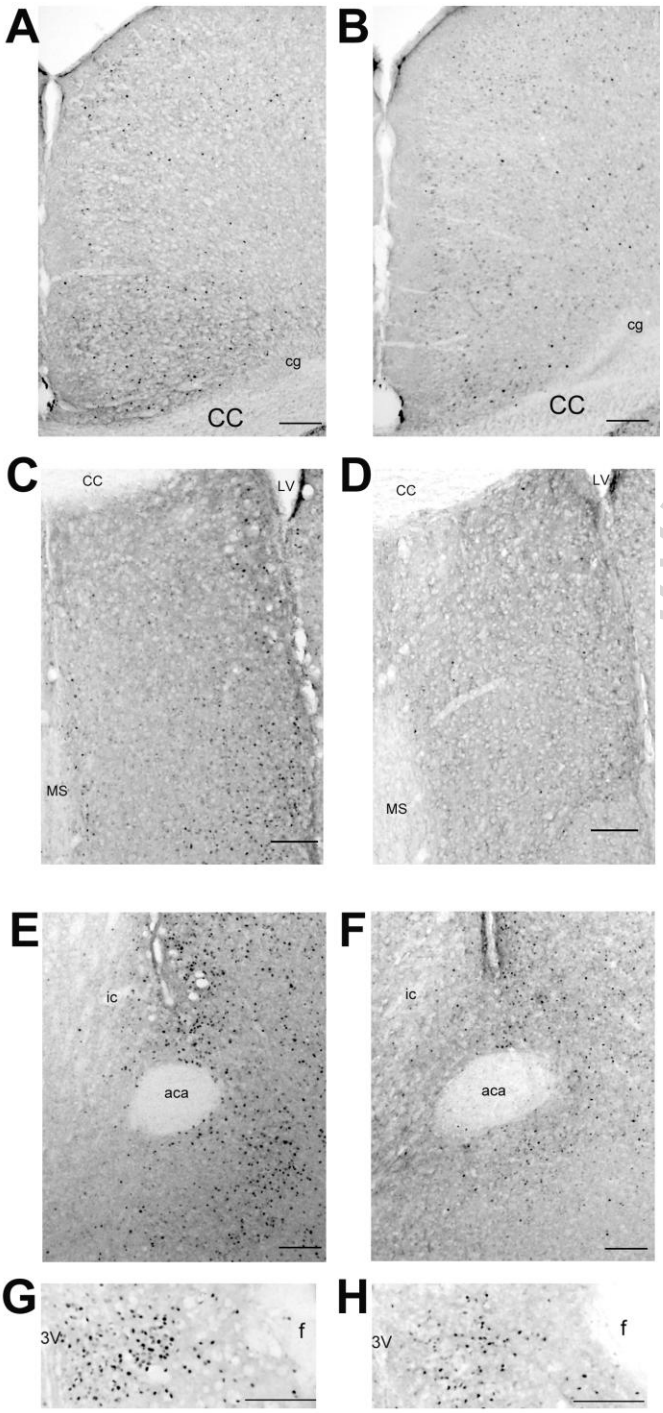


Figure 6

**Table 1.** Monoamine and metabolite concentrations (pg/mg of tissue) after dietary exposure at PND 60.

**Frontal Cortex**

| Groups | NE | DA | 5-HIAA | HVA | 5HT |
|---|---|---|---|---|---|
| KO-KD | 672.3 ± 48.3 | 2837.3 ± 581.4 | 299.1 ± 34 | 323.5 ± 21.8 | 228.0 ± 16.2 |
| KO-CD | 755.8 ± 70.2 | 3955.5 ± 691.2 | 239.2 ± 24.7 | 355.8 ± 25.6 | 249.8 ± 17.2 |
| WT-KD | 779.6 ± 41.7 | 2871.6 ± 502.6 | 269.3 ± 22.6 | 351.0 ± 23.0 | 218.6 ± 14.1 |
| WT-CD | 923.7 ± 112.8 | 3176.5 ± 706.5 | 261.6 ± 26.3 | 317.4 ± 26.7 | 204.9 ± 16.6 |

**Hippocampus**

| Groups | NE | DA | 5-HIAA | 5HT |
|---|---|---|---|---|
| KO-KD | 946.6 ± 91.3 # | 798.8 ± 501.9 | 983.0 ± 214.1 | 244.9 ± 31.5 # |
| KO-CD | 1068.2 ± 163.6 | 1266.1 ± 586 | 1777.3 ± 971.8 | 264.0 ± 51.4 |
| WT-KD | 1448.2 ± 225.8 | 613.8 ± 202.3 | 1225.3 ± 341.4 | 322.5 ± 52.0 |
| WT-CD | 1476.1 ± 344.3 | 829.8 ± 254.5 | 2078.1 ± 573.9 | 457.0 ± 113.6 |

**Hypothalamus**

| Groups | NE | DA | 5-HIAA | HVA | 5HT |
|---|---|---|---|---|---|
| KO-KD | 3961.6 ± 418.2 | 839.2 ± 88.8 # | 1176.0 ± 165.4 | 62.4 ± 8.8 | 406.9 ± 47 # |
| KO-CD | 4145.5 ± 886.9 | 662.5 ± 105.7 | 1116.3 ± 176.5 | 80.6 ± 17.0 | 370.4 ± 49.4 |
| WT-KD | 5327 ± 745.1 * | 1228.1 ± 195.0 | 2312.5 ± 394.3 | 135.1 ± 28.0 | 623.2 ± 75.7 |
| WT-CD | 2861.5 ± 446.5 | 1030.2 ± 285.2 | 1351.8 ± 195.0 | 90.0 ± 19.3 | 443.5 ± 79.3 |

**Cerebellum**

| Groups | NE | 5-HIAA | HVA | 5HT |
|---|---|---|---|---|
| KO-KD | 1275.8 ± 100.2 # | 269.8 ± 20.7 | 13.0 ± 2.1 | 112.5 ± 14.6 # |
| KO-CD | 1215.6 ± 108.1 | 355.7 ± 32.8 | 14.7 ± 2.2 | 129.7 ± 18.0 |
| WT-KD | 996.6 ± 92.5 | 312.1 ± 39.4 | 9.3 ± 0.7 | 64.0 ± 6.3 |

Male *En2* KO and WT mice fed a ketogenic or control diet from PND 21 to 60. Values are means ± SEM. # indicates genotype effect with KO differences from WT ($p<0.05$). * indicates differences from WT-CD ($p<0.05$). Abbreviations NE: norepinephrine, DA: dopamine, 5-HIAA: 5-hydroxyindole acetic acid, HVA: homovanillic acid, 5HT: 5-hydroxytryptamine

Highlights

- Ketogenic diet (KD) has neuroprotective effects
- *Engrailed 2 (En 2)* null mice have impaired brain monoamines and social behaviors
- Juvenile KD elevated hypothalamic norepinephrine in *En2*$^{+/+}$, not *En 2*$^{-/-}$ mice
- Juvenile KD rescued novel mouse and object interaction in *En2*$^{-/-}$
- Neural activation was differential affected by juvenile KD