Effects of the 3.2:1 Ketogenic Diet on Behavioral Symptoms of Autism in the BTBR Mouse Model

by

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I. ACKNOWLEDGEMENTS

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II. ABSTRACT

BACKGROUND: Autism spectrum disorders (ASD) are a group of pervasive developmental brain disorders characterized by three hallmark symptoms: deficits in sociability, difficulties with communication, and restricted, stereotyped, and repetitive patterns of behavior. The prevalence of ASD diagnoses increases each year. Now more than ever, more effective treatments are needed. Recent research has shown that a highly restrictive, 6:1 ketogenic diet (fat: carbohydrate + protein composition) significantly alleviates the three core symptoms of autism in the BTBR T+ tf/J (BTBR) mouse model of autism (Masino et al., 2012). Although these findings are promising, implementing this stringent diet in the ASD clinical population could be quite challenging. The purpose of this current study was to evaluate whether a less stringent diet comprised of a 3.2:1 (fat: carbohydrate + protein) ratio significantly reduces behavioral symptoms of autism in BTBR T+ tf/J mice.

METHODS: This study evaluated the effects of a 3.2:1 ketogenic diet on two experimental groups of BTBR T+ tf/J mice, a model which displays autism-like behaviors. The first group (n =14) received the ketogenic diet with a 3.2:1 (fat: carbohydrate + protein) ratio, and the second group (n =13) received a control diet that matched the ketogenic diet in protein, mineral, and vitamin content but differed in fat and carbohydrate content. The mice remained on either the ketogenic diet or the control diet for 21 days before undergoing behavioral tasks. After applying the respective diets, we conducted four behavioral measures to evaluate the three hallmark symptoms of ASD: the three-chambered test of sociability, grooming, marble burying (repetitive behaviors), and the social transmission of food preference.
tasks (communication). In addition, we collected glucose and ketone measurements to confirm metabolic effects of the diet and to potentially lend insight into the mechanisms of the ketogenic diet. We performed statistical analyses using SPSS software to compare observed behaviors between the two experimental groups of BTBR mice.

RESULTS: The three-chambered test of sociability revealed no significant difference in sociability between the BTBR mice fed the 3.2:1 ketogenic diet and those fed a matched, control diet. Similarly, the ketogenic diet did not influence the amount of time the BTBR mice engaged in self-grooming behaviors. Furthermore, the ketogenic diet did not influence the animals’ communication abilities, as assessed by the social transmission of food preference task. Animals fed the ketogenic diet engaged in slightly more marble burying in comparison to those fed the modified, control diet, suggesting that this diet composition does not effectively reduce repetitive behaviors. The 3.2:1 ketogenic diet produced unexpected metabolic effects: significantly increased blood ketone levels (as measured by β-hydroxybutyrate) but did not significantly affect change blood glucose.

CONCLUSIONS: In contrast to previous work with a 6.6:1 ketogenic diet, our data reveal that the 3.2:1 ketogenic diet did not significantly alter any of the hallmark symptoms of autism in the BTBR mouse model of autism. However, this diet composition did not produce expected metabolic effects, which may lend mechanistic insights into how the diet works. We conclude that further studies are needed to determine critical differences between behavioral effects of the 6.6:1 and 3.2:1 ketogenic diet. It may be beneficial to pursue other models of autism, or test
experimental groups where fat content is systematically manipulated to determine the lowest fat content that will produce significant behavioral rescue of the autistic phenotype in the BTBR mouse model of autism.

III. INTRODUCTION

A. AUTISM SPECTRUM DISORDERS

Autism spectrum disorders (ASD) are a group of pervasive, developmental brain disorders with similar, but not identical, behavioral manifestations (National Institute of Mental Health, 2011). Disorders along this spectrum include autistic disorder (classic autism), Asperger’s disorder (Asperger syndrome), pervasive developmental disorder not otherwise specified (PDD-NOS), Rett’s disorder (Rett syndrome), and childhood disintegrative disorder (CDD).

As of 2008, estimates suggested that one in 88 children had been identified with ASD (Centers for Disease Control and Prevention, 2012). In the past decade, there have been significant increases in the prevalence of ASD. According to reports from the Autism and Developmental Disabilities Monitoring (ADDM) Network, the prevalence of ASD increased 23% between 2006 and 2008 and 78% between 2002 and 2008 (Centers for Disease Control and Prevention, 2012). Researchers have attributed part of this increasing prevalence to a broadening of criteria for the diagnosis of ASD and increased awareness for autistic symptoms (Muhle et al., 2004).

Multiple factors influence ASD symptoms. Boys are four to five times more likely to receive an ASD diagnosis than girls (1 in 54 males, 1 in 252 females)
Racial and ethnic backgrounds also influence the prevalence of an ASD diagnosis. Diagnosis rates are highest in non-Hispanic white children, while diagnosis of non-Hispanic black children is slightly lower, and the lowest rate of diagnosis is found among Hispanic children (National Institute of Mental Health, 2011).

**Behavioral Characterization**

The current Diagnostic and Statistical Manual of Mental Disorders (DSM-IV-TR), which is used to diagnose ASD, has slightly different criteria for each disorder along the spectrum and does not categorize them under one diagnosis. The three hallmark symptoms most common among the ASD are (1) impaired social interactions; (2) difficulties with communication; and (3) restricted, repetitive, and stereotypic behavior. Diagnosis under the current DSM-IV-TR criteria also requires that onset of autistic symptoms - marked by delays or abnormalities in social interaction, language as part of social communication, or imaginative play - occurs prior to three years of age. Generally, children with autistic disorder (four years) are diagnosed earlier than those diagnosed with broadly defined autism spectrum diagnoses (four years, five months), and even earlier than those with Asperger disorder (six years, three months) (Centers for Disease Control and Prevention, 2012).

An updated manual, the DSM-5, which is expected for publication in May 2013, will incorporate several changes to the current ASD diagnostic criteria. Most importantly, the diagnosis of “autistic disorder” will now be termed “autism spectrum disorder” and will expand to incorporate classic autism, Asperger’s syndrome, CDD,
and PDD-NOS. Additionally, the three hallmark symptoms of autism will be
condensed into two categories: social/communication deficits and fixated
interests/repetitive behaviors. Unusual sensory behaviors will be explicitly included
as criteria within the domain of the second category. Finally, the proposed revision
acknowledges that although all symptoms must be present from infancy or early
childhood, they may not manifest themselves completely until later in life when
societal demands increase and less support is available for individuals at risk.

Despite a unified grouping in the forthcoming diagnostic manual, slight
behavioral differences can be identified among developmental disorders along the
autistic spectrum. Although patients with Asperger’s disorder display deficits in
sociability and restricted patterns of interest, these individuals demonstrate normal
language development at ages two and three and, typically, do not have comorbid
mental retardation (Buitelaar and Willemsen-Swinkels, 2000; Muhle et al., 2004).
Another important distinction lies in the rarity of CDD and Rett’s disorder in
comparison to other disorders on the ASD spectrum. Patients with CDD commonly
demonstrate normal early development, including language, until at least two years of
age, and then exhibit significant regression in behavioral, cognitive, and language
abilities. Furthermore, unlike classic autism, which most likely results from the
interaction of multiple genetic and environmental causes, Rett’s Disorder is caused by
a single-gene defect, usually de novo mutations or microdeletions of the methyl-CpG-
binding protein 2 (MeCP2) gene on the chromosome band, Xq28. Due to the location
of the affected gene on the X chromosome, this disorder has only been reported in
females (Buitelaar and Willemsen-Swinkels, 2000; Muhle et al., 2004). Individuals
diagnosed with Rett’s disorder display normal development until five months of age, when they begin to demonstrate a deterioration of social and communicative skills, purposeful hand movements as well as a deceleration of head growth (Buitelaar and Willemsen-Swinkels, 2000). Finally, patients diagnosed with PDD-NOS display the severe developmental problems typical of pervasive developmental disorders, but do not fit any specific diagnostic category along the autistic spectrum (Buitelaar and Willemsen-Swinkels, 2000; Muhle et al., 2004).

**Brain Abnormalities in Structure and Function**

In addition to characteristic behavioral manifestations, post-mortem samples and brain imaging techniques reveal regional abnormalities in ASD patients (Amaral et al. 2008). Although findings are inconsistent with regards to the locations of neurological abnormalities, the most common regions include areas of the cerebral cortex, brainstem, cerebellum, and many parts of the limbic system (Lawler et al., 2004).
Several post-mortem studies revealed consistent reductions in cell size as well as increased cell packing density in many parts of the brain, including the hippocampus, amygdala, entorhinal cortex, mammillary body, medial septal nucleus, and the anterior cingulate gyrus (Kemper and Bauman, 1993; Bauman, 1996). Researchers hypothesize that these abnormalities, specifically those in the cerebellum and the limbic system, are strongly associated with characteristic behaviors of ASD including deficits in sociability and communication (Bauman, 1996). The variety of atypical neuropathological findings in the ASD brain and their widespread

**Figure 1.** Brain areas that might be implicated in ASD (Amaral et al., 2008).
distribution in multiple brain regions implicates multiple neural networks. It is still unclear to what extent these neurological abnormalities are caused by genetic influences and how large a role they play in the etiology of ASD.

In addition to regional abnormalities in neural circuits, 10-20% of autistic individuals have enlarged heads (macrocephaly) and increases in total brain tissue volume associated with expansion of occipital and/or parietal lobes (Woodhouse et al., 1996). MRI studies of very young children with ASD (between 18 months and four years of age) showed a transient trend for 5%-10% larger brain volumes that normalize later in development (Courchesne et al., 2001; Hazlett et al., 2005). In contrast, head circumferences in mentally retarded children or adults without autism are typically smaller than average. The cause(s) of more rapid brain growth in ASD are unknown, but several mechanisms have been proposed including: 1) increased neurogenesis, 2) decreased neuronal death, or 3) increased production of non-neuronal brain tissue, which may include glia or blood vessels (Buitelaar and Willemsen-Swinkels, 2000).

Role of Neurochemical Systems

Serotonin (5-hydroxytryptamine; 5-HT) is a biogenic amine (Purves et al., 2001). The serotonergic system is of particular interest in relation to the cause of ASD because of serotonin’s role in embryogenesis, maturation of the brain, and several processes such as learning, sleep, and sensory perception. One of the most robust neurobiological findings in patients with ASD is a significant elevation of whole blood 5-HT concentration in comparison to normal subjects (Anderson et al., 1987; Buitelaar and Willemsen-Swinkels, 2000). Thirty percent of all patients with
autism demonstrate this neurobiological change. Although observations of ASD include both increased activity of the 5-HT transporter of platelets and decreased binding to the 5-HT$_2$ receptor, the exact mechanism and significance of hyperserotonemia still remains unclear (Buitelaar and Willemsen-Swinkels, 2000). Other neurochemical systems implicated in ASD include those of dopamine, glutamate, GABA, and oxytocin (Lawler et al., 2004).

Common Comorbidities

An ASD diagnosis rarely occurs in isolation. Persons with ASD often suffer from one or more comorbidities (National Institute of Mental Health, 2011). The most prominent comorbidities include epilepsy (prevalence estimates ranging from 5% to 46%), abnormalities in epileptiform electroencephalograms (EEGs) (prevalence rates soar as high as 60%, even in individuals without seizures), and mental retardation (about 75% of autistic individuals suffer from cognitive disabilities) (Caplan and Austin, 2000; Spence, 2009). Other common comorbidities include sensory problems (such as sensitivity to light and noise, claustrophobia, or adverse reactions to particular foods or clothing rubbing against the body), sleep disturbances (difficulty falling or staying asleep), cognitive disabilities (most often associated with problem solving tasks that are language-based), immune dysfunction, or gastrointestinal complications. In addition, at least 70% of patients with ASD suffer from psychiatric comorbidities, most often diagnosed as social anxiety disorder, attention deficit/hyperactivity disorder, oppositional defiant disorder, or depression (Masino et al., 2009; National Institute of Mental Health, 2011).
Associated Medical Conditions: Mono-genetic disorders

Autism is common in patients with fragile X syndrome and tuberous sclerosis complex. However, these single-gene defects, along with diagnosable medical conditions or cytogenetic abnormalities account for less than 10% of ASD cases (Muhle et al., 2004).

Causes of Autism

Despite its prevalence, the cause of autism remains unknown. Many individuals wrongly believe that vaccines, specifically the measles-mumps-rubella (MMR) vaccine that many individuals receive at a young age, cause the development of ASD. However, as of 2010, no scientific studies have proven this association (National Institute of Mental Health, 2011). More generally, no individual genetic or non-genetic causes have been identified, most likely due to the myriad of genetic and genomic disorders that may contribute to similar behavioral manifestations. The etiological heterogeneity of ASD makes it difficult to develop a treatment that is effective for a range of cases (Betancur, 2011).

It is very likely that genetic susceptibility, environmental factors, and an individual’s stage of brain development all contribute to the development of ASD (Kelada et al., 2003). Researchers hypothesize that environmental factors influence the expression of ASD susceptibility genes (Lawler et al., 2004). In addition, it is possible that the behavioral manifestations of ASD and underlying brain abnormalities may exacerbate the extent to which an individual is influenced by
environmental factors (Lawler et al., 2004). Thus far, it remains difficult to parse out the extent to which any one factor contributes to ASD.

**Genetic Predispositions**

Several findings provide evidence for the role of a genetic predisposition in the etiology of autism. Same-sex twin studies demonstrate a significantly higher concordance rate for identical versus nonidentical twins: one study found a 60% concordance for classic autism in monozygotic twins and a 0% concordance in dizygotic twins (Bailey, Le Couteur et al., 1995). More importantly, when considering the broader autistic phenotype (by incorporating criteria beyond that of classic autism) the difference in concordance rates between identical and nonidentical twins increases even more. In addition, there is a high biological reoccurrence risk for autism following the birth of an autistic child (60 to 150 times the population base rate) (Buitelaar and Willemsen-Swinkels, 2000).

Several other genetic abnormalities increase autism susceptibility, including reduced MET gene expression, which encodes for the MET receptor tyrosine kinase, and a common polymorphism in neurexin superfamily member CNTNAP2. Reduced MET gene expression is thought to contribute because this signaling pathway is involved in neocortical and cerebellar growth and maturation, immune function, and gastrointestinal repair, all of which are implicated in ASD (Campbell et al., 2006). CNTNAP2 (contactin-associated protein-like 2) is thought to increase familial risk for ASD because of its role in mediating cell-cell interactions in the nervous system as well as its hypothesized role in axon differentiation (Arking et al., 2008).
Environmental Susceptibility

Extensive research examines the environmental factors that contribute to ASD. Several prenatal and perinatal characteristics have been identified as risk factors for increased occurrence of ASD in offspring (Kolevzon et al., 2007). These characteristics include obstetric conditions (specifically, intrapartum hypoxia), advanced maternal and paternal age (most likely due to the increased risk of obstetric complications), and maternal exposure to stress, infection, anti-depressant medications, thalidomide or valproic acid (Hyman, 2005; Kolevzon et al., 2007; Kinney et al., 2008; Patterson, 2009; Patterson, 2011). Maternal metabolic conditions during pregnancy (such as diabetes, hypertension, and obesity) have also been found to be risk factors for ASD and other neurodevelopmental disorders (Krakowiak et al., 2012). Collectively, these findings provide strong evidence for the role that the maternal intrauterine environment plays in the development of autistic symptoms.

Similarly, parental psychiatric disorders are also potential contributors to ASD (Daniels et al., 2008). A study conducted in 2008 found that parents of autistic children were more likely to have been hospitalized for a mental disorder than parents of control subjects. This correlation supports the notion of familial aggregation of psychiatric conditions (Daniels et al., 2008). Research has found similar genetic loci underlying several psychiatric disorders, specifically ASD, schizophrenia, and bipolar disorder (Carroll and Owen, 2012). This suggests that a common biological pathway may exist among these disorders; possessing certain genetic loci and alleles may put an individual at risk for all three of these disorders. It is then more likely for psychiatric symptoms and/or disorders to aggregate within families.
Several other environmental influences may play a role in the development of ASD. Neurotoxicants may perturb critical developmental processes such as neurogenesis, proliferation, migration, synapse formation, and myelination (Lawler et al., 2004). The relationship between the immune system, nervous system, and molecular substrates (specifically, serotonin, neuropeptides, autoantibodies, and cytokines) is also of interest (Lawler et al., 2004).

*Current Treatment Options for Autism Spectrum Disorders*

Presently, scientists have not identified a cure for ASD. This inability to find a cure stems from uncertainty surrounding the etiology of this group of pervasive disorders. Therefore, current treatment options serve only to alleviate symptoms of the disorders. Due to the various ways in which ASD affects the individual as well as the prevalence of comorbidities, clinicians tend to take a multi-faceted approach to treatment.

Psychopharmacological interventions constitute one major approach. Pharmacological treatments target autistic symptoms, such as repetitive/stereotyped behaviors and thoughts, aggression, hyperactivity/inattention, or self-injurious behavior, rather than the core features of ASD (Buitelaar and Willemsen-Swinkels, 2000; National Institute of Mental Health, 2011). Stimulants, such as methylphenidate (Ritalin), are often prescribed to treat hyperactivity and impulsiveness. Selective serotonin reuptake inhibitors (SSRIs) are thought to target rigidity, stereotyped behaviors, and rituals. SSRIs, such as fluoxetine (Prozac®) or sertraline (Zoloft®), also are prescribed to help alleviate comorbid depression, aggression, and anxiety. However, the efficacy of these medications is still uncertain.
In 2009, a research study revealed that the antidepressant citalopram (Celexa®) was no more effective in reducing stereotyped, repetitive behaviors than a placebo (National Institute of Mental Health, 2011). In addition, SSRIs may be more efficacious for adolescents and adults with ASD, rather than children (Rogers and Vismara, 2008). Atypical antipsychotics, β-blockers, and anticonvulsants serve to reduce self-injury and aggression. Anticonvulsants also help manage comorbid epilepsy. Buspirone, clonidine, and atypical antipsychotics are prescribed to alleviate anxiety and affective symptoms.

Dietary intervention constitutes another treatment approach. The prevalence of comorbid gastrointestinal complications and metabolic disturbances in patients with ASD supports the implementation of a variety of modified diets. Some of these interventions include gluten-free, casein-free, gluten-free/casein-free, specific carbohydrate, and body ecology diets (Srinivasan, 2009). Although there have been anecdotal reports of success with these diets, valid scientific data is limited. Dietary interventions are notoriously difficult to adhere to and expensive to maintain, especially for children and adolescents who are routinely exposed to peers’ food in uncontrolled environments.

A third approach to treatment takes a psychosocial approach. Applied behavior analysis (ABA), which focuses on the reinforcement of new, desirable behaviors and the reduction of undesirable behaviors, is widely employed. This intervention focuses on the development of verbal behaviors, ranging from simple skills, such as echoing, to more complex skills necessary for effective communication in social situations. Another type of ABA, pivotal response training, seeks to
strengthen essential pivotal skills for engaging in social interactions (National Institute of Mental Health, 2011).

Given the prevalence of ASD and the limited success of any particular treatment – individual or combined – new approaches are necessary.

B. KETOGENIC DIET

The high-fat, low-carbohydrate ketogenic diet was first introduced in the 1920s as a first or second-line treatment for severe childhood epilepsy, but quickly fell out of favor with the rise of anticonvulsant medications, specifically phenytoin in 1938 (Gasior et al., 2006; Rho and Stafstrom, 2012). Since the 1990s, the ketogenic diet has regained recognition for its neuroprotective properties in the context of several neurologic disorders, metabolic disturbances, and other health conditions (Rho and Stafstrom, 2012).

Currently, the ketogenic diet demonstrates clinical efficacy for pediatric epilepsy and medically refractory epilepsy. A prospective study conducted at Johns Hopkins Hospital found that the ketogenic diet resulted in 10-15% of cases to be seizure free one year later, 30% of cases to have greater than 90% reduction in seizures, and 40-50% of cases found the diet to be too difficult to continue or to be ineffective (Freeman et al., 1998). The ketogenic diet has also been shown to provide therapeutic effects in cases of adult epilepsy, select cases of Dravet syndrome, myoclonic-astatic epilepsy, Rett syndrome, migrational disorders, and tuberous sclerosis (Freeman et al., 2007; Dhamija et al., 2013).

The high-fat, low-carbohydrate composition of the ketogenic diet causes significant metabolic changes in the body, which are thought to be responsible for the
diet’s neuroprotective effects (Politi et al., 2011). Table 1 outlines a typical day of food intake for a child on a 4:1 ketogenic diet consisting of 1500-calories. The diet has both anticonvulsant and antiepileptic properties (Freeman et al., 2007).

Anticonvulsant effects refer to the diet’s ability to stop discrete seizures, whereas antiepileptic effects refer to the diet’s ability to stop a propensity to develop recurrent or unprovoked seizures (Freeman et al., 2007). These properties, as well as the associated metabolic effects, will be discussed in more detail in the section entitled ‘Proposed Mechanisms of the Ketogenic Diet.’

<table>
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<th>Table 1. Typical Day of Food for Child on 4:1 1500 calorie Ketogenic Diet (Turner and Kossoff, 2006)</th>
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<td><strong>Breakfast</strong></td>
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<td><strong>Dinner</strong></td>
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Although the diet offers potential therapeutic effects, drawbacks do exist. In the case of epilepsy, the suggested duration of dietary intervention is lengthy (typically one to two years) in order to see beneficial effects (Turner and Kossoff, 2006). The diet’s stringent composition can be expensive, difficult to maintain, and time-consuming to prepare. Parents must check their child’s urine ketone levels several times per week (either in the home or doctor’s office setting). In addition, individuals on the ketogenic diet must seek help from a registered nutritionist to account for nutritional deficiencies (Turner and Kossoff, 2006).
Adverse side effects, such as hunger, constipation, lethargy, lack of weight gain (distinct from weight loss), low-grade acidosis, and hypoglycemia may appear. Fortunately, these complications are not long-lasting and can be alleviated by modifying the diet or taking supplemental medications. For example, slight adjustments in diet, stool softeners, or laxatives can improve constipation. A few less-common side effects of the ketogenic diet include the development of kidney stones in 5-7% of children, diminished growth (most often height, especially in young infants), and dyslipidemia, an abnormal amount of lipids in the blood (Turner and Kossoff, 2006; Masino et al., 2009).

Proposed Mechanisms of the Ketogenic Diet

Although researchers have hypothesized mechanisms for the diet, it has been difficult to link any individual neurochemical change associated with the diet to its neuroprotective and anticonvulsant effects (Politi et al., 2011). The proposed mechanisms generally examine the ability for certain nutrients and metabolic substrates to regulate, normalize, and even enhance brain functioning (Rho and Stafstrom, 2012).

The ketogenic diet provides adequate protein to maintain proper growth; however, the diet does not provide sufficient carbohydrates to meet metabolic needs. To compensate, the body enters a state of chronic ketosis and derives energy from fatty acid oxidation in the mitochondrial matrix. As a result, significant metabolic effects have been observed including -- an increase in circulating levels of ketone bodies (i.e. acetoacetate, ß-hydroxybutyrate and acetone, all produced in the liver) as well as a reduction in blood glucose levels, and an increase in mitochondrial function
(Gasior et al., 2006). The body converts both body fat and fats delivered via the diet into ketone bodies, which become the preferred source of energy. Ketone bodies, which readily cross the blood-brain barrier, serve as a more efficient source of fuel given significantly reduced glucose levels (due to the lack of carbohydrate consumption) (Hartman et al., 2007). Increased levels of ketone bodies also cause an increase in the production of adenosine triphosphate (ATP), via the Krebs cycle in brain mitochondria (Masino et al., 2009).

Several hypothesized mechanisms concerning the neuroprotective properties of the ketogenic diet exist. Research has explored whether increases in ketone bodies, which result from the degradation of free fatty acids present in the blood through β-oxidation, are responsible for the diet’s effects. While studies have failed to link β-hydroxybutyrate to the diet’s anticonvulsant effects, acetoacetate and acetone, two ketones produced in response to the degradation of β-hydroxybutyrate, have anticonvulsant properties in animal models (Hartman et al., 2007).

Another prominent avenue of experimental research examines whether significant alterations in the production and metabolism of major excitatory (glutamate) and inhibitory (GABA) neurotransmitters in the brain may be responsible for the diet’s anticonvulsant effects. Low aspartate levels, resulting from reduced availability of oxaloacetate, leads to an enhanced availability of glutamate. Research has shown that the ketogenic diet causes small regional increases in brain glutamate, the precursor of GABA via the GABA shunt (Hartman et al., 2007). Researchers believe that an increased flux of glutamate would also reflect an increase in the production of GABA, the major inhibitory neurotransmitter in mammals. One study
found that rats fed a ketogenic diet demonstrated greater angular bundle-evoked paired-pulse inhibition in the dentate gyrus, in comparison to animals fed a normal diet (Bough et al., 2003). This finding suggests that the ketogenic diet may enable enhanced, rapid synaptic inhibition, which may be mediated by GABA. The overarching hypothesis from this avenue of research suggests that the diet’s anticonvulsant effects may result from the net effect of increased GABAergic influence (Politi et al., 2011).

Polyunsaturated fatty acids (PUFAs), which result from an elevation in free fatty acids, may also play a role in the diet’s effects. The underlying mechanisms are quite complicated: one such hypothesis proposes that PUFAs increase the expression and activity of uncoupling proteins in the mitochondria by limiting the generation of reactive oxygen species (ROS) (Politi et al., 2011). Alternatively, certain PUFAs may block voltage-gated sodium or calcium channels, effectively regulating neurons’ membrane excitability (Stafstrom and Rho, 2012).

Another proposed mechanism examines the diet’s effects on energy metabolism. The diet upregulates hippocampal genes encoding energy metabolism and mitochondrial enzymes, which then leads to the stimulation of mitochondrial biogenesis (Gasior et al., 2006; Rho and Stafstrom, 2012). These changes suggest that ketone bodies are a more efficient source of fuel, which then make more energy available for ATP synthesis, and the stimulation of mitochondrial biogenesis is hypothesized to stabilize synaptic function and heighten energy reserves (Masino et al., 2009; Rho and Stafstrom, 2012). This perspective suggests that the ketogenic diet
should increase energy production capacity and reserves, which would then allow neurons to respond better to metabolic challenges.

In a related vein, the ketogenic diet increases ATP levels, which enables the production of energy reserves. ATP can either be broken down into adenine nucleotides or adenosine nucleosides or be converted to phosphocreatine for energy storage. Adenosine is thought to play a role in reducing both physiological and behavioral symptoms common in ASD (Masino et al., 2012). More specifically, extracellular adenosine offers anticonvulsant effects by inhibiting neuronal excitation via A1 receptors in several brain regions, controlling seizures by keeping them localized, and altering seizure threshold (Masino et al., 2009). Increased levels of adenosine may also help address sleep disturbances, frequent seizures, or comorbid anxiety disorders (Masino et al., 2009).

**Historical Use and Alternative Uses of the Ketogenic Diet**

Since the 1990s, the ketogenic diet has been explored as a potential cure for a plethora of related health conditions, many of which are common comorbidities of ASD. Animal model research has posited the diet as a possible treatment option for several neurological disorders, such as Alzheimer disease, Parkinson’s disease, traumatic brain injury and stroke, metabolic disorders, such as GLUT-1 deficiency, pyruvate dehydrogenase deficiency and phosphofructokinase deficiency, and other illnesses including cancer and cardiac ischemia (Baranano and Hartman, 2008).

In the context of aging and neurologic conditions related to aging, researchers have hypothesized that the ketogenic diet may retard the degeneration of neural structures and functions through the amelioration of age-related mitochondrial
dysfunction or the reduction of oxidative stress (Rho and Stafstrom, 2012). The diet has also been studied in relation to amyotrophic lateral sclerosis (ALS), which results from the degeneration of motor neurons in the cortex and anterior horn of the spinal cord (Rho and Stafstrom, 2012). In a mouse model of ALS, mice on the ketogenic diet had increased motor neuron counts and improved motor function on the rotarod performance test in comparison to animals fed a normal, control diet (Zhao et al., 2006).

Although the ketogenic diet has only recently been investigated in the field of oncology, several animal models have found promising effects, such as decreased tumor growth rates in animals with experimentally produced brain tumors, reduction of reactive oxygen species (ROS) production in malignant glioma cells, and a reduction in the expression of genes that affect glioma growth downstream (Stafford et al., 2010). It is not clear whether these effects result from ketogenic diet-induced ketosis or mere caloric restriction (i.e. reduced blood glucose levels). Clinical evidence supporting the effectiveness of ketogenic diets in cancer is limited and quite variable depending on the type of tumor and the organ system of interest (Politi et al., 2011).

Several animal studies have also evaluated the effectiveness of the ketogenic diet in relation to brain injury and related, long-term consequences of traumatic brain injury such as epilepsy. While findings surrounding the diet and anti-epileptogenesis following head injury are extremely mixed, animal studies have demonstrated that pre-treatment with the ketogenic diet significantly reduced cortical contusion volume in a controlled cortical impact (CCI) injury model, prevented apoptotic
neurodegeneration in a weight drop model, and enabled significant tissue sparing in
the brain following CCI injury (which was due to ketosis rather than hypoglycemia in
fasting) (Appelberg et al., 2009; Rho and Stafstrom, 2012).

Ketogenic Diet and Autism Spectrum Disorders

In 2003, Evangeliou et al. conducted a pilot study evaluating the effects of an
intermittent ketogenic diet on children between four and 10 years of age displaying
autistic behaviors. A modified John Radcliffe diet, a variant of the ketogenic diet, was
administered to 30 children living on the island of Crete for six months, alternating
between four-week intervals of ketogenic diet and two-week intervals of a normal,
control diet. Participants completed the Childhood Autism Rating Scale (CARS)
before and after dietary intervention, and the data revealed that 10 of the 18
participants who adhered to the diet for the entire duration of the study demonstrated
moderate or significant improvement (Evangeliou et al., 2003; Rho and Stafstrom,
2012).

Recent research has demonstrated that a 6.6:1, highly restrictive, ketogenic
diet significantly alleviates the three core symptoms of ASD in the BTBR T<sup>+</sup> tf/J
mouse model of autism (Masino et al., 2012). Mice fed the ketogenic diet
demonstrated increased sociability in the three-chambered test of sociability,
decreased self-directed repetitive behaviors (i.e. self-grooming), and improved social
transmission of food preference (STFP; a measure of communication). Mice fed the
ketogenic diet also exhibited significant changes in blood chemistry, most
importantly increased ketones and decreased glucose levels (Masino et al., 2012).
While the metabolic changes that result from the ketogenic diet have been
consistently reported, thus far the ways in which autistic behaviors are alleviated remain unknown.

C. BTBR T<sup>+</sup> tf/J MOUSE MODEL OF AUTISM

Behavioral Characterization

The BTBR T<sup>+</sup> tf/J (BTBR) strain of mice displays a behavioral phenotype that corresponds to the three core diagnostic symptoms of ASD in humans. Several relevant behavioral assays, such as the three-chambered test of social approach (also called a test of sociability), STFP, and test of self-grooming, demonstrate the strain’s deficits in sociability, difficulties with communication, and stereotypic, repetitive behaviors (McFarlane et al., 2008).

In the social approach apparatus, BTBR males fail to show preference for the side chamber containing the stranger mouse (i.e. the social chamber) (Moy et al., 2004; McFarlane et al., 2008). Similarly, BTBR mice engage in significantly less social interaction when given the opportunity to interact with another juvenile mouse (McFarlane et al., 2008). BTBR mice display significant reductions in social interactive behaviors (approach, follow, flight, grooming another, huddling) and significant increases in non-dyadic behaviors (self-grooming and isolation) (Pobbe et al., 2011).

Furthermore, the BTBR strain exhibits difficulties with communication. In the STFP, BTBR mice ate less of the familiarized food (whose flavor the demonstrator mouse carried) in comparison to the control mice (C57BL/6J; commonly referred to as B6), which interacted more with the demonstrator mouse when placed in the same
cage. Furthermore, BTBR mice sniffed the whiskers and mouse of the demonstrator mouse less frequently and for shorter amounts of time (McFarlane et al., 2008).

In addition, BTBR mice display atypical isolation-induced ultrasonic vocalizations (typically emitted by pups after they have been separated from their mother and littermates during the first two weeks of life), reduced interaction-induced ultrasonic vocalizations (typically emitted by juvenile mice when engaging in social investigation during weeks three and four of life), and minimal ultrasonic vocalizations in response to female urine of both BTBR and B6, or control, mice (Wöhr, 2012). The absence and/or decrease in these vocalizations provide strong evidence for BTBR’s deficits in communication.

Finally, the BTBR strain consistently displays stereotypic and/or repetitive behaviors. In general, BTBR mice spend significantly more time engaging in self-directed grooming in comparison to control mice and fail to reverse spatial habits in the Morris water maze task (Moy et al., 2007). Research has explicitly dissociated the social deficits mentioned above from an abnormal stress reactivity response (Silverman et al., 2010). Furthermore, BTBR mice display normal measures in terms of general health, motor functions, and sensory abilities (most importantly, olfaction) (Moy et al., 2007). These findings confirm that the strain’s decreased performance on the STFP task results from low sociability, rather than worsened sensory abilities.

It is important to note that these distinctive, phenotypic qualities vary between gender but, overall, the evidence supporting the BTBR strain’s validity as a mouse model for autism is quite extensive. As with ASD, the mechanisms that underlie each
of these behavioral characteristics still remain unknown. Future research should investigate what factors contribute to these deficits.

*Anatomical and Genetic Characterization*

In addition to behavioral similarities, the BTBR strain displays several genetic, neuroanatomical, and physiological features that are similar to those found in patients with ASD. The myriad of the abnormalities that have been identified in the BTBR strain accurately mirrors the complexity of ways in which ASD may manifest in the clinical population, and acknowledges the inability for the etiology of ASD to be defined by any one genetic malfunction, neuroanatomical abnormality, or physiological symptom.

Most often, B6 control mice, an inbred strain with high sociability, serve as the comparison strain to BTBR. However, the B6 strain is not a perfect genetically-matched control. The BTBR strain was bred by crossing mice carrying the wildtype T (brachyury) gene with the stock carrying tufted (tf) mutation (Meyza et al., 2012). Then, the BTBR strain was out-crossed to the 129P1/ReJ strain mice to enhance breeding ability (Clee et al., 2005). The significant anatomical and genetic differences that exist between the BTBR and the B6 strain make it extremely difficult to determine causal relationships between any one abnormality and the behavioral manifestations that are observed. The most relevant characteristics of the BTBR strain are those that closely parallel characteristics observed in the clinical population with ASD.

The BTBR strain displays several single nucleotide polymorphisms (SNPs). The strain’s altered *Kmo* encoding kynurenine 3-hydroxylase is a conserved domain
that is also found in humans. This SNP is particularly relevant to ASD because kynurenine 3-hydroxylase is involved in the synthesis of kynurenic acid, which is an antagonist of glutamate and nicotinic receptors and may indirectly influence neuroprotection, dendritic spine formation, and dopamine release (Meyza et al., 2012).

In addition, the BTBR strain carries a Disc1, or Disrupted in Schizophrenia, mutation, which has been found to be associated with several psychiatric disorders, especially schizophrenia, in humans. A significant association was found to exist between the Disc1 mutation and infantile autism as well as Asperger syndrome (Kilpinen et al., 2008). It has been hypothesized that this abnormality may affect proper formation of the corpus callosum and influence the ability for newly born neurons to migrate and become incorporated into this brain structure (Meyza et al., 2012).

Another gene of interest, exostosin 1 (Ext1), is down-regulated in the BTBR strain. This gene is thought to be responsible for the synthesis of guidance molecules that are necessary for neuronal fibers to cross the midline of the corpus callosum during development. The down-regulation of Ext1 provides a potential explanation for the strain’s complete absence of a corpus callosum. While the clinical population does not demonstrate this severe of a deficit, research has found that individuals with ASD have a significantly smaller corpus callosum in comparison to controls (once individuals with macroencephaly were excluded from analyses) (Kilian et al., 2008). It is possible that decreased myelination or excessive axonal pruning cause this reduction in corpus callosum size in those with normocephalic ASD (Kilian et al.,
Ext1 is also involved in the biosynthesis of heparan sulfate, which will be discussed next.

Similar to individuals with ASD, the BTBR strain also demonstrates diminished concentrations of laminin and heparan sulfate levels (Meyza et al., 2012). Both of these fractions, which are components of the extracellular matrix of the subventricular zone of the lateral ventricles, are thought to influence growth and guidance factors during development. Heparan sulfate is critical in the development of the central nervous system and plays an important role in the organization of neural connections during development, which are implicated in ASD (Blanchard et al., 2012).

Finally, substantial forebrain reductions were noted in several neurodevelopmental proteins (i.e. PSA-NCAM, NG2, NeuroD and DCX). The downregulation of these proteins provides a potential explanation for decreased neurogenesis in the dentate gyrus of the hippocampus and impaired development of the hippocampal commissure, which may help explain the failure for the BTBR mice to develop typical, social behaviors (Kusek et al., 2007; Meyza et al., 2012).

The Limitations of Translational Research

While several behavioral and physiological similarities exist between the BTBR strain and humans diagnosed with ASD, there are many limitations inherent in translational studies. An animal model is unable to replicate a human disease and, especially in the case of ASD, one mouse model cannot fully encompass the heterogeneous manifestations of a spectrum of disorders that is centered on human sociability, communication, and behavior. However, the BTBR strain does provide a
conceptual analogy to the three core deficits in ASD and can be helpful in gaining a better understanding of the biological and genetic underpinnings of these disorders.

D. THESIS OVERVIEW AND HYPOTHESIS

The current study investigated the effects of a 3.2:1 ketogenic diet on the BTBR mouse model of autism. Previous research, which demonstrated the efficacy of a 6.6:1 ketogenic diet in the BTBR mouse model, prompted interest in a less stringent, more clinically relevant diet composition. We predicted that the 3.2:1 ketogenic diet would improve sociability and communication and reduce stereotypic, repetitive behaviors, thus alleviating the three core symptoms of ASD.

IV. METHODS

E. Animals

Animal procedures were performed in accordance with the NIH Guide for Care and Use of Laboratory Animals. All procedures were approved by the Institutional Review Boards of Trinity College. All animals used were BTBR T+ tf/J mice. Original breeders were purchased from Jackson Laboratories (Bar Harbor, ME), and the animals used in the experiments were bred on-site. Mice were housed with their mother until weaning at three weeks of age. Home cages were polycarbonate and 26.67 cm in length, 48.26 cm in width, and 15.24 cm in height (Ancare, N40 Series). During this time, all mice were fed normal chow (CD; LabDiet 5001, W.F. Fisher & Son, Somerville, NJ), which was available in the cage. At weaning mice were separated from their mother, grouped by sex, and fed with normal
chow until five weeks of age. At this time, one half of the mice were assigned to the ketogenic diet with a 3.2:1 fats to carbohydrates and protein ratio (product no. F5848; Bio-Serv, Frenchtown, NJ) and remained on this diet for at least 21 days before undergoing any behavioral testing. The other half of the mice were switched from LabDiet 5001 to a modified, control diet (product no. F6588; Bio-Serv, Frenchtown, NJ), which was matched to the F5848 test diet for protein, mineral, and vitamin content. The modified, control diet had a fat: carbohydrate + protein ratio of less than 0.1:1.

Animal weights were recorded before any dietary intervention and after being on the ketogenic diet or the modified, control diet for three weeks. Tail blood was taken from animals after at least three weeks of dietary intervention for glucose and ketone analysis. The behavioral tests were conducted when the mice had been on the experimental diets for three to five weeks. After all behavioral tests were conducted all of the mice that had been on the ketogenic diet (n=14) were switched to a modified, control diet for one week. The three-chambered test of sociability was completed a second time to evaluate the effects of diet reversal after one week. Different stranger mice were used to maintain social novelty.

F. Behavioral Tasks

*Three-Chambered Test of Sociability*

The three-chambered sociability test was conducted to compare relative sociability in BTBR mice before and after dietary intervention. The three-chambered sociability apparatus (Fig. 1) was made of transparent Plexiglas with manually
removable doors, which divided the space into three separate chambers. Wire cups were placed in both side chambers for the entire duration of the experiment. In phase two, one of the wire cups housed a stranger mouse and the other was left empty. These wire cups had a diameter of 10.41 cm, a height of 10.92 cm, and 1.02 cm bar intervals (Galaxy Pencil Cup).

Figure 2. Three-chambered sociability apparatus (Moy, n. d.)

Before beginning the test of sociability, stranger mice were enclosed in the wire cup for 30 minutes to habituate to the environment and apparatus. Simultaneously, the test mice were habituated to the test room. After this habituation period, the stranger mice returned to their home cage, and the apparatus was washed with hot water and soap, and dried thoroughly.
Figure 3. **Schematic of the three-chambered test of sociability:** A) 30-min. habituation of stranger mice to apparatus B) 10-min. habituation of test mouse to center chamber only, followed by 10-min. period when test mouse has access to entire apparatus to measure if any baseline side preference exists (phase one) C) Stranger mouse is placed in one side chamber for 10 min., measuring sociability of test mouse (phase two) (Svedova, 2011).

The test mouse was then habituated to the center chamber of the sociability apparatus (both side chambers were inaccessible) for 10 minutes. Afterwards, the side doors were lifted, and the test mouse was allowed to explore the entire apparatus (all three chambers) for 10 minutes (phase one). This 10-minute session, in which the test mouse has access to the entire apparatus, served as a baseline measure to determine if the mouse had an initial preference for any one chamber. Following phase one, the test mouse returned to the center chamber, the side chambers were closed off, and a stranger mouse was placed under the wire cup in one of the side chambers. The doors
were lifted, and the test mouse had access to all three chambers (one of which housed the stranger mouse) for 10 minutes (phase two). This session served as a measure of relative sociability by comparing how much time the test mouse spent in the chamber with an empty wire cup versus the chamber with a stranger mouse housed in the wire cup. The three-chambered sociability tests were video recorded to allow for (1) blind scoring and (2) a more thorough analysis of behaviors observed.

Evaluation of Repetitive Behaviors

Both the self-grooming test and the marble-burying test were conducted to measure repetitive behaviors in BTBR mice. In the self-grooming test, eight to ten week-old mice were habituated in an empty transparent cage (19.05 cm in length, 29.21 cm in width, and 12.7 cm in height) for 10 minutes. Following this habituation period, the mice were videotaped for 10 minutes in the apparatus. Scorers blind to the diet treatment recorded the time mice spent self-grooming. Self-grooming was defined as any time the mouse is engaging in licking, scratching, or stroking its body. Self-grooming was also recorded in the three-chambered test of sociability. Time spent engaging in repetitive behaviors during phase two was scored blind and expressed in terms of total time in apparatus (phase two = 600 seconds).

The second test of repetitive behaviors was the marble-burying test, in which fifteen marbles were placed evenly in a three by five fashion, in a transparent, plastic cage (10.5” x 19” x 8”) that was filled with five cm of flattened wood chip bedding. The test mouse was placed in the cage for 30 minutes. After this 30 minute period, a digital picture was taken vertically above the cage, and Adobe Photoshop was used to count pixels of marbles using the “Quick Selection” tool, and the total outline of the
bedding area was measured using the “Polygonal lasso” tool. Marble pixels were expressed as a percent of total bedding area pixels.

_Social Transmission of Food Preference_

Before beginning the STFP task, all mice were habituated to eating from jars for 48 hours prior to testing. On day one, all mice were fasted for 18 hours with cagemates. After this period of fasting, the demonstrator mouse was placed in a new home cage with one flavored food for two hours. The weight of the flavored food and jar was recorded before this two-hour period. The three flavors tested were 2% cocoa, 1% cinnamon, and 0.25% cumin, all of which were administered in powered diet form in a glass jar. After two hours, the amount of flavored food was weighed a second time to confirm that the demonstrator mouse had consumed at least 0.5 grams of the flavored food provided. The demonstrator mouse was then put into a food-free cage with no more than four observer mice for 30 minutes. After 30 minutes, the observer mice were removed and each put into separate cages, each with two jars containing a different flavored food. One jar contained the trained food, and the second jar contained one of the other two, untrained flavors listed earlier. The two jars were weighed before and after the two-hour period when the observer mice had access to the two flavored options. The amount of each flavored food that was consumed by the observer mouse was recorded.

_Statistical Analyses_

In the three-chambered test for sociability, chamber preference was expressed as the ratio of time spent in one side chamber in relation to the total time spent in both
side chambers. A repeated-measures analysis of variance was employed to analyze (1) the time mice spent in each of the two side chambers of the three-chambered sociability apparatus to evaluate relative sociability (phase two), with factors of diet and phase after the three-week dietary intervention (N=27) and after the one-week reversal to the modified, control diet (N=14); (2) social transmission of food preference, with factors of diet and flavor (trained versus untrained); and (3) animal weights, with factors of diet and time point (baseline and after three-week dietary intervention). A Student t-test was employed to analyze (1) time spent grooming in the three-chambered test of sociability, (2) time spent grooming in the self-grooming test, and (3) blood chemistry data. A Mann-Whitney test was employed to analyze marble-burying data.

V. RESULTS

Body Weight Analysis

Following a three-week period of dietary intervention, both the ketogenic diet and the modified, control diet produced a similar pattern of weight gain. There were no significant differences between control diet-fed and ketogenic diet-fed animals at either time point (see Fig. 4). This finding illustrates that the ketogenic diet neither impeded growth nor promoted obesity in comparison to the control diet. The two-factor analysis of variance did show a significant main effect for time point (baseline vs. three-weeks), $F(1, 25) = 117.875, p < .001$; did not show a significant main
effect for diet, $F(1, 25) = .332, p > .5$; and did not show a significant interaction between time point and diet, $F(1,25) = .633, p > .4$.

**Figure 4: Average body weight (grams) before and after three-week dietary intervention.** Both dietary treatments caused a significant increase in body weight after the three-week period. * Indicates a significant increase in body weight of both treatment groups over the three-week span. There was no significant difference between the average weight of animals on the modified, control diet (n=13) and those placed on the ketogenic diet (n=14) at the three-week weigh in.

**Blood Chemistry Analysis**

Following ketogenic diet intervention, mice demonstrated a slight increase in glucose levels, although this finding only produced a weak trend ($p = .083$). In contrast, $\beta$-hydroxybutyrate levels increased significantly in mice fed the ketogenic diet for three weeks ($p \leq .001$).
Table 2: Blood chemistry data

<table>
<thead>
<tr>
<th>Diet</th>
<th>Control diet</th>
<th>Ketogenic diet</th>
<th>Sig. (2-tailed)</th>
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</thead>
<tbody>
<tr>
<td>Glucose (mg/dl)</td>
<td>$X = 132.5$, $SE = 4.4$</td>
<td>$X = 145.9$, $SE = 5.8$</td>
<td>.083</td>
</tr>
<tr>
<td>ß-hydroxybutyrate (mmol/L)</td>
<td>$X = 0.162$, $SE = 0.018$</td>
<td>$X = 0.643$, $SE = 0.092$</td>
<td><strong>.000</strong>*</td>
</tr>
</tbody>
</table>

* Indicates a significant increase in ketone levels (as measured by ß-hydroxybutyrate) in mice fed the ketogenic diet in comparison to mice fed the modified, control diet.

Three-chambered Test of Sociability

![Figure 5](image)

**Figure 5. Relative sociability as measured by the three-chambered test of sociability.** There was no significant difference in time spent with the stranger mouse versus an empty cage (phase two) between mice fed the modified, control diet (n= 13) and those fed the ketogenic diet (n= 14). Neither group demonstrated a baseline preference for either of the side chambers, as indicated by phase one.

The ketogenic diet did not seem to improve relative sociability in the BTBR T+ tf/J mice. Although mice fed the ketogenic diet spent slightly more time in the chamber with the stranger mouse than those fed the modified, control diet, this
difference in relative sociability was not statistically significant (see Fig. 5). The two-factor analysis of variance did not show a significant main effect for phase, $F(1, 25) = .609$, $p > .4$; did not show a significant main effect for diet, $F(1, 25) = .142$, $p > .7$; and did not show a significant interaction between phase and diet, $F(1,25) = 1.851$, $p > .18$.

![Graph showing time in target chamber vs. control and ketogenic diets](image)

**Figure 6. Relative sociability following one-week reversal to control diet.** Reversal back to a modified, control diet after three-week treatment of the ketogenic diet (n=14) did not significantly affect time spent with the stranger mouse versus the empty cage in phase two.

As expected, since the ketogenic diet did not have a significant effect on sociability, mice switched back to the modified, control diet for one-week displayed no significant change in sociability (see Fig. 6). The two-factor analysis of variance did not show a significant main effect for phase, $F(1, 26) = 2.121$, $p > .16$; did not
show a significant main effect for diet, $F(1, 26) = .553$, $p = .47$; and did not show a significant interaction between phase and diet, $F(1, 26) = .858$, $p > .37$. Thus, switching to or from the ketogenic diet did not alter sociability.

*Evaluation of Repetitive Behaviors*

The self-grooming test was conducted to evaluate the effect of the ketogenic diet on repetitive behaviors in BTBR mice. After the three-week period of dietary intervention, the ketogenic diet did not have a significant effect on the amount of time a mouse spent engaging in self-grooming behaviors ($p > .3$) (see Table 3). Self-grooming was also measured in phase two of the three-chambered test of sociability. The ketogenic diet did not have a significant effect on the amount of time a mouse spent engaging in self-grooming behaviors in this task either ($p > .62$).

<table>
<thead>
<tr>
<th>Table 3: Grooming Data (all in seconds)</th>
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<tbody>
<tr>
<td><strong>Test</strong></td>
</tr>
<tr>
<td>Single-chamber</td>
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<tr>
<td></td>
</tr>
<tr>
<td>Three-chambered test (phase 2)</td>
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</table>

The marble-burying test was also conducted to evaluate the ketogenic diet’s effect on repetitive behaviors. Contrary to our hypothesis, animals fed the ketogenic diet demonstrated a significant increase in marble-burying, $p = .048$. However, this finding just reached significance (see Fig. 7).
Figure 7: Rank of Amount Buried. In this plot, each animal was ranked by amount of marble burying, which was determined by the percent of visible marble pixels in relation to total bedding area. A larger percentage of visible marble pixels indicates less burying (further to the left on the X axis), whereas a smaller percentage of visible marble pixels indicates more burying (further to the right on the Y axis). Animals fed the ketogenic diet (n=14) buried slightly more than those fed the modified, control diet (n=13). The Mann-Whitney test indicated that this difference in marble burying just reached significance, with $Z(25) = -1.990$, $p = .048$. 
Social Transmission of Food Preference

Figure 8: Average amount of trained vs. untrained flavor consumption. The repeated-measures analysis of variance test revealed that the ketogenic diet had no significant effect on consumption of the trained flavor versus the untrained flavor. No significant differences in flavor consumption existed between animals fed the modified, control diet (n=8) and those fed the ketogenic diet (n=7), p = .269.

Following the three-week period of dietary intervention, communication abilities of all mice were evaluated with the STFP task. The two-factor analysis of variance did not demonstrate a significant main effect for diet, $F (1, 13) = .255$, $p > .6$; did not demonstrate a significant main effect for flavor, $F (1, 13) = .057$, $p > .8$; and did not show a significant interaction between diet and flavor, $F (1, 13) = 1.332$, $p = .269$. Therefore, these findings confirm that there is no STFP in this strain of mice, and that the ketogenic diet had no significant influence on the BTBRs’ communication abilities (see Fig. 8).
VI. Discussion

The current study evaluated the effectiveness of a 3.2:1 ketogenic diet on behavioral symptoms of ASD in the BTBR T^{+} tf/J mouse model of autism. The experiments failed to demonstrate any alleviation of abnormal sociability, improvement of impaired communication, or reduction of repetitive behaviors. Furthermore, following the three-week dietary intervention, mice did not demonstrate the metabolic changes that are typically observed as a result of the ketogenic diet. While blood ketone levels were significantly increased, there was no significant alteration in blood glucose. Furthermore, the increase in ketones on the 3.2:1 ketogenic diet administered here was significantly smaller in magnitude than the increase in ketones observed when BTBR mice were fed the 6.6:1 ketogenic diet (Masino et al., 2012).

Why a metabolic treatment approach for ASD?

Due to the prevalence of metabolic dysfunction in individuals with ASD, it was hypothesized that a metabolic treatment would effectively alleviate behavioral symptoms of ASD. Several neurometabolic disorders, including phenylketonuria (PKU), disorders of purine metabolism, biotinidase deficiency, disorders of cerebrospinal fluid neurotransmitters, and Smith-Lemli-Opitz syndrome, have been classified as having an autistic phenotype (Zecavati and Spence, 2009). Furthermore, research has found a disproportionately high proportion of mitochondrial diseases in those with ASD (Oliveira et al., 2005). Therefore, the ketogenic diet, which has been shown to improve mitochondrial functioning and biogenesis, may offer therapeutic
effects (Bough et al., 2006; Gasior et al., 2006). Furthermore, it has been hypothesized that a ketogenic diet increases adenosine (Masino and Geiger, 2008) and that increased adenosine may alleviate symptoms of ASD (Masino et al., 2009). Preliminary work supports a ketogenic diet-induced increase in adenosine (Masino et al., 2011), a positive relationship between adenosine and ASD (Masino et al., 2011), and the potential for a ketogenic diet to reduce symptoms of ASD (Evangelio et al., 2009; Masino and Rho, 2012).

Metabolic Changes and the Ketogenic Diet

In addition to a difference in the overall dietary ratio (fat: carbohydrate + protein), the 3.2:1 ketogenic diet contained a higher relative amount of polyunsaturated fatty acids (PUFAs) compared to the matched, control diet. Therefore, it is possible that any observed behavioral effects may result from metabolic changes related to ketosis or to PUFA content. However, the absence of behavioral effects, despite significant increases in β-hydroxybutyrate and probable increases in PUFAs, rules out the possibility of either of these compounds directly mediating the therapeutic effects of the ketogenic diet. Instead, this experiment suggests that even during successful treatment (as observed with the 6.6:1 ketogenic diet) these compounds serve as intermediaries; if they contribute to the diet’s effects on sociability and other autism-related behaviors, it is likely indirectly. This is interesting considering previous research, some of which suggests that acetoacetate, acetone, and L-(+)-β-hydroxybutyrate in vitro exert direct anticonvulsant effects (Rho et al., 2002). However, the dose applied in vitro may not extrapolate directly to in vivo metabolic changes precipitated by the ketogenic diet and suggest a cautionary
approach to in vitro studies of a ketogenic diet. At this time, these issues remain unresolved.

Regarding metabolic changes, it may be that increased ketones and decreased glucose are required simultaneously, or that the decrease in glucose is particularly necessary. Furthermore, it is possible that the unexpected metabolic response (i.e. increase in ketone levels, but no significant change in glucose levels) observed in response to the 3.2:1 ketogenic diet was specific to the BTBR strain – a strain that tends towards obesity – and typical blood chemistry changes would be observed in another mouse model of autism. However, it is also possible that mice, in general, are unresponsive to this diet composition. Additional experiments to pursue this are underway. To determine whether mice, as a species, do not respond to the 3.2:1 diet composition, rat models of autism should be tested. Rats prenatally exposed to valproic acid demonstrate behavioral impairments similar to those of ASD, and, therefore, are often used as a model of autism (Bambini-Junior et al., 2011).

Finally, minor modifications to the current protocol may make for a more comprehensive understanding of the 3.2:1 composition’s effects on blood chemistry and would better pinpoint if and when metabolic changes occur. In the future, tail blood should be taken at more frequent time intervals, rather than just after the three-week dietary intervention. It would be advantageous to analyze blood chemistry every few days over the course of the dietary intervention. Perhaps, significant alterations would be observed within the first few days of intervention and then fade over the course of the rest of the protocol. To better understand the relationship between changes in blood chemistry and behavioral effects, it would be beneficial to test for
behavioral improvements at time points when changes in blood chemistry are observed.

**Magnitude of Blood Chemistry Changes**

In this experiment, despite significant increases in β-hydroxybutryate, the 3.2:1 ketogenic diet did not produce significant behavioral effects. However, it is possible that the magnitude of the change in ketones was not significant enough to result in behavioral changes. In this study, β-hydroxybutryate levels increased four-fold in animals fed the ketogenic diet compared to those fed the matched, control diet (see Table 2). In contrast, in a previous study, animals fed the 6.6:1 ketogenic diet demonstrated a ten-fold increase in β-hydroxybutryate levels compared to animals fed the control diet (Masino et al., 2012). Therefore, one possibility is that the 3.2:1 ketogenic diet did not alter ketone levels enough to effectively cause change in behavior, and that there is a dose-dependent relationship between ketones and autistic behaviors.

There is intense interest in developing ketone-enhancing strategies for a number of neurological disorders (Cahill and Veech, 2003; Veech, 2004); for example, ketone esters are currently in development as a dietary supplement (Kashiwaya et al., 2013). To elucidate any dose-dependent relationship relating ketones to improvements in behaviors associated with ASD, future research should study the effects of this less stringent diet composition in combination with an alternative method for specifically increasing ketone levels. Previous research has employed a combined treatment approach to epilepsy using both the ketogenic diet and pharmacological inhibition of CYP2E1, which causes an increase of blood
acetone levels (a ketone that is also increased by the ketogenic diet). This increase in acetone levels may significantly increase antiepileptic effects of the ketogenic diet (Palmer, 2013). A similar approach could be taken with the 3.2:1 ketogenic diet and its ability to alleviate behavioral symptoms of ASD. Perhaps, the effectiveness of this less stringent composition can be augmented when supplemented with another method of increasing ketones, such as a ketone ester or inhibitors of CYP2E1.

*The Confound of Caloric Restriction*

There has been a long-standing confound in ketogenic diet research that perhaps the diet’s therapeutic effects are due, at least partially, to caloric restriction, rather than the composition (particularly very high fat content and very low carbohydrate content). For example, caloric restriction, which reduces total food intake without causing nutritional deficiencies, delays onset and reduces seizure incidence in juvenile and adult EL mice (a model of idiopathic epilepsy) (Greene et al., 2001). Furthermore, in this same study, caloric restriction is a more effective antiseizure dietary therapy than the ketogenic diet in juvenile mice.

The confound of caloric restriction remains a question in this current study. In contrast to the 6.6:1 ketogenic diet, mice fed the 3.2:1 ketogenic diet gained as much weight as those fed the matched, control diet. Therefore, the 6.6:1 composition resulted in restricted weight gain, but the 3.2:1 composition did not. If the ketogenic diet in this experiment had been successful in alleviating behavioral symptoms of ASD, the findings would have served as a piece of evidence against the necessity of caloric restriction. Unfortunately, this was not the case, and the confound of caloric restriction still remains an issue.
Alternative Assessments of Effectiveness

While the 3.2:1 ketogenic diet was not effective in alleviating symptoms relevant to ASD, it is possible that the diet offers therapeutic effects in different domains. In the future, alternative tests should be conducted to evaluate the effectiveness of the 3.2:1 ketogenic diet outside the realm of ASD. As a starting point, it would be beneficial to determine the diet’s effects on epilepsy. If the 3.2:1 composition is to have therapeutic effects at all, it would most likely be observed in models of epilepsy, because several different varieties of the ketogenic diet have been shown to cause significant improvement in patients with intractable epilepsy (Dhamija et al., 2013). Unfortunately, it is possible that this less stringent composition will not even be effective against induced seizures in BTBR mice, a model with mixed efficacy of the ketogenic diet in epilepsy. Overall, some combination of these confounds of dietary composition, metabolic effects, and species issues will help explain why, unlike the 6.6:1 ketogenic diet, the 3.2:1 ketogenic diet was unable to alleviate deficits in sociability, communication, and stereotypic, repetitive behaviors.

If this diet composition is shown to offer antiepileptic effects, further research should be conducted to test its efficacy in different domains. For example, previous research has found the 6.6:1 ketogenic diet to demonstrate anti-inflammatory and analgesic effects in juvenile and adult rats (Ruskin et al., 2009). These experiments employed a hotplate test to measure analgesia (reduced response to thermal pain), and more recent research found that a 3.2:1 diet composition (different composition than the diet tested here) similarly reduces thermal pain responses. Perhaps this balanced,
clinically relevant 3.2:1 composition tested here in a mouse model of autism can also offer these therapeutic effects for other conditions.

VII. Conclusion

The current study demonstrated that the 3.2:1 ketogenic diet (fat to protein + carbohydrate ratio) does not significantly alleviate the three core symptoms of autism in BTBR T+ tf/J mice. This diet composition was less stringent, and therefore more clinically relevant, in relation to the 6.6:1 ketogenic diet, which has proved efficacious in previous research (Masino et al., 2012).

Despite the absence of behavioral changes, application of the 3.2:1 ketogenic diet resulted in elevated ketone bodies (as measured by β-hydroxybutyrate), and emerging data on the relationship between metabolic and behavioral effects may help elucidate key mechanisms underlying the ketogenic diet. Similarly, application of the 3.2:1 ketogenic diet putatively resulted in elevated levels of polyunsaturated fatty acids, which have been suggested to reduce seizure frequency and intensity, but exact mechanisms and critical levels of PUFAs remain unclear. The lack of expected decrease in blood glucose may provide the biggest clue regarding key mechanisms of the ketogenic diet. Here, elevated ketone levels, and a likely elevation in PUFAs, alone, were not sufficient to significantly alleviate behavioral symptoms of autism in the BTBR mouse model.
VIII. References


