NEURAL BEHAVIORAL OUTCOMES OF A KETOGENIC DIET IN

ENGRAILED-2 NULL MALE MICE

By

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ABSTRACT OF THE DISSERTATION

Neural Behavioral Outcomes of a Ketogenic Diet In *Engrailed-2* Null Male Mice

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Dr. Nicholas T. Bello

A ketogenic diet (KD), high in fat and low in carbohydrates, reduces seizure activity in pediatric epilepsy and improves autistic-related behaviors, but the neural mechanisms involved in these improvements are unknown. In the following studies *Engrailed-2 (En2)* knockout (KO, *En2*−/−) and wild-type (WT) male mice were fed either a KD or control diet (CD) from postnatal day (PND) 21 to PND 60 (childhood to young adulthood). We hypothesized that a KD fed during this critical time period would alter biogenic amine concentration, metabolism, behavioral deficits, and ultimately neural activation in *En2*−/− mice. Biogenic amine levels of norepinephrine (NE), dopamine (DA), and serotonin (5-HT) were reduced in forebrain regions and increased in the cerebellum of KO mice, consistent with previous findings. A KD increased hypothalamic NE in WT mice, but not in KO mice. Social behaviors, i.e. frontal contact, were increased and repetitive grooming behaviors reduced in KO-KD. Regardless of previous diet, KO mice displayed increased chow intake post-restraint stress, indicative of a coping response. A KD restored body weight in KO mice by increasing lean mass to WT-KD, but also increased fat mass. KO-KD had enhanced fat metabolism, increased blood glucose response, and reduced blood pressure. KO-KD exposed to a stranger mouse had increased c-Fos
immunoreactivity in the cingulate cortex, septal region, and paraventricular nucleus of the hypothalamus (PVN). KO-CD spent more time interacting with a novel object and had increased c-Fos immunoreactivity in the bed nucleus of the stria terminalis (BNST) and PVN. The unique activation of the cingulate and septal region in KO-KD with exposure to a stranger mouse, suggests that these areas could be critical for social behaviors. This research has implications for understanding the impact of a KD on neural development and autistic-like behaviors.
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INTRODUCTION

Autism spectrum disorder (ASD) is a neurodevelopmental disorder that was first described in 1943. It was originally defined by a triad of deficits: impaired social interactions, impaired communication, and restricted or repetitive behaviors. Clinical studies have revealed growth abnormalities in the cerebellum, cerebrum, amygdala, and hippocampus. These impairments may contribute to the behavioral and metabolic dysfunctions, such as abnormal stress responses, elevated levels of cortisol, and body weight homeostatic imbalance observed in ASD. According to the Center for Disease Control (CDC), 1 in 68 children are diagnosed with ASD, with boys having a higher prevalence rate (5 times more common). Although the exact cause for the development of ASD is unknown, it is thought that a combination of genetic and environmental factors may contribute to improper neural development. Not only are medical expenses for ASD patients higher than those without ASD (4.1-6.2 times greater), behavioral interventions cost $40,000 to $60,000 per child per year. Thus, research into ASD is crucial to attempt to better understand this disorder and discover new treatments. This doctoral project aims to look at the effects of a ketogenic diet in the Engrailed-2 knockout mouse, which displays autistic-like behaviors, to determine if behavioral, metabolic, and neural outputs can be positively altered.
Autism Spectrum Disorder Prevalence

The prevalence of autism spectrum disorder (ASD) has increased twenty-to thirty-fold since the late 1960s, when it was estimated that one in 2,500 children were diagnosed with ASD (Baio 2014). The term autism is derived from the Greek word "autos", which means self, from the characteristic social avoidance behavior of the disorder. Initially thought to be a form of childhood schizophrenia, individuals were promptly institutionalized. Leo Kanner was the first to describe autism as an unique developmental disorder, distinct from schizophrenia, in 1943 by his observations of autistic patients at Johns Hopkins child psychiatry hospital. In his paper, he identified two critical features of autism: aloneness and sameness. (Eisenberg et al., 1956; Kanner 1968). By 1980, ASD was defined in the Diagnostic and Statistical Manual of Mental Disorders (DSM) III as a pervasive developmental disorder, distinct from schizophrenia. The three domains included: lack of responding to others, impairments in communication, and bizarre responses to the environment, all of which developed within the first 30 months of age (Pichot 1986). Even thirty years ago it was understood that ASD develops very young and yet diagnostic measures have not been improved to diagnose before 18 months of age.

Today ASD in the DSM-V is defined as deficits in social communication and social interaction across multiple contexts, which persist, and severity is determined by social communication impairments and restricted, repetitive patterns of behavior. Deficits in social-emotional reciprocity range from reduced sharing of interests and abnormal social approach to complete failure of verbal communication. Nonverbal communicative behavioral deficits include abnormal eye contact and body language, lack of facial
expressions, and inability to understand nonverbal gestures. Most notably, deficits in developing, maintaining, and understanding relationships is disabling in ASD. These deficits even impact early relationships by lacking the ability to imaginative play with friends or siblings to a complete lack of interest in other peers. These symptoms are further defined by severity, in which individuals must manifest at least two of the following symptoms: stereotyped or repetitive behaviors, sameness or ritualized routines, restricted and intense fixated interests, and hyper- or hypo-reactivity to sensory input. Individuals must have symptoms that are present early in development, which significantly impacts daily life, and are not better explained by an intellectual disability. In addition, this diagnosis can be accompanied with comorbidities, such as intellectual or language impairment, medical or genetic conditions, catatonia, or another mental or behavioral disorder (American Psychiatric Association 2013).

Currently 1 in 68 children are identified on the autistic spectrum with prevalence being higher in boys (1 in 42) than girls (1 in 189) (Baio 2014). The alarming increase in the number of children diagnosed with ASD has spurred researchers to investigate causative gene and environmental factors. It is recognized that this increased prevalence rate could be partially attributed to improved recognition and diagnosis. Nevertheless, there is very little that can be done to improve quality of life, due to a lack of understanding of basic mechanisms that result in ASD. In addition, children are typically not diagnosed until age 4, when developmental milestones are missed, but researchers have shown that a reliable diagnosis can be determined by 18 months of age by well-trained professionals (Ventola et al., 2008). Typical screenings for developmental delays and disabilities are completed at 9, 18, 24 or 30 months of age, as recommended by the American Academy of Pediatrics. There are many screening tools for ASD including, but not limited to, the Ages and Stages Questionnaire (ASQ),
Modified Checklist for Autism in Toddlers (MCHAT), Communication and Symbolic Behavior Scales (CSBS), and Screening Tool for Autism and Young Children (STAT). Further specific diagnostic tools can be used to classify and determine severity of the autism. These include: Childhood Autism Rating Scale (CARS), Autism Diagnosis Observation Schedule-Generic (ADOS-G), and Gilliam Autism Rating Scale-Second Edition (GAR-2) (Srour et al., 2006). Interestingly, ASD symptoms appear during very early neural development, during which time there is an increase in synaptic density, brain glucose metabolism, and gray matter density followed by a significant elimination or pruning of neurons (Spear 2000; Andersen 2003; Bingham et al., 2011), figure 1. These alterations have been found to coincide with psychiatric diseases, such as schizophrenia, bipolar disorder, eating disorders, and attention deficit disorder (Paus et al., 2008). ASD may be a result of improper synaptic pruning during development, but there is lack of evidence to support this theory (Saugstad 2008; Thomas et al., 2011). It is also unknown whether genetic or environmental factors could influence neural development resulting in ASD symptoms, but if a critical window of development could be determined, an intervention during this time may improve ASD symptoms.

**Pharmacotherapy for ASD**

Despite having behavioral similarities to other psychiatric diseases, pharmaceutical medications for ASD do not target core symptoms. Pharmacotherapies are prescribed to relieve comorbid symptoms, such as aggressive tendencies, hyperactivity, anxiety, and self-injury. The only two medications that are approved for individuals with ASD by the US Food and Drug Administration (FDA) are risperidone and aripiprazole. Both these medications act on biogenic amine receptors for dopamine (D2), serotonin (H1, 5-HT1A, and 5-HT2A), and adrenergic (alpha 1 and alpha 2) receptors.
Risperidone is an antagonist for D2, 5-HT2A, alpha 1, alpha 2, and H1 receptors and was first implicated as a treatment for schizophrenia (Leysen et al., 1994; Marder et al., 1994; Farde et al., 1995). Similarly, aripiprazole is a 5-HT2A antagonist and is thought to act on the mesocortical pathways in schizophrenia, but it is also a partial D2 and 5-HT1A agonist (Stahl 2001; Potkin et al., 2003; Hirose et al., 2004; McIntyre 2011). These atypical antipsychotics have been shown to treat irritability, including aggression, self-injury, and severe tantrums in ASD patients. Yet, these medications can increase risk for metabolic syndrome, dyslipidemia, and hyperglycemia. Aripiprazole can even increase aggressive behaviors. More importantly, these medications only treat side-effects and not the disease itself. Thus, these medications are only recommended as a last resort option (Martin et al., 2004; Dinnissen et al., 2015). Although only risperidone and aripiprazole are approved for ASD, these are not the only two medications prescribed to ASD patients. Unfortunately, many pharmacotherapies are given to help calm ASD patients to alleviate stress for parents and caregivers, but the few studies analyzing efficacy of these treatments are confounded by small sample size, research design and intolerance to the medications. Thus, physicians must be sure pharmacotherapy is really in the best needs of the patient. Methylphenidate, a modulator of dopamine and noradrenaline (Maxwell 1965; Solanto 1986; Scahill et al., 2004; Jahromi et al., 2009), and atomoxetine, a selective noradrenaline reuptake inhibitor (Christman et al., 2004; Liu et al., 2008; Garnock-Jones et al., 2009; Bari et al., 2013), are prescribed to treat attention deficit hyperactivity disorder symptoms, but response rates are typically low and ASD children have higher adverse side-effects than non-ASD children (Research Units on Pediatric Psychopharmacology Autism 2005). In addition, antidepressants are prescribed for ASD patients. For instance, the antidepressant citalopram, a selective serotonin reuptake inhibitor (SSRI), does not improve ASD behaviors significantly different from placebo (King et al., 2009). Even though there is a lack of evidence for the
efficacy of antidepressants for ASD, they are the most commonly prescribed treatment (Kolevzon et al., 2006; Camacho et al., 2014).

Without a definitive pharmacotherapeutic intervention available, researchers have begun to analyze alternative treatment options; one of note is the use of the neurohormone oxytocin. This nine-amino-acid peptide is synthesized in both the paraventricular and supraoptic nucleus of the hypothalamus and plays a large role facilitating uterine contractions during labor and in milk let-down during lactation (Sawchenko et al., 1984; Lee et al., 2009). It has been hypothesized that oxytocin may play a role in social behaviors and in one clinical trial, adults with ASD who received intravenous infusion of oxytocin had a reduction in repetitive behaviors and improved social/emotional recognition (Hollander et al., 2003). Yet, efficacy for this treatment option in children and adolescence is not clear. Studies have shown conflicting reports as to whether oxytocin can improve ASD symptoms (Gordon et al., 2013; Dadds et al., 2014). Therefore, without studies with proper controls and appropriate sample size, it is unclear whether current pharmacotherapy treatments and alternative treatments, such as oxytocin, for modulating and treating ASD symptoms are efficacious.

**Nutritional Comorbidities with ASD**

Although pharmacotherapeutic strategies have shown little efficacy in regards to treating ASD symptoms, some success has been observed using nutritional interventions and diet therapies (Witwer et al., 2005; Marti 2010). This type of treatment is justified by the large number of comorbid nutritional disorders with ASD including phenylketonuria, glucose-6-phosphatase deficiency, propionic acidemia, adenosine deaminase deficiency, Smith-Lemli-Opitz syndrome, and branched chain ketoacid
dehydrogenase kinase deficiency (Ciaranello et al., 1982; Evangeliou et al., 2001; Schaefer et al., 2006; Kayser 2008; Ghaziuddin et al., 2013; Spilioti et al., 2013). In addition, autistic individuals typically have food sensitivities, leading to a higher risk of having reduced body weight and a lower body mass index (BMI). In some ways this may be due to the “sameness” that is observed in autistic individuals with low tolerance to trying new foods, but many also have physical gastrointestinal intolerance reducing the type of foods they can consume (Kang et al., 2014). It has been shown that this unintentional diet restriction may result in nutritional deficiencies and inadequate intake of fiber, vitamin D, vitamin B12, calcium, and folate (Zimmer et al., 2012; Graf-Myles et al., 2013). Common dietary interventions used for ASD patients include gluten-free diet, casein-free diet, specific carbohydrate diet, and a ketogenic diet (KD) (Srinivasan 2009). It has been estimated that 15.5% of ASD patients are on a modified diet, but studies are confounded by small sample size and a heterogeneous study group (Witwer et al., 2005). These modified diets have low adverse effects, unlike the medications previously described, and discontinued use will not result in withdrawal or an adverse event. For instance, if not managed carefully, withdrawal symptoms of aripiprazole and risperdal can include severe anxiety, delusions, depression, insomnia, mood swings, suicidal thoughts, and nausea to name a few (Stein-Reisner et al., 2004; Kim et al., 2010).

Elimination diets, such as a gluten and/or casein-free diet, may be efficacious in individuals that lack enzymes to digest gluten and casein. It is thought that some individuals may have increased gut permeability resulting in increased sensitivity to specific foods. In a chronic 1-8 year study of 70 autistic children following a Gluten Free Casein Free (GFCF) diet, 81% of patients improved significantly by the third month of diet intervention and had continued improvements in autistic-like behaviors. Of the 19% that did not improve, about 1/3 did not continue with the dietary intervention (Shattock et
al., 2002; Reichelt et al., 2009; Pennesi et al., 2012). This study and others (Knivsberg et al., 2002; Whiteley et al., 2010; Pennesi et al., 2012) have shown improved ASD symptoms from a casein-free and gluten-free diet, but the mechanism for this improvement is unknown. More research involving the brain-gut axis is needed to understand why this restriction diet may be beneficial. Unlike an intervention period, this diet may need to be permanent for these individuals to have sustained clinical improvement in ASD signs and symptoms. Gastrointestinal disturbances in ASD are supported by a study at the Harvard Medical School, which collected intestinal biopsy samples from 199 children and adults with ASD. Results showed that 62% had deficiencies in lactase, 16% were deficient in sucrase, and 10% were deficient in maltase (Kushak et al., 2011). Although identification of these nutritional problems and intestinal intolerances do not provide a cure for autism, it will reduce gastrointestinal disturbances and increase quality of life. Because of these demonstrated clinical improvements, more rigorous studies are necessary to understand the mechanisms by which diet may improve ASD symptoms.

**Efficacy of the Ketogenic Diet in Neurological Disorders**

Before being coined the “Adkins diet”, the KD had been used to treat children with drug-resistant epilepsy since the 1920’s (Vining 1999; Lefevre et al., 2000; Keene 2006). It is thought that even prior to the KD being used, a fasting diet was implemented to control seizure activity (Wheless 2008). Both fasting and the KD result in low glucose levels, the primary energy substrate for brain metabolism, but the resulting production of ketone bodies (acetoacetate and β-hydroxybutyrate) can be used as energy substitutes for the brain (Stafstrom et al., 2003). The KD is very low or absent in carbohydrates and
high in fat, but differs from a high fat diet (HFD) in that it does not result in rapid weight gain and metabolic impairments (Roncon-Albuquerque et al., 2008; Bartolomucci et al., 2009; Auvinen et al., 2012; Sharma et al., 2013). For pediatric epilepsy, the KD is based on a ratio of 3:1 or 4:1 (fat:carbohydrate) with protein levels kept at age requirements determined by the World Health Organization (Vining 1999). The classic KD uses long-chain triglycerides (LCT), but medium-chain triglycerides (MCT) result in a higher ketone production, because octanoic and decanoic acids are more easily transported into cells (Huttenlocher 1976; Liu 2008). Using MCT ketogenic diet allows for a more balanced diet (with higher carbohydrate content), but has increased side-effects including abdominal bloat and diarrhea. A classic KD also has adverse side effects, including constipation, dyslipidemia, lethargy, hypoglycemia, and acidosis (Johnstone et al., 2008; Paoli et al., 2013). Even though a KD may be difficult to tolerate long-term, the reduction or complete elimination of seizure activity can greatly improve quality of life and prevent further neural damage.

The efficacy of the KD for epilepsy in large populations is difficult to determine, since patient number is typically low, pathologies differ, and intervention duration can vary. One of the first published studies in 1925 determined the KD eliminated seizure activity in 60% of patients and 35% had a 50% reduction in seizures (Peterman 1925). Yet, shortly after this observation, the first anticonvulsant, diphenylhydantoin, was discovered and use of the KD was dramatically reduced. It was not until the 1990s that the KD was re-discovered as a treatment for patients resistant to pharmacotherapies (Vamecq et al., 2005). In 1998, a large study at Johns Hopkins Hospital was conducted. The KD was fed to 150 patients for 3 months, which resulted in 3% of patients becoming seizure free and after 12 months this rate increased to 7%. Overall 20% had more than a 90% reduction in seizures and 23% had at least a 50% reduction in seizure activity.
(Vining et al., 1998). It is still unknown how the KD results in reducing seizure activity, but it is still widely used in patients that do not respond to pharmacotherapy.

Pediatric epilepsy is not the only disorder for which the ketogenic diet has been shown to have efficacy. The KD may benefit individuals with Parkinson’s disease (Gasior et al., 2006), infantile phosphofructokinase deficiency (Swoboda et al., 1997), McArdle disease or glycogenosis type V (Busch et al., 2005), migraine sufferers (Di Lorenzo et al., 2015), type 2 diabetes mellitus, Alzheimer’s disease (Henderson 2008), hypercholesterolemia (Dashti et al., 2007; Al-Khalifa et al., 2009; Paoli et al., 2013), and ASD (Evangeliou et al., 2001; Evangeliou et al., 2003) to name a few. Unfortunately many of these studies have low sample size, diverse pathologies, and a lack of controls. ASD is especially intriguing since 20-30% of children with autism will develop epilepsy by adulthood, thereby providing justification for such dietary interventions (Chavez et al., 2007). Evangeliou and colleagues (2003) evaluated the use of a KD in 30 children ages 4-10 years with ASD in a pilot study. Children were fed a modified John Radcliffe diet consisting of 19% energy from carbohydrates, 10% from protein, and 71% from fat for 6 months. Although not a very low carbohydrate ketogenic diet, 60% of the children demonstrated improvements in their CARS scores (Evangeliou et al., 2003). After this study, Evangeliou and colleagues (2013) sought to determine possible reasons for KD efficacy in these children with ASD. They screened 187 ASD children (105 males and 82 females between 4-14 years old) for inborn errors of metabolism. In six patients with elevated blood serum β-hydroxybutyrate post-glucose challenge, the KD improved CARS scores. In one patient, pharmacotherapies (risperidone and hydroxyzine) were ceased and the individual was able to return to a public school without any clinical problems (Spilioti et al., 2013). Although this was only one child out of 6, this study demonstrates that some individuals with ASD may have inborn errors of metabolism that
result in ASD symptoms and the KD may rescue these impairments. It is important to note that a dietary intervention may be a difficult application in children with ASD due to the gastrointestinal comorbidities associated with the disorder (Ghaziuddin et al., 2013; Samsam et al., 2014).

The efficacy of the KD has also been shown in a mouse model that displays autistic-like behaviors. The BTBR T+ tfij (BTBR) mouse model displays low sociability, reduced communication, and increased self-directed behaviors. Juvenile BTBR mice fed the KD for three weeks demonstrated increased sociability in a three-chamber social test, decreased self-directed repetitive behavior, and improved social communication of a food preference. Yet these animals do not suffer from seizures or have an abnormal EEG, possibly because they lack a corpus callosum and have a severely reduced hippocampal commissure, thus the justification of the KD in the BTBR mouse model is unclear. In addition, this study suffers from a lack of controls. Nevertheless, it is interesting to note that a dietary intervention in a mouse model with autistic-like behaviors can demonstrate improvements in social behaviors (Mcfarlane et al., 2008; Bohlen et al., 2012; Ruskin et al., 2013).

Actions of the Ketogenic Diet

Consumption of the KD results in production of ketone bodies including β-hydroxybutyrate, acetoacetate, and acetone by the β-oxidation of fatty acids in the liver. This can occur normally during fasting, prolonged exercise and in neonates and pregnant women. Acetoacetate and β-hydroxybutyrate are transported in the blood to extrahepatic tissues, converted to acetyl-CoA and oxidized in the citric acid cycle. This provides energy to the skeletal system, cardiac muscle, and renal cortex. Also, the brain
can use ketone bodies as an energy sources when glucose is unavailable. This process begins in the liver when two molecules of acetyl-CoA condense. Acetoacetyl-CoA then condenses with acetyl-CoA and forms β-hydroxy-β-methylglutaryl-CoA (HMG-CoA). HMG-CoA is cleaved to acetoacetate and acetyl-CoA. Acetoacetate is then reduced to acetone, which can be exhaled, and β-hydroxybutyrate. In extrahepatic tissues, β-hydroxybutyrate is oxidized to acetoacetate. Acetoacetate becomes acetoacetyl-CoA, which is cleaved by thiolase to yield two acetyl-CoAs. These acetyl-CoAs can then enter the citric acid cycle to produce glucose (Laffel 1999). Furthermore, β-hydroxybutyrate, the main endogenous ligand of hydroxyl-carboxylic acid 2 (HCA2) receptor, is activated during periods of fasting or on the KD. When activated, adenylyl cyclase is inactivated and intracellular cAMP levels decline. This leads to the inhibition of lipolysis and may function as a negative feedback loop to regulate free fatty acid formation during starvation (Taggart et al., 2005; Gille et al., 2008; Rahman et al., 2014). In this way, the KD regulates energy homeostasis, but there are other actions of the KD to be explored.

It has been hypothesized that the KD may provide neuroprotective properties. Several theories have been proposed including that ketone bodies increase ATP levels, reduce reactive oxygen species (ROS) production, improve mitochondrial function, and increase anti-apoptotic molecules (Bough et al., 2006; Kim Do et al., 2007; Maalouf et al., 2007). The KD has been shown to upregulate hippocampal genes encoding energy metabolism and mitochondrial enzymes. In one study by Bough and colleagues (2006), electron micrographs from the dentate/hilar region of the hippocampus found a 46% increase in mitochondria in rats fed the KD for three weeks (Bough et al., 2006). It has also been shown in rat hippocampus, that the KD may reduce ROS by inducing glutathione peroxidase activity (Ziegler et al., 2003). Similar reductions in ROS have been seen in juvenile mice fed the KD, which resulted in increased mitochondrial
uncoupling proteins (UCPs) in the dentate gyrus and reduced the mitochondrial membrane potential (Sullivan et al., 2004). On the other hand, the KD may protect neurons against apoptosis and neuroinflammation. It has been hypothesized that the ketogenic diet may have an anti-apoptotic effect by blocking activation or activity of protein phosphatase 2A, which can trigger apoptosis by inactivating the anti-apoptotic factor Bcl2 (Maalouf et al., 2009). Lastly, β-hydroxybutyrate can bind HCA2 on neutrophils, microglia, and monocyte-derived cells to reduce neuroinflammation (Yang et al., 2010; Jeong et al., 2011; Kim Do et al., 2012; Rahman et al., 2014). Thus, these neuroprotective properties of the KD may be critical during neural development.

Interestingly, the KD may reduce pain reactivity and peripheral inflammation. Inhibitory pathways in the central and peripheral nervous systems may be regulated by levels of adenosine, GABA, and potassium conductance (due to high polyunsaturated fatty acid, PUFA, content) through PPAR activation to reduce pain reactivity and inflammation (Woolf 1983; Yang et al., 2004; Masino et al., 2009; Liu et al., 2014). In adult and juvenile rats fed a 79% KD diet for 3-4 weeks, pain reactivity was reduced, as measured by hindpaw thermal nociception, and reduced inflammation, measured by complete Freund’s adjuvant-induced local hindpaw swelling and plasma extravasation. The anti-inflammatory and hypoalgesic effect was significantly more effective in juveniles (Ruskin et al., 2009). A dietary intervention may be more beneficial during early development. In addition, the transporter for ketone bodies in astrocytes, monocarboxylic acid transporter proteins (MCTs), has a peak in expression during suckling that declines significantly with the switch to glucose (Rafiki et al., 2003). Thus, the KD may have increased efficiency in children versus adults, because younger individuals have a less developed brain and have a greater capacity to transport and utilize ketone bodies for energy. Thus, if the KD has neuroprotective properties during
development, then the KD may alleviate or reduce some of the debilitating symptoms of ASD. However, to target this critical period of development, better diagnostic tools for younger children are needed.

**Diet Impact on Stress and Feeding Behaviors**

In addition to epilepsy, many other comorbidities are present in ASD including abnormal sensory perception, sleep disturbances, cognitive disabilities, immune dysfunction, gastrointestinal complications, and numerous psychiatric manifestations, such as anxiety disorder, attention deficit/hyperactivity disorder, and oppositional defiant disorder (Gadow *et al.*, 2009; Kotagal *et al.*, 2012; Kang *et al.*, 2014; Matson *et al.*, 2014). Children with ASD have improper responses to stress, which can vary from mild to severe, and this abnormal reactivity can interfere with their daily life and make it difficult to interact with their peers (Herman *et al.*, 1989; Young *et al.*, 1990; Kennedy *et al.*, 2002; Maguire *et al.*, 2013). These stress-related disorders change in symptoms from childhood through adulthood, which coincide with hormonal and synaptic changes (Dorn *et al.*, 1997). During early development neurons are overproduced, then are selectively eliminated during two major pruning events, which occur prior to birth and in periadolescence. This period of programmed cell death is thought to increase neural signaling efficiency to match the needs of the environment (Jacobson 1976; Landmesser 1980; Paus *et al.*, 2008). These developmental events, which occur prior to birth and in periadolescence in humans, result in a 40% reduction in synapses in the frontal cortex (Huttenlocher 1975, 1979; Huttenlocher *et al.*, 1982; Huttenlocher 1984). The reorganization of the dopamine (DA) reward system during these developmental pruning events have been well characterized, but other monoamine systems, such as norepinephrine (NE) and serotonin (5-HT), have not been well defined (Lidow *et al.*,}
1991; Lidow et al., 1992; Andersen 2003). It has been hypothesized that deficits in cell migration, unbalanced excitatory-inhibitory networks, and improper synapse formation and pruning contribute to ASD (Takeuchi 2008; Thomas et al., 2011). For instance, in rodent studies it has been shown that pups of dams exposed to the organophosphate insecticide, chlorpyrifos (CPF), have long-term adverse effects on synaptic development and function (Qiao et al., 2004; Slotkin 2004). Improper, too much or too little, synaptic pruning has been hypothesized to be involved in a number of disorders (Johnston 2004; Paus et al., 2008). It is unknown as to whether these effects on neural development can be reversed or whether there is any correlation with ASD. Children with ASD have neurotransmission alterations in limbic and neocortical areas of the cerebral cortex, the brainstem, cerebellar, thalamic and basal ganglia (Hashimoto et al., 1995; Carper et al., 2005; Hazlett et al., 2005; Rojas et al., 2014). Taken together, these studies suggest that abnormal synaptic pruning of neural pathways that modulate the hypothalamic-pituitary adrenal (HPA) axis may be associated with or contribute to the development of ASD.

The homeostatic control of food intake and body weight regulation is altered by dietary interventions, such as a HFD. A HFD attenuates the HPA axis in response to a stressor. Normally stress reduces food intake, but access to a highly palatable food, specifically dietary fats, during a stress period results in hyperphagia or binge eating in humans and animals (Kinzig et al., 2008; Bartolomucci et al., 2009; Bello et al., 2012). Overeating of a high fat food activates the dopaminergic reward pathway and depresses the HPA axis, which reduces the stress response and stress-related hormones (Dallman et al., 2005). In response to a physiological stressor, the paraventricular nucleus (PVN) stimulates corticotrophin releasing hormone (CRH), which stimulates release of adrenocorticotropic hormone (ACTH) from the anterior pituitary and glucocorticoids (GCs) from the adrenal cortex (Hanson et al., 1995; Nieuwenhuizen et al., 2008). It is thought that in response to an acute stress, GCs stimulate motivation for “comfort
foods,” such as foods high in fat. These “comfort foods” may reduce autonomic and HPA responses to repeated stressors in rats (Dallman et al., 2005). On the other hand, diet-induced obesity (DIO) caused by long-term exposure to a HFD, results in a dysregulation in the homeostatic mechanisms (Schwartz et al., 2004). Corresponding impairments are also seen in feeding-related hormones, such as ghrelin and glucagon-like peptide-1 (GLP-1), which act directly or indirectly to influence the hypothalamic pathways involved in controlling food intake and body weight regulation (Wisse et al., 2004). Another feeding-related hormone, leptin, is secreted from adipose tissue in response to a meal and acts directly on anorexigenic hypothalamic pro-opiomelanocortin (POMC) neurons and orexigenic neuropeptide Y (NPY)/agouti-related peptide (AGRP) neurons to reduce food intake and increase energy expenditure, in order to maintain body weight (Yaswen et al., 1999; Woods et al., 2000; Gropp et al., 2005; Luquet et al., 2005). Obesity results from disturbances in neuronal and hormonal pathways associated with feeding behavior leading to the loss of appetite control and inappropriate counter-responses to dietary challenges. Resistance to the ability of leptin to reduce food intake is a major contributor to the development of obesity and excessive body weight gain (Joyner 1996), although leptin resistance does not occur with prolonged exposure to KD (Kinzig et al., 2010). While the mechanisms leading to obesity in DIO have been well-studied, this is not the case with a KD, which results in a similar degree of adiposity. It possible that the KD, which is high in fat, may also blunt the HPA axis, providing a coping mechanism during stressful conditions and for children with ASD this could help modulate anxiety-related behaviors (Shin et al., 2010).

**Noradrenergic and Serotonergic Controls of Stress and Feeding**

Homeostatic signals in the central nervous system (CNS) interact with peripheral
hormones to modulate stress and feeding behaviors. Clinical studies have found variable levels of monoamines in the urine and blood plasma of ASD patients (Gillberg et al., 1987; Martineau et al., 1992), but there is limited data on monoamine levels in the CNS in ASD. Thus, despite many medications that focus on targeting monoamines in the CNS, results have generally only shown minor improvements (Volkmar 2001). NE is a major neurotransmitter involved in stress- and anxiety-related behaviors. The NE-containing neurons are located in seven clusters in the brainstem (A1-A7) (Itoi et al., 2010). Projections from the locus coeruleus (LC; A6 and A4) make up the dorsal noradrenergic bundle (DNAB) and projections from the caudal hindbrain (A1 and A2) make up the ventral noradrenergic bundle (VNAB) (Sawchenko et al., 1982, 1983). The DNAB projects from the LC and is the main source of NE in the cortex, hippocampus and cerebellum (King 2006). NE is a neurotransmitter tightly involved in the stress response, maintaining attention states, and sleep cycles (Sara 2009; Rinaman 2010). Studies have demonstrated that destruction of VNAB neurons specifically, by hypothalamic lesions, results in hyperphagia (Ahlskog et al., 1973; Ahlskog et al., 1975). It has also been shown that following ab libitum feeding, rats have increases in NE in hypothalamic and medial prefrontal cortical regions (Hoebel et al., 1989). Abnormal increases in NE in the hypothalamus and reductions in sympathetic tone have been shown in DIO in rats, which may contribute to dysregulation of the HPA axis and reinforced feeding of a diet high in fat (Levin et al., 1983, 1986). These studies and studies from our lab (Bello et al., 2013), suggest that NE levels may be altered by dietary intervention. As previously mentioned, the KD has been shown to reduce seizure activity in children with epilepsy and although this mechanism is not completely understood, it is clear that NE is involved in this pathway (Szot et al., 2001; Ruskin et al., 2009). When seizure activity is compared in animals with and without deficits in the noradrenergic system, it was found that the noradrenergic system is required for the anticonvulsant
protective effect of the KD (Szot et al., 2001). However it has not been determined whether the high fat content in the KD or the lack of carbohydrates contributes to reductions in behavioral deficits seen in ASD.

Another monoamine that is critical for stress and feeding behaviors is 5-HT. The 5-HT-containing neurons are organized into nine nuclei (B1-B9) and are located in the mid and hindbrain areas. The dorsal raphe (B7), in particular, is a midbrain nucleus that contains a substantial portion of the total brain 5-HT and has distinct projections to hypothalamic nuclei and other feeding- and stress-related forebrain areas (Medeiros et al., 2005). Activation of serotonergic neurons causes hyperpolarization of NPY/AgRP neurons and depolarization of POMC/CART neurons, leading to hypophagia and the promotion of satiety (Mackenzie et al., 1979; Mongeau et al., 1997). Reductions in brain 5-HT induces abnormalities in the HPA axis, which has been associated with depression and hyperphagia (Mongeau et al., 1997). Diets high in fat and carbohydrates have been shown to increase levels of 5-HT, which results in a mood elevation and alleviation of depression (Markus et al., 1999; Markus et al., 2000; Moorhouse et al., 2000; Homberg et al., 2010). In ASD, 5-HT has become a particular biogenic amine of interest, because 5-HT plays a role in embryogenesis, brain maturation, sleep, learning and sensory inputs. It has been shown that ASD patients have a significant elevation of whole blood 5-HT, increased activity of 5-HT transporter in platelets, and decreased binding to the 5-HT$_{2A}$ receptor (Anderson et al., 1987; Hranilovic et al., 2009; Nakamura et al., 2010). Although NE and 5-HT signaling are clearly involved in ASD symptoms, there is little evidence that medications targeting these neurotransmitter pathways alleviates behavioral deficits (Volkmar 2001; West et al., 2009). Due to the potential of dietary interventions to work on both the peripheral and central nervous system to stabilize anxiety-related behaviors, it is important to examine the effects of a dietary intervention on a developmental animal model for ASD. This study will determine if the KD alters
expression of these monoamines in brain regions that are critical to stress and feeding behavior.

Interaction of Genes and Environment in ASD

With the significant increase in ASD diagnoses, research on the etiology of ASD has greatly intensified and emerging data suggests that ASD may be the result of both genetic and environmental factors, although the exact cause remains unknown. Many environmental factors, such as infectious diseases, alcohol, and exposure to heavy metals during pregnancy have been implicated (Ciaranello et al., 1982; Ghaziuddin et al., 2013). The association with heavy metals has gained particular attention, since it has been well documented that heavy metals, such as mercury can lead to neurological defects, and learning disabilities (Marsh et al., 1980; Carman et al., 2013). For example, in one study of 45 children in Egypt (age 2-10), blood levels of mercury and lead were significantly higher in ASD children than in non-ASD control children, although a cause and effect was not established. However, administration of DMSA (meso-2,3-dimercaptosuccinic acid), a chelating agent, was associated with a reduction in autistic symptoms, which correlated with the removal of these heavy metals (Yassa 2014). Although this study was small, isolated, and perhaps outdated, it is intriguing that behavioral symptoms improved as a result of a chelating agent. Whether increases in environmental toxins are associated with increased incidences of ASD in future generations is extremely difficult to pinpoint, but it cannot be discounted as a plausible mechanism.

In addition to environmental causes, there have been many common susceptibility alleles implicated in ASD and more research is beginning to show that ASD
has a large genetic predisposition. Twin studies do demonstrate an increased rate for ASD in identical twins, specifically a 60% concordance for classic autism (Bailey et al., 1995). Parents are cautioned after the birth of their first autistic children, because there is a reoccurrence risk of 60 to 150 times the population rate (Buitelaar et al., 2000). Even non-ASD siblings have language delay (Gamliel et al., 2007; Ben-Yizhak et al., 2011) and social impairments (Constantino et al., 2005). Yet, determining gene causality is not an easy task, since genetic contributions to autism are heterogenous and even the most common genetic forms only account for 1-2% of ASD cases (Abrahams et al., 2008). Some genetic disorders, such as Tuberous Sclerosis, Joubert Syndrome, Smith Lemli Opitz, Cowden, Fragile-X, Angelman, 16p11.2 deletion, Cohen, Amith-Magenis, and Phelan-McDermid syndrome have high rates of autistic-like behaviors (Cohen et al., 2005). There are over 100 genes that may contribute to ASD and these may share common pathways and functions. Genes implicated in ASD include those involved in social and emotional responsivity, sex hormone biosynthesis, metabolism and transport, and neural development and connectivity. Some genes thought to be involved in social and emotional responsivity are those involved in social bonding (oxytocin receptor, OXTR) and neurotransmission (gama-aminobutyric acid A receptor beta 3, GABRB3). Genes involved in sex hormone biosynthesis and metabolism, such as cytochrome P450, and receptors for estrogen (17 beta-estradiol) have been implicated as well. Although these genes may certainly be involved in autistic symptoms, genes involved in neural development and connectivity are the more likely cause of the neural abnormalities associated with ASD. These include genes involved in neural differentiation and growth (ubiquitin protein ligase 3A, UBE3A; neurotrophic tyrosine kinase receptor type 1, NTKR1), synapse formation and maintenance (neuroligin genes, NLGN3 and NLGN4X), synapse communication (shank proteins, Shank1-3) and hindbrain/cerebellar development (Engrailed-2, EN2; Homeobox A1, HOXA1)
(Chakrabarti et al., 2009; Peca et al., 2012). It is critical to study these genes to determine how they are involved in the pathophysiology of ASD, but it is important to recognize that these genes may only be deleterious in combination or in specific pathways and there is no one autism gene. The role of genetics, environment, and epigenetic factors need to be further studied to understand how they are playing a role in brain circuit dysfunction and how they can be linked to behaviors and disease.

**Engrailed Genes and Implications for ASD**

It has been shown that the engrailed genes, *En1* and *En2*, are important homeobox transcription factors for mid-hindbrain innervation, cerebellar development, and neurotransmitter growth and maturation (Loomis et al., 1996; Hanks et al., 1998; Alberi et al., 2004; Simon et al., 2005; Gherbassi et al., 2006; Sgado et al., 2006; Halladay et al., 2009). The discovery that midbrain dopaminergic (mDA) neurons express *En1* and *En2* have resulted in their use as therapeutic proteins in a mouse model of Parkinson’s disease (PD). These genes protect mDA neurons by increasing translation of nuclear-coding subunits of mitochondrial complex I (Alvarez-Fischer et al., 2011). Furthermore, the similarities in behavioral deficits between *Engrailed-2* (*En2*) null mice and autism have resulted in this model becoming highly relevant to use in studying behavioral and neural pathways involved in ASD. The *EN2* single-nucleotide polymorphism, rs1861972-rs1861973 A-C haplotype, is genetically associated with autism (72% carry the A-C haplotype). This A-C haplotype is necessary for transcriptional activation and is mediated by CUX1 and NFIB. (Benayed et al., 2005; Benayed et al., 2009; Choi et al., 2012). In an analysis of 13 frozen post-mortem cerebellar cortex samples from ASD individuals, it was determined that there were abnormal expression and methylation patterns of the *En2* gene in all 13 samples (James
et al., 2013). EN2 rs1861972-rs1861973 A-C haplotype produces a gain in function (Choi et al., 2012), and it is thought that mutations producing a loss or gain in function can result in similar behavioral impairments and be deleterious to the central nervous system by affecting neural homeostasis (Ramocki et al., 2008; Auerbach et al., 2011).

The engrailed gene was first classified in Drosophila for its importance as a developmental regulator (Davidson et al., 1988). Humans and mice have two engrailed genes and they both work to modulate CNS development. The engrailed genes regulate gene expression by binding to AT-rich DNA cis-sequences. This repressor can either actively block the trans-activation of activators by binding to nearby cis-sequences or the engrailed proteins compete for the binding of the basal transcriptional machinery to TATA box sequences (Horikoshi et al., 1990; Ohkuma et al., 1990; Jaynes et al., 1991). During early embryogenesis, both genes are expressed in the border between mid-brain and hindbrain, but these two genes play slightly different roles in development (Davidson et al., 1988; Gardner et al., 1992). Mutations in the engrailed genes affect the ventral mid-hindbrain nuclei, the LC, and the raphe nuclei (RN) ultimately resulting in abnormal levels of NE and 5HT in both the forebrain and hindbrain structures (Cheng et al., 2010). Specifically, En1 is expressed in the spinal cord, somites, and limbs by embryonic day 8. En1 null (En1<sup>−/−</sup>) mice morphological deficits are apparent in the mid and hindbrain by embryonic day 9. These animals lack the inferior colliculus, the caudal superior colliculus, and part of the cerebellum. Later expression of En1 is not required for cerebellar foliation, but En2 is required and results in a delay in formation of the secondary fissure and premature initiation of the prepyramidal fissure (Millen et al., 1994; Sgaier et al., 2007). In addition, En1 is highly expressed by dopaminergic neurons in the substantia nigra and ventral tegmentum, but only a double knockout of both engrailed genes results in deficits in dopamine (Simon et al., 2001; Simon et al., 2004).
En1/2 double mutants die at birth due to a lack of almost the entire midbrain and anterior hindbrain, which results in a loss of the dorsal RN and LC (Liu et al., 2001; Sgado et al., 2013). En2 is initially expressed at embryonic day 8.5 in the mesencephalon (midbrain) and rhombomere 1, and expression continues throughout embryonic and postnatal development (Joyner 1996; Herrup et al., 2005). Heterozygous mutants for En1, En2 or both demonstrate that these two genes can partially compensate for the deletion of the other, but En1 seems to be more critical for development. En1 can compensate for En2 deletion, but lack of En1 also reduces expression of En2 (Simon et al., 2005). Thus, the Engrailed genes have a critical role in neural development and alterations in their expression may affect brain regions that lead to neural developmental disorders.

There are two En2 mouse mutations, including a traditional knock-out and a transgenic misexpression mutant. The traditional knockout, developed by Joyner and colleagues in 1991 (Joyner et al., 1991), uses En2\textsuperscript{tm1Alj/tm1Alj} (En2), generated on a 129S2/SvPas background. The En2 null or knockout (En2\textsuperscript{−/−}) mouse is created by homologous recombination whereby 300 base-pairs (bp) of the En2 intron and 700 bp of the homeobox exon is replaced with a neo expression vector (111), figure 3. En2\textsuperscript{−/−} mice 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. In addition, animals have deficits in NE in the hippocampus, reduced NE (~25%) and 5-HT in the forebrain, increased NE and 5-HT in the hindbrain, and reductions in tyrosine hydroxylase. En2\textsuperscript{−/−} also have an underdeveloped cerebellum and 30-40% reduction in all the major cerebellar cell types (Ohkuma et al., 1990; Joyner et al., 1991; Millen et al., 1994; Vogel et al., 1996; Kuemerle et al., 1997; Benayed et al., 2005; Cheh et al., 2006; Benayed et al., 2009; Brielmaier et al., 2012; Sgado et al., 2013). Studies have also shown that En2\textsuperscript{−/−} mice have an increase seizure susceptibility, which is also observed in
ASD. In *En2−/−* mice, it is thought that increased seizure susceptibility is due to improper development of γ-aminobutyric acid (GABA)-positive neurons, leading to reduced GABAergic neurons in the hippocampus and cerebral cortex (Tripathi et al., 2009; Sgado et al., 2013; Provenzano et al., 2014a). Interestingly, *En2−/−* mice have impaired behavior involving social interaction, memory, sensory-motor gating, decreased play, reduced social sniffing, reduced aggressiveness and depression compared with wild-type (WT, *En2+/+*) mice. Also, *En2−/−* mice have deficiencies in spatial learning and memory, which may be due to reduced neurofibromin expression and increased pERK levels in the hilus (Cheh et al., 2006; Provenzano et al., 2014b). Recently, DiCicco-Bloom and colleagues (2012), also demonstrated behavioral deficits in *En2−/−* mice. Both *En2−/−* and heterozygous, *En2+/−*, display fewer social interactions, such as sniffing, following, and front approach, between same-sex juveniles and opposite-sex adult mice. In a three-chambered social approach task, *En2−/−* mice fail to show sociability (Brielmaier et al., 2012). These findings are consistent with lesions in central brain areas associated with sociability, such as in the hypothalamus, frontal cortex, hippocampus, striatum and thalamus (Mcmillen et al., 1988; Fowler et al., 2002; Valencia-Alfonso et al., 2004; Branchi et al., 2006; Bibancos et al., 2007). *En2−/−* mice also demonstrate cognitive and fear conditioning deficits in water maze training and lack of selective quadrant search in a probe trial (Brielmaier et al., 2012). In depression-related experiments, *En2−/−* mice displayed higher levels of immobility on forced swim, suggestive of impairments in both 5-HT and NE (Brielmaier et al., 2012). Remarkably, chronic disipramine, a selective NE reuptake inhibitor, significantly reduced immobility in a tail suspension and forced swim task, restored sociability, and reversed impairments in contextual fear conditions (Brielmaier et al., 2014). This collective set of data indicates that the development of the serotonergic and noradrenergic pathways are regulated by the Engrailed proteins and
may contribute to behavioral deficits seen in $\text{En2}^{+/\text{-}}$ mice. Thus, it is of interest to use this animal model to study behavioral alterations resulting from deficits in the CNS.
Summary

This doctoral thesis project aims to determine the effects of a KD fed during development (postnatal day 21-60) on metabolic, neural, and autistic-like behaviors in adult En2−/− mice. Although there are many animal models that are used to study autistic-like behaviors, En2−/− mice are unique, because they have deficits in neural pathways and increased seizure susceptibility, which has been implicated in neural developmental disorders, specifically ASD. Furthermore, the KD has been shown to be beneficial for pediatric epilepsy, which is a comorbidity for many developmental disorders, including autism. The pathways that function to improve seizure activity in these children have yet to be elucidated, but our hypothesis is that the KD can improve synaptic development and maturation, figure 2. Therefore, the objective of this study is to determine if autistic-like behaviors can be improved in En2−/− mice fed the KD during development and, if so, which brain regions and pathways are being modulated. Thus, it was hypothesized that alterations caused by a ketogenic diet would improve the neural and behavioral deficits associated with En2−/− male mice. This knowledge generated from this study may benefit human health in two ways. First, these studies will investigate the role for En2 in developmental circuits and behaviors. Second, it will be the first to provide a mechanistic understanding of a role for dietary interventions, specifically ketogenic diets, as potential treatment strategies for individuals in the continuum of ASD.
Literature Cited


**Common Abbreviations**

ASD = autism spectrum disorder  
DSM = Diagnostic and Statistical Manual of Mental Disorders  
ASQ = Ages and Stages Questionnaire  
MCHAT = Modified Checklist for Autism in Toddlers  
CSBS = Communication and Symbolic Behavior Scales  
STAT = Screening Tool for Autism and Young Children  
CARS = Childhood Autism Rating Scale  
ADOS-G = Autism Diagnosis Observation Schedule-Generic  
GAR-2 = Gilliam Autism Rating Scale-Second Edition  
FDA = Food and Drug Administration  
SSRI = selective serotonin reuptake inhibitor  
BMI = body mass index  
KD = ketogenic diet  
GFCF = gluten-free casein-free  
HFD = high fat diet  
LCT = long-chain triglycerides  
MCT = medium-chain triglycerides  
HMG-CoA = β-hydroxy-β-methylglutaryl-CoA  
HCA₂ = hydroxyl-carboxylic acid 2  
ROS = reactive oxygen species  
UCPs = uncoupling proteins  
MCTs = monocarboxylic acid transporter proteins  
DA = dopamine  
NE = norepinephrine  
5-HT = serotonin
CPF = chlorpyrifos
HPA = hypothalamic-pituitary adrenal
PVN = paraventricular nucleus
CRH = corticotrophin releasing hormone
ACTH = adrenocorticotropic
GCs = glucocorticoids
DIO = diet-induced obesity
GLP-1 = glucagon-like peptide
POMC = pro-opiomelanocortin
NPY = neuropeptide Y
AGRP = agouti-related peptide
CNS = central nervous system
LC = locus coeruleus
DNAB = dorsal noradrenergic bundle
VNAB = ventral noradrenergic bundle
DMSA = meso-2,3-dimercaptosuccinic acid
OXTR = oxytocin receptor
GABRB3 = gama-aminovutyric acid A receptor beta 3
UBE3A = ubiquitin protein ligase 3A
NTKR1 = neutrophic tyrosine kinase receptor type 1
NLGN = neuroligin genes
En2 = Engrailed-2 gene
HOXA = homeobox gene
En1 = Engrailed-1 gene
mDA = midbrain dopaminergic
PD = Parkinson’s disease
RN = raphe nuclei

$En1^{+/} = En1$ null

$En2^{+/} = En2$ null

γ-aminobutyric acid = GABA

$En2^{+/+} = En2$ wildtype
Figures

Figure 1. Timeline of human and mouse development, demonstrating the changes in central nervous system and synaptic development with onset of mental disorders. Courtesy of: Anderson 2003. DOI: 10.1016/S0149-7634(03)00005-8 Copyright © 2003 Neuroscience and Biobehavioral Reviews (adapted) and Cryan et al., 2014. DOI: 10.1016/j.molmed.2014.05.002 Copyright © 2014 Trends in Molecular Medicine (adapted).

Figure 2. Proposed effects of the KD on the trajectory of synaptic growth and maturation. The KD is proposed to have beneficial effects, yet unknown, on synaptic development. Courtesy of Woolfrey et al., 2011. DOI: 10.1038/nn.2741 Copyright © 2011 Nature Neuroscience (adapted).

Figure 3. Structure of the En2 protein, wild-type (WT) and mutant loci. En2 protein with four en-conserved domains indicated by shaded boxes. The 60-amino acid homeobox is labeled. The arrow indicates the intron in the En2 gene. En2 wild-type locus (middle) and mutant (bottom) loci shown with En2 exons in thick-lined rectangles with translated sequences checkered and homeobox solid. The neo-containing vector is shown as a thin-lined rectangle. Pr is the 500-bp human β-actin promoter sequences. The one transcript of the WT En2 gene and two transcripts of the mutant En2 locus from both the En2 promoter and the β-actin promoter are indicated with narrow rectangles indicating the exon sequences. Restriction sites are B, Bam HI and Bg, Bgl II. Courtesy of: Rossant et al., 1991. DOI: 10.1126/science.1672471 Copyright © 1991 Science (adapted).
Figures

Fig 1.

<table>
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Neuronal complexity through the lifespan

Synaptic density

Stages of brain development

Age of onset of mental disorders

- ADHD
- Autism
- Schizophrenia
- Anxiety disorders
- Mood disorders
- Impulsivity disorders

Fig 2.

Proposed effects of KD on Neural Development

Synaptic Development

Synapse formation

Maintenance

ASD

ASD on KD

Typical

Pruning

Birth | Childhood | Adolescence | Adulthood
**Fig 3.**

*En-2 Protein*

*En-2 wild-type locus*

*En-2 mutant locus*
Research Objectives and Hypothesis

1) Examine KD effects on biogenic amine concentrations in forebrain and hindbrain regions in adult male KO and WT En2 mice fed from PND 21 to 60.

*Hypothesis for Aim 1: En2⁻/⁻ mice fed a KD will have improved biogenic amine signaling in the frontal cortex.*

2) Determine the consequences of pre-adulthood exposure (PND 21-60) to a KD on social and stress-related behaviors in adult male KO and WT En2 mice.

*Hypothesis for Aim 2: Feeding En2⁻/⁻ male mice a KD from post-weaning to adulthood will improve social behaviors and stress-related impairments in adulthood.*

3) Analyze changes in metabolic outputs (e.g. body weight, metabolism, glucose response, blood pressure) from feeding En2 KO and WT male mice a KD.

*Hypothesis for Aim 3: Due to neural alterations in En2⁻/⁻ mice, mice will have alterations in metabolic outputs that will impact adult body weight.*

4) Define the role of the KD in mediating social behavior in En2 KO and WT exposed to a stranger mouse or novel object by measuring neural activation.

*Hypothesis for Aim 4: Exposure to a KD will result in increased c-Fos expression in brain regions that are critical to social behavior in En2⁻/⁻ mice.*
CHAPTER TWO: A ketogenic diet increases hypothalamic norepinephrine of En2 wild-type male mice.

ABSTRACT

A ketogenic diet (KD) acts on both the central and peripheral nervous system, as shown by reduced seizure activity in pediatric epilepsy and decreased pain sensitivity in rodent models. The following studies used Engrailed-2 (En2) knockout (KO) and wild-type (WT) male mice, which have been used to study neural systems affected by this transcription factor and autistic-like behaviors. En2 mutations affect neural development, resulting in abnormal noradrenergic and serotonergic systems in both forebrain and hindbrain structures, which may result in the observed behavioral deficiencies. To determine if the KD could differentially alter central biogenic amine concentration in the brain, KO and WT mice were fed either ketogenic diet (KD; 80% fat, 0.1% carbohydrates) or control diet (CD; 10% fat, 70% carbohydrates) from postnatal day (PND) 21 to adulthood (PND 60). Increased ketone bodies (+85.7%) and blood glucose (+16.2%) were observed in the KO-KD mice compared with KO-CD at sacrifice confirming the expected dietary effects. Although there were no effects of the KD on biogenic amine concentrations in any of the brain regions of KO mice, KD increased hypothalamic norepinephrine in WT-KD mice (+86.2%) compared with WT-CD. The results of this study show that the KD fed from post-weaning to adulthood can increase NE in the hypothalamus of WT mice, but does not affect brain biogenic amine levels in the brain of En2 KO male mice.
Introduction

Autism spectrum disorder (ASD), a developmental disability observed with increasing frequency in the population, displays many comorbidities such as: generalized anxiety disorder, attention deficit, hyperactivity, and behaviors that may cause self-injury (Badaruddin et al., 2007; Hurwitz et al., 2012; Matson et al., 2014; Bitsika et al., 2015). In non-ASD individuals, these disorders are partially controlled and treated by pharmaceutical therapies, which target neurotransmission and biogenic amines. For example, reuptake inhibitors of biogenic amines, which typically involve the noradrenergic, serotonergic, and dopaminergic pathways, have been shown to relieve anxiety (Bambico et al., 2010) and depression (Frazer 2000; Mathew et al., 2008).

Individuals with ASD suffer from similar disorders, but clinical studies analyzing biogenic amines in the blood and urine of individuals with ASD are inconsistent (Gillberg et al., 1987; Martineau et al., 1992), thus it is unclear if altered peripheral levels of biogenic amines correlate with anxiety in autistic individuals (Martineau et al., 1992; Nakamura et al., 2010). Despite this lack of evidence, two medications that are approved for use by the US Food and Drug Administration (FDA) in individuals with ASD are risperidone and aripiprazole. These have been shown to treat irritability, aggression, self-injury, and tantrums in ASD patients (Martin et al., 2004; Clos et al., 2007; Kim et al., 2010). Yet, the mechanism of action of these medications specifically for ASD have yet to be elucidated. Thus, more research is necessary to determine the role of biogenic amines in ASD.

One often overlooked moderating factor is the influence of nutrition on the trajectory of neural development. Depression and anxiety-related behaviors are modulated by the central nervous system (CNS) and can be influenced by diet (Levin et al., 1986). Biogenic amines, such as norepinephrine (NE), serotonin (5-HT), and...
dopamine (DA) have been implicated in psychological disorders, such as depression and generalized anxiety disorder (Lopez et al., 1999; Frazer 2000; Lira et al., 2003), as well as eating disorders, including bulimia nervosa and obesity (Ahlskog et al., 1973; Kaye et al., 1991; Bonisch et al., 2006; Billes et al., 2007; Bello et al., 2011). The relationship between diet and neural signaling is bidirectional, because while diet can influence neural signaling, neural signaling can also influence food intake and dietary preference. Consuming a diet high in fat and carbohydrates can increase levels of 5-HT and reduce symptoms of depression acutely (Markus et al., 2000; Homberg et al., 2010). Yet, obesity may also cause dysregulation of the dopaminergic reward system and reinforce intake of palatable foods, specifically those high in fat and carbohydrates, to reduce depression-like symptoms (Tracy et al., 2008; Bello et al., 2010; Cone et al., 2013; Sharma et al., 2013). Diet can also directly influence neural signaling in physiological disorders. A notable example of diet influencing neural signaling is the use of the ketogenic diet (KD) to treat drug-resistant pediatric epilepsy (Vining 1999; Lefevre et al., 2000; Keene 2006). It is well known that a diet high in fat can increase NE in hypothalamic regions and reduce sympathetic tone (Levin et al., 1983, 1986), but rodent studies have also shown that the noradrenergic system is required for the seizure reduction properties of the KD (Szot et al., 2001). Because 20-30% of children with autism will develop epilepsy, a KD intervention could be beneficial in certain ASD populations (Dicicco-Bloom et al., 2006; Chavez et al., 2007). Evangeliou and colleagues (2003, 2013) have shown that ASD individuals fed the KD have improvements in autistic-like behaviors (Evangeliou et al., 2003; Spilioti et al., 2013). Although this dietary intervention shows efficacy in these small studies, the mechanism of action of the KD has yet to be determined and more research is needed to understand how the KD is influencing neural signaling.
ASD has a large genetic component and there are many genes implicated in the disorder. One gene, *Engrailed-2 (EN2)*, has been found to contain a single-nucleotide polymorphism and the rs1861972-rs1861973 A-C haplotype, which is genetically associated with ASD (Benayed *et al.*, 2005; Benayed *et al.*, 2009; Choi *et al.*, 2012). *EN2* is a homeobox transcription factor that is critical for mid-hindbrain innervation, cerebellar development, and neurotransmitter system maturation (Loomis *et al.*, 1996; Hanks *et al.*, 1998; Alberi *et al.*, 2004; Simon *et al.*, 2005; Gherbassi *et al.*, 2006; Sgado *et al.*, 2006; Rossman *et al.*, 2014). *Engrailed-2* null (*En2<sup>-/-</sup>*) mice have impaired social interaction, deficits in fear conditioning and sensory motor gating, and decreased play behaviors (Cheh *et al.*, 2006; Brielmaier *et al.*, 2012). Mutations in *En2* affects the monoaminergic brainstem nuclei, the locus coeruleus, and the raphe nuclei resulting in abnormal noradrenergic and serotonergic systems in both forebrain and hindbrain structures (Simon *et al.*, 2005; Brielmaier *et al.*, 2014). Since the KD is known to modulate biogenic amine systems and *En2<sup>-/-</sup>* mice have impairments in these systems, there is justification to use this mouse model to study the effects of a dietary intervention on biogenic amine signaling.

In this study, knockout (KO) and wild-type (WT) *En2* male mice were fed the KD or control diet (CD) from postnatal day (PND) 21-60 to investigate if the KD could alter biogenic amine concentration, specifically regarding DA, 5-HT, NE, in forebrain and hindbrain regions. We hypothesized that *En2<sup>-/-</sup>* mice would display altered biogenic amine signaling in the frontal cortex, which would be improved by pre-adulthood exposure to the KD. Results from this study will provide foundation for the following chapters, which will further describe developmental effects of the KD on *En2* KO male mice.
Materials and Methods

Animals.

En2<sup>tm1Alj/tm1Alj</sup> mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and delivered to the University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School (UMDNJ-RWJMS) in Piscataway, NJ. En2<sup>tm1Alj/tm1Alj</sup> mice were generated on a 129S2/SvPas background as previously described (Joyner et al., 1989). En2 heterozygous (En2<sup>+/−</sup>) breeding pairs were imported from UMDNJ to the G.H. Cook Campus of Rutgers University (New Brunswick, NJ) for the following study and placed on a 12:12 h light:dark cycle with lights off at 1800 h. Heterozygous breeding pairs were utilized for all studies to prevent possible social and stress-related deficient imprinting behaviors. To prevent genetic drift, every ten generations, En2<sup>+/−</sup> mice were crossed to B6129SF2/J mice for creation of new En2<sup>+/−</sup> 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.

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 utilized for PCR: GTTCACAGTCCTGTGAAATGCAGC, common
to both $En2^{+/+}$ and $En2^{-/-}$ mice; ACCAACAGGTACCTGACAGAC, 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 (Ohkuma et al., 1990; Jaynes et al., 1991; Joyner et al., 1991; Joyner 1996).

Experimental diet.

To determine the effects of the KD on biogenic amines, a total of 56 $En2$ 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 matched control diet (CD; 10% fat, 20% protein, 70% CHO, 3.85 Kcal/g; D12450K; Research Diets) from PND 21 to 60, which encompasses a developmental period analogous to middle to late childhood in humans; see Chapter 1 Table 1. It is important to note that neither diet contained sucrose to prevent preference of a sweeter food and inappropriate consumption. At PND 60, blood ketone bodies were measured by a ketone meter (Precision Plus Ketone Meter, Abbott Laboratories, North Chicago, Il, USA). A separate group of mice was fed the KD through PND 60, then placed on standard chow diet (Purina Mouse Diet 5015, 25.34% fat, 19.81% protein, 54.86 CHO, 3.7 Kcal/g) for 2 days to determine if changes in ketone body concentrations were restored to normal.

KD exposure on forebrain biogenic amines.

At PND 60, KO-KD, KO-CD, WT-KD, WT-CD ($n = 20$/group) mice were fasted for 5 hours (0800h-1300h), then sacrificed by decapitation for analysis of biogenic amines and metabolites in regional brain tissue. Brains were dissected to isolate the hypothalamus (-1.06 to -2.06 Bregma), hippocampus (-1.06 to -2.06 Bregma), frontal cortex (+2.46 to +1.98 Bregma), and cerebellum (-5.40 to -8.24 Bregma). For the
forebrain regions, a brain matrix was utilized for standardized dissection. For the cerebellum, the entire cerebellum was removed by blunt dissection (Franklin 2008). Biogenic amines for each individual brain section were extracted and analyzed as previously described (Verpeut et al., 2013) 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 biogenic amine relative to milligrams (mg) of wet tissue weight per sample. At sacrifice, blood glucose was measured by a glucometer (Alphatrack, Abbott Laboratories) using trunk blood. A separate group of KO (n=4) and WT (n=4) male mice, both fed the KD, was used to determine blood ketone bodies (β-hydroxybutyrate) by a ketone meter (Precision Plus Ketone Meter, Abbott Laboratories, North Chicago, IL, USA) at PND 60 and 62 with a tail nick.

*Statistical Analysis.*

Regional brain mass and biogenic amines, blood glucose, and β-hydroxybutyrate levels were analyzed with a factorial analysis of variance (ANOVA). To determine changes in β-hydroxybutyrate from PND 60-62, a within repeated measures two-way ANOVA was utilized to determine possible dietary changes. Post-hoc comparisons were made, when appropriate, with a Newman-Keuls test. All statistical analyses were performed using Statistica 7.1 software (StatSoft, Tulsa, OK, USA) and significance was set at α = 0.05.
Results

Effects of the KD on biogenic amines in En2 mice

Post-sacrifice brains were sectioned and weighed for HPLC analysis in En2−/− and WT mice to determine possible genotype and diet effects.

**Forebrain:** There were no significant differences in levels of NE, DA, 5-HIAA, HVA, or 5-HT in the frontal cortex of En2 KO and WT mice fed the KD or CD.

**Hippocampus:** There was a genotype effect for both NE [F(1, 69) = 4.3, p < 0.05] and 5-HT [F(1, 53) = 5.0, p < 0.05]; KO mice had lower levels compared with WT. There were no significant differences in DA or 5-HIAA concentrations in the hippocampus of KO or WT mice fed the KD or CD. Levels of HVA were not detectable in the hippocampus.

**Hypothalamus:** There was a diet x genotype effect for NE in the hypothalamus [F(1, 61) = 4.1, p < 0.05]. Post-hoc analysis determined that WT-KD had increased levels of NE compared with WT-CD (p < 0.05). There was also a genotype effect for DA [F(1, 62) = 4.3, p < 0.05] and 5-HT [F(1, 59) = 4.8, p < 0.05], whereby KO mice had lower levels of DA and 5-HT compared with WT, regardless of diet. There were no significant differences in 5-HIAA or HVA in the hypothalamus with respect to genotype or diet.

**Cerebellum:** There was a genotype effect for NE in the cerebellum [F(1, 62) = 4.3, p < 0.05] and 5-HT [F(1, 52) = 11.8, p < 0.01], whereby KO mice had higher levels compared with WT, regardless of diet. There were no significant differences in 5-HIAA or HVA in the cerebellum with respect to genotype or diet. However, the ratio of 5-HT to its metabolite 5-HIAA showed a genotype effect [F(1, 50) = 6.2, p < 0.05] and a diet x genotype effect [F(1, 50) = 5.3, p < 0.05]. Post-hoc analysis determined WT-KD had higher 5-HIAA/5-HT ratio compared with all other groups (p < 0.05); see Table 2. Although not a major endpoint, it was noted there was a genotype effect for the mass of the cerebellum [F(1, 72) = 10.3, p < 0.01] where KO mice had a 44% reduction in cerebellar mass compared to WT (data not shown). There were no significant
differences in brain mass in any other region with respect to genotype or diet. Overall biogenic amines were found to be reduced in forebrain regions and increased in the cerebellum of KO mice regardless of diet. A KD increased hypothalamic NE of WT mice compared to WT-CD.

*Ketone bodies (β-hydroxybutyrate) and blood glucose alterations as a result of the KD in En2 KO mice.*

At PND 60, WT and KO mice fed the KD had increased blood β-hydroxybutyrate \([F(1,59) = 26.0, p < 0.0001]\) and blood glucose levels \([F(1,74) = 30.3, p < 0.0001]\) compared with CD-fed counterparts; see Table 3. After two days on standard chow, there were no longer significant differences in ketone body concentrations in KO or WT mice previously fed the KD relative to the corresponding groups fed the CD for the duration of the study \([F(1,44) = 1.6, p = 0.3]\), data not shown. Future studies utilized PND 62 to begin testing as acute effect of β-hydroxybutyrate will not be present.

**Discussion**

This study utilized *En2* knockout (KO) and wild-type (WT) male mice to determine changes in biogenic amine levels in brain regions resulting from exposure to the KD. Previous studies in other labs have shown that *En2* KO mice have deficits in biogenic amine concentrations in forebrain regions and increased levels in hindbrain regions, specifically in NE and 5-HT (Cheh *et al.*, 2006; Brielmaier *et al.*, 2014). It has been suggested that abnormal synaptic development in this model results in an inability of biogenic amines to transmit to forebrain regions. These deficits may result in the autistic-like behaviors displayed in *En2* KO mice. Recently, Dicicco-Bloom and colleagues demonstrated that desipramine, a selective NE reuptake inhibitor, reversed behavioral abnormalities in *En2*KO mice (Brielmaier *et al.*, 2014). If NE can restore
autistic-like behaviors and a high-fat diet can alter central NE (Levin et al., 1986; Bello et al., 2013), then the KD, which is also high in fat, may restore concentrations of central biogenic amines and autistic-like behaviors. In addition, due to the potential neuroprotective properties of the KD (Masino et al., 2009; Jeong et al., 2011), this study explored whether the KD would restore biogenic amine concentration, relative to WT mice, during development. We hypothesized that exposing KO mice to the KD from weaning (PND 21) to adulthood (PND 60) would alter biogenic amine levels in adulthood, but this hypothesis was not confirmed.

At PND 60, mice fed the KD (KO and WT) had higher levels of β-hydroxybutyrate than CD-fed mice confirming the expected dietary effect. Blood glucose levels on PND 60 were also significantly increased in mice fed the KD compared with those fed the CD, independent of genotype. To determine if β-hydroxybutyrate returned to normal after two days on standard chow, β-hydroxybutyrate was measured again on PND 62 in KO and WT mice previously exposed to the KD from post-weaning (PND 21) to PND 60. After two days on standard chow there were no differences in β-hydroxybutyrate levels between mice previously fed the KD compared with those fed the CD for the entire study duration, independent of genotype, clearly demonstrating that the KD-induced ketosis is rapidly reversible. The recognition that KD-induced ketosis is a reversible and acute effect is an important consideration for the design of future studies. The rapid reversal of ketosis is consistent with observations from Ellenbroek and colleagues (2014), who reported that rats fed a KD for 8 weeks had decreased sensitivity to peripheral insulin and impaired glucose tolerance, but that these effects were reversed when animals were switched to a non-ketogenic diet (Ellenbroek et al., 2014). While peripherally this may be the case, central ketone bodies may still be increased, as observed by Levin and colleagues (2014). Rats previously fed a HFD were found to have increased release of
β-hydroxybutyrate from astrocytes in the ventral medial hypothalamus, after peripheral levels were normalized (Le Foll et al., 2014). Therefore, although we saw peripheral ketone body levels return to normal after two days on standard chow at PND 62, central levels may remain elevated. Additional studies are necessary to test this hypothesis.

While lean rodents may develop increased body fat and indicators of metabolic syndrome, such as abnormal blood glucose levels, from a KD (Ribeiro et al., 2008), clinical studies of a KD in obese humans leads to a reduction in body weight and normalization of blood glucose levels (Dashti et al., 2007; Johnstone et al., 2008). Thus, KD effects on body composition appear to be dependent on the metabolic state. This current study was conducted to determine if a KD could result in protection of neural development based on normalization of biogenic amines in the brains of KO mice. Major neural plasticity during development only continues until adulthood, thus a dietary intervention during this time is hypothesized to result in permanent behavioral improvements and any negative metabolic outputs would be reversible (Huttenlocher 1984; Swann et al., 1999). Nevertheless, studies in subsequent chapters will examine KD effects on metabolic outputs, such as hemodynamic measurements and respiratory exchange.

To investigate whether forebrain concentrations of biogenic amines were differentially altered by dietary exposure, KO and WT mice were fed a KD or CD from PND 21 to PND 60. On PND 60, mice were decapitated and brains were sectioned for regional analysis of biogenic amines. KO mice, regardless of dietary exposure, had decreased NE and 5-HT in the hippocampus and lower levels of DA and 5-HT in the hypothalamus compared with WT mice. Experiments in Chapter 3 and 4 explored how these changes in biogenic amines may be important in food intake or anxiety-related behaviors. As previously shown (Cheh et al., 2006), KO mice had higher levels of NE
and 5-HT compared with WT in the cerebellum. It was noted that KO mice also had a 44% reduction in cerebellar mass, which could account for what seemed like an increase in biogenic amines in the cerebellum. Since biogenic amines were calculated as a weighted analysis, and there may be increased density of neurons in the brains of KO mice, it is difficult to conclude whether there are actually differences in biogenic amine content based on neuronal density. Alternatively, differences in the cerebellum could also be attributed to the method of tissue collection, since the entire cerebellum was removed by blunt dissection and was not standardized by use of the brain matrix. Further studies are needed to determine the relationship between neural growth and density with biogenic amine levels in the cerebellum of KO mice. On the other hand, in WT mice, the KD increased NE in the hypothalamus and increased the 5-HIAA/5-HT ratio in the cerebellum. This was not surprising since a high fat diet has been shown to increase NE in hypothalamic brain regions (Levin et al., 1983). Yet, it was surprising that there was an increased turnover rate of serotonin. Although stress has been shown to increase the 5-HIAA/5-HT ratio in plasma of rats exposed to water-immersion restraint stress or nicotine administration (Takada et al., 1995), this does not explain the increased turnover rate in the cerebellum of the WT-KD mice since mice were not exposed to a stressor prior to decapitation. Perhaps the increase in neural NE resulted in a heightened stress response at sacrifice. In the following chapters, blood plasma corticosterone and food-intake response to an acute coping restraint stress will be described.

Even though we expected the KD to normalize biogenic amine concentrations in KO mice relative to WT, this was not the case. There are several reasons that may explain the lack of changes in biogenic amine levels in KO mice compared with WT. Notably, animals were on dietary intervention from post-weaning to adulthood, and the
critical window of biogenic amine alteration may have been missed. In addition, our dissections removed whole tissue sections, but we might have seen more acute differences if specific neural regions were isolated, which could be achieved by in vivo microdialysis. Moreover, our collaborators demonstrated that desipramine reversed behavioral abnormalities in En2−/− mice. Specifically, there was a reduction in immobility in a tail suspension and forced swim test, restored sociability in a three-chamber social test, and reversed impairments in contextual fear conditioning (Brielmaier et al., 2014). Perhaps KO mice have lost the mechanism to respond to KD that is upstream or independent of biogenic amine production. Due to the heterogeneity found in clinical ASD patients, this loss in mechanism may not be relevant. Thus, it is still possible that biogenic amines play a role in behavioral impairments in En2−/− mice, but the KD does not seem to alter these concentrations in whole brain regions.

In conclusion, En2−/− male mice exposed to the KD from PND 21 to 60 had increased ketone bodies and blood glucose, but the KD did not restore biogenic amine concentrations to WT levels. Future studies are needed to determine if there are other metabolic impairments resulting from KD exposure. Also, En2 WT mice have increased NE in the hypothalamus and increased 5-HIAA/5-HT in the cerebellum as a result of KD exposure. Although changes in NE in the hypothalamus have been shown in animals fed a high-fat diet, we show for the first time that the KD, high in fat and low in carbohydrates, can also increase NE in the hypothalamus. This study suggests that this increase in NE is associated with increases in dietary fat and not carbohydrates. En2−/− mice are resistant to high fat diet changes in hypothalamic NE and no significant changes were observed in biogenic amines in other brain region. In the following chapters, the effects of the KD on behaviors, metabolic outputs, and neural activation of c-Fos in determined brain regions will be described in En2 mice.


### Tables

**Table 1:** Experimental groups and respective diets.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Diet (Research Diets, New Brunswick, NJ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KO-KD</td>
<td>Ad Lib; 80% Fat, 20% Protein, 0.1% Carbohydrates (D03022101)</td>
</tr>
<tr>
<td>KO-CD</td>
<td>Ad Lib; 10% Fat, 20% Protein, 70% Carbohydrates (D12450K)</td>
</tr>
<tr>
<td>WT-KD</td>
<td>Ad Lib; 80% Fat, 20% Protein, 0.1% Carbohydrates (D03022101)</td>
</tr>
<tr>
<td>WT-CD</td>
<td>Ad Lib; 10% Fat, 20% Protein, 70% Carbohydrates (D12450K)</td>
</tr>
</tbody>
</table>

All groups had unlimited access to respective diets from PND 21 to 60.
Table 2. Biogenic amine concentrations (pg/mg of tissue) after dietary exposure.

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>KO-CD (n = 20)</th>
<th>KO-KD (n = 20)</th>
<th>WT-CD (n = 20)</th>
<th>WT-KD (n = 20)</th>
<th>Diet Effect (p value)</th>
<th>Genotype Effect (p value)</th>
<th>Diet x Genotype Effect (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frontal Cortex</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NE</td>
<td>755.8 ± 70.2</td>
<td>627.3 ± 48.3</td>
<td>923.7 ± 112.8</td>
<td>779.6 ± 41.7</td>
<td>0.121</td>
<td>0.062</td>
<td>0.678</td>
</tr>
<tr>
<td>DA</td>
<td>3955.5 ± 691.2</td>
<td>2837.3 ± 581.4</td>
<td>3176.5 ± 706.5</td>
<td>2871.6 ± 502.6</td>
<td>0.257</td>
<td>0.552</td>
<td>0.516</td>
</tr>
<tr>
<td>5-HIAA</td>
<td>239.2 ± 24.7</td>
<td>299.1 ± 34</td>
<td>261.6 ± 26.3</td>
<td>269.3 ± 22.6</td>
<td>0.215</td>
<td>0.890</td>
<td>0.337</td>
</tr>
<tr>
<td>HVA</td>
<td>355.8 ± 25.6</td>
<td>323.5 ± 21.8</td>
<td>317.4 ± 26.7</td>
<td>351.0 ± 23.0</td>
<td>0.977</td>
<td>0.824</td>
<td>0.179</td>
</tr>
<tr>
<td>5-HT</td>
<td>249.8 ± 17.2</td>
<td>228.0 ± 16.2</td>
<td>204.9 ± 16.6</td>
<td>218.6 ± 14.1</td>
<td>0.798</td>
<td>0.095</td>
<td>0.272</td>
</tr>
<tr>
<td>Hippocampus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NE</td>
<td>1068.2 ± 163.6</td>
<td>946.6 ± 91.3</td>
<td>1476.1 ± 344.3</td>
<td>1448.2 ± 225.8</td>
<td>0.732</td>
<td>0.040</td>
<td>0.830</td>
</tr>
<tr>
<td>DA</td>
<td>1266.1 ± 586</td>
<td>798.8 ± 501.9</td>
<td>829.8 ± 254.5</td>
<td>613.8 ± 202.3</td>
<td>0.426</td>
<td>0.469</td>
<td>0.769</td>
</tr>
<tr>
<td>5-HIAA</td>
<td>1777.3 ± 971.8</td>
<td>983.0 ± 214.1</td>
<td>2078.1 ± 573.9</td>
<td>1225.3 ± 341.4</td>
<td>0.132</td>
<td>0.613</td>
<td>0.956</td>
</tr>
<tr>
<td>5-HT</td>
<td>264.0 ± 51.4</td>
<td>244.9 ± 31.5</td>
<td>457.0 ± 113.6</td>
<td>322.5 ± 52.0</td>
<td>0.212</td>
<td>0.030</td>
<td>0.347</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NE</td>
<td>4145.5 ± 886.9</td>
<td>3961.6 ± 418.2</td>
<td>2861.5 ± 446.5*</td>
<td>5327 ± 745.1*</td>
<td>0.087</td>
<td>0.951</td>
<td>0.048*</td>
</tr>
<tr>
<td>DA</td>
<td>662.5 ± 105.7</td>
<td>839.2 ± 88.8</td>
<td>1030.2 ± 285.2</td>
<td>6128.1 ± 195.0</td>
<td>0.306</td>
<td>0.041</td>
<td>0.953</td>
</tr>
<tr>
<td>5-HIAA</td>
<td>1163 ± 176.5</td>
<td>1176.0 ± 165.4</td>
<td>1351.8 ± 195.0</td>
<td>2312.5 ± 394.3</td>
<td>0.142</td>
<td>0.051</td>
<td>0.194</td>
</tr>
<tr>
<td>HVA</td>
<td>80.6 ± 17.0</td>
<td>62.4 ± 8.8</td>
<td>90.0 ± 19.3</td>
<td>135.1 ± 28.0</td>
<td>0.540</td>
<td>0.067</td>
<td>0.154</td>
</tr>
<tr>
<td>5-HT</td>
<td>370.4 ± 49.4</td>
<td>406.9 ± 47</td>
<td>443.5 ± 79.3</td>
<td>623.2 ± 75.7</td>
<td>0.106</td>
<td>0.032</td>
<td>0.282</td>
</tr>
<tr>
<td>Cerebellum</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NE</td>
<td>1215.6 ± 108.1</td>
<td>1275.8 ± 100.2</td>
<td>1069.1 ± 108.4</td>
<td>996.6 ± 92.5</td>
<td>0.952</td>
<td>0.042</td>
<td>0.520</td>
</tr>
<tr>
<td>5-HIAA</td>
<td>355.7 ± 32.8</td>
<td>269.8 ± 20.7</td>
<td>269.5 ± 35.7</td>
<td>312.1 ± 39.4</td>
<td>0.560</td>
<td>0.554</td>
<td>0.088</td>
</tr>
<tr>
<td>HVA</td>
<td>14.7 ± 2.2</td>
<td>13.0 ± 2.1</td>
<td>19.6 ± 9.4</td>
<td>9.3 ± 0.7</td>
<td>0.447</td>
<td>0.939</td>
<td>0.586</td>
</tr>
<tr>
<td>5-HT</td>
<td>129.7 ± 18.0</td>
<td>112.5 ± 14.6</td>
<td>90.7 ± 11.9</td>
<td>64.0 ± 6.3</td>
<td>0.091</td>
<td>0.001</td>
<td>0.708</td>
</tr>
<tr>
<td>5-HIAA/5-HT</td>
<td>3.2 ± 0.4</td>
<td>2.8 ± 0.5</td>
<td>3.3 ± 0.4</td>
<td>5.2 ± 0.5*</td>
<td>0.155</td>
<td>0.016</td>
<td>0.026*</td>
</tr>
</tbody>
</table>

Male En2 KO and WT mice fed a ketogenic or control diet from PND 21 to 60. Values are means ± SEM. Data were analyzed with a factorial ANOVA and with post-hoc Newman-Keuls test. Same letter indicates significant difference between groups and * indicates overall significance.


Table 3. Blood ketone bodies and glucose levels after dietary exposure.

<table>
<thead>
<tr>
<th></th>
<th>KO-KD</th>
<th>KO-CD</th>
<th>WT-KD</th>
<th>WT-CD</th>
<th>Diet Effect (p value)</th>
<th>Genotype Effect (p value)</th>
<th>Diet x Genotype Effect (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ketone Bodies</strong>,</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mmol/L</td>
<td>1.1 ± 0.1&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>0.68 ± 0.1&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>1.13 ± 0.1&lt;sup&gt;c,d&lt;/sup&gt;</td>
<td>0.74 ± 0.1&lt;sup&gt;b,d&lt;/sup&gt;</td>
<td>&lt;0.001*</td>
<td>0.55</td>
<td>0.86</td>
</tr>
<tr>
<td><strong>Blood Glucose</strong>,</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mg/dl</td>
<td>181.8 ± 6.0&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>148.0 ± 6.1&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>192.4 ± 6.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>155.6 ± 7.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&lt;0.001*</td>
<td>0.16</td>
<td>0.82</td>
</tr>
</tbody>
</table>

Values are means ± SEM. Data was analyzed with a factorial ANOVA with post-hoc Newman-Keuls test. Same letter indicates significant difference between groups and * indicates overall significance.
CHAPTER THREE: A ketogenic diet during post-weaning period increases social behaviors in young adult male EN2 null mice.

ABSTRACT

The ketogenic diet (KD), shown to improve clinical neurological disorders, may have beneficial implications for autism spectrum disorder (ASD). Engrailed-2 (En2) knockout (KO) and wild-type (WT) male mice were fed either KD (80% fat, 0.1% carbohydrates) or control diet (CD; 10% fat, 70% carbohydrates) from weaning at postnatal day (PND) 21 to young adulthood (PND 60) to determine social and stress-related behaviors. In a three-chamber social test, KO-KD mice displayed increased social behavior, measured by frontal contact (+26.4%), and reduced repetitive self-grooming (-81.4%) compared with KO-CD. Baseline corticosterone, prior to a 1-h stressor, was significantly lower in KO-KD compared to KO-CD (-15.7%). At the end of the 1-h restraint stress, KO-CD had higher levels of corticosterone than WT-CD (+8.9%). Post-restraint stress food intake was significantly increased in KO mice compared with WT over the 4-h intake period (+13.3%). Increased stress reactivity in KO-CD compared to WT supports the use of the model, but more importantly the KD reduced the stress response in KO mice. In a forced swim paradigm, to measure depressive-like behaviors, no changes in immobility were observed in KO mice. This study confirms the utility of KO mice as a relevant model for ASD based on deficits in behavior and stress endpoint compared with WT mice. In addition, KO mice fed the KD from post-weaning to young adulthood had improved social performance, reduced repetitive behaviors, and lower plasma corticosterone levels in response to stress when compared with En2<sup>-/-</sup> mice fed the CD.
Introduction

Cardinal features of autism spectrum disorder (ASD) include deficient human relationships, abnormal nonverbal communication, repetitive/restricted behaviors, hyper/hypo-reactivity to sensory input, and impaired communicative skills (American Psychiatric Association 2013). During early childhood, development of these symptoms coincides with increases in synaptic density, brain glucose metabolism, and gray matter volume followed by a significant elimination or pruning of neurons (Spear 2000; Andersen 2003; Bingham et al., 2011). One often overlooked moderating factor is the influence of nutritional sources or diet on the trajectory of these neurodevelopmental events. A notable example is the use of a ketogenic diet (KD) to treat drug-resistant pediatric epilepsy (Vining 1999; Lefevre et al., 2000; Keene 2006). Efficacy of the KD treatment in ASD has been observed in small samples of children and adolescents with improvements in clinical ASD-related outcomes (Witwer et al., 2005; Srinivasan 2009; Marti 2010), but the mechanism involved in these improvements are unknown.

One comorbidity of ASD, epilepsy, will develop in 20-30% of autistic children. Both of these neurological disorders have overlapping biological pathways, including shared genes important for transcription (FOXG1, MECP2, MEF2C), cell growth and proliferation (PTEN; tuberous sclerosis complex, TSC1/2), and synapse development, stability, and function (SCN2A, CASK, CDKL5, FMR1, and SHANK3). Abnormalities in these genes and pathways result in a wide range of symptoms including, but not limited to, severe intellectual disorders (MECP2, MEF2C, FMR1) and tumor growth (PTEN, TSC1,2) (Mefford et al., 2011; Olson et al., 2014; Pinto et al., 2014). Therefore, it is unclear exactly what mechanisms are shared by both ASD and epilepsy to result in autistic-like behaviors. The triad of deficits in ASD, deficient human relationships, abnormal nonverbal communication, and repetitive/restricted behaviors, are most likely a
result of improper neural development resulting from abnormalities in many genes. It is known that neural pathways are more sensitive to endogenous influences in early development (Swann et al., 1999; Muller et al., 2011; Saugstad 2011; Tang et al., 2014). For instance, dysfunctions in GABAergic signaling have been associated with both ASD and epilepsy (Carvill et al., 2015; Cochran et al., 2015). GABAergic signaling regulates synapse elimination and axon pruning by acting on local dendritic Ca\(^{2+}\) signaling (Hayama et al., 2013; Hayama et al., 2014; Brandao et al., 2015). Reduced expression of GABAergic marker mRNA in the hippocampus and cerebral cortex has been found in Engrailed-2 null (En2\(^{-/-}\)) mice, which not only are susceptible to seizure activity, but display autistic-like behaviors.

The objective of this study was to determine if the KD can improve stress-related behaviors and social interaction to rescue autistic-like behaviors of young adult male En2\(^{-/-}\) mice. In the following study, knockout (KO) and wild-type (WT) En2 male mice were fed the KD or control diet (CD) from postnatal day (PND) 21 to 60 to determine if a dietary intervention, the KD, could improve behavioral deficits, reduce stress-related behaviors, and alter depressive-like behavior associated with En2\(^{-/-}\) mice in adulthood.

**Materials and Methods**

*Animals and Genotyping.*

En2\(^{-/-}\) mice were maintained by heterozygous breeding pairs and group housed with mixed litter and non-littermates to normalize social and stress-related behaviors for experimental procedures, as described in Chapter 2.
**Experimental diet.**

To determine the effects of the KD on behavior, stress, and depression, $En2^{−/−}$ (KO, $n = 30$ total) and $En2^{+/+}$ (WT, $n = 26$ total) mice were fed *ab libitum* 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 control diet (CD; 10% fat, 20% protein, 70% CHO, 3.85 Kcal/g; D12450K; Research Diets) from PND 21 to 60, which encompasses a developmental period analogous to middle to late childhood in humans; see Chapter 2 Table 1. It is important to note that neither diet contained sucrose to prevent preference of a sweeter food and inappropriate consumption. At PND 60, all animals were placed on standard chow (Purina Mouse Diet 5015, 25.34% fat, 19.81% protein, 54.86 CHO, 3.7 Kcal/g) until sacrifice.

**Assessment of social behaviors.**

At PND 62, KO-KD ($n = 14$), KO-CD ($n = 13$), WT-KD ($n = 12$), WT-CD ($n = 13$), fed either KD or CD, 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 (Brielmaier et al., 2012). Two days post-dietary intervention was chosen, since ketone body levels are normalized to CD fed mice, as determined in Chapter 2. During three 10-min recorded phases the following was quantified: time spent in each chamber, frontal contact (time spent between experimental and stranger mice), and self-grooming. In phase 1, all mice had 10 min to explore and acclimate to all three chambers. In phase 2, a stranger mouse (adult male $En2^{−/−}$ non-litter mate) was placed in a wire cage in chamber 1 and the experimental mouse could choose between interaction or continual exploration of the chambers and empty wire cage in chamber 3, for 10 min. In phase 3, the stranger mouse was now familiar and a second stranger 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. Also, the three-chamber apparatus was cleaned with disinfectant
(Labsan 120) between each test. All behavior was recorded and analyzed using a time-
sampling computer program, Hindsight (version 1.3) and each mouse was scored by
three observers blind to the treatment conditions and genotypes.

*Measurement of acute restraint stress.*

At PND 70 half of the mice were exposed to a 1-h restraint stress, as previously
described (Bello et al., 2010; Sharma et al., 2013; Bello et al., 2014a; Bello et al.,
2014b). All mice were food restricted 24 h prior to experiments and placed in clean
cages. Prior to the start of the stressor, non-stressed animals were placed in individual
clean cages. Mice exposed to the stress were placed in well-ventilated Plexiglas
restrainers by which 50 μl of blood was collected via a tail nick at baseline and 60 min.
Blood samples were maintained on ice until centrifugation at 3,000 rpm for 10 min at
4°C, then plasma was stored at -80°C. A radioimmunoassay was performed to
determine plasma corticosterone (sensitivity: 25 ng/ml; MP Biomedicals, Santa Ana, CA,
USA) levels. At the end of the 1-h restraint stress, animals were returned to individual
cages and re-fed standard chow. Standard chow intake was measured at 0.5, 1, 2, and
4 h post-restraint. The 2-h and 4-h post-restraint intakes were during the dark cycle.
After the 4-h intake, mice were returned to appropriate group housing. A week later, at
PND 77, groups were crossed-over with the non-stressed animals exposed to the
stressor. The experiment was repeated as previously described.
Forced swim test.

At PND 91, mice were exposed to a six-minute forced swim test, as previously described (Can et al., 2012). All mice were placed individually in an inescapable Plexiglas transparent tank with water set to room temperature (23-25°C) to measure immobility latency and time spent immobile. Mobility was defined as any movement other than those necessary to balance and keep the head above the water, which may include slight movement of the feet and tail. Mice may also drift after swimming and this was not counted as mobility. Tap water in the tank was filled so mice could not touch the bottom with their feet or tail, but also could not climb out of the tank. Water level was marked on the tank so the volume was consistent for each animal. After the six minute forced swim test, mice were patted dry and placed in an empty cage lined with paper towels over a heating pad to prevent hypothermia. The tank was cleaned between each animal with disinfectant (Labsan 120). All behavior was recorded and analyzed using a time-sampling computer program, Hindsight (version 1.3) and each mouse was scored by three observers blind to the treatment conditions and genotypes.

Statistical Analysis.

Food intake post restraint stress and corticosterone measurements were determined by two-way repeated measures analysis of variance (ANOVA). Measurements in the three-chambered test were analyzed by a multivariate ANOVA (MANOVA). Behavioral measurements in the forced swim test were analyzed with a factorial ANOVA. Post-hoc comparisons were made, when appropriate, with a Newman-Keuls test. All statistical analyses were performed using Statistica 7.1 software (StatSoft, Tulsa, OK, USA) and significance was set at α = 0.05.
Results

Social behaviors in En2^{−/−} mice increased 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-KD had increased social contact over all three phases compared with KO-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-CD had increased self-grooming behaviors compared with KO-KD (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. KO-KD trended to spend as much time with the stranger mouse in phase 2 as WT-CD; see Fig. 2a. Although not statistically significant, for the middle chamber, chamber 2, phase x diet effect was close to significance [F(2, 144)=2.9, p=.056], with KO-CD tending to spend increased time in the middle chamber 2 during phase 2 when there was a stranger mouse in one chamber; see Fig. 2b. There was a phase effect [F(2, 144)=10.0, p<0.001], genotype x diet effect [F(1, 144)=4.5, p<0.05], and phase x genotype x diet effect [F(2, 144)=3.8, p<0.05] for chamber 3, which was empty in phase 1 and 2 until a stranger mouse was place in a wire cage in phase 3. Post-hoc analysis determined that KO-CD spent significantly more time in chamber 3 with the stranger mouse in phase 3 compared with WT-CD (p < 0.05); see Fig. 2c. A KD increased social behaviors (i.e. frontal contact) and reduced repetitive self-grooming behaviors in KO mice.
Stress parameters were reduced in En2−/− mice fed the KD.

In the restraint stress test, there was a significant genotype x diet effect \( [F(1,108) = 7.2, p < 0.01] \) and time effect \( [F(1,108) = 1093.8, p < 0.001] \) for blood corticosterone levels. Post-hoc analysis showed KO-CD had significantly higher levels of corticosterone than KO-KD at baseline (\( p < 0.05 \)). All mice displayed increased corticosterone compared to baseline as a result of the acute restraint stress; see Fig. 3a. A significant time effect \( [F(3,153) = 93.0, p < 0.0001] \) and time x genotype effect \( [F(3,153) = 3, p < 0.05] \) was detected for post-restraint stress food consumption. Post-hoc analysis demonstrated KO mice consumed more chow post-restraint compared with WT over the 4-h period (\( p < 0.05 \)); Fig. 3b-c. A KD reduced baseline corticosterone, but not food intake post-restraint stress in KO mice.

Immobilty is increased in mice fed the ketogenic diet.

In the forced swim test there was a diet effect \( [F(1, 52) = 8.7, p < 0.01] \) and genotype effect \( [F(1, 52) = 5.5, p < 0.05] \) for time spent immobile. There was a trend where KO-CD spent less time immobile compared with KO-KD; Fig. 4a. There were no significant effects observed for immobility latency; Fig. 4b. Immobility was not significantly altered by a KD in KO mice.

Discussion

The objective of this study was to determine if a KD, fed from weaning (PND 21) to young adulthood (PND 60), would affect behavior of male En2−/− mice based on measurements of social behaviors, response to stress, and depressive-related behaviors. In order to control for the acute effects of the diets, all behavioral testing was completed after animals were removed from experimental diets and fed standard chow for several days (>48-h). This short period of standard chow feeding has been shown to
restore the metabolic alterations (i.e., elevated ketone bodies) caused by the KD. As shown in Chapter 2, NE was increased in WT-KD at PND 60, which could be a result of acute elevated ketone bodies (β-hydroxybutyrate) or a chronic effect of dietary exposure during development. Since the purpose of this study was to determine if a dietary intervention can have long lasting implications on neural transmission and adult behaviors, removal of possible acute dietary effects was necessary. Ketone bodies were restored to CD level after 48-h post-KD. This was critical for experiments to control for possible acute ketone body effects on neurotransmission. Previous studies using En2−/− mice have described behaviors only when animals were fed a standard chow, thus our groups were placed back on a standard chow prior to behavioral testing.

It has been hypothesized that dysfunctional noradrenergic and serotonergic pathways contribute to the behavioral impairments demonstrated in ASD (Lam et al., 2006). Since En2−/− mice have abnormalities in noradrenergic and serotonergic neurons in mid- and hindbrain structures (Simon et al., 2005) and reduced social behaviors (Brielmaier et al., 2012), they are considered a relevant animal model for studying ASD. Also, it is well established that a high-fat diet can alter brain NE and 5-HT activity (Levin et al., 1986; Billes et al., 2007) and stress responses by the HPA axis (Shin et al., 2010). Therefore, to test the hypothesis that the KD can alter neural pathways critical to social behaviors, animals were placed in a three-chamber social testing apparatus. This allows experimental mice the choice to freely interact with a stranger mouse or to explore the two empty chambers with or without a wire cage. In phase 1, chambers 1 and 3 have an empty wire cage. In phase 2, a stranger mouse was randomly placed in chamber 1 or 3. In phase 3, the stranger mouse remains and a second stranger mouse is placed in the remaining wire cage. To avoid both stranger mice, which would be an indicator of social avoidance or a heightened fear response, the experimental mouse would have to remain in the middle chamber 2. Remaining in the middle chamber results in a fear response in
a normal animal, because without anywhere to hide, the mouse is exposed. This
instinctual reaction to hide is a response as a prey species to avoid a predator. Feeding
the KD to $En2^+/+$ mice increased social contact, i.e., frontal contact and reduced repetitive
behaviors, as defined by self-grooming. In this paradigm, increased grooming by KO
mice is most likely a coping mechanism during the three-chambered social test, which
reduced the amount of time the mouse could spend interacting with the stranger mouse.
The control diet had no effect on sociability in $En2^+/+$ mice, which displayed decreased
frontal contact with a stranger mouse. In phase 3, when two stranger mice were present
on opposite ends of the chamber, WT-CD preferred to stay with the stranger mouse
introduced in phase 2, but KO-CD spent significantly more time with the new stranger
mouse. Further behavioral analysis is needed to determine if KO mice have increased
exploratory behaviors. Nevertheless, these results support previous findings in the BTBR
T +tf/J (BTBR) mouse model, that demonstrated increased sociability in a three-chamber
test when male juvenile mice (PND 35) were fed a KD for three weeks. BTBR mice also
showed increased self-directed repetitive behaviors (self-grooming) (Mcfarlane et al.,
2008). BTBR mice typically have reduced sociability and communication and increased
repetitive behavior, so the results of the study reported by McFarlane and colleagues
provides evidence that a KD can positively influence behavior. The KD improved social
behaviors in both the $En2$ and BTBR mice. Understanding the neural mechanisms that
result in these behavioral changes may lead to future clinical treatment plans for certain
ASD populations.

To determine response to an acute coping stressor, animals were exposed to a
restraint stress at PND 70. Interestingly, pre-exposure to the KD from weaning to young
adulthood decreased baseline corticosterone levels in adult $En2^+/+$ mice. Restraint stress
increased corticosterone levels from baseline to 60 min in all groups. Typically, an acute
stressor will reduce intake in animal models (Calvez et al., 2011) but in chronic stress
conditions, animals may cope with stress by demonstrating hyperphagia, as observed in binge-eating disorder (BED) and bulimia nervosa (BN) (Nieuwenhuizen et al., 2008; Bartolomucci et al., 2009). Thus, food intake as a result of the 1-h restraint stress was measured post-stressor up to 4 h. It was hypothesized that the KD would blunt the HPA axis and reduce the overall stress response. Instead, no differences were observed in mice previously exposed to a KD or CD. Interestingly, KO mice did have a significant increase in food intake post-stressor compared with WT, which may be interpreted as a coping response to the acute stress, as in BED or BN. These results are of particular importance since a previous study showed that En2/− mice do not have differences in stress-related behaviors based on measurements of an elevated plus-maze and a light/dark exploration task (Brielmaier et al., 2012). However, that study did not measure stress-related food intake or acute coping behaviors. Increased food intake could also be due to reduced 5-HT in the hypothalamus, as found in Chapter 2 (Mackenzie et al., 1979; Hoebel 1985; Koopman et al., 2013). Thus, En2/− mice may have increased acute stress parameters, i.e., baseline plasma corticosterone and enhanced food intake as a result of an acute restraint stress. Only baseline plasma corticosterone could be reduced in En2/− mice pre-exposed to the KD. Further experiments are needed to elucidate if the HPA axis is indeed altered in En2/− mice.

Lastly, mice were exposed to a forced swim test to determine if the KD could rescue depressive-like behaviors. From Chapter 2 it was determined that regional brain biogenic amine levels were not altered by the KD, but these acute measurements do not rule out effects of the KD on neural transmission. Previously it has been shown that reduced noradrenergic concentrations in En2/− mice are accompanied by increased immobility in the forced swim test (Brielmaier et al., 2012). Also, desipramine, a selective NE reuptake inhibitor, can significantly reduce immobility in forced swim and restore sociability in the three-chambered social test of En2/− mice (Brielmaier et al., 2014). In
the present study, KO mice were not significantly different from WT. Statistically, KO-CD presented reduced immobility time compared with WT-KD, but this is not biologically significant. There was a trend that KO-CD spent less time immobile than KO-KD. A lack of mobility differences in KO-CD may be due to a number of factors most notably the order effect of behavioral tests. DiCicco-Bloom and colleagues (Brielmaier et al., 2012) exposed mice to various forms of social and stress-related tests prior to the forced swim test, which was similar to our study, but the addition of the acute restraint stress may have resulted in a heightened stress response. KO-CD had increased corticosterone at baseline of the acute restraint stress test, compared with KO-KD, which may have impacted the forced swim test. In addition, environmental factors may be altered between the two studies, since our behavioral experiments were performed in our local animal facility. For instance, our behavioral testing area could have more noise or light, which could influence stress levels of the mice and result in different outcomes. Thus, in this study En2−/− mice were not found to have depressive-like behaviors and the KD had no influence on immobility in a forced swim test.

In conclusion, feeding En2−/− mice a KD from PND 21 to 60 increased social behaviors, reduced repetitive self-grooming behaviors, and lowered baseline corticosterone levels. Overall, this study determined KD affects social, stress, and depressive-like behaviors in En2−/− mice. The intricate neural mechanisms involved in these changes have yet to be elucidated, but the KD dietary intervention from post-weaning to young adulthood in the En2−/− mice may have long lasting neural effects. It has been hypothesized that the KD provides neuroprotective properties by reducing neural inflammation or glutamate-induced free radical formation. The KD may reduce glutamate-induced free radical formation by increasing NADH oxidation in the mitochondrial respiratory chain in neocortical neurons, which may protect neurons from improper pruning events (Abdelwahab et al., 2010; Vallejo et al., 2010). Neural
protection during development could potentially explain improved social and stress-related behaviors observed in $En2^{−/−}$ mice fed the KD in the current study. Additional studies are needed to determine if seizure susceptibility and markers of epileptic activity can also be altered by the KD and the mechanism by which the KD improves autistic-like behaviors in young adult $En2^{−/−}$ mice.
Literature Cited


depression-related, social and cognitive deficits in Engrailed-2 knockout mice. *Genes Brain Behav*, 13, 286-298.


Figure Captions

**Figure 1.** Social behaviors in a three-chamber social interaction test 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. KO-KD (n = 14), KO-CD (n = 13), WT-KD (n = 12), and WT-CD (n = 13) 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 mates. Average times are mean ± SEM. a. Average total time (30 min) engaging in frontal contact with the adult male En2+/− non-litter mates. Same letter indicates significant difference (A, p < 0.05) from KO-CD. Same letter indicates significant difference (B, p < 0.05) from WT-CD. b. Average total time (30 min) engaging in self-grooming. Same letter indicates significant difference (A, p < 0.05) from KO-CD.

**Figure 2.** Duration of time spent in specific chambers during three-chamber social interaction test. Average times are mean ± SEM. a. Average total time (10 min per phase) spent in chamber that has an adult male En2+/− non-litter mate during phase 2 and 3. During phase 1 this chamber was empty. b. Average total time (10 min per phase) spent in empty chamber during each of the 3 phases. There was a trend where KO-CD spent increased time alone in chamber 2 during phase 2 when there was an adult male En2+/− non-litter mate in one chamber. c. Average total time (10 min per phase) spent in each chamber with an adult male En2+/− non-litter mate during phase 3. Same letter indicates significant difference (A, p < 0.05) from WT-CD in phase 3.

**Figure 3.** Chow intake and corticosterone response to restraint stress for KO-KD (n = 16), KO-CD (n = 14), WT-KD (n = 13), and WT-CD (n = 13). One week following the three-chamber social test at PND 70, all animals were food restricted for 24 h followed
by an immobilization stress (restraint stress) or no stress for 1 h prior to refeeding with standard chow. One week later, non-stress animals were exposed to the restraint stress. All data are mean ± SEM. a. Plasma corticosterone (ng/ml) at baseline and after restraint stress (60 min). Same letter designates KO-CD from KO-KD (A, p < 0.05) at baseline. # indicates significant elevation (p < 0.001) from baseline. b. Post-restraint all animals were re-fed standard chow in individual cages. c. Due to a lack of a diet x genotype effect, the data were expressed to show the genotype effect. * indicates significance (p < 0.05) from WT.

**Figure 4.** Forced swim test to examine depressive-like behaviors in KO male mice. a. Time spent immobile (%). All data are mean ± SEM. b. Time to immobility. There were no significant differences between groups in this region.
Figures

Fig 1a.

![Graph showing Frontal Contact (sec) for KO-KD, KO-CD, WT-KD, and WT-CD.]

Fig 1b.

![Graph showing Self-grooming (sec) for KO-KD, KO-CD, WT-KD, and WT-CD.]
Fig 2a.

![Graph showing Time Spent in Chamber (sec) across different phases (condition).]

- KO-KD
- KO-CD
- WT-KD
- WT-CD

Phase (condition)

Fig 2b.

![Graph showing Time Spent in Chamber (sec) across different phases (condition).]

- KO-KD
- KO-CD
- WT-KD
- WT-CD

Phase (condition)
Fig 3b.

Fig 3c.
Fig 4a.

Fig 4b.
CHAPTER FOUR: Ketogenic diet fed post-weaning to young adulthood rescues lean body mass and alters blood glucose response in EN2 null mice.

ABSTRACT

Individuals with autism spectrum disorder (ASD) often have difficulties maintaining body weight homeostasis along with complications in growth and development. A ketogenic diet (KD), used to treat drug-resistant pediatric epilepsy, has been shown to reduce body weight, improve glucose tolerance, and increase energy expenditure in an obese state. The following studies set to establish the metabolic phenotype of Engrailed-2 (En2) knockout (KO) mice, which display autistic-like behaviors, and to determine if the KD improves metabolic endpoints. KO and wild-type (WT) male mice were fed either a KD (80% fat, 0.1% carbohydrates) or control diet (CD; 10% fat, 70% carbohydrates) from weaning at postnatal day (PND) 21 to young adulthood (PND 60). Increased body weight (+18.1%), as a result of increased lean mass, was observed in the KO-KD mice compared with KO-CD at PND 60. Blood pressure trended to be reduced in KO-KD compared to KO-CD. The KD increased fat mass in both KO and WT mice (approximately 50%). WT-KD had increased activity during the dark cycle (+42.9%) compared with KO-KD. An oral glucose tolerance test revealed an increased blood glucose response in KO-KD mice compared to all other groups. This study demonstrated that a KD fed to En2 null mice from post-weaning to young adulthood can normalize body weight to WT mass by increasing lean body mass, but may dysregulate blood glucose metabolism.
Introduction

The fundamental features of autism spectrum disorder (ASD), which include deficient human relationships, abnormal nonverbal communication, and repetitive/restricted behaviors typically overlook the metabolic and growth comorbidities associated with this developmental disorder (American Psychiatric Association 2013). ASD children are at a high risk of being underweight with a low body mass index (BMI) and suffer from numerous gastrointestinal disturbances, such as food intolerance (Mari Bauset et al., 2012; Kang et al., 2014). These gastrointestinal disturbances can be controlled, but not necessarily treated. Dietary interventions aimed at controlling autistic-like behaviors are mostly anecdotal, but recently it has been shown that the ketogenic diet (KD), high in fat and low in carbohydrates, improved autistic-like behaviors (Ghaziuddin et al., 2013; Spilioti et al., 2013). Although this small study required extremely tedious personal care for parents and caretakers, it suggests that a KD may positively influence autistic-like behaviors.

Despite numerous online blogs and discussion groups, which claim that certain diets may help children with ASD, there is a lack of scientific peer-reviewed evidence to show efficacy for any dietary treatment strategy. However, there does seem to be growing evidence to support a possible benefit of a KD in ASD, since the KD has been shown to improve social behaviors in animal models of ASD (Mantis et al., 2009; Ruskin et al., 2013). The KD has also been shown to influence neural pathways and has been used to treat drug-resistant pediatric epilepsy (Vining 1999; Lefevre et al., 2000; Keene 2006; Abdelwahab et al., 2010; Jeong et al., 2011). Since 20-30% of children with autism will also develop epilepsy (Dicicco-Bloom et al., 2006; Chavez et al., 2007), it is plausible that ASD and epilepsy share common pathways of neurological dysfunction that could be amenable to dietary treatments. A KD has been reported to improve
clinical outcomes (reduce autistic-related behaviors) in small samples of children and adolescents with ASD (Evangelion et al., 2003; Witwer et al., 2005; Srinivasan 2009; Marti 2010). Evangelion and colleagues (2003) discovered that a six-month KD treatment resulted in improvements in autistic-like behaviors (based on CARS scores). This is the first study to determine efficacy of a KD dietary intervention to treat autistic-related behaviors. However, it is still unknown how the KD is influencing neural signaling, autistic behaviors, and to what extent it is altering metabolic outcomes.

Engrailed-2 (En2) is a transcription factor critical to neural development. The En2 null mouse model has been used to study neural systems related to ASD, as described in previous chapters. In mice, mutations in En2 result in abnormal forebrain and hindbrain structures and autistic-like behaviors. Recent studies have shown that En2 may regulate insulin-like growth factor 1 (IGF-1) signaling which, along with its role in glucose metabolism, acts to promote brain development and plasticity. IGF-1 has been found to be increased in the blood, but decreased in cerebrospinal fluid of ASD children (Riikonen et al., 2006; Mills et al., 2007). These observations may be due to dysfunctional growth hormone (GH). En2−/− mice have decreased GH mRNA expression, which may result in the observed upregulation of IGF-1 mRNA in the liver and down-regulation in the hippocampus (Provenzano et al., 2014). Due to the numerous metabolic pathways that GH and IGF-1 influence, it is likely that En2−/− mice have abnormalities in growth and metabolism, but these changes have not been well characterized. Therefore, the following study was conducted to determine if En2 null mice have altered glucose metabolism and reduced lean body mass relative to WT mice and, if so, whether a KD can normalize these parameters.

In the current study, KO (En2−/−) and WT (En2+/+) male mice were fed a KD or control diet (CD) from postnatal day (PND) 21 to 60 and various metabolic endpoints
(e.g. body weight, lean mass, fat mass, gas exchange, locomotor activity, glucose response, and blood pressure) were measured. We have previously shown that a KD can improve social behaviors in En2 KO mice (Chapter 3), but whether a KD can also improve the metabolic state in these mice is unknown. In obese and/or diabetic individuals, a KD can reduce body weight and improve metabolic syndrome (Astrup et al., 2004; Al-Khalifa et al., 2009), but a KD has also been shown to cause metabolic impairments and dysregulation of blood glucose in rodent models (Ellenbroek et al., 2014). Thus, we hypothesized that En2−/− mice exposed to the KD will have improved metabolic outputs, as measured by an EchoMRI, indirect calorimeter, oral glucose tolerance test, and a noninvasive blood pressure system.

**Materials and Methods**

*Animals and Genotyping.*

En2−/− mice were maintained by heterozygous breeding pairs and group housed for experimental procedures, as described in Chapter 2.

*Experimental diet.*

To determine the effects of the KD on metabolism, a total of 144 Engrailed-2 KO and WT mice were *ab libitum* fed either a lard based ketogenic diet, as described in Chapter 3.

*Growth and Hemodynamic measurements.*

Body weight (measured to the nearest 0.1g) was measured in the four groups of mice (KO-KD, n=16; KO-CD, n=14; WT-KD, n=13; WT-CD, n=13) weekly. At PND 84, hemodynamic measurements for all mice were recorded by a noninvasive blood pressure (BP) CODA system (Kent Scientific, Torrington, CT, USA) to determine
possible effects on hemodynamic measurements of the experimental diets. This automated system measured systolic and diastolic BP, mean BP, and heart rate via tail volume pressure recording. One day prior to recording experimental data, animals were habituated to the system for one trial period (~ 30 min). Measurements were determined by averaging three successfully recorded trials. A separate group of mice (n = 4 per group), exposed to the same experimental diets, were measured for body length post-sacrifice from nose to anus at PND 62.

**KD exposure body composition.**

In a separate group, metabolism and body composition of KO and WT mice as a result of exposure to the KD or CD from PND 21-60 was analyzed. At PND 60, body composition (lean versus fat mass) was determined using an EchoMRI 3-in-1 Body Composition Analyzer (Echo Medical Systems, Houston, TX, USA) in all mice (n = 32/group). Mice were allowed a 5-min habituation period to the holding tube before being placed in the EchoMRI. Each mouse was measured twice with a 5-min break between each run. Results were only considered if lean mass measurements were within 0.2 g. One portion of this group (n = 20/group) was sacrificed for biogenic amine analysis, as explained in Chapter 2, and gross body fat dissection. To quantify body fat, a midabdominal incision was performed on each carcass to identify and remove subcutaneous, retroperitoneal, and epididymal fat pads. Fat pads were weighed to the nearest 0.01 g.

**Metabolic measurements in an Indirect Calorimeter.**

After body composition in the EchoMRI was determined, mice (n = 12/group) were placed in an indirect calorimeter, the Oxymax: Comprehensive Lab Animal Monitoring System (CLAMS) (Columbus Instruments, Columbus, OH, USA) for 48 h to
measure vO2, vCO2, respiratory exchange ratio or energy expenditure (RER, vCO2/vCO2), and locomotor activity. Standard chow was fed to all mice during this 48-h time period and intake was measured. Only the last 24 h was used for analysis to account for acclimation to the chambers and solitary housing. Locomotor activity was measured by counting the number of infrared (IR) beam breaks in the plane. This was determined by x-axis beam breaks (x total) and vertical beam breaks (z total).

Oral glucose tolerance test post KD exposure.

A separate group of KO and WT mice (n = 10/group) on the same dietary intervention were exposed to an oral glucose challenge at PND 62 and PND 69. At PND 62 (two days after dietary switch to standard chow) an oral glucose tolerance test (OGTT) was performed. Six hours prior to testing, mice were placed in clean cages, weighed, and food-restricted. At the start of the test, mice were placed in Plexiglas restrainers and a tail nick was performed to obtain a baseline glucose reading using a glucometer. Immediately thereafter, mice were gavaged with a bolus of glucose (2.0 g/kg body weight) and placed in an individual clean cage without food and water. Blood samples were collected at 15, 30, 60, and 120 min post-gavage. After the 120 min blood collection, mice were returned to home cages and re-fed standard chow. One week later, at PND 69, the OGTT was repeated to determine if effects of the experimental diet persisted.

Statistical Analysis.

Body weight, indirect calorimeter results (vO2, vCO2, RER, locomotor activity), and blood glucose as a result of an OGTT were measured by two-way repeated-measures analysis of variance (ANOVA). Body length, hemodynamic measurements (blood pressure and heart rate), EchoMRI measurements, and gross fat weights were
analyzed with a factorial ANOVA. Area under the curve (AUC) was determined for each OGTT measurement and analyzed with a factorial ANOVA. Post-hoc comparisons were made, when appropriate, with a Newman-Keuls test. All statistical analyses was performed using Statistica 7.1 software (StatSoft, Tulsa, OK, USA) and significance was set at α = 0.05.

Results

En2−/− mice have low body weight on CD, but KD exposure results in body weight similar to WT mice.

For the four groups (KO-KD, n=16; KO-CD, n=14; WT-KD, n=13; WT-CD, n=13) body weight was measured from PND 21 to 60 on specific diets and then from PND 60 to 91 on standard chow. Body weight from PND 21-60 showed a significant genotype effect \([F(1,52) = 5.4, p < 0.05]\), diet effect \([F(1,52) = 27.4, p < 0.0001]\), time effect \([F(6,312) = 1348.8, p < 0.0001]\), time x diet effect \([F(6,312) = 14.2, p < 0.0001]\) and time x genotype x diet effect \([F(6,312) = 2.5, p < 0.05]\). A post-hoc test revealed at PND 60, that the KD normalized the low body weight of KO mice and KO-CD had significantly lower body weight than the other three groups \((p < 0.05)\). At PND 62, mice were switched to standard chow during behavioral testing, until sacrifice at PND 91. Body weight from PND 62-91 on standard chow demonstrated a significant genotype effect \([F(1,52) = 5.4, p < 0.05]\), genotype x diet effect \([F(1,52) = 4.7, p < 0.05]\), time effect \([F(4,208) = 132.8, p < 0.0001]\), and time x diet effect \([F(4,208) = 7.0, p < 0.0001]\). Post-hoc analysis determined that at sacrifice, PND 91, KO-CD had significantly lower body weight compared with WT-CD \((p < 0.05)\). KO-KD body weight remained similar to WT; see Fig. 1. A subset of mice \((n = 4/group)\) were measured from nose to anus to determine overall length in relationship to growth. There was a genotype effect \([F(1,12)\)
and post-hoc analysis determined that KO-CD was significantly shorter than WT-KD (p < 0.05) and KO-KD trended similar to WT; see Fig. 2. There were no differences in BMI between groups (body weight / length²); data not shown. A KD increased body weight of KO mice relative to WT and restored overall growth.

*Hemodynamic measurements were reduced in En2−/− mice fed the KD.*

Hemodynamic measurements were recorded for systolic BP, diastolic BP, mean arterial pressure, and heart rate at PND 84. There was a genotype effect for systolic BP [F(1,51) = 5.6, p < 0.05], and a post-hoc test determined KO mice had significantly higher systolic BP than WT mice (p < 0.05). There was a diet effect for diastolic BP [F(1,51) = 7.0, p < 0.01], for which a post-hoc test revealed KO-CD had significantly higher diastolic BP than WT-KD. There was a significant genotype effect for mean arterial BP [F(1,51) = 5.0, p < 0.05], and a post-hoc analysis revealed KO-CD had significantly higher mean arterial BP than WT-KD (p < 0.05). No significant differences were found for heart rate measurements; see Table 1. Blood pressure trended to be reduced to WT for KO mice fed a KD.

*Body composition was altered as a result of the KD.*

Changes in body composition in separate groups of KO and WT mice exposed to the same dietary intervention was determined. Body composition of fat and lean mass was determined by an EchoMRI (n = 32/group). There was a diet effect [F(1,248) = 159.0, p < 0.0001] for fat mass and post-hoc analysis determined the KD significantly increased fat mass regardless of genotype; see Fig. 3a. There was a diet [F(1,248)=33.3, p < 0.0001] and genotype effect [F(1,248)=20.0, p < 0.0001] for lean mass. Post-hoc analysis revealed KO-KD had restored lean mass to WT and KO-CD had decreased lean mass (p < 0.05) compared with all other groups; see Fig. 3b.
Immediately after body composition analysis, mice were housed individually in the indirect calorimeter system for 48 h (n = 12/group) or sacrificed for analysis of biogenic amines (n = 20/group; see Chapter 2). Animals that were sacrificed for biogenic amine analysis were also dissected for gross fat pad mass. These measurements were taken to determine if there were differences in fat distribution. For subcutaneous fat pad mass there was a diet effect \[F(1,73) = 19.5, p < 0.0001\]. KO-KD had similar levels of subcutaneous fat mass compared to WT. Post-hoc analysis determined WT mice previously fed the KD had increased subcutaneous fat mass compared with CD fed mice regardless of genotype \(p < 0.001\); see Fig. 4a. Measurements of retroperitoneal fat pad mass showed a diet effect \[F(1,73) = 61.4, p < 0.0001\]. Post-hoc analysis determined KD fed KO and WT mice had significantly more retroperitoneal fat pad mass compared with WT-CD and KO-CD \(p < 0.0001\); see Fig. 4b. For epididymal fat pad mass there was a diet effect \[F(1,73) = 26.5, p < 0.0001\]. Post-hoc analysis determined KD fed KO and WT mice had significantly more epididymal fat pad mass compared with CD fed WT and KO mice \(p < 0.01\); see Fig. 4c. Body fat was increased in all fat pad regions of mice fed a KD and lean mass was restored in KO compared to WT.

*Ketogenic diet alters metabolic outputs in an indirect calorimeter.*

Immediately after EchoMRI analysis, KO and WT mice were housed individually in the indirect calorimeter for 48-h (n = 12/group) and fed a standard mouse chow. The last 24-h interval was analyzed for night versus day \(vO_2\), \(vCO_2\), heat, RER, and locomotor activity. There was a diet effect \[F(1,44) = 16.2, p < 0.0001\] and time effect \[F(1,44) = 166.6, p < 0.0001\] for \(vO_2\). Post-hoc analysis all animals had increased \(vO_2\) at night versus during the day \(p < 0.01\). In addition, KD fed mice had significantly lower \(vO_2\) compared to WT-CD in both day and night time periods \(p < 0.05\); see Fig. 5a. There was a diet effect \[F(1,44) = 35.7, p < 0.0001\] and time effect \[F(1,44) = 260.5, p <
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Post-hoc analysis determined that all animals had increased vCO2 at night versus during the day (p < 0.01). In addition, KD fed mice had significantly lower vCO2 compared to WT-CD during the day. All groups had significantly lower vCO2 compared to WT-CD at night (p < 0.05); see Fig. 5b. A factorial ANOVA of night RER values showed a diet effect, where animals previously fed a KD had a RER closer to 0.7 (p < 0.05); see Fig. 5c. Locomotor activity on the x-axis and z-axis were determined by total number of beam breaks. Analysis showed a genotype effect [F(1,44) = 9.3, p < 0.01], time effect [F(1,44) = 104.5, p < 0.0001], and a time x genotype effect [F(1,44) = 8.9, p < 0.01] for x total activity. Post-hoc analysis revealed KO and WT-CD mice had significantly lower levels of x total activity at night compared with WT-KD (p < 0.001); see Fig. 5d. There was a diet effect [F(1,44) = 4.7, p < 0.05], genotype effect [F(1,44) = 9.4, p < 0.01], time effect [F(1,44) = 77.4, p < 0.0001], and a time x genotype effect [F(1,44) = 9.1, p < 0.01] for z total activity. Post-hoc analysis revealed WT-KD had significantly higher levels of z total activity at night compared with all other mice (p < 0.001); see Fig. 5e. These effects were independent of acute metabolic effects from the KD, as after two days there were no significant differences in ketone body concentration between KD and CD animals, as described in Chapter 2. Metabolism of KD fed mice was indicative of enhanced fat metabolism.

**KD results in glucose regulation impairments in En2^-/- mice.**

In a separate group of KO and WT mice on the same dietary intervention, blood glucose concentrations after an oral gavage of glucose (2 g/kg) were determined at PND 62 and PND 69 (n = 10/group). At PND 62 there was a time effect [F(4,148) = 108.4, p < 0.0001], time x diet effect [F(4,148) = 3.3, p < 0.05], and a time x genotype effect [F(4,148) = 2.6, p < 0.05]. Post-hoc analysis determined KO-KD had significantly higher blood glucose compared with WT-CD and KO-CD (p <
0.01) at 30 min; see Fig. 6a. At PND 69, there was a diet effect \[ F(1,37) = 4.9, p < 0.05 \] and genotype effect \[ F(1,37) = 5.5, p < 0.05 \]. Post-hoc analysis determined KO-KD had significantly higher blood glucose compared with WT-CD \( p < 0.005 \) at 15 min and KO-CD \( p < 0.05 \) at 30 min; see Fig. 6b. Blood glucose (AUC) concentrations showed a significant diet effect \[ F(1,37) = 5.1, p < 0.05 \], genotype effect \[ F(1,37) = 4.2, p < 0.05 \], and time effect \[ F(1, 37) = 4.5, p < 0.05 \]; data not shown. This significant difference led us to complete a factorial ANOVA for PND 69, which showed a diet effect \[ F(1,37) = 4.5, p < 0.05 \] and genotype effect \[ F(1,37) = 4.7, p < 0.05 \]. A post-hoc test revealed KO-KD had significantly higher AUC blood glucose than all other groups at PND 69 \( p < 0.05 \); see Fig. 6c. Acute effects of the KD are still present at PND 69 in KO mice.

**Discussion**

This study utilized developing male \textit{En2} mice to determine metabolic changes resulting from a KD. We hypothesized that exposing \textit{En2} male mice to the KD from weaning (PND 21) to young adulthood (PND 60) would elevate low body weight and alter metabolic outputs compared to WT mice. As described in Chapter 3, \textit{En2} mice have increased baseline corticosterone, thus hemodynamic measurements were also determined. The results of the study showed that KO-CD had significantly lower body weight than KO-KD but there were no differences in body weight between KO-KD and WT-KD. Thus, the KD increased the low body weight of the \textit{En2} mice relative to WT-KD mice. In addition, a KD, high in fat and low in carbohydrates, can normalize body weight in \textit{En2} mice relative to WT mice. Body length, the measurement from mouse to anus, was also determined. WT-KD was significantly longer than KO-CD, but KO-KD was not significantly longer than KO-CD. Thus the KD may restore growth and development in KO mice. Our present findings indicate that the KD increased low weight
in En2−/− male mice relative to WT mice. Future experiments could examine these outcomes comparing a KD with a high-fat/high-carbohydrate diet in En2−/− male mice.

KO-CD at PND 84 had increased diastolic and mean arterial blood pressure compared with WT. These differences were not seen in KO-KD mice, suggesting that the KD may have had a protective effect for En2−/− mice. More studies are necessary to determine if En2−/− mice have hemodynamic differences relative to WT mice. On the other hand, despite attempts to acclimate mice to the CODA system, the restraint during the test may have induced a greater stress response in KO mice. Behavioral studies in Chapter 3 indicated that En2−/− mice had increased baseline corticosterone and when exposed to an acute restraint stress, animals had increased food intake post-restraint stress. This may be indicative of food-related stress reactivity or acute coping behavior. En2−/− mice have been shown to display deficits in social interactions as juveniles and adults, impaired learning, but no differences in stress-related behaviors. Brielmaier and colleagues (2012) measured stress-related behaviors by an elevated plus-maze and a light/dark exploration task, which cannot measure food-related stress reactivity or acute coping behaviors (Brielmaier et al., 2012). Thus, additional studies are necessary to determine if En2−/− mice have changes in hemodynamic measurements, that are impacted by stress.

To determine if changes in body mass were related to fat or lean mass, mice were placed in an EchoMRI and gross fat pad mass was weighed post-dissection. Due to the high fat content of the KD it was hypothesized that animals would have increased fat mass. Increased fat mass, specifically visceral fat, has been shown in other animal models fed the KD (Ribeiro et al., 2008; Srivastava et al., 2013), thus specific fat pad weights were determined in En2−/− mice. KD-fed mice, regardless of genotype, had increased retroperitoneal and epididymal fat compared with those fed CD. WT-KD had significantly increased subcutaneous fat pad mass compared with mice fed the CD.
Other studies have shown that mitochondrial oxidative phosphorylation proteins, uncoupling protein 1, and overall brown adipose tissue are increased in mice fed the KD (Srivastava et al., 2012; Srivastava et al., 2013). Future studies could analyze this possible sympathetic activation. In addition to analyzing fat mass, lean mass was determined by the EchoMRI system. Interestingly, we found that $En2^{-/-}$ mice have reductions in body weight due to reduced lean mass, not fat mass. This is intriguing since the KD was able to normalize lean mass in $En2^{-/-}$ mice to WT. It has been shown that the KD reduces fat levels without changing lean body mass, so it has been used as a diet for athletes (Paoli et al., 2012; Paoli et al., 2015). These data suggest that the KD could be beneficial for increasing body weight in ASD individuals without harming lean body mass.

To analyze further alterations in metabolic outputs and possible changes in locomotor activity due to deficits in lean body mass in $En2^{-/-}$ mice, animals were placed in an indirect calorimeter. All mice fed the KD had lower levels of $vO_2$ and $vCO_2$, compared with CD mice in both day and night time frames. RER levels closer to 1.0 are suggestive of increased metabolism of carbohydrates versus fat. Mice fed the KD trended to have RER closer to 0.7, indicative of enhanced fat metabolism. This suggests that the prior exposure to KD had effects on metabolism that continued after mice were switched to a standard chow diet. Despite this trend, biologically this was not significant and the KD did not have long-lasting effects on RER. In addition to respiratory measurements, the indirect calorimeter was able to determine locomotor activity.

Previously, $En2^{-/-}$ mice have been shown to have mild rotorod abnormalities (Brielmaier et al., 2012), which could suggest mild coordination problems. In the present study, we only observed locomotion and there were no differences between $En2^{-/-}$ mice fed the KD and CD, nor were there differences between $En2^{-/-}$ mice and WT-CD mice. However, for x-axis horizontal beam breaks, KO mice did have trending reduced activity compared to
WT, but the spike in WT-KD activity may be distorting the overall result significance. There are a number of reasons for this possible spike in locomotor activity in WT-KD including the novel environment, enhanced food seeking behaviors, and switch from group to individual housing. While it is acknowledged that all mice experienced these changes, nevertheless, there were no changes in locomotor activity as a result of KD exposure in En2\(^{-/-}\) mice.

Lastly, possible glucose impairments in En2\(^{-/-}\) mice were measured using an oral glucose challenge. Other studies have shown that the KD can result in glucose intolerance and metabolic syndrome (Feinman et al., 2003; Ribeiro et al., 2008; Ellenbroek et al., 2014). In addition, a recent study by Provenzano and colleagues (2014) determined that En2\(^{-/-}\) mice have abnormal levels of IGF-1 in the liver and hippocampus, suggesting En2 modulates IGF-1 activity during development. Due to the role of IGF-1 in glucose homeostasis, we hypothesized that En2\(^{-/-}\) mice would have abnormal glucose response to an oral glucose challenge. In our study, two days after dietary intervention and after being switched to standard chow, KO-KD demonstrated higher blood glucose compared with WT-CD and KO-CD at 15 min. At 30 min, KO-KD still had increased blood glucose compared with KO-CD. After a week on standard chow, KO-KD blood glucose had increased blood glucose compared with WT-CD at 15 min and KO-CD at 30 min. AUC of week 2 (PND 69) blood glucose revealed KO-KD had significantly higher AUC blood glucose than all other groups. From this it can be concluded that the KD does result in blood glucose impairments post-oral glucose challenge in En2\(^{-/-}\) mice. Interestingly, there were no differences in blood glucose between WT mice fed either the KD or CD after the glucose challenge. Thus, there were no changes in hemodynamic measurements nor blood glucose levels as a result of KD exposure during development in WT mice. Furthermore, En2\(^{-/-}\) mice may be sensitive to external factors, such as a KD, that can impact metabolic parameters and result in
impairment in glucose homeostasis. Future experiments looking at the lasting effects on glucose homeostasis are necessary to determine the consequences of KD in En2\textsuperscript{-/-} mice.

In conclusion, exposing En2\textsuperscript{-/-} mice to the KD from PND 21 to 60 normalized low body weight by increasing lean body mass, but also increased blood glucose response after an oral glucose challenge. Body weight changes are reversible, as En2\textsuperscript{-/-} mice fed the KD trended to decrease in body weight as they remained on standard chow. En2\textsuperscript{-/-} mice were found to have novel impairments including deficits in lean body mass and increased diastolic blood pressure. In an indirect calorimeter, mice previously fed the KD had much lower intake of standard chow (data not shown), thus future studies using a BioDaq system (Research Diets) are necessary to analyze real-time food consumption. Overall, this study determined KD effects on metabolic outcomes in En2\textsuperscript{-/-} mice. Studies beyond the scope of this thesis are needed to determine if observed metabolic changes are a result of En2 modulating GH and IGF-1 or by an unknown mechanism. The changes in blood glucose and blood pressure that persisted on the standard chow diet suggest that the KD may result in chronic changes in hemodynamic outputs and glucose homeostasis. The KD, as a dietary intervention for ASD, may improve low body weight and reduce stress-related parameters, but also result in impaired glucose homeostasis.
Literature Cited


Figure 1. Body weight of KO and WT mice from PND 21 to 60 during dietary exposure (KD or CD) and from PND 60 to 91 on standard chow. Body weight (g) are mean ± SEM. * indicates significance (p < 0.05) from KO-CD at PND 60.

Figure 2. Length of KO and WT mice (n = 4/group) at PND 62 after dietary exposure (KD or CD) from PND 21-60. Length (cm) is mean ± SEM. Same letter indicates significant difference (A, p < 0.05) from KO-CD.

Figure 3. EchoMRI of body composition (fat and lean mass) in KO and WT mice (n = 32/group) with different dietary exposures (KD or CD) at PND 60. Average body composition measurements are mean ± SEM. a. Fat mass as determined by EchoMRI. *, # indicates significance (p < 0.05) from all other groups. b. Lean mass as determined by EchoMRI. * indicates significance (p < 0.05) all other groups. Same letter indicates significance (A, p < 0.05) from WT-CD.

Figure 4. Gross fat pad weights in KO and WT mice (n = 20/group) with different dietary exposures (KD or CD) at PND 60. Average fat pad mass is mean ± SEM. a. Gross subcutaneous fat pad mass. Same letter indicates significance (A,B; p < 0.001) from CD fed mice. b. Gross retroperitoneal fat pad mass. Same letter indicates significance (A,B; p < 0.0001) from KO-KD. Same letter indicates significance (C,D; p < 0.0001) from WT-KD. c. Gross epididymal fat pad mass. Same letter indicates significance (A,B; p < 0.01) from KO-KD. Same letter indicates significance (C,D; p < 0.01) from WT-KD.

Figure 5. Average metabolic outputs in an indirect calorimeter (oxymax/CLAMS) in KO
and WT mice with different dietary exposures (KD or CD). At PND 60, mice (n = 12/group) were individually housed in an indirect calorimeter for 48-h with access to standard chow to determine metabolic activity. The last 24 h was analyzed for night versus day vO2, vCO2 heat, RER, and locomotor activity. Average measurements are mean ± SEM. a. Average vO2 for 24 h. Same letter indicates significance (A,B; p < 0.05) from KD mice during the day. Same letter indicates significance (C,D; p < 0.05) from KD fed mice at night. # indicates significance (p < 0.0001) between day and night. b. Average vCO2 for 24 h. Same letter indicates significance (A,B; p < 0.05) from KD fed mice. # indicates significance (p < 0.05) of all groups from WT-CD at night. # indicates significance (p < 0.0001) between day and night. c. Average respiratory exchange ratio (RER) for 24 h. d. Average x total activity (x-axis) for 24 h. * indicates significance (p < 0.001) from WT-KD. # indicates significance (p < 0.0001) between day and night. e. Average z total activity (z-axis) for 24 h. * indicates significance (p < 0.001) from WT-KD. # indicates significance (p < 0.0001) between day and night.

Figure 6. Blood glucose as a result of an OGTT. KO and WT mice (n = 10/group) with prior exposure to KD or CD were food restricted for 6-h and a baseline (0-min) blood glucose measurement was obtained. Mice were gavaged with an oral bolus of glucose (2 mg/kg) and blood glucose was measured at 15, 30, 60, and 120 min. Average measurements are mean ± SEM. a. Blood glucose response to an oral glucose challenge at PND 62. # indicates significance (p < 0.05) between KO-KD and CD fed mice at 15 min. $ indicates significance (p < 0.01) between KO-KD and KO-CD at 30 min. b. Blood glucose response to an oral glucose challenge at PND 69. # indicates significance (p < 0.01) between KO-KD and WT-KD at 15 min. $ indicates significance (p < 0.05) between KO-KD and KO-CD at 30 min. c. AUC of PND 69 blood glucose response to an oral glucose challenge. * indicates significance (p < 0.05) from KO-KD.
Figures

Fig 1.

Ketogenic or Control Diet

Standard Chow

Body Weight (g)

PND

KO-KD (n=16)
KO-CD (n=14)
WT-KD (n=13)
WT-CD (n=13)

Fig 2.

Length (cm)

KO-KD
KO-CD
WT-KD
WT-CD

Groups (n = 4/group)
Fig 4a.

Subcutaneous Fat Pad Weight (g)

KO-KD  KO-CD  WT-KD  WT-CD

A, B  A  B

Fig 4b.

Retroperitoneal Fat Pad Weight (g)

KO-KD  KO-CD  WT-KD  WT-CD

A, B  A, C  C, D  B, D
Fig 4c.

![Graph showing Epididymal Fat Pad Weight (g) for KO-KD, KO-CD, WT-KD, and WT-CD groups.]

Fig 5a.

![Graph showing VO2 (ml/kg/h) for KO-KD, KO-CD, WT-KD, and WT-CD groups during Day and Night.]

- KO-KD
- KO-CD
- WT-KD
- WT-CD

Note: Bars with different letters indicate significant differences.
Fig 6a.

PND 62 Blood Glucose (mg/dl)

Time (min)

KO-KD
KO-CD
WT-KD
WT-CD

Fig 6b.

PND 69 Blood Glucose (mg/dl)

Time (min)
Fig 6c.

AUC Blood Glucose at PND 69

KO-KD  KO-CD  WT-KD  WT-CD
### Table 1. Hemodynamic measurements after experimental diets.

<table>
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<tr>
<th></th>
<th>KO-KD (n=16)</th>
<th>KO-CD (n=14)</th>
<th>WT-KD (n=13)</th>
<th>WT-CD (n=13)</th>
<th>Diet Effect (p value)</th>
<th>Genotype Effect (p value)</th>
<th>Diet x Genotype Effect (p value)</th>
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</thead>
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<tr>
<td>Systolic BP, mmHg</td>
<td>115.6±4.9</td>
<td>112.7±6.1</td>
<td>106.3±4.8</td>
<td>104.9±7.3</td>
<td>0.622</td>
<td>0.022*</td>
<td>0.462</td>
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<tr>
<td>Diastolic BP, mmHg</td>
<td>78.3±4.2</td>
<td>93.8±6.2</td>
<td>73.2±4.6</td>
<td>85.4±5.7</td>
<td>0.011*</td>
<td>0.199</td>
<td>0.761</td>
</tr>
<tr>
<td>Mean Arterial BP, mmHg</td>
<td>90.5±4.4</td>
<td>101.4±4.7</td>
<td>83.2±3.7</td>
<td>88.9±4.6</td>
<td>0.066</td>
<td>0.03*</td>
<td>0.558</td>
</tr>
<tr>
<td>Heat Rate, bpm</td>
<td>303.3±17.0</td>
<td>345±49.4</td>
<td>371.5±36.4</td>
<td>407.2±63.2</td>
<td>0.365</td>
<td>0.131</td>
<td>0.944</td>
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</tbody>
</table>

Values are means ± SEM. Data was analyzed with a factorial ANOVA with post-hoc Newman-Keuls test. Same letter indicates significant difference between groups and * indicates overall significance.
CHAPTER FIVE: **Ketogenic diet fed post-weaning to young adulthood increased c-Fos immunoreactivity in brain regions critical to autistic-like behaviors in *EN2* null mice.**

ABSTRACT

Autism spectrum disorder (ASD) is a neural developmental disorder in which individuals have deficits in social behavior, repetitive/restricted behaviors, and abnormal communication. The ketogenic diet (KD) has been shown to restore social interaction in animal models displaying autistic-like behaviors. The following studies used *Engrailed-2* (*En2*) knockout (KO) and wild-type (WT) male mice fed either KD (80% fat, 0.1% carbohydrates) or control diet (CD; 10% fat, 70% carbohydrates) to determine whether a KD fed from weaning at postnatal day (PND) 21 to adulthood (PND 60) would alter immunoreactivity of neural c-Fos, an early immediate gene, in forebrain regions of mice following exposure to a stranger mouse or a novel object in a three-chamber social test. KO-KD mice displayed increased c-Fos immunoreactivity in the cingulate cortex (+67.2%) and septal region (+54.0%) compared with KO-CD when exposed to a stranger mouse. When exposed to a novel object, KO-KD had normalization of c-Fos immunoreactivity compared to WT mice, but KO-CD demonstrated an increase in the anterior bed nucleus of the stria terminalis (BNST, +38.4%) and paraventricular nucleus (PVN, +46.5%) compared to KO-KD. Thus, a KD may alter neural response in forebrain regions, specifically the cingulate cortex and septal region, to restore social behaviors in *En2* null mice.
Introduction

The neural developmental disorder, autism spectrum disorder (ASD), involves alterations in several brain regions critical for social behaviors, communication, language, and sensory processing (American Psychiatric Association 2013). These symptoms typically coincide with varying levels of cognitive impairment estimated to impact 80-90% of the ASD population (Steffenburg 1991). Thought to be a result of abnormal neural development, such as improper synaptic pruning (Müller et al., 2011; Saugstad 2011), analysis of post-mortem human brain tissue has begun to elucidate structural anomalies in the cerebellum (Courchesne et al., 1988), amygdala-hippocampal complex (Abell et al., 1999), front-temporal regions (Mcalonan et al., 2005), and the caudate nuclei (Bauman et al., 2005; Carper et al., 2005; Langen et al., 2012) of ASD individuals. Structural impairments in the brain can result in a wide variety of alterations in function (some compensation may take place), thus more research is necessary to integrate clinical imaging findings with neuropsychological and molecular genetic research to determine a possible mechanism involved in ASD.

Several brain regions have been implicated in autistic-like behaviors through studies in rodent models and by functional neural imaging in humans (Courchesne et al., 1988; Adolphs 2001; Courchesne et al., 2005; Waiter et al., 2005; Atmaca et al., 2007; Kuemerle et al., 2007; Groen et al., 2010; Kumar et al., 2012; Pobbe et al., 2012; Sgado et al., 2013; Provenzano et al., 2014). Regions implicated in social behaviors and emotional response include the medial prefrontal cortex, superior temporal cortex, cingulate cortex, parietal cortex, fusiform gyrus, and the amygdala (Brothers et al., 1990; Adolphs 2001; Langen et al., 2012). For instance, boys with autism have a 13-16% abnormal enlargement of the amygdala, which may explain the severe anxiety and mood disturbances found in ASD. Due to the importance of the amygdala in the regulation of
emotion, this abnormality may lead to social impairments through inability to control emotions and react appropriately to stimuli (Sparks et al., 2002; Schumann et al., 2004; Schumann et al., 2009; Groen et al., 2010). In addition, impairments in these forebrain regions may affect executive functions and result in the cognitive inflexibility and repetitive limited behaviors found in ASD (Dziobek et al., 2011). These repetitive behaviors share similarities to individuals with obsessive-compulsive disorder, in which behaviors may result from dysfunctional neural signaling in the orbitofrontal cortex, cingulate cortex, and thalamus (Whiteside et al., 2004). Comorbidities with various other psychological disorders, including anxiety, depression, impulsivity, and hyperactivity have resulted in pharmaceutical therapies that have shown to treat these disorders and alleviate some autistic-related symptoms (Gordon et al., 1993; Hollander et al., 2000; Wynn et al., 2009; Dove et al., 2012; Doyle et al., 2012; Hurwitz et al., 2012; Dinnissen et al., 2015).

Interestingly, epilepsy is a common comorbidity in ASD and both share similar dysfunction in the cerebral cortex, amygdala, cerebellum, and hippocampal regions (Jones et al., 2015). Drug-resistant pediatric epilepsy has been effectively treated using a dietary intervention, the ketogenic diet (KD) (Peterman 1925; Huttenlocher 1976; Lefevre et al., 2000; Bough et al., 2007; Wheless 2008). In rodent models, it has been shown that the KD acts directly on neural transmission through norepinephrine and although an exact mechanism is unknown, studies have shown that the noradrenergic system is required the anticonvulsant KD effect (Szot et al., 2001; Weinshenker et al., 2001). These studies and others (Yudkoff et al., 2007; Abdelwahab et al., 2010; Deng-Bryant et al., 2011; Di Lorenzo et al., 2015) demonstrate direct effects of the KD on central neural signaling and processes, but the KD may also have implications for peripheral signaling. Ruskin and colleagues (2009), have shown that the KD significantly
Reduced pain and inflammation in rats, which are peripheral signaling pathways. Reduced pain and inflammation are amplified in adolescent rats fed the KD (Ruskin et al., 2009), providing justification for the use of a dietary intervention during early development. Due to the comorbidity of epilepsy in autism, common pathways may be affected in both disorders. Thus, a KD could result in improvements in symptoms in certain ASD populations, since a KD has demonstrated benefits in treating epilepsy (Dicicco-Bloom et al., 2006; Chavez et al., 2007). Spilioti and colleagues (2013) fed a KD to ASD children with elevated levels of β-hydroxybutyrate post-glucose challenge and demonstrated improvements in autistic-related behaviors. Despite being on a very small scale, this study is the first to determine efficacy of a KD treatment for ASD individuals with possible metabolic impairments (Spilioti et al., 2013). It is still unknown how the KD is influencing common brain regions and ultimately changing neural signaling and autistic-like behaviors.

Mutations in En2 result in abnormal forebrain and hindbrain neural signaling, due to improper developmental of the locus coeruleus, raphe nuclei, and monoaminergic brainstem nuclei (Loomis et al., 1996; Hanks et al., 1998; Alberi et al., 2004; Simon et al., 2005; Gherbassi et al., 2006; Sgado et al., 2006; Rossman et al., 2014). Similar to ASD patients, En2 null (En2−/−) mice have abnormalities in the amygdala, which has been reported as an anterior shift of the position of the amygdala in the cerebral cortex (Kuemerle et al., 2007), hypoplasia of cerebellar vermal lobules (Courchesne et al., 1988), and reductions in Purkinje cell number in the cerebellum (Bauman et al., 1985; Kemper et al., 1993; Kuemerle et al., 1997; Dicicco-Bloom et al., 2006). Irregular neural structures and signaling may result in the autistic-like behaviors found in the En2−/− mouse, including impaired social interaction, deficits in fear conditioning, and decreased play behaviors (Simon et al., 2005; Cheh et al., 2006; Brielmaier et al., 2012; Brielmaier
et al., 2014). Despite these descriptive studies, the exact mechanism that results in autistic-like behaviors in the En2−/− mouse remains unknown. As demonstrated in previous chapters, the KD alters metabolism and social behaviors in En2−/− mouse, but this does not result from changes in regional biogenic amines. To begin to elucidate what neural mechanisms are involved in changes in autistic-related behaviors, c-Fos immunoreactivity will be determined by immunohistochemical staining in forebrain regions critical to social behavior. The immediate early gene, c-Fos, is activated by a number of stimuli, including cytokines, UV radiation, psychological drugs, and mating (Sagar et al., 1988; Manzo et al., 2008; De Santana et al., 2015). In this study, c-Fos expression was determined in certain brain regions of mice following exposure to a stranger animal in the 3-chambered social test. As previously shown (Avale et al., 2011), exposure to a novel mouse can elicit expression of the immediate-early gene, c-Fos. This indirect marker of neural activity was used to determine which brain regions are critical for social behaviors and if the KD mediates neural activation in En2 mice.

In the subsequent study, we utilized KO (En2−/−) and WT (En2+/+) male mice fed the KD or control diet (CD) from postnatal day (PND) 21 to 60 to elucidate changes in neural reactivity in mice exposed to a stranger mouse or novel object in a three-chamber social test. Our studies (Chapter 3) and work of others (Ruskin et al., 2013) have shown that the KD can improve social behaviors in a mouse model displaying autistic-like behaviors, but the extent to which the KD is altering neural mechanisms is unknown. To attempt to understand these changes in forebrain regions, reactivity of c-Fos was determined by immunohistochemical staining in the En2 mouse exposed to a stranger mouse or a novel object for 10 min. We hypothesized that social improvements in En2−/− mice would be accompanied by increased c-Fos immunoreactivity in forebrain regions, which would be absent in mice exposed to a novel object.
Materials and Methods

Animals and Genotyping.

*En*2<sup>−/−</sup> mice were maintained by heterozygous breeding pairs and group housed for experimental procedures, as described in Chapter 2.

Experimental diet.

To determine the effects of the KD on behavior, stress, and metabolism, a total of 40 *Engrailed*-2 KO and WT mice were *ab libitum* fed either a control or lard based ketogenic diet, as described in Chapter 3.

Social behaviors in *En*2 mice with diet exposures.

At PND 62, *En*2<sup>−/−</sup> (KO, n = 12 total) and *En*2<sup>+/+</sup> (WT, n = 12 total) mice fed either KD or CD, were placed in a three-chamber social paradigm (Stoelting Co, Wood Dale, Illinois, USA) and c-Fos expression was determined in certain brain regions following exposure to a stranger mouse. Effects of the KD on social behavior was analyzed similar to Chapter 3 (Avale *et al.*, 2011; Brielmaier *et al.*, 2012). Two days post-dietary intervention was chosen to eliminate confounding factors associated with acute ketogenic effects, since ketone body levels are normalized to CD fed mice during this period, as determined in Chapter 2. During two 10-min recorded phases the following was quantified: time spent in each chamber, frontal contact (time spent with experimental and stranger mice), and self-grooming. In phase 1, all mice had ten min to explore and acclimate to all three chambers. In phase 2, a stranger mouse (adult male *En*2<sup>−/−</sup> non-litter mate) was placed in a wire cage in one of the side chambers. The free mouse could choose to spend time with the mouse or explore the chamber for ten minutes. It is important to note that the stimulus (mouse) switched side chambers randomly confounding side preference and an empty wire cage was always present.
opposite of the stimulus. Also, the three-chamber apparatus was cleaned between each test with disinfectant (Labsan 120). In Chapter 3, it was determined that only one interaction phase with a stranger mouse was necessary to determine changes in frontal contact, thus c-Fos positive cell counts were determined after only one 10-min exposure to a stranger mouse. All behavior was 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.

Novel object exploratory behaviors in En2 mice with diet exposures.

At PND 62, a separate set of En2<sup>-/-</sup> (KO, n = 8 total) and En2<sup>+/+</sup> (WT, n = 8 total) fed either KD or CD, were placed in a three-chamber social test (Stoelting Co, 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 stranger 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 stranger 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. It is important to note that the stimulus (novel object) switched side chambers randomly confounding side preference and an empty wire cage was always present opposite of the stimulus. Also, the three-chamber apparatus was cleaned between each test with disinfectant (Labsan 120). All behavior was 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.
Neural activation in forebrain regions in En2 mice with diet exposures.

After the 20 min social exposure test, mice were returned to home cages for 90 min to allow for synthesis of the c-Fos protein in the nuclei of activated neurons (Bisler et al., 2002). Mice were sacrificed with 0.1% Euthasol (pentobarbital sodium and phenytoin sodium) solution intraperitoneal (IP) and perfused through the heart with 100 ml of phosphate buffered saline (PBS) then with 60 ml 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 ice-cold precision cryostat (Leica Microsystems, Rijswijk, The Netherlands). Free-floating sections in a 24-well plate were stored in Cryoprotectant until immunohistochemistry was performed. Unless otherwise noted, sections were placed on a shaker for all wash and incubation steps at room temperature. 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% H₂O₂ in H₂O. 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 3 x 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 3 x 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 3 x 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 30 sec to stain Fos-like products black. PBS was added immediately after desired stain was reached and sections were washed 3 x 10 min in PBS to halt the Ni-DAB reaction. After the last wash, 0.1% sodium azide in PBS was added to each well and plates were stored in a 4⁰ refrigerator. 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 (Bello et al., 2013).

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

Imaging was performed using a Olympus FSX-BSW and FSX100 software (Olympus videoscope, Tokyo, Japan). Quantification was performed automatically by identifying c-Fos positive black nuclei using Image J software system (NIH, Bethesda, MD) image analysis software (Bello et al., 2013). Three anatomically matched tissue slices of each region (unilateral) of each mouse was used in data analysis. Cells were counted by two blinded observers.

A total of 15 forebrain regions were analyzed and quantified, but statistical significance was only found in four regions. Regions of interest chosen for c-Fos quantification, in which statistical significance was found, were the anterior cingulate cortex (1.42, 1.10, 0.86 mm Bregma), septal region (0.86 mm Bregma), anterior bed nucleus of the stria terminalis (BNST; 0.26, 0.14, 0.02 Bregma), paraventricular nucleus (PVN; -1.06, -1.22 mm Bregma) (Franklin 2008). These regions are involved in feeding behavior, stress responses, and social behaviors. Regions that did not achieve statistical significance included the piriform cortex, anterior hypothalamus, ventral medial hypothalamus, dorsal medial hypothalamus, lateral hypothalamus, peripheral
hypothalamus, dentate gyrus, pre-frontal cortex, nucleus accumbens, amygdala, and arcuate nucleus of the hypothalamus.

Statistical Analysis.

KO and WT were compared for each statistical analysis. Behavioral measurements in the three-chambered social test and c-Fos positive cell counts were analyzed with a factorial analysis of variance (ANOVA). Post-hoc comparisons were made, when appropriate, with a Fisher LSD test. All statistical analyses were performed using Statistica 7.1 software (StatSoft, Tulsa, OK, USA) and significance was set at $\alpha = 0.05$.

Results

En2−/− mice spent significantly more time with a novel object.

Two days after the switch to standard chow, at PND 62, mice were placed in a three-chamber test exposed to either a stranger mouse ($n = 6$/group) or a novel object ($n = 4$/group) prior to analysis for c-Fos positive nuclei. For frontal contact with a stranger mouse, there was a significant diet effect [$F(1, 20) = 8.4, p < 0.01$]. KO-KD had a trending increase in frontal contact, but it did not reach statistical significance as in Chapter 3. There was a phase effect [$F(1, 43) = 6.4, p < 0.05$], phase x diet effect [$F(1, 43) = 12.4, p < 0.01$], and genotype x diet [$F(1, 43) = 4.3, p < 0.05$] for the chamber that was empty in phase 1 then contained a stranger mouse in phase 2. There was a trend, which showed that KO-KD spent significantly more time in the chamber with the stranger mouse compared to KO-CD; see Fig 1a-b. For novel object contact, there was a diet effect [$F(1, 12) = 11.4, p < 0.01$], whereby KO-KD spent little time with the novel object, similar to WT mice, but KO-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 then contained a novel object in phase 2, there was a phase effect \( F(1, 24) = 29.2, p<0.0001 \). KO-KD interaction with the novel object was similar to WT mice. KO-CD spent more time in this chamber compared with all other groups \( (p < 0.01) \); see Fig 2b. To determine whether mice spent more time with the novel object, which had previously been housed with stranger male mice, or any novel object, i.e. wire cage, the time spent with the novel object versus the wire cage was determined. There was a significant diet effect \( F(1, 12) = 15.5, p < 0.01 \). Post-hoc analysis determined that interaction with the novel object versus the wire cage was normalized in KO-KD compared to WT mice, but KO-CD spent more time with the novel object versus the wire cage compared with KO-KD \( (p < 0.05) \); Fig 2c. KO mice have reduced frontal contact with a stranger mouse, but increased time spent with a novel object.

Alterations in neural activation in forebrain regions of En2\(^{−}\)/ mice.

After exposure to a stranger mouse or a novel object, all mice were perfused for c-Fos immunoreactivity in forebrain regions, including the cingulate cortex, septal region, BNST, and PVN. In the cingulate cortex of animals exposed to a stranger mouse, there was a significant diet effect \( F(1, 20) = 18.5, p < 0.0001 \). Post-hoc analysis determined KD exposure increased the number of immunopositive cells in KO and WT mice compared with both KO-CD \( (p < 0.01) \) and WT-CD \( (p < 0.05) \); see Fig 3a-b. No significant differences were found in the cingulate cortex with novel object exposure; see Fig 3c-d. In the septal region of mice exposed to a stranger mouse, there was a significant diet effect \( F(1, 20) = 17.2, p < 0.0001 \). Post-hoc analysis determined KD exposure increased the number of immunopositive cells in KO mice compared with KO-CD \( (p < 0.05) \) and in WT mice compared with both KO-CD \( (p < 0.0001) \) and WT-CD \( (p < 0.01) \); see Fig 4a-b. No significant differences were found in the septal region with novel
object exposure; see Fig 4c-d. A KD increased neural activation of c-Fos in KO mice exposed to a stranger mouse in the cingulate cortex and septal region, which are regions critical to social behavior.

In the BNST of mice exposed to a stranger mouse, there was a significant diet effect \[F(1, 20) = 6.5, p < 0.05\] and KD exposure increased the number of immunopositive cells in WT mice compared with WT-CD \(p < 0.01\); see Fig 5a-b. Novel object exposure demonstrated a significant genotype x diet effect \[F(1, 12) = 5.6, p < 0.05]\] in the BNST. KO-CD had significantly increased number of immunopositive cells compared with KO-KD and WT-CD \(p < 0.05\); see Fig 5c-d. In the PVN of mice exposed to a stranger mouse, there was a significant diet effect \[F(1, 20) = 12.8, p < 0.05\] and KD exposure increased the number of immunopositive cells in KO and WT mice compared with WT-CD \(p < 0.05\); see Fig 6a-b. Novel object exposure resulted in a genotype x diet effect \[F(1, 12) = 26.5, p < 0.0001\] in the PVN. KO-CD had a significantly increased number of immunopositive cells compared with all other groups \(p < 0.05\); see Fig 6c-d. Exposure to a novel object increased neural activation in the PVN and BNST, both regions implicated in stress-reactivity, of KO-CD mice.

**Discussion**

This study used adult \(En2^{-/-}\) mice to determine changes in neural c-Fos response resulting from dietary exposure. We hypothesized that exposing \(En2^{-/-}\) male mice to the KD from weaning (PND 21) to adulthood (PND 60) would differentially alter neural activation in response to a stranger male mouse. Indeed, increased c-Fos immunoreactivity was found in the cingulate cortex, septal region, and PVN of both KO and WT mice fed the KD. In WT mice, KD exposure increased c-Fos immunoreactivity in the BNST. In response to a novel object, increased c-Fos immunoreactivity was found in
KO-CD in the BNST and PVN. Interestingly, KO-KD had unique increased c-Fos immunoreactivity in the septal region and cingulate cortex, which may be indicative of changes in autistic-like behaviors in this mouse model, as a result of a dietary intervention, which was previously described in Chapter 3. Since genotype effects were not found, it was assumed that KO mice do not have deficient cell numbers that could impact c-Fos activation in the described regions. Thus, effects from a KD were assumed to be an increase in expression, not overall cell number. Additional studies are necessary to prove this hypothesis. These results show that the KD fed from PND 21 to 60 can enhance neural response in forebrain regions of En2 mice exposed to a stranger mouse.

The main goal of this study was to further elucidate the findings in Chapter 3, which demonstrated increased frontal contact in En2−/− male mice fed a KD from PND 21-60. Behavior was reanalyzed in this separate group of mice to confirm behaviors found in Chapter 3 and to determine possible changes in time spent with novel object prior to staining for c-Fos immunoreactivity. Since the main endpoint of this study was to determine changes in c-Fos immunoreactivity, animal numbers were powered for c-Fos analysis. KO-KD did not display normalized frontal contact compared with KO-CD, as found in Chapter 3, since animal numbers were not powered for behavioral analysis. This study hoped to determine if KD exposure resulted in altered neural c-Fos response in brain regions associated with social behaviors. As a result of exposure to a stranger mouse in the social behavior test, c-Fos immunoreactivity was normalized in the cingulate cortex, septal region, and PVN of KO-KD mice compared with KO-CD. These regions have been implicated in autistic-like behaviors for their roles in repetitive behaviors, stress reactivity, and energy homeostasis (Courchesne 1997; Adolphs 2001; Welch et al., 2003; Cascio et al., 2014). Thus, the KD restored neural reactivity in
regions critical to autistic-like behaviors in En2^-/- male. On the other hand, in WT mice, the KD increased c-Fos immunoreactivity in the cingulate cortex, septal region, BNST, and PVN. It is unknown whether changes in these regions are due to the high-fat content of the diet, changes in body weight, or critical to the social behaviors we observed in the three-chambered social test. For example, animals exposed to a high-fat diet (Cifani et al., 2012) or a binge-eating schedule (Bello et al., 2014) composed of sweetened fat display increased activation in the PFC. Thus, a combination of dietary fats and increased body weight could have altered neural reactivity in the PVN, which is involved in the homeostatic control of feeding and body weight (Stricker-Krongrad et al., 1996; Cowley et al., 2001; Bello et al., 2013). Since norepinephrine was altered in the hypothalamus of WT-KD mice (see Chapter 2), which was not found in KO-KD, increased c-Fos immunoreactivity may be a result of the high-fat component of the KD and not critical to social behaviors. Additional studies are needed to elucidate changes resulting from dietary fat versus ketone body production. Also, future studies can determine the role of the cingulate cortex and septal region in autistic-like behaviors in En2^-/- male mice.

In addition, a separate group of mice were exposed to a novel object to serve as a control for the stranger mouse exposure paradigm. Surprisingly, KO-CD displayed increased time with a novel object, which was significant from time spent with the wire cage. Interestingly, KO-CD displayed increased c-Fos immunoreactivity in the BNST and PVN. These regions did not have increased c-Fos immunoreactivity in KO-CD when mice were exposed to a stranger mouse. Thus, this increased engagement with a novel object is interesting and perhaps less threatening to interact with in comparison to a stranger male mouse. All other groups did not spend more time with the novel object than the wire cage and had little c-Fos immunoreactivity in brain regions, post-exposure.
This suggests that novelty behaviors in KO mice were normalized by feeding a KD. Perhaps the novel object elicited a stress-response in KO-CD, since the object had been exposed to unfamiliar male mice, thus activating c-Fos in regions pertaining to the hypothalamic-pituitary-adrenal (HPA) axis (Lopez et al., 1999; Nieuwenhuizen et al., 2008). Previously, we showed that KO-CD had increased baseline corticosterone levels, see Chapter 3, which could explain enhanced c-Fos immunoreactivity in the BNST and PVN, HPA regions, but a lack of increased immunoreactivity when mice were placed with a stranger mouse is perplexing. Perhaps avoiding the stranger mouse and spending time in the two empty chambers acted as a coping mechanism for these mice. To analyze these possible baseline differences, mice could be placed in a single chamber and forced to be in closer proximity to the stranger mouse prior to analyzing for c-Fos immunoreactivity. Time spent exploring the chambers could affect c-Fos immunoreactivity, thus more detailed behavioral studies are necessary.

This study determined that increased frontal contact with a stranger mouse in the three-chamber test enhanced c-Fos immunoreactivity in limbic areas, including the cingulate cortex, septal region, and PVN of $En2^{-/-}$ mice exposed to the KD compared to KO-CD. When this stimulus was switched to a novel object, $En2^{-/-}$ mice fed a control diet displayed increased c-Fos immunoreactivity in the BNST and PVN. Activation of the PVN was consistent in both KO-KD and KO-CD, which may have initiated the coping behavior found in Chapter 3 post-restraint stress chow intake. The cingulate cortex and septal region were unique regions activated in KO-KD mice, suggesting they may be critical for autistic-like behaviors. Since there were no changes in c-Fos immunoreactivity in WT-CD, the increased neural activation in WT-KD mice in the cingulate cortex, septal region, BNST and PVN, suggest a dietary effect. This study not only determined that the KD can exert an influence on neural activation in forebrain regions, but elucidated two
neural regions, the cingulate cortex and septal region, that may be influenced by a
dietary intervention to enhance social behaviors in \( En2^- \) mice.
Literature Cited


Figure Captions

Figure 1. Social behaviors in a three-chamber social interaction test at PND 62 when exposed to a stranger mouse. Two days after switching from experimental diets (KD or CD) to standard chow, mice were exposed to a three-chamber social test. KO-KD, KO-CD, WT-KD, and WT-CD (n = 6/group) were placed in the three-chamber test for a total of 20 min with two 10-min phases with or without adult male En2^{+/−} non-litter mates. Average times are mean ± SEM. a. Average total time (20 min) engaging in frontal contact with the adult male En2^{+/−} non-litter mates. b. Average total time (10 min per phase) spent in chamber that had an adult male En2^{+/−} non-litter mate during phase 2. During phase 1 this chamber was empty. * indicates significant difference from all in phase 1 (p < 0.05).

Figure 2. Behavior in a three-chamber apparatus at PND 62 when exposed to a novel object. 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 (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 stranger male mice. Average times are mean ± SEM. a. Average total time (20 min) engaging in contact with a novel object. * indicates significant difference from all (p < 0.05). b. Average total time (10 min per phase) spent in chamber that has a novel object in phase 2. * indicates significant difference from all (p < 0.0001). c. Ratio of the average time spent with the novel object versus the wire cage. Same letter indicates significant difference of KO-CD from KO-KD (A, p < 0.05).
Figure 3. Average immunoreactive c-Fos counts in the cingulate cortex of KO and WT mice with different dietary treatments (KD or CD) as a result of exposure to a stranger mouse (n = 6/group) or a novel object (n = 4/group). Counts are mean ± SEM. a. Exposure to a stranger mouse. Same letter indicates significant difference of KO-KD from KO-CD (A, p < 0.01) and WT-CD (B, p < 0.05). Same letter indicates significant difference of WT-KD from KO-CD (C, p < 0.01) and WT-CD (D, p < 0.01). b. Exposure to a novel object. There were no significant differences between groups in this region. c-d. Representative cingulate cortex micrographs of c-Fos (black) staining of mice exposed to a stranger mouse (c) and a novel object (d) in a three-chamber apparatus. Sections are 0.86 mm from Bregma. Scale bars are 153 μm.

Figure 4. Average immunoreactive c-Fos counts in the septal region of KO and WT mice with different dietary treatments (KD or CD) as a result of exposure to a stranger mouse (n = 6/group) or a novel object (n = 4/group). Counts are mean ± SEM. a. Exposure to a stranger mouse. Same letter indicates significant difference of KO-KD from KO-CD (A, p < 0.05). Same letter indicates significant difference of WT-KD from KO-CD (B, p < 0.05) and WT-CD (C, p < 0.01). c. Exposure to a novel object. There were no significant differences between groups in this region. Representative septal region micrographs of c-Fos (black) staining of mice exposed to a stranger mouse (b) and a novel object (d) in a three-chamber apparatus. Sections are 0.86 mm from Bregma. Scale bars are 153 μm.

Figure 5. Average immunoreactive c-Fos counts in the BNST of KO and WT mice with different dietary treatments (KD or CD) as a result of exposure to a stranger mouse (n = 6/group) or a novel object (n = 4/group). Counts are mean ± SEM. a. Exposure to a
stranger mouse. Same letter indicates significant difference of WT-KD from WT-CD (A, \( p < 0.01 \)). c. Exposure to a novel object. Same letter indicates significant difference of KO-CD from KO-KD (A, \( p < 0.05 \)) and WT-CD (B, \( p < 0.05 \)). Representative BNST micrographs of c-Fos (black) staining of mice exposed to a stranger mouse (b) and a novel object (d) in a three-chamber apparatus. Sections are 0.14 mm from Bregma. Scale bars are 153 μm.

Figure 6. Average immunoreactive c-Fos counts in the PVN of KO and WT mice with different dietary treatments (KD or CD) as a result of exposure to a stranger mouse (n = 6/group) or a novel object (n = 4/group). Counts are mean ± SEM. a. Exposure to a stranger mouse. Same letter indicates significant difference of WT-CD from KO-KD (A, \( p < 0.05 \)) and WT-KD (B, \( p < 0.05 \)). c. Exposure to a novel object. * indicates significant difference from all groups (\( p < 0.05 \)). Representative PVN micrographs of c-Fos (black) staining of mice exposed to a stranger mouse (b) and a novel object (d) in a three-chamber apparatus. Sections are -1.06 mm from Bregma. Scale bars are 153 μm.
Figures

Fig 1a-1b.

Fig 2a-2b.

Fig 2c.
Fig 3a.

![Graph showing c-Fos positive counts in different groups.]

Fig 3b.

![Images showing tissue sections labeled KO-KD, KO-CD, WT-KD, and WT-CD.]

**Stranger Mouse**
Fig 3c.

![Bar chart showing Cingulate Cortex c-Fos positive (counts) for KO-KD, KO-CD, WT-KD, and WT-CD.](image)

Fig 3d.

![Immunohistochemistry images for KO-KD, KO-CD, WT-KD, and WT-CD.](image)
Fig 4a.

![Bar chart showing Stranger Mouse data](chart1.png)

- KO-KD
- KO-CD
- WT-KD
- WT-CD

Fig 4b.

![Images showing Stranger Mouse](images1.png)
Fig 4c.

Novel Object

Septal Region c-Fos positive (counts)

KO-KD  |  KO-CD  |  WT-KD  |  WT-CD

Fig 4d.

Novel Object

KO-KD  |  KO-CD  |  WT-KD  |  WT-CD
Fig 5a.

![Graph showing c-Fos positive counts for Stranger Mouse.

KO-KD > KO-CD > WT-KD = WT-CD

Fig 5b.

![Images of Stranger Mouse brain regions.

KO-KD, KO-CD, WT-KD, WT-CD]
Fig 6a.

![Stranger Mouse](image)

Paraventricular Nucleus c-Fos positive (counts)

KO-KD | KO-CD | WT-KD | WT-CD

Fig 6b.

![Stranger Mouse](image)
Fig 6c.

Novel Object

Paraventricular Nucleus c-Fos positive (counts)

KO-KD  KO-CD  WT-KD  WT-CD

Fig 6d.

Novel Object

KO-KD  KO-CD  WT-KD  WT-CD
Overall Discussion and Summary

The diagnosis of a neurological developmental disorder can be devastating to a family, especially autism spectrum disorder (ASD), which presents as a seemingly sudden heterogeneity of behavioral disabilities with a prognosis that little can be done to improve symptoms. Currently, 2 years of age is the earliest possible behavioral diagnosis for ASD because there is no definitive physiological test. This has led to many hypotheses citing environmental factors for causation of symptoms, such as pesticides (Keil et al., 2014; Shelton et al., 2014), severe acute stress (Boksa 2010), and even vaccinations. In 1998 a falsified research paper was published claiming the measles, mumps, and rubella (MMR) vaccination caused ASD. This paper was found to be a complete medical fabrication, but was not retracted in full until 2010 (Halsey 2002; Zetterstrom 2004). Unfortunately, this publication resulted in a fury of media attention and public outcry against vaccinations. Despite scientific efforts to dispel the notion and publish proper epidemiological studies, the number of unvaccinated children has grown, leading to outbreaks and unnecessary endangerment of individuals (Weber 2008; Poland et al., 2010; Deer 2011; Holton et al., 2012; Taylor et al., 2014). Although, the cause of ASD is still unknown, research has determined that a heterogeneity of genetic factors may be involved in autistic-behaviors. This new research suggests individuals are born predisposed to ASD, although an unknown environmental factor could be involved (Jiang et al., 2013; Hormozdiari et al., 2015; Yuen et al., 2015). With little known as to the causation of ASD, there is no cure. Treatments for ASD only alleviate some symptoms, thus research is necessary to increase understanding of the mechanisms involved in ASD in attempts to find a causation and cure.
The comorbidities of ASD, including increased seizure susceptibility, suggest similar neural mechanisms are involved in both disorders. A ketogenic diet (KD), high in fat and low in carbohydrates, has been shown to be beneficial for pediatric epilepsy (Peterman 1925; Yudkoff et al., 2004), providing justification for the use of this diet for ASD. Specifically, levels of the ketone body, beta-hydroxybutyrate, in the blood of human patients have been shown to positively correlate with seizure reduction (Van Delft et al., 2010). Furthermore, human clinical studies have shown improvements in small populations of children and adolescents with ASD, but a larger clinical study has yet to be conducted (Spilioti et al., 2013). Diets high in fat have been shown to impact neural transmission, such as increasing NE and DA turnover, and alter psychological state by increasing stress-related eating behaviors (Levin et al., 1986; Levin et al., 2000; Cone et al., 2013). The KD, and in particular high ketone body production, may have similar effects improving ASD symptoms. To study the effects of the KD on autistic-like behaviors, the Engrailed 2 null (En2−/−) mouse was utilized, due to its genetic association with ASD, increased seizure susceptibility, and display of autistic-like behaviors.

Therefore, the objectives of this doctoral thesis research was to determine whether the KD fed during development (postnatal day 21 to 60) had any effect on biogenic amine concentration, social behavior, metabolic outputs, and c-Fos immunoreactivity of forebrain regions in adult En2−/− male mice.

Although there are numerous rodent models to study ASD (Halladay et al., 2009; Chadman et al., 2012), EN2 was chosen due to the relevance of this transcription factor in neural development and its genetic association with ASD (Benayed et al., 2009; Choi et al., 2012). As mentioned, ASD is most likely a result of heterogeneity of genetic factors, but to begin to understand the complexity of this disorder, it is important to understand the individual function of each implicated gene. EN2, a homeobox
transcription factor is critical for neural development and signaling. Mutations in En2 result in abnormal brain structures, such as a smaller cerebellum, and dysregulation of biogenic amine neural transmission in both forebrain and hindbrain structures (Joyner et al., 1991; Millen et al., 1994; Simon et al., 2005b; Cheh et al., 2006; Viaggi et al., 2015). Although some of these structural abnormalities, such as changes in the cerebellum (Skefos et al., 2014), are similar in autistic brains, the heterogeneity of autism confounds the role of En2 in neural development. Moreover, En2−/− mice display autistic-like behaviors, such as impaired social interaction, deficits in fear conditioning and sensory motor gating, and decreased play behaviors (Cheh et al., 2006; Brielmaier et al., 2012). Due to the importance of these neural structures and transmission in psychological states, such as depression, stress, and social behaviors, and the extent to which diet can alter biogenic amine signaling, this study investigated knockout (KO) and wild-type (WT) En2 male mice fed the KD or control diet (CD) from postnatal day (PND) 21 to PND 60.

Initially we determined how the En2 mutation alters biogenic amine levels in mice fed either a KD or CD from PND 21 to 60. Possible alterations in biogenic amine concentration, specifically regarding dopamine (DA), serotonin (5-HT), norepinephrine (NE), 5-hydroxyindoleacetic acid (5-HIAA), and homovanillic acid (HVA) in forebrain and hindbrain regions were analyzed. This study did not find robust changes in biogenic amine concentrations resulting from KD exposure in KO mice. There was, however, a diet x genotype effect for NE in the hypothalamus. This interaction was due to WT-KD having a higher NE content compared to WT-CD. En2−/− mice, regardless of diet, had significantly lower concentrations of NE and 5-HT in the hippocampus, as well as lower levels of DA and 5-HT in the hypothalamus. In contrast, En2−/− mice had increased NE and 5-HT in the cerebellum. These results support previous findings (Simon et al.,
2005a; Cheh et al., 2006) describing changes in biogenic amine concentrations in En2\(^{-}\) mice. Although we only measured these biogenic amine concentrations at PND 60, other studies using En2\(^{-}\) mice (Viaggi et al., 2015) have shown that the 5-HT concentrations are age-dependent. Viaggi and colleagues (2015) determined that there are early neurotransmitter deficits (1-3 months of age) in frontal and occipital cortex 5-HT content with late compensatory recovery (6 months of age) in En2\(^{-}\) mice (Viaggi et al., 2015).

Conversely, WT mice fed the KD had increased NE in the hypothalamus, which was not found in En2\(^{-}\) mice. It has been shown that a high-fat diet can alter noradrenergic signaling in the hypothalamus in rats (Ahlskog et al., 1973; O’donohue et al., 1978; Bello et al., 2013). This suggests an impaired ability for the KD to increase NE in the hypothalamus of En2\(^{-}\) mice and a possible deficiency in the noradrenergic pathway. To unravel this question, further studies are required to investigate synaptic growth and maturation as a result of the KD. Thus, while the KD can alter biogenic amines in WT mice, this study could not determine any dietary effects on biogenic amines in En2\(^{-}\) mice. However, this study did show that En2\(^{-}\) mice have deficits in NE, 5-HT, and DA in forebrain regions, which could result in alterations in social, depression-like, and stress-related behaviors; see Table 1. In-vivo microdialysis to determine real-time changes in biogenic amines post stressor could extend these findings, since changes in biogenic amines may have been overlooked by analyzing whole brain regions at PND 60. Whether any subtle changes in biogenic amine levels were missed because whole brain homogenates were analyzed is unknown and additional studies are necessary to further elucidate changes in biogenic amine receptors, transporters, and concentrations in discrete brain regions to completely understand the potential dietary consequences.

As previously mentioned, En2\(^{-}\) mice have impaired social behavior, which may result from neural deficits, specifically in biogenic amine signaling (Cheh et al., 2006;
Brielmaier et al., 2012). In the three-chambered test, this study confirmed $En2^{−/−}$ mice have decreased frontal contact with a stranger mouse and increased repetitive grooming behaviors. Similar to a previously reported study using the BTBR mouse model, which also displays autistic-like behaviors (Ruskin et al., 2013), a KD was able to restore behavioral impairments in frontal contact and reduce repetitive self-grooming behaviors in $En2^{−/−}$ mice. Thus, although there were no changes in regional biogenic amine concentrations, $En2^{−/−}$ mice fed the KD displayed changes in social and repetitive behavior, which suggests an unknown mechanism, possibly independent of biogenic amine concentration, such as synaptic signaling, receptor level, and tone. Interestingly, $En2^{−/−}$ mice spent more time with a novel object which had been previously housed with stranger male mice. This increased contact was in preference to a wire cage. This behavior suggests that exploration of novel objects is not deficient in $En2^{−/−}$ mice. In addition to deficits in social behavior, individuals with ASD have heightened stress-reactivity, which can negatively affect social interactions and daily activities. This study enhanced knowledge regarding stress-related changes in $En2^{−/−}$ mice with and without KD exposure. KO-CD were found to have increased baseline corticosterone compared with KO-KD at baseline and 1 h compared with WT-CD prior to the restraint-stress experiment. Also, $En2^{−/−}$ mice had increased chow intake post-restraint stress, suggesting an acute coping mechanism, which could be enhanced by reduced 5-HT in the hypothalamus. $En2^{−/−}$ mice may have an impaired hypothalamic-pituitary-adrenal (HPA) axis, but since corticosterone is produced by the adrenal-cortex, which can be stimulated by ACTH and fluctuations based on diurnal circadian rhythm, additional research is necessary to understand these pathways in $En2^{−/−}$ mice (Fu et al., 2003). Lastly, depression-like behaviors were measured in a forced swim test. $En2^{−/−}$ mice displayed increased mobility in the forced swim test, which may have resulted from a heightened stress response despite efforts to reduce stress in these testing parameters. From our
biogenic amine analysis, we expected KO mice to have decreased immobility from the lower levels of DA found in the forebrain based on HPLC analysis. Instead, no changes in immobility were found. Nevertheless, depressive-like behaviors were not well characterized using only the forced-swim test and future studies could examine if the KD changes depression-like behaviors in En2−/− mice. The KD reduced baseline corticosterone in En2−/− mice, but did not reduce food intake post-stress. It would be of interest to further determine changes in stress reactivity in the En2−/− mice to see if the KD changes other behavioral outputs, such as in a light-dark box and open arm maze. From these behavioral studies, we were able to determine that the KD can partially restore social behaviors in adulthood and reduce baseline stress in En2−/− mice in adulthood; see Table 2.

Feeding a KD from PND 21 to 60 can improve autistic-like behaviors in adulthood of En2−/− mice, which may be independent of changes in biogenic amines in the CNS. In addition to deficient social parameters, individuals with ASD that have difficulty maintaining body weight (Evans et al., 2012) and this study shows that the KD could potentially increase weight gain in these individuals by virtue of its high fat content and caloric density. The En2−/− mice fed the KD post-weaning to adulthood had increased body weight, which was a result of recovered lean mass. KO-KD mice had a trend of increased body length relative to the KO-CD mice, although this was not a statistically significant effect. Mice fed the KD had overall increased fat compared with mice fed CD. After mice were switched to a standard chow diet, KO mice previously fed the KD had a trend of reduced body weight, suggesting that metabolic effects on body weight are not persistent. Although the KD did increase body weight and restore lean mass in En2−/− mice, there is also the potential for the KD to result in metabolic syndrome (Feinman et al., 2003; Ellenbroek et al., 2014). Severe metabolic impairments could lead to
individuals failing to complete dietary treatment and noncompliance in adolescents fearing obesity or diabetes. In this study, several metabolic parameters including body weight, body fat, lean mass, blood pressure, metabolism in an indirect calorimeter, and blood glucose response to an oral glucose tolerance test were measured at PND 60, to determine if there were adverse metabolic changes as a result of the KD. As expected, KO-KD and WT-KD had a respiratory exchange ratio (RER) indicative of fat metabolism, compared with CD mice. KO mice had increased blood pressure (+22%), which was normalized with the KD. Blood pressure normalization resulting from a KD has also been shown in human weight loss studies (Perez-Guisado 2008). It has been shown that a KD can result in elevated blood glucose and insulin insensitivity in human studies (Park et al., 2011; Ellenbroek et al., 2014). Although insulin sensitivity was not measured in this study, a blood glucose post-challenge test was performed. The results of this test showed that the KD does elevate blood glucose response in En2−/− mice, but not in WT mice; see Table 3. These changes may persist, as seen by blood glucose impairment response extending to PND 69 in En2−/− mice previously fed the KD, but further studies are needed to determine long-term consequences of the diet. The restoration of lean body mass and blood pressure in En2−/− mice previously fed the KD may outweigh the potential negative metabolic effects in clinical ASD if autistic-like behaviors are resolved. Also, negative metabolic effects could be reduced by regulating the length and dietary composition of the treatment as well as with co-medications.

To further elucidate neural regions involved in social behavior, mice were exposed to a stranger mouse or a novel object, then perfused for c-Fos immunoreactivity in forebrain regions. Exposure to a novel mouse elicited expression of the immediate-early gene, c-Fos, in both WT and KO mice as previously shown (Avale et al., 2011). This indirect marker of neural activity identified forebrain regions that could be critical for
social behaviors and identified that the KD alters this activation in *En2* mice. Time spent with the stranger mouse increased c-Fos immunoreactivity in limbic regions, specifically the cingulate cortex and septal region of *En2* KO and WT mice previously fed the KD. Interestingly, the cingulate cortex and septal region have been implicated in autistic-like behaviors (Adolphs 2001; Rosene et al., 2004; Simms et al., 2009). No changes were found in the cingulate cortex or septal region of KD-fed mice exposed to a novel object. In regards to stress-related regions, increased c-Fos immunoreactivity was found in the bed nucleus of the stria terminalis (BNST) and paraventricular nucleus of the hypothalamus (PVN) of KO-KD and WT-KD compared with WT-CD in animals exposed to a stranger mouse. No differences were found between KO-KD and KO-CD. Thus, these regions may be stimulated by social exposure and further enhanced in mice previously fed the KD. When exposed to a novel object, KO-CD had increased c-Fos immunoreactivity compared with KO-KD and WT-CD in the BNST and with all groups in the PVN. Since changes were found between KO-KD and KO-CD, but not KO-KD and WT-KD, this suggests some normalization of c-Fos immunoreactivity. Increased c-Fos immunoreactivity in the KO-CD mice was associated with increased contact time with a novel object. Therefore, this study determined that social behaviors may be regulated by the cingulate cortex and septal regions of *En2* mice and the KD may improve autistic-like behaviors by acting on critical limbic regions. Enhanced c-Fos immunoreactivity in the BNST and PVN of *En2* mice exposed to a novel object may also be indicative of HPA axis activation. This study determined two critical regions, the cingulate cortex and medial septal region, that may be altered by social exposure in *En2* mice fed the KD, but further studies are necessary to determine the exact mechanism involved and how the KD is altering c-Fos immunoreactivity in these regions; see Table 4.
This results of this study demonstrated changes in autistic-like behaviors, metabolism, and c-Fos immunoreactivity in forebrain regions of En2 KO mice fed the KD. KO-KD mice had restored frontal contact behaviors and reduced repetitive behaviors when exposed to a stranger mouse that coincided with increased c-Fos immunoreactivity in limbic regions, specifically the septal region and cingulate cortex. These regions have been implicated in social behaviors in humans. There were no changes in biogenic amine concentrations in KO-KD. Yet, the KD did alter biogenic amine concentrations in WT mice, suggesting a disrupted or missing pathway in the KO mice. Increased neural reactivity in septal region, cingulate, and PVN was observed in KO-KD compared to KO-CD, suggesting an enhanced response, due to diet, when exposed to a stranger mouse. When exposed to only a novel object, KO-CD had increased c-Fos immunoreactivity in the BNST and PVN relative to WT-CD mice, which may be indicative of an increased stress response. However, these regions were not activated when KO-CD was exposed to a stranger mouse. Nevertheless, unique increased c-Fos immunoreactivity in the cingulate cortex and septal region of KO-KD suggest an unknown mechanism of the KD acting on neural signaling. Also, as expected, the KD restored lean body mass, increased fat mass, altered respiratory exchange indicative of fat metabolism, and resulted in an enhanced blood glucose response in En2−/− mice. Overall, the KD reduced autistic-like behaviors and increased body weight in En2−/− mice, when fed post-weaning to adulthood.

Future experiments are needed to further elucidate the specific biochemical and physiological mechanisms that are involved with restoring social behaviors and changing metabolic parameters in En2 mice. Since, this study determined that En2−/− mice have deficiencies in lean body mass, examining the effects of exercise in these mice would be of interest to see if exercise can normalize lean body mass to WT. The oxymax (indirect
calorimeter) could be utilized to determine metabolic output after animals were exposed to a treadmill. Mice with less lean mass would be expected to have a reduced ability to run on a treadmill, but these studies could also determine if the KD could change exercise-induced expenditure. Reduced lean mass may also reduce overall strength. This could be measured by a grip-strength test. In addition to body weight changes, the oral glucose tolerance test (OGTT) determined that KO mice may have changes in glucose tolerance or impaired insulin sensitivity, which could be measured more precisely by a hyperinsulenic-euglycemic clamp test. Changes in glucose homeostasis could be indicative of alterations in hypothalamic areas and regulation of glucose uptake by the brain or other tissues, but there could also be changes in regulation of glucose metabolism in the pancreas or liver. On the other hand, our collaborators described restored social behaviors in En2−/− mice, as a result of treatment with desipramine, long-term modulation of biogenic amines may be important in regulating social behaviors in adult En2−/− mice. Besides biogenic amines, neural receptors implicated in ASD could be analyzed. For example, β2-subunit nicotinic receptors (β2-nAChRs) are of particular interest, since they share a pathway with β-hydroxybutyrate and are involved in regulating social behavior. β2-nAChRs null mice (β2−/−) display increased time with a stranger mouse and increased contact with a novel object, demonstrating reduced inhibition or increased novel exploration (Avale et al., 2011). In the current study, increased ketone bodies in the blood, as a result of the KD, may increase nicotinic receptors and result in the same increase in sociability. Lastly, it is unknown from the current study if the high fat diet component of the ketogenic diet or ketone bodies resulted in the changes found, but future studies could determine this distinction.

The knowledge generated from this study may benefit basic research and human health in the following ways. First, these studies further investigated the role for En2 in
developmental circuits critical for neural development, metabolism, behaviors, and ketone body and En2 signaling interaction As En2 has been implicated in the continuum of ASD, it is critical to understand the role of this transcription factor. Second, these studies demonstrated that a ketogenic diet could normalize social and metabolic outputs and therefore, could be considered a potential treatment strategy for individuals with ASD. In addition, it is unknown whether ketone bodies, resulting from the ketogenic diet, lead to the changes found, but future studies could test this new hypothesis. If ketone bodies, and not overall fat consumption, result in the same changes, then a dietary intervention using ketone bodies or a ketogenic pill could be prescribed (Rho et al., 2008; Viggiano et al., 2015) for ASD children during development to avoid the adverse metabolic effects of a high fat diet. In conclusion, this study supports a role for the KD in altering autistic-like behaviors, metabolism, and neural reactivity in En2 null mice exposed post-weaning (PND 21) to young adulthood (PND 60).


### Table 1. Biogenic amine concentration summary of results.

<table>
<thead>
<tr>
<th></th>
<th>Genotype Effect</th>
<th>Diet Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>KO-CD vs WT-CD</td>
<td>KO-KD vs KO-CD</td>
</tr>
<tr>
<td>Forebrain Norepinephrine</td>
<td>↓</td>
<td></td>
</tr>
<tr>
<td>Cerebellum Norepinephrine</td>
<td>↑</td>
<td></td>
</tr>
<tr>
<td>Forebrain Serotonin</td>
<td>↓</td>
<td></td>
</tr>
<tr>
<td>Cerebellum Serotonin</td>
<td>↑</td>
<td></td>
</tr>
<tr>
<td>Forebrain Dopamine</td>
<td>↓</td>
<td></td>
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</tbody>
</table>

Male En2 KO and WT mice fed a ketogenic or control diet from PND 21 to 60. Arrow represents direction of change; i.e. ↑ significant increase and ↓ significant decrease.

### Table 2. Social and stress-related summary of results.

<table>
<thead>
<tr>
<th></th>
<th>Genotype Effect</th>
<th>Diet Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>KO-CD vs WT-CD</td>
<td>KO-KD vs KO-CD</td>
</tr>
<tr>
<td>Frontal Contact with Mouse</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>Frontal Contact with Novel Object</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>Self-Grooming</td>
<td>↓</td>
<td></td>
</tr>
<tr>
<td>Plasma Corticosterone at Baseline</td>
<td>↓</td>
<td></td>
</tr>
<tr>
<td>Chow Intake Post-Stress</td>
<td>↑</td>
<td></td>
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</tbody>
</table>

Male En2 KO and WT mice fed a ketogenic or control diet from PND 21 to 60. Arrow represents direction of change; i.e. ↑ significant increase and ↓ significant decrease.
**Table 3.** Metabolic output summary of results.

<table>
<thead>
<tr>
<th></th>
<th>Genotype Effect KO-CD vs WT-CD</th>
<th>Diet Effect KO-KD vs KO-CD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Weight</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>Fat Mass</td>
<td></td>
<td>↑</td>
</tr>
<tr>
<td>Lean Mass</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>OGGT Blood Glucose</td>
<td></td>
<td>↑</td>
</tr>
<tr>
<td>Blood Pressure</td>
<td>↑</td>
<td>↓</td>
</tr>
</tbody>
</table>

Male En2 KO and WT mice fed a ketogenic or control diet from PND 21 to 60. Arrow represents direction of change; i.e. ↑ significant increase and ↓ significant decrease.

**Table 4.** Neural activation summary of results.

<table>
<thead>
<tr>
<th></th>
<th>Genotype Effect KO-CD vs WT-CD</th>
<th>Diet Effect KO-KD vs KO-CD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cingulate Cortex</td>
<td>↑ with Stranger Mouse</td>
<td></td>
</tr>
<tr>
<td>Septal Region</td>
<td>↑ with Stranger Mouse</td>
<td></td>
</tr>
<tr>
<td>BNST</td>
<td>↑ with Novel Object</td>
<td>↑ with Stranger Mouse and ↓ Novel Object</td>
</tr>
<tr>
<td>PVN</td>
<td>↑ with Novel Object</td>
<td></td>
</tr>
</tbody>
</table>

Male En2 KO and WT mice fed a ketogenic or control diet from PND 21 to 60. Arrow represents direction of change; i.e. ↑ significant increase and ↓ significant decrease.