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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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HIGHLIGHTS

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

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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 improve 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 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 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.

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 in ketone 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) and lead to improvements in mitochondrial function [7–10]. Emerging evidence suggests that KD or modified KD could have clinical utility for other neurological and developmental

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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 midbrain to hindbrain regionalization, cerebellar development, and neural growth and maturation [25–31]. During early embryogenesis, *En* genes are expressed in the midbrain 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 midbrain and hindbrain nuclei, the locus coeruleus (LC) and the raphe nuclei (RN), ultimately resulting in abnormal levels of norepinephrine (NE) and serotonin (5-hydroxytryptamine; 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 studies, 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, and 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 three-chamber 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.

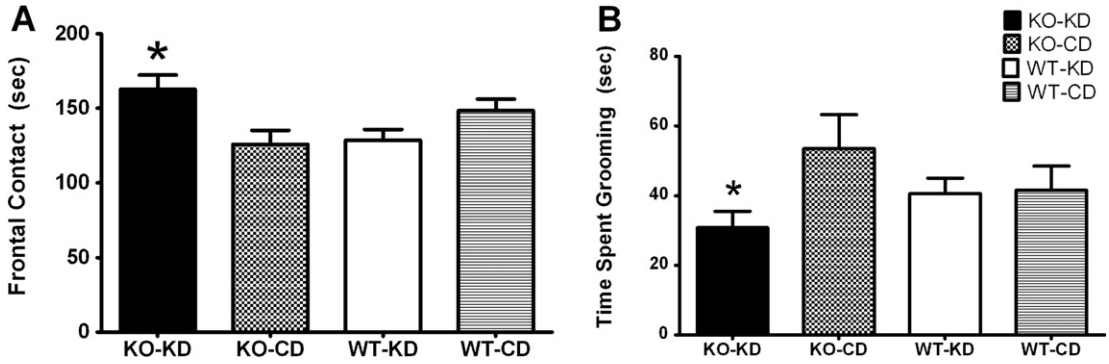

Fig. 1. Social behaviors in a three-chamber social interaction test with a novel mouse at PND 62. Two days after switching from experimental diets (KD or CD) to standard chow, mice were exposed to a three-chamber social test. Groups were designated KO-KD (n = 14), KO-CD (n = 13), WT-KD (n = 12), and 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-littermate (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.