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TRINITY COLLEGE

PERSISTENT EFFECTS OF THE KETOGENIC DIET ON THE
CORE SYMPTOMS OF AUTISM IN BTBR MICE

BY

ELIZABETH MAE FOLEY

A THESIS SUBMITTED TO THE FACULTY OF THE NEUROSCIENCE PROGRAM IN
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**Persistent Effects of the Ketogenic Diet on the
Core Symptoms of Autism in BTBR Mice**

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ABSTRACT

BACKGROUND:

The Ketogenic Diet (KD), is a high fat, low carbohydrate dietary regimen, that has been shown to treat a diverse array of medical conditions, most notably the seizure associated with epilepsy. Recent studies have demonstrated that in addition to reducing seizure frequency, the KD has antiepileptogenic effects that continue after reversal to control diet (CD). Such results indicate that the KD does not only suppress seizures in adult mice and rats, but it is potentially effective in attenuating long-term disease progression. Furthermore, studies on autism spectrum disorder (ASD) in a murine mouse model (BTBR mice) found that KD treatment improved autistic behaviors by increasing sociability in a three-chamber test and decreasing self-directed repetitive behavior. This study will use the BTBR mouse model to determine if improvement in ASD-associated behaviors persists when animals are returned to a CD.

METHODS:

BTBR mice at 5 weeks of age were randomly assigned to one of three diet groups: CD, KD, and KD reversal. All mice underwent three-chamber behavioral testing (a test of sociability and self-directed repetitive behaviors in autistic models of mice) followed by two periods lasting three weeks. During the first period, both KD group and KD reversal groups were switched to KD for three weeks and underwent three-chamber behavioral testing. Subsequently, both groups underwent three additional weeks of KD, however in the last 5 days of the second period the reversal group was reverted to the CD and underwent their final three-chamber behavioral test. Body weights and blood chemistry (glucose and ketone levels) of each mouse was measured before treatment (five weeks of age; Test 1) as well as after three weeks of diet administration (eight weeks of age; Test 2) and after 6 weeks of diet administration (eleven weeks of age; Test 3).

RESULTS:

Although it was expected that enhanced sociability would be observed and continue after reversal to the CD, no significant improvements in sociability (characterized by ratio of phase 2, preference for sociability phase, compared to phase 1, the non-social phase) were found in any treatment group. Blood analysis revealed the hallmark characteristics of KD treatment, including weight loss, decreased glucose, and increased β -hydroxybutyrate levels were found in the KD and KD reversal group in Test 2. Additionally, weight glucose, β -hydroxybutyrate levels returned to baseline levels after 5-day reversal to CD.

CONCLUSION:

Despite detected metabolic changes induced by the KD, no notable improvements in sociability were detected when the entire sample size was analyzed. These data conflict with previous research thus ongoing research will consider methodological differences and include additional measures of sociability including frontal contact and self-directed repetitive behaviors.

INTRODUCTION

Autism Spectrum Disorder **Background**

Autism Spectrum Disorder (ASD) is a chronic neurodevelopmental syndrome that emerges in early childhood and affects day to day functioning (Christensen, 2012). ASD, as described in the Diagnostic and Statistical Manual of Mental Disorders, 5th edition (DSM-5), is characterized two main symptoms; persistent social communication impairment and restricted and repetitive patterns of behavior (Melmed & Cubells, 2016). Within the past two decades the global prevalence of ASD has increased twentyfold; in the United States alone, the Autism and Developmental Disabilities Monitoring (ADDM) Network estimates prevalence of 1 in 68 children aged 8 years (Simonoff et al, 2007; Jon Baio, 2010).

Impact

According to the Center for Disease Control (CDC), ASD occurs in all racial, ethnic, and socioeconomic groups and is about 4.5 more common in males (Christensen, 2012). The economic burden of ASD is tremendous and additive throughout life as disorders on the autistic spectrum are lifelong conditions with both direct (medical care) and indirect costs (parental loss in productivity). Reportedly, the total direct cost per year for children with ASD is between \$11.5-\$70 billion US dollars (Lavelle et al., 2014). Moreover, the physiological and behavioral problems associated with ASD - sleeping disturbances, tantrums, self-harm, etc. - are correlated with poor psychological health of other family members, namely mothers (Goin-Kochel, 2007; Hastings, 2003).

Emergence of Autism

The medical diagnosis of autism has been used for over a century beginning in the 1900s with the psychiatrist Eugene Bleuler. Although he studied schizophrenia, Bleuler coined the term autism to denote the social withdrawal symptoms associated with schizophrenia (Melmed & Cubells, 2016). In 1943, the term autism was adapted by psychiatrist, Leo Kanner, who studied mentally disabled children. Kanner published a paper that described case studies of highly intelligent children that were preoccupied with their own internal world and displayed Bleuler's concept of autism. Importantly, Kanner made a distinction between the developed autism in schizophrenic patients and children born with "infantile autism" who displayed disruptions in emotional expression and lack of empathy (Kanner, 1943). Around this same time, Hans Asperger discovered a condition that appeared to be a less severe form of autism which also included high functioning intellectual abilities. However, lack of social and emotional reciprocity and other social communication impairments among patients led to the separate diagnosis of Asperger's syndrome (Asperger & Firth, 1991).

Diagnostic Criteria

Autism was not incorporated into the DSM as a separate diagnostic category until the third edition in 1987. At this point, a checklist of criteria for diagnosing autism was included and focused on three main deficits: (a) social reciprocity, (b) communication, and (c) special interests/repetitive behaviors. In 1994, the fourth edition of the DSM was created and included a revised definition of autism which now fell into the overarching category of pervasive developmental disorders (PDD). PDD included five subcategories: (a) autistic disorder; (b)

Asperger disorder; (c) Rett’s syndrome; (d) childhood disintegrative disorder; and (e) PDD-not otherwise specified (NOS) (Melmed &Cubells, 2016).

The most recent change to the fifth edition of the DSM was published in 2013 and eliminated all subcategories. As of now, the previous PDD subcategories create a single condition known as Autism Spectrum Disorder. The chart in Table 1 outlines the severity levels that create the spectrum. Thus, ASD is not a single condition, but a collection of neurodevelopmental disorders. There are no current physiological or diagnostic tests to determine if individuals fall on the spectrum, only behavioral methods are available for detecting ASD. Thus, careful parental monitoring of unusual patterns of early development is the first line for screening for ASD (Melmed &Cubells, 2016).

Severity level	Social communication	Restricted, repetitive behaviors
Level 3 “Requiring very substantial support”	Severe deficits in verbal and nonverbal social communication skills cause severe impairments in functioning, very limited initiation of social interactions, and minimal response to social overtures from others. For example, a person with few words of intelligible speech who rarely initiates interaction and, when he or she does, makes unusual approaches to meet needs only and responds to only very direct social approaches	Inflexibility of behavior, extreme difficulty coping with change, or other restricted/repetitive behaviors markedly interfere with functioning in all spheres. Great distress/difficulty changing focus or action.
Level 2 “Requiring substantial support”	Marked deficits in verbal and nonverbal social communication skills; social impairments apparent even with supports in place; limited initiation of social interactions; and reduced or abnormal responses to social overtures from others. For example, a person who speaks simple sentences, whose interaction is limited to narrow special interests, and how has markedly odd nonverbal communication.	Inflexibility of behavior, difficulty coping with change, or other restricted/repetitive behaviors appear frequently enough to be obvious to the casual observer and interfere with functioning in a variety of contexts. Distress and/or difficulty changing focus or action.
Level 1 “Requiring support”	Without supports in place, deficits in social communication cause noticeable impairments. Difficulty initiating social interactions, and clear examples of atypical or unsuccessful response to social overtures of others. May appear to have decreased interest in social interactions. For example, a person who is able to speak in full sentences and engages in communication but whose to- and-fro conversation with others fails, and whose attempts to make friends are odd and typically unsuccessful.	Inflexibility of behavior causes significant interference with functioning in one or more contexts. Difficulty switching between activities. Problems of organization and planning hamper independence.

Table 1: Severity levels for autism spectrum disorder (Melmed &Cubells, 2016).

Etiology of ASD

Many studies have aimed to pinpoint the biological underpinnings of ASD, yet a known molecular mechanism of the disorder remains unidentified. Despite this uncertainty, both genetic and environmental factors have been shown to play a role in the pathogenesis of ASD. Studies on siblings have been instrumental in uncovering the heritability of neurodevelopmental disorders. A recent study found concordance rates for ASD among identical twins (that share 100% of their genetic material) to be significantly higher than concordance rates of both fraternal twins and non-twin siblings (both share 50% of their genetic material): 80%, 30%, and 20%, respectively (Rosenberg et al. 2009).

Genetic Role

Although the high concordance rate suggests a strong genetic role, it has been found that autism is genetically heterogeneous, as there is only 5% of a shared genetic alteration (Voineagu et al. 2011). Furthermore, a comprehensive study in the genomic DNA of patients with ASD found that within their sample of 264 families, no genomic variants were identified more than twice, suggesting genetic alterations at multiple loci can contribute to the pathogenesis of ASD (Sebat et al, 2007). With this study in mind, Morrow et al. mapped several loci and determined that a common mechanism that ties together the numerous distinct gene mutations is activity-dependent gene expression (Morrow et al 2008). Although many different mutations are implicated, it is the shared effect on synaptic development that may be responsible for the symptoms of ASD. This idea is the basis of the Rare Allele-Common Disease Theory, which proposes that rare “private mutation” within families ultimately creating a genetic susceptibility for common higher order impairments associated with ASD (Schork et al. 2009).

Additional studies have uncovered changes in social behavior in individuals with autism-risk alleles that carry genetic risk factors yet are unaffected by ASD. For example, it was found that certain identified risk alleles have been associated with higher cognitive abilities based on IQ. This suggests a relationship between genetics and disorders on the autism spectrum, such as Asperger syndrome (Clarke et al., 2016). Furthermore, a parent-rated scale of social and communication impairments indicated that a genetic risk for ASD was associated with altered behavior despite clinical diagnoses of ASD (Robinson et al., 2016). Together, these studies suggest a genetic component of ASD that makes an individual susceptible to the disorder, yet additional environmental influences are responsible for the actual onset of the disorder (Nardone & Elliott, 2016).

Environmental Role

Since 1970, there has been a reported increase in the rate of diagnosed ASD within the United States. This trend has been similarly identified in the U.K. and has fueled the idea that some environmental factors may play a strong role in the increasing incidence of autism (Blaxill, 2004). Studies analyzing exposure to certain environments have identified many factors in association with increased frequency of ASD diagnoses. Notably, exposure to specific teratogens, including thalidomide and valproic acid, have been linked to an increased risk of ASD. In the 1960s, thalidomide was prescribed to treat anxiety disorders as well as morning sickness for pregnant women (Miller, 1991). However, it was discovered that along with congenital disorders inducing malformation of the limbs, approximately 5% of the children exposed to thalidomide in their prenatal environment also developed ASD (Matsuzaki et al., 2012). Similarly, it was found that pregnant women using valproic acid to treat disorders

including epilepsy, migraines, and bipolar disorder, had an 8.9% increased likelihood of having have a child with ASD (Rasalam et al., 2005). Although the exact mechanism is unknown, it is clear that thalidomide and valproic acid in the prenatal environment is associated with increased risk of ASD.

In addition to environmental toxins, maternal diabetes, advanced parental age, low birth weight, and multiple births are all prenatal risk factors for ASD (Hallmayer et al. 2011). Postnatally, gastrointestinal abnormalities, viral or bacterial infection, zinc deficiencies, and abnormal melatonin synthesis have all been associated factors of individuals with disorders on the autistic spectrum (Grabrucker, 2012).

Recent studies regarding prenatal risk factors have led to assessment of maternal health in relation to offspring risk for development of ASD. In a study surveying Danish births from 1980 to 2005, it was found that expectant mothers hospitalized for viral infections (i.e. flu, chickenpox, rubella, etc) during their first trimester of pregnancy had a threefold increase in the incidence of giving birth to a child diagnosed with ASD (Atladóttir et al., 2010). Similarly, a report of Swedish births found a 30% increase in ASD when the mothers were hospitalized for viral infections during pregnancy (Lee et al., 2014). However, unlike the Danish study, this investigation reported a significant effect for an association between ASD diagnosis and viral infection in all trimesters of pregnancy. Such studies regarding viral infections and immune activation supports possible immune-mediated mechanisms as the biological underpinnings of ASD.

In addition to maternal viral infection, a recent study suggests that the presence of maternal autoimmune disorders may also be a risk factor for ASD. Atladottir et al. examined nearly 700,000 Swedish births and discovered that children diagnosed with ASD was associated

with maternal autoimmune disorders such as Rheumatoid Arthritis, Celiac Disease, and those with a family history of Type 1 Diabetes (Atladóttir et al., 2009). Furthermore, an overall study using the Swedish health registry, Keil et al. analyzed maternal health in relation to prenatal health and discovered there was a 60% increase in the odds for developing ASD for children born of mothers with autoimmune disorders (Keil et al., 2010). Although these studies link maternal immune activation and the development of offspring with ASD, they do not determine the exact mechanism.

It is speculated that immune response activation plays a role in the etiology of ASD through epigenetics. Epigenetic regulation represents changes in gene expression by factors other than DNA sequence (Simmons, 2008; Egger et al., 2004). As stated by Goldberg et al., epigenetics is a recently studied phenomenon that represents changes in the final outcome of a locus or chromosome without changing the underlying DNA sequence (Goldberg et al., 2007). DNA methylation and histone modification are two commonly studied epigenetic markers as they impact gene expression without altering the sequence of DNA (Nardone and Elliott, 2014). DNA methylation is responsible for alteration in DNA-protein interaction which increases gene silencing and may also influence levels of gene transcription, while histone methylation modify chromatin structure to influence patterns of gene expression (Jones & Takai, 2001; Goldberg et al., 2007). These markers are present during brain development and are associated with periods of neural plasticity, thus is speculated that environmental factors such as viral infections during natal development, toxins, or maternal autoimmune disease may induce epigenetic changes that do not alter genotype yet have a profound effect of phenotype (Spiers et al., 2015; Goldberg et al., 2007). Ultimately, these findings suggest that both the maternal environment and external

environmental factors play a role in epigenetic changes that may be responsible for the biological underpinnings of ASD (Egger et al., 2004).

Neuroanatomical effects of ASD

Despite an inconclusive mechanism, research has demonstrated genetic, environmental, and epigenetic basis of the etiology of ASD. Although the biological underpinnings remain unclear, neuroanatomical differences have been identified. In a comprehensive case study of five adults with ASD, post mortem ultrastructural brain tissue was studied in three core areas implicated in the pathology of ASD: the anterior cingulate cortex, orbitofrontal cortex, and lateral prefrontal cortices. When comparing the anterior cingulate cortex (ACC), a decrease in diameter of long-range axons was discovered among the individuals with ASD (Casanova, 2004).

Compared with control tissue, higher density of medium and short axons within the ACC due to increased axonal branching was discovered suggesting an over-connection of prefrontal regions in ASD. This finding is consistent with previous studies that propose that increased density and branching of short and medium axons is a compensatory mechanism to maintain connectivity because of decreased long-range axons that affect signal transmission delays (Casanova, 2004). Furthermore, the excessive cortico-cortical connectivity is inadequately selective and poorly synchronized leading to impairments in integrating information (Courchesne & Pierce, 2005).

Within the orbitofrontal cortex (OFC), axons of the individuals with ASD had decreased myelin thickness which suggests decreased signal conduction speed. Within the lateral prefrontal cortices (LPFC) no significant differences were discovered (Casanova, 2004). Together, the ultrastructural brain findings provide an outcome of the biological mechanism that underlies

ASD. Inadequate long-range axons, excessive expression of short and medium axons, and decreased myelin thickness in prefrontal regions may help explain the etiology of ASD and the resulting impairments in social communication and repetitive patterns of behavior (Zikopoulos & Barbas, 2010).

Comorbidity

Mental retardation and epilepsy are distinct neurological conditions that often co-occur with ASD. In a comprehensive study, 30% of patients experienced no mental impairment, 30% experienced mild to moderate impairment, while 40% experienced profound mental retardation (Fombonne, 2003). In terms of epilepsy, it was found that 40% of children diagnosed with ASD have some form of epilepsy (Gabis et al., 2005). Other behavioral conditions associated with ASD include hyperactivity, anxiety, depression, and sleeping disturbances (Shaw et al., 2014). Reportedly, children with ASD have an increased likelihood of experiencing a variety of ailments that effectively worsen the core symptoms of ASD. For example, individuals with disorders on the autistic spectrum are 1.8 times more likely to have asthma and food allergies, 1.6 times more likely to have skin allergies, 2.5 times more likely to have severe headaches, and 3.5 times more likely to have colitis (Melmed & Cubells, 2016).

Treatment Methods for ASD

Background

There is currently no known cure for ASD. Moreover, because it is often difficult for clinicians to address the variable manifestations of ASD, an array of treatment methods has been created to address the core symptoms of autism: persistent social communication impairment and

restricted and repetitive patterns of behavior. Current treatment methods include most commonly behavioral therapy, educational intervention, and drug treatment. A combination of early behavioral intervention, including speech and language therapy, and educational programming is regarded as the most common and effective treatment for ASD, targeting the core symptoms (Autism Speaks). Drug treatments such as the atypical antipsychotic risperidone may improve sensory motor behaviors of autism however it had no effect of social communication impairments (McDougle et al. 2005). Alternative treatments include specific diets, mind-body interventions, energy therapies using electromagnetic fields, and homeopathy (Lofthouse, 2012).

The Ketogenic Diet

Background

The ketogenic diet (KD) is an alternative metabolic therapy developed in the 1920s to treat refractory epilepsy. The diet was created after Dr. Conklin, an American osteopathic physician, found that starving the body of food reduced seizure frequency in his pediatric patients (Freeman, Kossoff, & Hartman, 2007). It was further determined by Russell Wilder of the Mayo Clinic in 1921 that a diet consisting of high fat-content foods, adequate protein, and insufficient levels of carbohydrate for metabolic needs would provide appropriate nutrition, yet mimic the biological process of starvation resulting in seizure reduction (Veech, 1004; Barborika, 1928). The original KD was composed of 4:1 lipid: non-lipid ratio with 80% fat, 15% protein, and 5% carbohydrate (Napoli et al., 2014).

The diet was used effectively by clinicians until the 1940s when new anticonvulsant drugs, such as Dilantin, were put on the market. It is notable that breakthrough seizures can occur when carbohydrates are reintroduced into the diet thus strict maintenance of the KD is

required for efficacy (Huttenlocher, 1976). With increased drug development, there was a push from pharmaceutical companies and physicians as well to move away from seemingly stringent treatment methods, such as the KD, and more towards drug treatments. As the KD took the backseat to various pharmaceuticals, it was prescribed less frequently thus physicians became less experienced with the diet. This lack of experience contributed to the misconception that the KD did not work and was difficult to tolerate (Vining et al., 1998).

Although the KD continued to be used clinically in a handful of seizure clinics, a larger reemergence of the KD began in the 1980s after a famous producer, Jim Abrahams founded the Charlie Foundation to Cure Pediatric Epilepsy, named after his son with refractory epilepsy (Vining et al., 1998). After trying multiple drug treatments for his son, Abrahams learned about the KD and brought his son to John Hopkins Hospital to begin the metabolic therapy. Shortly after diet administration, Abrahams was astounded that his son's seizures stopped. Amazed that a dietary regimen could have such a profound impact on his son's health, Abrahams began the Charlie Foundation to spread awareness regarding the beneficial effects of the diet.

In 1994, with funding from the Charlie Foundation, a multicenter study of the efficacy of the KD was conducted in order to create a standardized protocol for the KD and assess outcomes. Careful analysis of 7 medical centers with a total of 51 pediatric epilepsy patients on the KD revealed that the majority of patients that remained on the KD for one year had a up to a 90% decrease in seizures. Remarkably, each center had at least 1 patient become seizure free. This study demonstrated that not only was the diet efficacious, but following the similar protocols the rate of success of the KD was similar among different centers. Despite new anticonvulsant medications, studies on the efficacy of the KD provided evidence that for children

with refractory epilepsy the success rate of the KD exceed the efficacy of pharmaceutical treatments (Vining et al., 1998).

Today, the Johns Hopkins Protocol still exists but has been modified since the early 1990s. Some of these modifications include: lowered lipid: non-lipid ratio, decreased caloric intake from fat (~60-70%), and fat provided as triglycerides esterified with medium-chain fatty acids to address deficits in carnitine metabolism (Kossoff et al., 2007; Liu et al., 2013). Studies on pediatric children of various ages have determined that a 4:1 ratio is typically more efficacious for younger children and a 3:1 ratio is typically more efficacious for older children (Zupec-Kania & Spellman, 2008). Below, Tabel 2, adapted from Zupec-Kania & Spellman (2008), shows an average day on the KD for a three year old pediatric patient.

Breakfast	40g 36% Heavy cream 32g Sausage links 24g Avocado- Haas 5g Canola oil
Lunch	40g 36% Heavy cream 18g Sliced turkey breast 22g Raw cucumber slices and celery sticks 22g Mayonnaise
Dinner	40g 36% Heavy cream 20g Baked cod 43g Roasted cauliflower 23g Butter
Snack	10g Sliced strawberries 28g 36% Whipped heavy cream

Table 2: A typical day on the ketogenic diet for a 3-year-old child. Note that the majority of fats are from heavy cream, vegetable oils, and butter, which represent medium and long chain triglycerides. Adapted from Zupec-Kania & Spellman, 2008.

Ketogenic Diet Mechanism of Action:

As previously mentioned, in the early 1920s Dr. Conklin found that starvation was an effect treatment for reduction of seizure frequency in his pediatric patients. It is generally

accepted that the KD mimics the chronic state of ketosis that is similarly induced by starvation. This occurs by changing the body's form of metabolism, switching from the use of carbohydrate and proteins as the primary metabolic fuel to using the high fat stores (Zupec-Kania & Spellman, 2008). When glucose is not available, mitochondrial β -oxidation of fatty acids in the liver produces ketone bodies in the form of acetoacetate and β -hydroxybutyrate (BHB) (Laffel et al., 1990).

The biochemical metabolism of ketone bodies include ketogenesis in the liver and allows for this fat-derived energy to be used in various organs, most notably the brain. A simple overnight fast would generate ketone bodies which would meet approximately 2-6% of the body's energy requirements, the rest would be provided by stores of glycogen. After a three day fast, about 30–40% of the energy needs would be supplied by ketones (Laffel et al., 1990). In addition to increased blood ketone levels, other metabolic effects of the KD include reduced blood glucose and increased mitochondrial function (Ruskin et al., 2013).

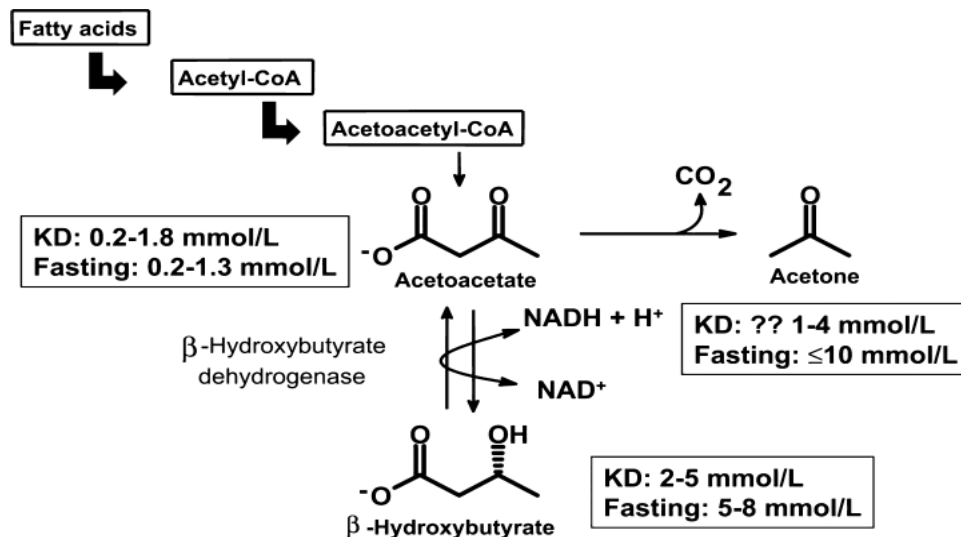


Figure 1: The image outlines the metabolic pathway responsible for the production of ketone bodies during fasting or treatment with the KD. During fasting and KD there is increased acetoacetate, acetone, and β -hydroxybutyrate (relative concentrations are present in the boxes). Due to this metabolic pathway, presence of β -hydroxybutyrate in the blood is used to indicate successful implementation of the KD (Bough & Rho, 2007).

Although it has been almost a century since the diet's conception, the exact mechanism of the KD remains poorly understood. It has been proposed that aside from ketone metabolism, the KD is responsible for other metabolic shifts that underlie the beneficial effects including: modification of the tricarboxylic acid cycle to increase neurotransmitter synthesis (adenosine and GABA), limit reactive oxygen species, and boost energy production in the brain. Such metabolic shifts are the basis for several hypotheses for the mechanism of action of the KD (Nylen, Likhoddii, & Burnham, 2009). Below, five of the most common hypotheses are briefly explored. The hypotheses share many similar components but attribute the clinical effectiveness to different biological aspects induced by the KD.

1. ***The Ketone Hypothesis:*** Research conducted by Juge et. al (2010) has noted that ketone bodies compete with the chloride ions needed for release of the excitatory neurotransmitter glutamate from vesicular glutamate transporters. In addition to this suppression of neuronal excitation, ketone bodies have also been shown to enhance receptor function of the inhibitory neurotransmitter, GABA (Yang et al., 2007). The ability of ketone bodies to suppress glutamate and activate GABA indicates that that ketone bodies decrease the characteristic hyperexcitability associated with seizures. This hypothesis predicts that the ketone bodies present due to KD administration are directly responsible for the anticonvulsant effect on the brain (Nylen, Likhoddii, & Burnham, 2009).
2. ***Metabolic Hypothesis:*** This hypothesis is rooted in the idea that ketone bodies produced by the KD are a more efficient energy source than glucose (Nylen, Likhoddii, & Burnham, 2009). In a study conducted by Appleton and De vivo (1974), it was reported that cerebral

energy reserves were significantly higher in their male albino rats on the KD versus a standard control diet. Appleton and De Vivo attributed this metabolic change to a high-energy state of the brain cells from an increased ratio of ATP: ADP triggered by a high concentration of available fats and reduced carbohydrates. In addition to refractory epilepsy, the KD is also clinically used to treat glucose transporter protein syndrome and pyruvate dehydrogenase deficiency, both of which result in cerebral energy failure (Nordli & De Vivo, 1997). Additional research by Bough et al. (2006) demonstrated that compared to control animals, mice fed the KD for three weeks had a 46% increase in hippocampal biogenesis of mitochondria. These KD fed mice showed an up regulation of transcription factors that encode for mitochondrial protein which suggested an increased ATP production (Bough et al., 2006). The Metabolic Hypothesis postulates that once the body has become accustomed to enzymatic and endocrine changes that accompany the KD, the increased activation of tricarboxylic acid cycle will increase cerebral energy via ATP production which stabilizes neuronal membrane potential, leading to greater seizure resistance (Zajac, et al., 2014; Bough et al., 2006).

3. ***Amino Acid Hypothesis:*** It is suggested that KD modifies the products of the tricarboxylic acid cycle and alters the ratio of amino acid neurotransmitters, enhancing GABAergic inhibition (Nylen, Likhodii, & Burnham, 2009). This hypothesis is rooted in the concept of GABA shunting, which is a regulating response thought to be mediated by the KD. In this theory, the GABA shunting reduces the amplitude of subsequent excitatory postsynaptic potentials and thereby reduces the hyperexcitability (McNally & Hartman, 2012). Depending upon the neuronal network, GABA may have an anticonvulsant or proconvulsant effect, although research has indicated that cerebral GABA levels in male

albino rats are not altered compared to rats fed a standard control diet, local or regional GABA concentration differences may have a large effect on neuronal excitability and thereby reduce seizure frequency (Holmes, 1995; Al-Mudallah et al., 1996).

4. ***Anti-Inflammatory and Lowered Reactive Oxygen Species Hypothesis:*** Previous research has shown that inflammation contributes to the development of chronic epilepsy and various other CNS diseases. Studies involving rodent models demonstrate that induced seizures trigger an inflammatory response that enhances neuronal excitability and propagates epileptic activity (Vezzani & Granata, 2005). Because inflammation is mediated by reactive oxygen species which are generated by polymorphonuclear neutrophils at the site of tissue damage, there is thought that the KD functions through an anti-inflammatory mechanism that effectively lowers reactive oxygen species (Mittal et al., 2014; Gasior et al., 2006). Specifically, a study by Maalouf et al. (2006) suggests that the ketone bodies produced through KD administration may decrease mitochondrial reactive oxygen species production by increasing mitochondrial uncoupling protein levels. This mechanism suggests that ketone bodies are more than a fuel source but they may have a cellular protective effect and function through an antioxidant mechanism (Kim et al., 2007).
5. ***The Adenosine Hypothesis:*** As outlined in the Metabolic Hypothesis, the KD is thought to increase mitochondrial biosynthesis which in turn increases cerebral energy and brain ATP levels. The Adenosine Hypothesis is based on the observation that increased intracellular ATP will equilibrate and result in increased extracellular concentrations of ATP which is dephosphorylated into the neuromodulator, adenosine (Masino & Geiger, 2008). Adenosine is a molecule typically present in the extracellular space of brain tissue

and is associated with neuronal inhibitory effects, sleep promotion, and seizure reduction (Dunwiddie and Masino, 2001). When adenosine binds to adenosine A1/A2 receptors, it has been shown to have an anticonvulsant effect; conversely, antagonism of A1/A2 receptors leads to stimulant effects of rodent locomotion (Marston et al., 1986). Thus, it is thought that the KD increases adenosine A1/A2 receptor activation which hyperpolarizes neurons, closes calcium channels, decreases excitatory glutamate release, and adequately decreases neuronal excitability (Dunwiddie and Masino, 2001).

Proposed Connection: Ketogenic Diet and ASD

The Adenosine Hypothesis identifies key components of the neuromodulator, adenosine, which is ubiquitous in the CNS (Dunwiddie and Masino, 2001). In addition to its role as an anticonvulsant, researchers Poleszak and Malec identified that adenosine A2 receptor activation attenuated amphetamine-induced stereotypy in male Wistar rats, including increases rat grooming and increased head movements that interfere with goal-directed behavior (Poleszak and Malec, 2003; Wolgin, 2012). With the core behavioral symptoms of ASD in mind – persistent social communication impairment and restricted and repetitive patterns of behavior – it has been proposed that activation of adenosine receptors may aid the core symptoms of ASD (Melmed & Cubells, 2016, Masino and Geigers, 2008). Based on the knowledge that epilepsy and ASD have a high rate of comorbidity, a shared underlying biological mechanism of reduced adenosine has been suggested (Brooks-Kayal, 2010). Figure 2 demonstrates the metabolic relationship between ketone bodies (BHB and acetoacetate) and the neuromodulator, adenosine.

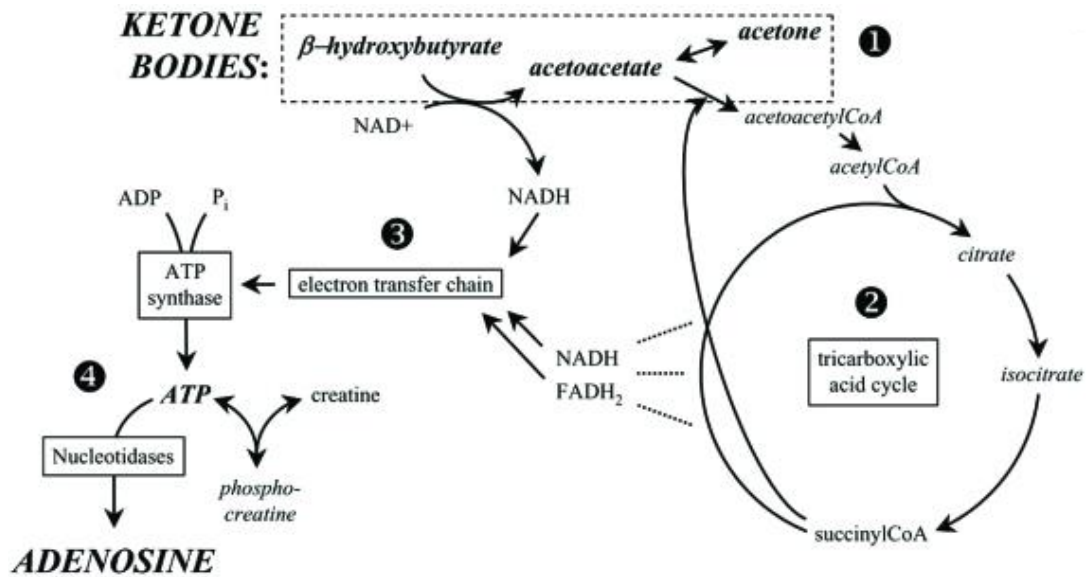


Figure 2: In step 1, ketogenic metabolism yields ketone bodies which are transported to other tissues. In the ketolytic metabolism shown step 2, the ketones have been converted into acetyl CoA which enters the tricarboxylic acid cycle which channels protons and electrons into the electron transport chain. In step 3, the electron transport chain drives a gradient across the outer membrane of the mitochondria which creates ATP via ATP synthase. In step 4, a large ATP/adenosine ratio intracellularly leads to an extracellular increase of adenosine (Masino et al., 2009).

In 2002, a pilot study was conducted on the island of Crete to assess the role of the ketogenic diet in 30 children (ages 4-10 years) with autistic behavior. This population was of interest to researchers Evangeliou et al. (2003) because metabolic testing during a glucose challenge revealed high level of ketone bodies. It was thought that individuals in the population may have a mitochondrial energy production disturbance affecting the tricarboxylic acid cycle. If this is the case, it is supposed that increases ketone body synthesis will be activated in order to generate sufficient ATP. At the beginning of the study all children were classified with mild-moderate (n=2) or severe ASD (n=28). Results indicated that of the patients that continued on the KD for a 6-month period experienced marked improvement in their social behavior, speech, hyperactivity, and other autistic behaviors (Evangeliou et al., 2003). Overall, there was a 60% of

the children on the KD improved on the Childhood Autism Rating Scale (Evangelidou et al., 2003).

Despite the promising results published by Evangelidou et al. (2003) suggesting the KD could help improve the behavioral symptoms to ASD, studies on children with ASD and the KD were not formally replicated until 2013 when researchers Herbert and Buckley conducted a case study of a young female diagnosed with both ASD and epilepsy (Herbert & Buckley, 2013). After pharmacological treatments failed to reduce seizures, she was switched to a gluten-free, casein-free diet and then added a low ratio KD (1.5:1). It was found that after 14 months of diet consumption her seizure frequency was greatly reduced and, most notably, her behavioral symptoms of ASD were markedly reduced; her score on the Childhood Autism Rating Scale decreased from 49 (severe autism) to 17 (score of 30 being the cutoff for ASD).

The beneficial effects of the KD have also been shown in laboratory studies with rodent models. Ruskin et al. (2013) fed juvenile BTBR mice, a murine autistic mouse model, and a strict 6:1 ratio of KD for three weeks. Following behavioral at 8 weeks of age, it was noted that the KD increased social interaction in a three-chamber test of sociability as compared with mice fed the control diet (Figure 3).

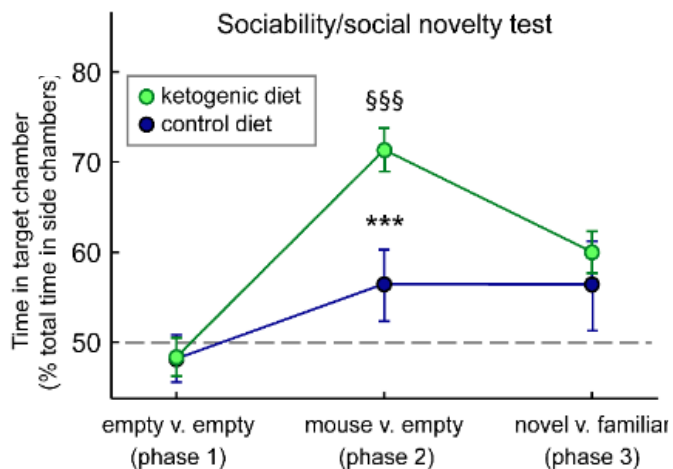


Figure 3: Results demonstrating the effect of the KD in improving behavioral symptoms in BTBR mouse models. Notably, after only 3-5 weeks on KD, improvement in sociability and decreased repetitive grooming behavior was found (Ruskin et al, 2013).

KD and Diet Reversal

More recent studies have used the KD to assess its efficacy in treating a myriad of different disorders including the behavioral symptoms of Rett syndrome, blood glucose levels and weight loss associated with type II diabetes, and the inflammatory environment of cancer (Evangelidou et al., 2003). Research by Lusardi et al., (2015) has considered the KD as a therapy to halt the development of epilepsy. In their study, rodents fed the KD for fifteen weeks showed a prolonged reduction of seizures which continued after a 4-day reversal to control diet. Methods of detecting epigenetic markers, such as a DNA methylation assay, were used to gather total genomic DNA from frozen tissue and assess global DNA methylation of KD and Reversal treatment groups compared to the CD group. Specifically, Lusardi et al. (2015), characterized the hippocampal 5-methylcytosine (5-mC) content with an ELISA based assay.

It was found that animals maintained on the CD had an increased global DNA methylation status in whole hippocampal isolates, whereas KD-fed epileptic animals were found to have reduced levels of DNA methylation. This reduction of global hippocampal 5-mC more similarly resembled the methylation in CD-fed and KD-fed non-epileptic controls. Interestingly enough, this reduction of global hippocampal 5-mC status found to be maintained in the Reversal group over 8 weeks, after the switch back to the CD. Such results indicate that the KD does not only suppress seizures in adult mice and rats, but it is potentially effective in attenuating long-term disease progression by its influence on epigenetics. Lusardi et al. (2015) provide evidence that the KD has an antiepileptogenic disease modifying effect capable of preventing the further development of epilepsy by impacting gene expression (Lusardi et al., 2015). This study provides evidence that there is a lasting effect of the diet that persists even when rodents are reversed to CD.

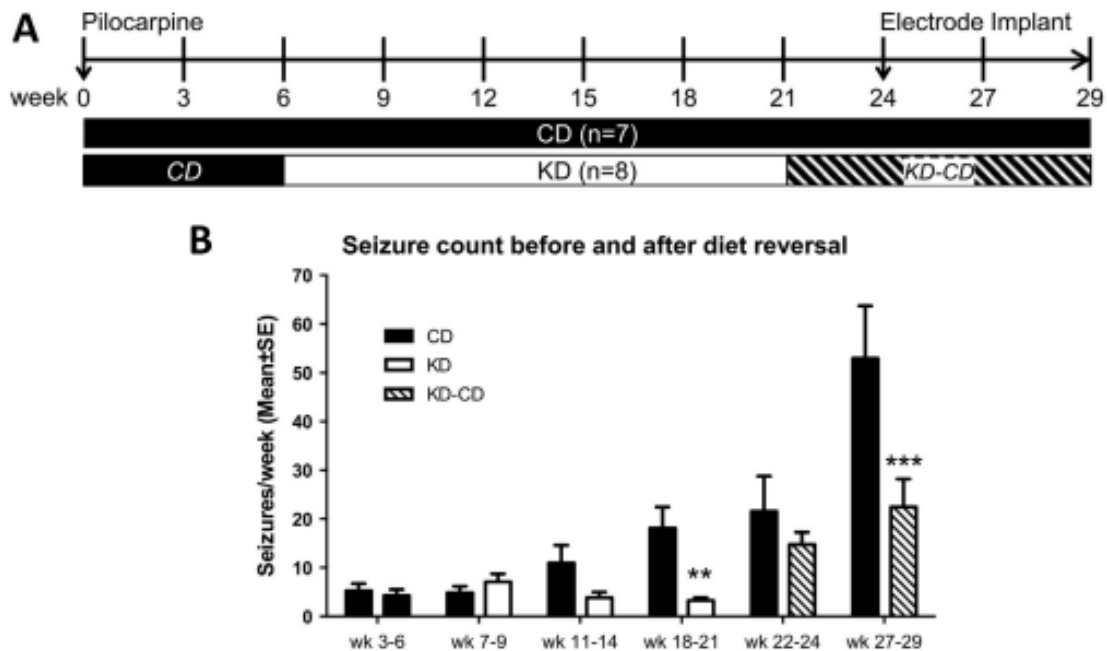


Figure 4: The image demonstrates the antiepileptogenic effects of a KD in the rat model of epilepsy. Experimental design is outlined in A – 6 weeks after drug induced status epilepticus, animals were randomly assigned to KD or CD. KD reversal to (KD-CD) was implemented in week 21. Shown in B, seizures in the KD-fed group were significantly reduced compared to control in weeks 18–21 while still on KD and this improvement persisted in week 27–29, after diet reversal (Lusardi et al., 2015).

Persistent Pro-Social Effect of the KD:

In this lab, a persistent effect of the diet has been noted in previous lab trials regarding the KD and male BTBR mice. In an unpublished study, it was found that after 12 weeks of KD administration, increased sociability was detected in mice that remained on the KD as well as with mice that underwent a 5-day reversal to the CD. Figure 5a demonstrates the timeline of the study, while Figures 5b and 5c outline the significant increase in pro-social behavior detected in phase 2 of the three-chamber test that was expected with mice remaining on the KD (5b) but unpredicted for mice reverted to the CD (5c). This suggests that like the Lusardi et al. (2105)

study, which demonstrated lasting seizure reduction despite diet reversal, the KD may have lasting pro-social effects that persist even when rodents are reversed to CD.

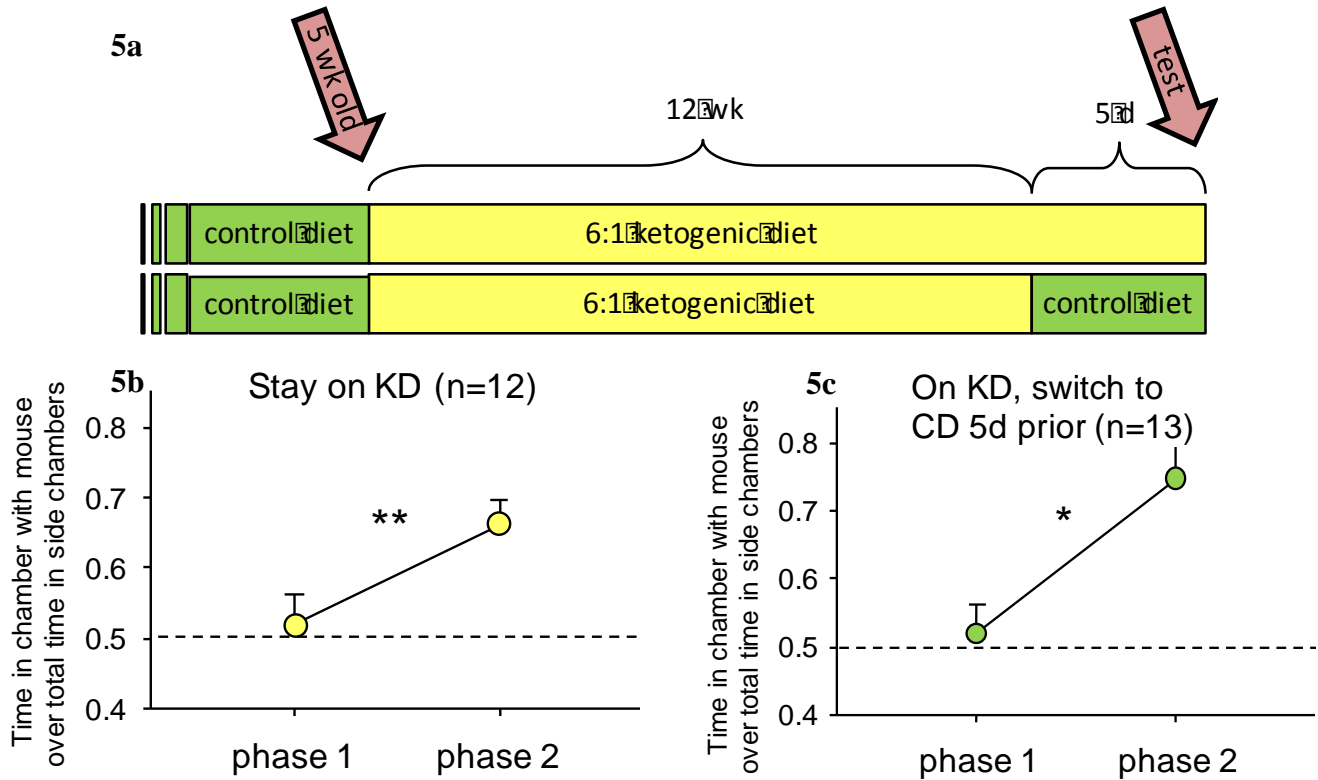


Figure 5: In 5a, the timeline demonstrates KD diet administration and 5-day reversal to CD with a single behavioral test after 12 weeks and 5 days. Figures 5b and 5c show the increase in pro-social behavior detected in both KD and Reversal groups.

THESIS OVERVIEW:

As previously mentioned, there are many methods of treatment that exist to improve the core symptoms of autism, yet none of the various methods have been shown to reverse the disease process. This study is interested in the improved behavioral effects of the KD on autistic mouse models that may outlast diet consumption. This idea is rooted in the findings of Lusardi et al. (2105), regarding prolonged antiepileptogenic effect of the KD as well as findings from our own lab that suggest pro-social behavior induced by the KD may have persistent effects. With these findings in mind, it is expected that enhanced sociability will continue even after reversal to the control diet. This will provide evidence that multiple benefits of the KD can endure beyond the time frame of diet administration. If this expectation holds true, further research may be done to demonstrate that the KD is more than a metabolic treatment that ameliorates symptoms, but a therapy that modifies the course of ASD.

MATERIALS AND METHODS

Ethics Statement

All procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and approved by the Animal Care and Use Committee of Trinity College (A3869-01).

Animals

All behavioral testing was performed in adult male Black and Tan Brachyury *T+ Itpr3tf/J* (BTBR) mice, a mouse model of autism (Jackson Laboratories, Bar Harbor, ME). The BTBR strain is a result of selective inbreeding to develop the favorable phenotypic dark black fur coat with a notable light brown underbelly. In addition to fur color, throughout their lifespan BTBR mice have noticeable hair loss in tufted patterns due to a mutation, *Itpr3tf* (inositol 1,4,5-triphosphate receptor 3; tufted), and they have short tail lengths due to *T mutation* (brachyury). Aside from their morphology, BTBR mice exhibit several symptoms of autism including: reduced social interactions, impaired play, low exploratory behavior, unusual vocalizations and high anxiety as compared to other inbred strains (McFarlane, 2008; Moy, 2007, Scattoni, 2008). Studies conducted for the Mouse Phenome Project indicate that BTBR have neurological difference including an absent corpus callosum and a severely reduced hippocampal commissure (Wahlsten D, 2003). These physiological differences may be the underlying causes of their displayed symptoms of autism.

Study Timeline

At weaning, male BTBR mice (Jackson Laboratories, Bar Harbor, ME) were housed socially (3–6 per cage) and fed the standard rodent chow (CD; LabDiet 5001, W.F. Fisher & Son, Somerville, NJ). At five weeks of age, cages were assigned randomly to remain on control diet (n=11), switched to the 6:1 KD (F3666; BioServ, Frenchtown, NJ) (n=12), or the KD-CD reversal group (n=11). Initial three-chamber behavioral testing began at five weeks of age to provide baseline sociability data for each test group. A second round of behavioral testing occurred in all test groups after three weeks after KD diet administration. Final testing occurred after 6 weeks of CD, KD, and after 5 days of reversal to CD for the KD-CD mice test group. Mice were all fed ad libitum and all measures were taken to minimize animal suffering.

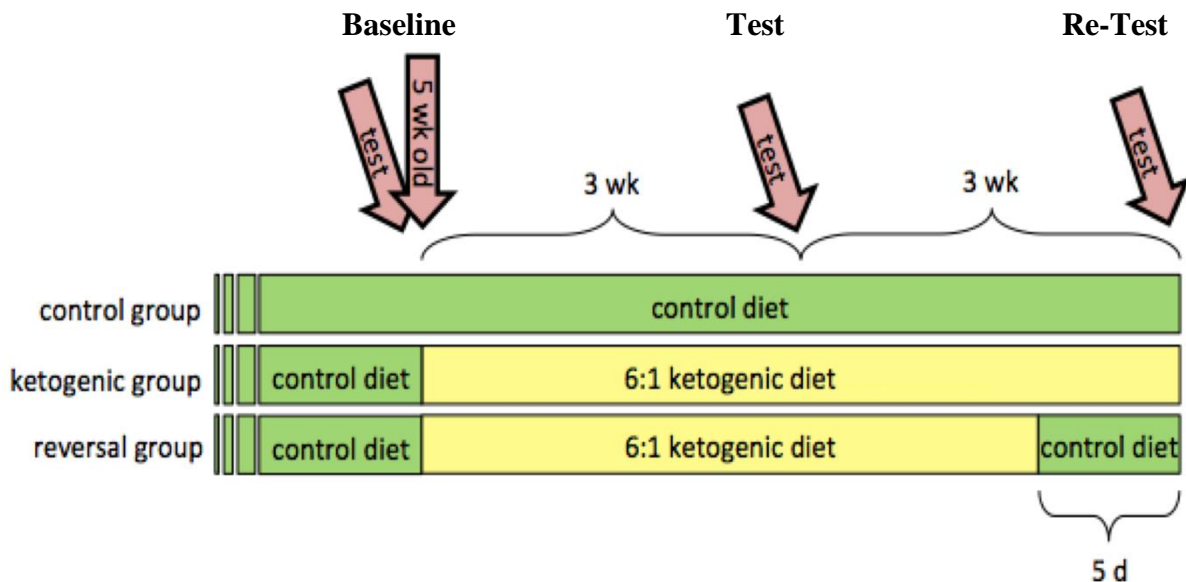


Figure 6: Study design timeline. All mice are male BTBR; Test 1 is a baseline at 5 weeks of age; Test is after 3 weeks on diet; Re-Test is after 6 weeks on diet and after 5-day reversal to CD.

Weight & Blood Analysis: *Ketone and Glucose Levels*

In order to measure blood, all mice were briefly anesthetized by inhalation of isoflurane, a general anesthetic. Blood was then collected by nicking the lateral tail vein. The blood ketone (BHB) and glucose was measured using a Precision Xtra ketone/glucose meter and strips and recorded for each mouse. Increased blood concentration of BHB was taken as evidence of a high concentration of ketone bodies in the blood, ketonemia, in animals fed with the KD. The mice were placed back into their home cage to wake up and recovery from anesthesia.

Behavioral Testing

Three-Chamber Testing

The three-chamber test is used to assess sociability and self-directed repetitive behavior in autistic mouse models. The three-chamber apparatus was made of Plexiglas and consisted of three equally-sized chambers (42.5 cm x 19.1 cm x 22.2 cm) separated by removable doors. These doors block entrance from the center chamber to the left and right side chambers. A wire cage (inverted pencil cup) was set against the back wall of each of the two side chambers. These were present for the entirety of testing. The three-chamber test is made up of two initial habituation phases and three recorded test phases and occurs for a total duration of 40 minutes. Before the start of a testing session, the cage of test mice were placed in the testing room, with the cage filter lid removed, to habituate to the testing room for 30 minutes. Simultaneously, the stranger mice (adult, male, from the same diet treatment as each test group) were placed under the two wire cups in the apparatus and left to habituate to the cups for 10 minutes (Figure 7A). Afterward, the stranger mice were returned to their cage and the apparatus was washed with warm water and soap to remove the scent of the stranger mice as well as any urine and feces

excreted from the stranger mice. Next, the subject mouse was placed in the center chamber with the doors blocking access to both left and right chambers. This lasted for 10 minutes and allowed the subject mouse to be familiar with the apparatus (Figure 7B). After habituation sessions for the stranger and subject mice were completed, a video-recording camera was setup to record the activity of the test mouse in the two 10 minute phases (Phase 1 and Phase 2) of the three-chamber test for each group: CD, KD, and CD-KD reversal.

In the first recorded video session, Phase 1, the doors of the side chambers were removed and the lid placed on top of the apparatus. The removal of the doors allowed the subject mouse to freely explore the left and right side chambers (Figure 7C) and this phase tested for side bias. At the end of the Phase 1 and before the start Phase 2, the subject mouse was gently guided back into the center chamber and the doors replaced, isolated the subject mouse in the center chamber. At this point, one stranger mouse was then placed under a wire cages in a side chamber and the cement block was placed to hold the wire cage in place. Next, the side doors were removed to allow the test mouse full access to the side chambers (Figure 7D). Phase 2 tested for social preference (time the subject mouse spent in the chamber with the stranger vs. time spent alone). At the end of the testing session, all mice were returned to their home cage and placed back in the mouse room. The entire three-chamber apparatus, including the removable doors, wire cages and cement blocks, was washed and dried.

Phase 2: Sociability

As mentioned above, phase 2 measures the tendency of the subject mouse to approach another mouse and engage in social investigation. In Figure 7C, one side chamber contains a stranger mouse that is contained in a wire cup that allows for visual, olfactory, auditory and some

tactile contact to be made while the side chamber contains the empty wire cage. Normal, healthy, mice are highly social animals that typically display social investigation of unfamiliar mice of the same species while BTBR mice demonstrate autistic-like behaviors (reduced social interactions, impaired play, low exploratory behavior) that were expected to interfere with sociability resulting in less time spent with the test mouse.

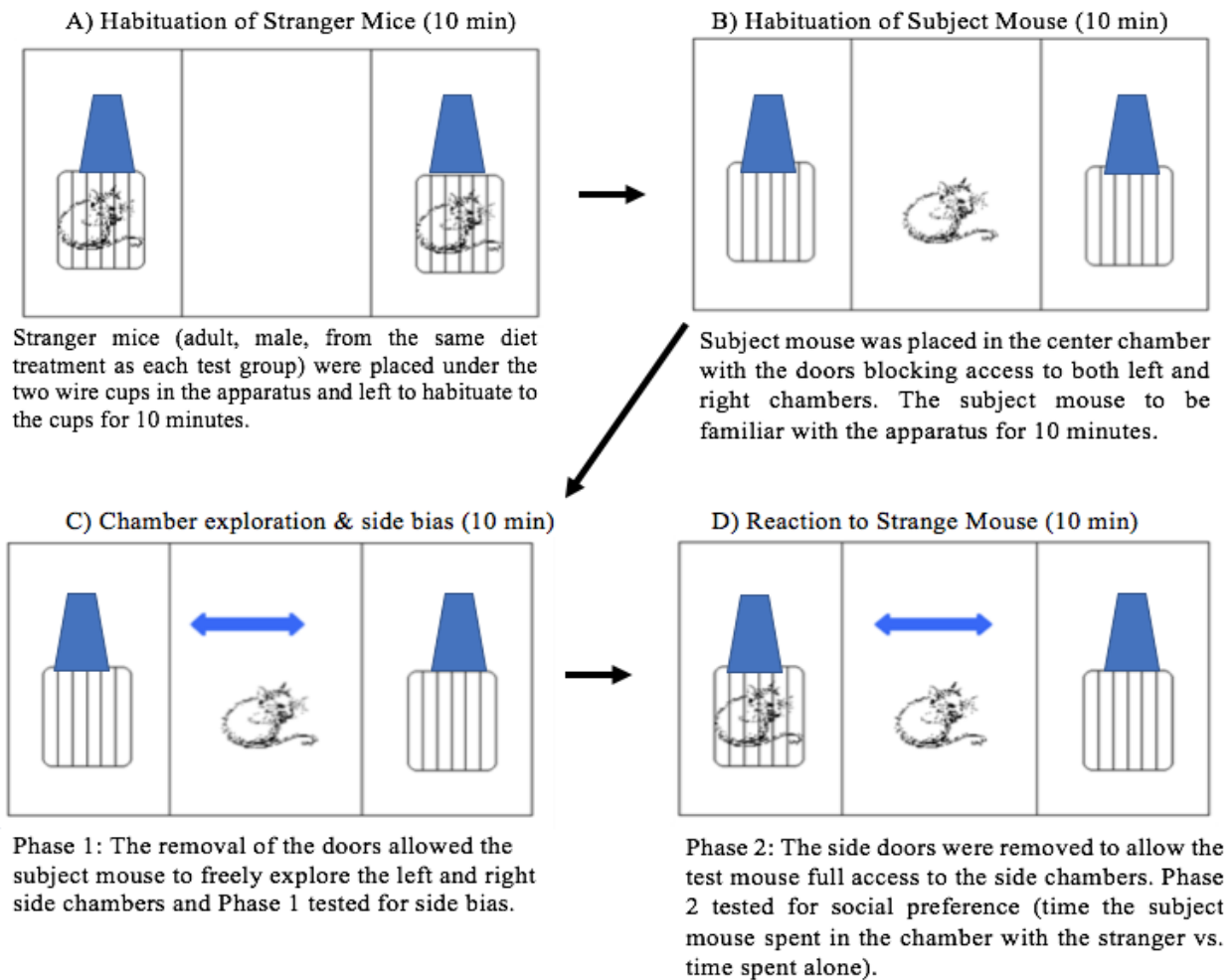


Figure 7: Outline of the three-chamber test. (Svedova, 2011)

Statistical Analysis

Data from the three chamber tests was scored by two independent researchers and at least one scorer was blind to dietary treatment. These data were entered into an Excel spreadsheet then analyzed based on time spent in each chamber. Sociability test in three-chamber Phase 2 test was defined as the ratio of time spent in the social chamber with the stranger mouse to the total time spent in side chambers. Mean, standard deviation, standard error of the mean and t-tests were run on all data and graphed with SigmaPlot software.

RESULTS

Weight & Blood: Ketone and Glucose Levels

Body weights and blood chemistry of each mouse was measured before treatment (five weeks of age; Baseline) as well as after three weeks of diet administration (eight weeks of age; Test) and after 6 weeks of diet administration (eleven weeks of age; Re-Test). Weight loss, increased ketone levels, and decreased glucose levels were found in KD and Reversal groups in the Test after 3 weeks of KD consumption. Additionally, weight, ketone, glucose levels returned to baseline after 5-day reversal to CD.

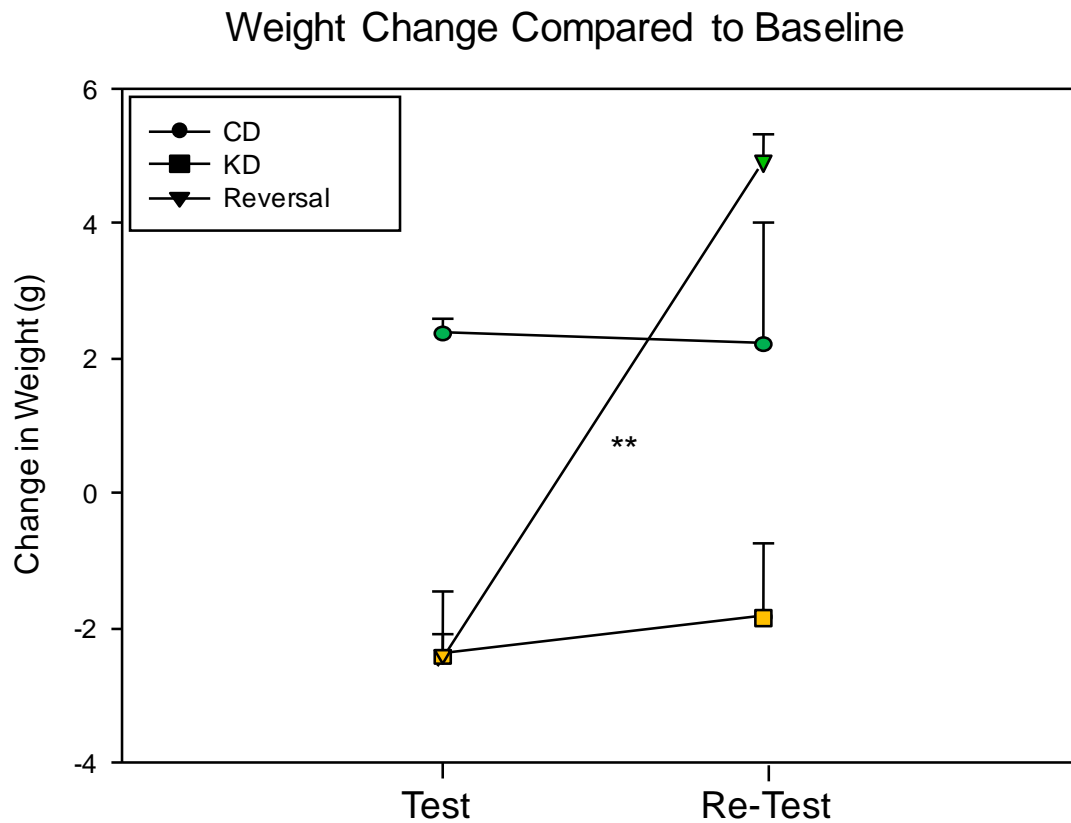


Figure 8: This figure shows change in weights at 5 weeks of age as compared 8 and 11 weeks of age for male BTBR mice in each treatment condition: CD, KD, and KD Reversal. There was a significant weight gain between Test and Re-Test as the Reversal group was switched to the CD (** $p < 0.01$).

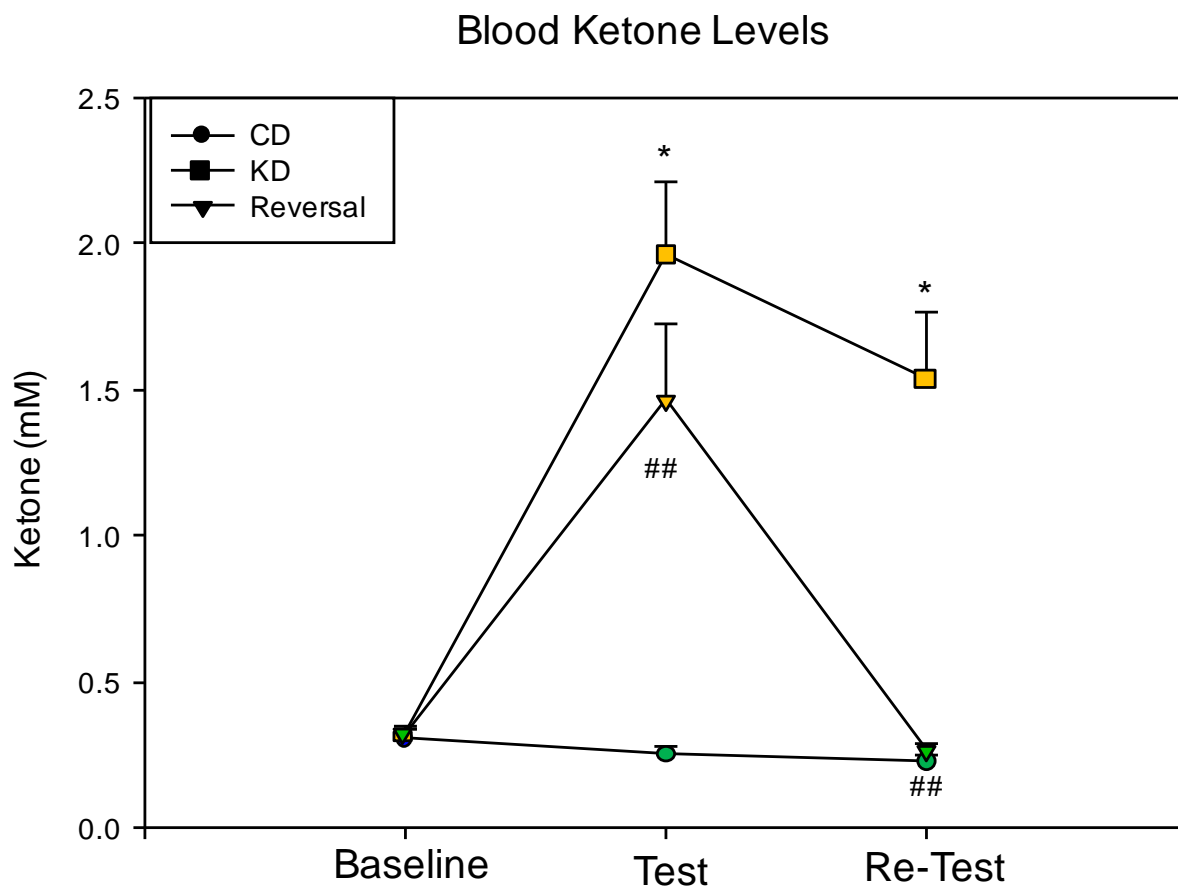


Figure 9: KD increases blood ketone levels while reversal to CD returns ketone levels to baseline. There was a significant increase in ketone levels between the Baseline and first Test in both the KD and Reversal groups (KD: * $p < 0.05$; Reversal ## $p < 0.01$). The KD-fed group continued to have elevated ketone levels, while the Reversal group returned to baseline ketone levels (KD: * $p < 0.05$; Reversal ## $p < 0.01$).

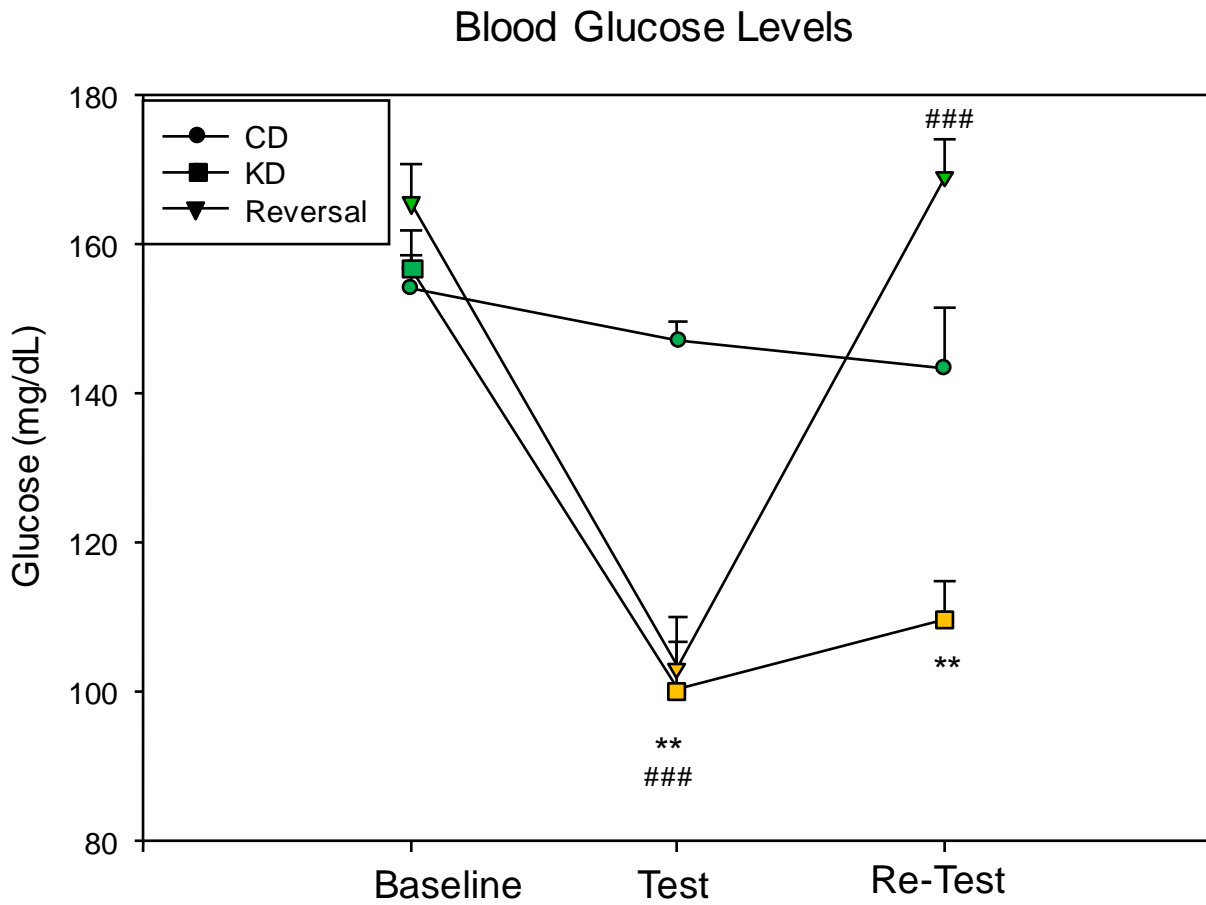


Figure 10: KD decrease blood glucose levels while reversal to CD returns glucose levels to baseline. There was a significant decrease in glucose levels between the baseline test and the first Test in both the KD and Reversal groups (KD: ** $p < 0.01$; Reversal ### $p < 0.001$). The KD-fed group continued to have decreased blood glucose levels, while the Reversal group returned to baseline ketone levels (KD: ** $p < 0.01$; Reversal ### $p < 0.001$).

Three-Chamber Sociability Testing

The ratio of Phase 2, preference for sociability phase, compared to Phase 1, the non-social phase was used to assess sociability in each treatment group: CD, KD, and Reversal. As expected, results suggest that the CD-fed BTBR mice did not spend significantly more time with a mouse-containing versus an empty chamber in any of the three tests, nor were the CD-fed mice found to be asocial. The first treatment group, KD-fed mice, showed neither an overall increase in sociability in the Test after three week of diet administration, nor in the Re-Test (after six weeks of diet administration) with was contrary to previous findings. Similarly, the Reversal group did not demonstrate significant changes in sociability in any of the three behavioral tests.

● = Control Diet

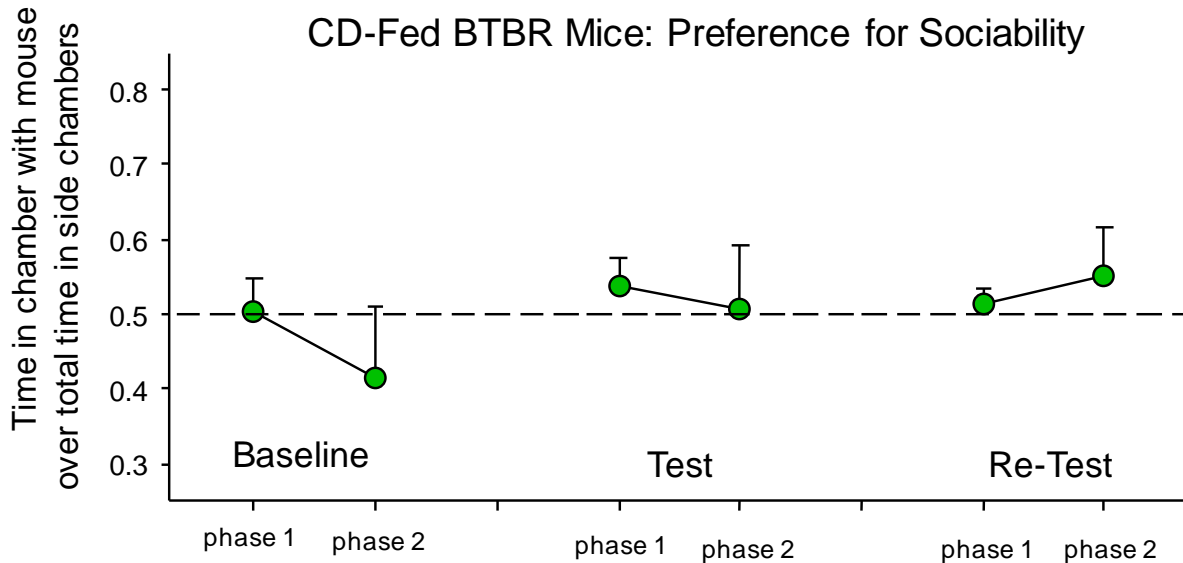


Figure 11: CD has no effect on social preference in CD-Fed male BTBR mice in three chamber test. Figure shows that as expected, CD-fed mice had no significant behavioral changes characterized by time in chamber with stranger mouse in Phase 2 over total time in side chambers (n=11).

- = Control Diet
- = Ketogenic Diet

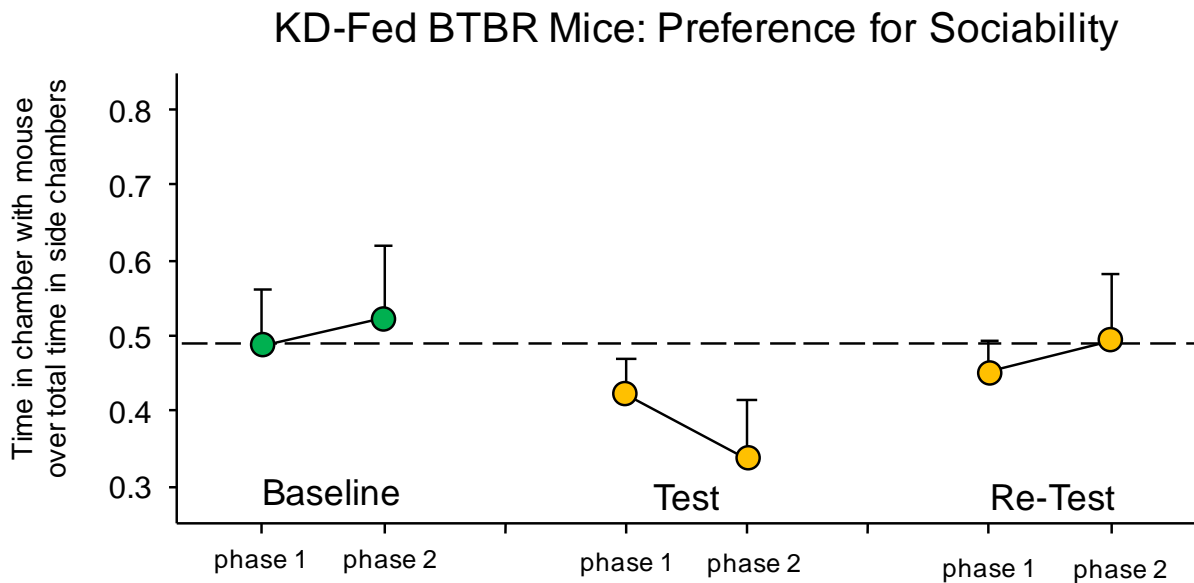


Figure 12: KD has no effect on social preference in KD-Fed male BTBR mice in three chamber test. KD-fed male BTBR mice did not exhibit significant pro-social behavior after 3 weeks of KD (Test) or 6 weeks of KD (Re-Test) (n=12). These data conflict with previous research findings of Ruskin et al. (2013).

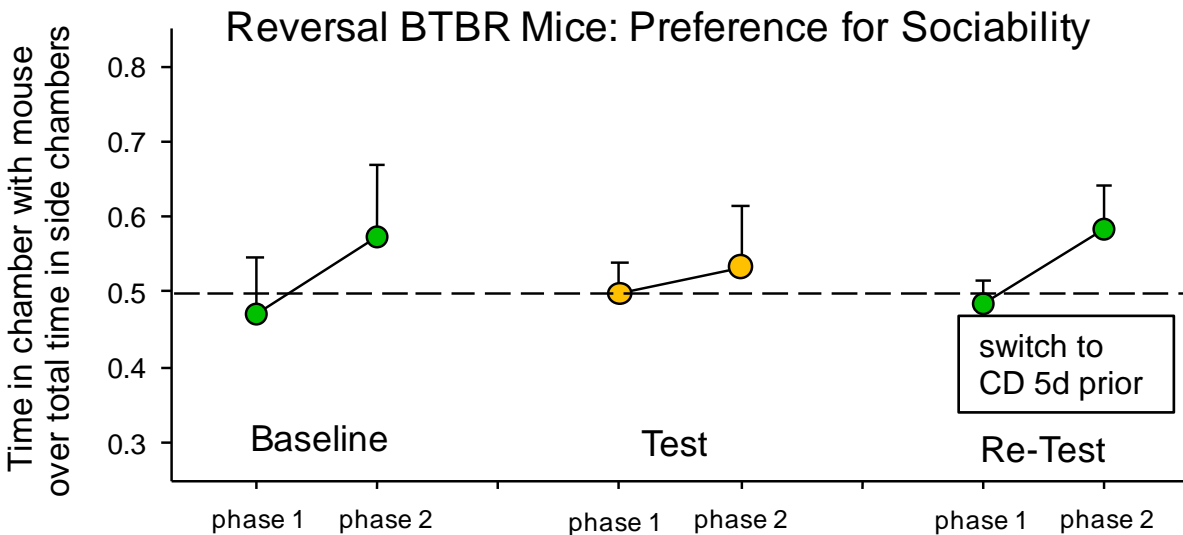


Figure 13: Neither the KD nor Reversal to CD had a significant effect on social preference of male BTBR mice in three chamber test. Data reveals male BTBR mice demonstrated no significant pro-social behavior after 3 weeks of KD (Test) or after a 5-day reversal to CD (Re-Test) (n=11).

Three-Chamber Sociability Testing – Mice Social on KD

As the data does not indicate improvement in social behavior for the Reversal group in the three chamber Test (after 3 weeks of KD) compared to sociability in Baseline test, it remains difficult to draw conclusions regarding the persistent effects of the diet. For this reason, the mice which demonstrated increased sociability (ratio > 0.5 in Phase 2 of the Test, after 3 weeks of KD administration) were selectively isolated and re-analyzed.

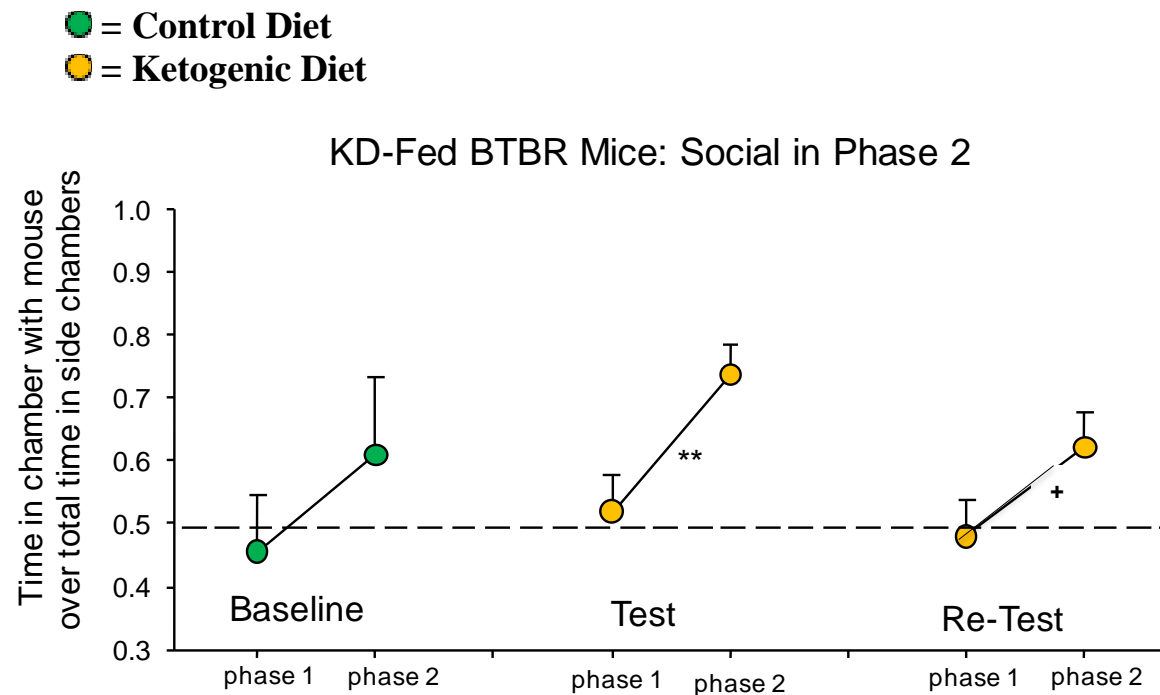


Figure 14: KD increase male BTBR sociability during three chamber testing. Data indicates KD-fed mice spent significantly more time in the target chamber during phase 2 than phase 1 in the Test after three weeks of KD (n=6, **p < 0.01). In the Re-Test, mice exhibited a tendency of increased sociability that approached significance (+ p < 0.09).

- = Control Diet
- = Ketogenic Diet

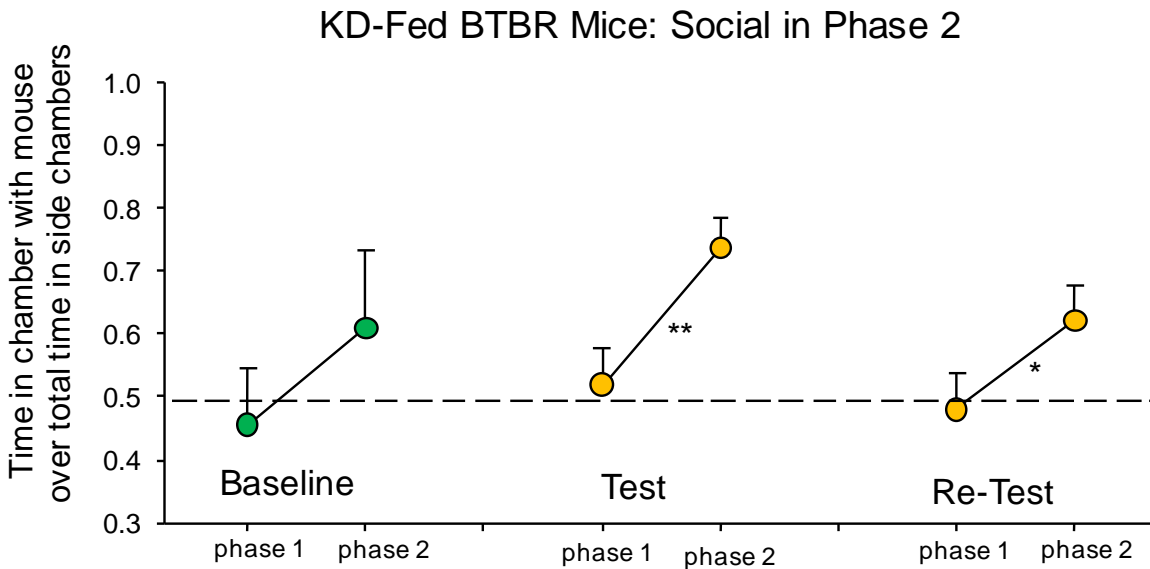


Figure 15: KD increase male BTBR sociability, while reversal to CD has no significant effect on behavior during three chamber testing. Data indicates while on the KD for three weeks (in Test), mice spent significantly more time in the in the chamber with the stranger mouse during phase 2 (n=5, **p < 0.01). However, after 5-day reversal to the CD, BTBR mice exhibited no significant pro-social behavior suggesting that the KD does not have persistent effects on sociability.

DISCUSSION

In this study, blood, weight, and behavioral effects of the KD on juvenile male BTBR mice, a mouse model of autism spectrum disorder (ASD) were assessed. BTBR mice exhibit several symptoms of autism including: reduced social interactions, impaired play, low exploratory behavior, unusual vocalizations and high anxiety as compared to other inbred strains (McFarlane, 2008; Moy, 2007; Scattoni, 2008). BTBR mice at 5 weeks of age were randomly assigned to one of three diet groups: CD, KD, and Reversal. All mice underwent three-chamber behavioral testing followed by two periods lasting three weeks. During the first period, both KD group and KD reversal groups were switched to KD for three weeks and underwent three-chamber behavioral testing. Subsequently, both groups underwent three additional weeks of KD, however in the last 5 days of the second period the reversal group was reverted to the CD and underwent their final three-chamber behavioral test.

It was expected that at our baseline test (five weeks of age) all dietary treatment groups would be non-social, as they had been on the CD. After three weeks of diet administration (eight weeks of age), it was expected that the CD group would remain non-social while the KD and Reversal groups would demonstrate pro-social behaviors as previous studies have indicated that KD-fed BTBR mice showed behavioral improvements including an increased sociability in a three-chamber test after simply three weeks of diet administration (Ruskin et al., 2013). Finally, after six weeks of diet administration (11 weeks of age), it was expected that the CD would again remain non-social, the KD and Reversal groups would maintain their pro-social behaviors. The idea of a continued, or persistent, effect of the diet was rooted in previous lab findings that mice on the diet for 12 weeks demonstrated lasting positive behavioral alterations after a 5-day dietary reversal.

Our initial expectation that all male BTBR mice would demonstrate non-social behaviors was supported by our data. Additionally, as expected, our study found significant impact of the KD on blood levels and weight change; just three weeks after KD administration, in our second behavioral test, results show a significant increase in ketone levels and significant decrease in glucose levels for both KD and Reversal groups. Results for the Re-Test, 5 days after reversal to the CD, results indicate the reversal group is no longer in ketosis. Although our blood analysis revealed the hallmark characteristics of KD treatment, including weight loss, decreased glucose, and increased ketone levels, we did not successfully replicate the pro-social effects of the KD on behavior previously found by Ruskin et al. (2013, and unpublished data). Even when the mice that demonstrated pro-social behavior after three weeks on the diet in phase 2 of the behavioral test were isolated, there was only a slight hint that the KD had effect on behavior in the KD group. In the Reversal group, despite a significant appearance of pro-social behavior after three weeks on the diet, the mice fell back to baseline non-social behavior once they were switched to the KD. In sum, our results suggest the KD does not have persistent effects on sociability.

It is unclear why we were unable to replicate improvement in sociability, which has been demonstrated in the laboratory setting on multiple replications. Some ideas include the composition of our KD distributed by the company BioServ. One thought is that ingredients making up the 6:1 ratio of the KD may have been altered or the provider for the ingredients may have changed. Additionally, it is known that in any behavioral testing the external environment including temperature, lighting, noise, and scents can have a profound effect on animal behavior. Specifically, exposure to scents in a lab setting has been impacted in changing the social status and competitive in inbred male mice, including BTBR mouse models (Bine et al., 2013). It could be proposed that a change in environment may have induced a stress response in our mouse

models and thereby affected the pro-social effects of the KD. Additionally, in the unpublished laboratory data presented in in Figure 7, the persistent effect is based on a single three-chamber test after a 5-day reversal. This is in contrast with our methodology in which three behavioral tests were run. It is possible that the increased behavioral testing influenced behavior within our population of BTBR mouse models.

As we move forward with this study, it is important to consider other behavioral tests for ASD-like behaviors. The core symptoms of autism include persistent social communication impairment and restricted and repetitive patterns of behavior. In this study, a single method of behavioral testing was used to assess sociability, however many other methods exist to test the social deficits that are characteristic of ASD including frontal contact analysis of the existing three-chamber videos and social transmission of food preference (STFP) tasks. STFP tests for social learning and the ability of a test mouse to observe and detect olfactory cues on a stranger mouse and learn to prefer a certain food based on the scent (Wrenn, 2004). Frontal contact is a measure of preference of reciprocal social interaction (Silverman et al., 2010). It was found that after three weeks on the KD, Ruskin et al. (2013) noted that male BTBR fed the KD had improved communication as they were trained to prefer a food based on social interaction and demonstrated a significant increase in frontal contact with stranger mice as compared to control diet-fed mice.

In addition, the BTBR mouse strain has been shown to have abnormally high repetitive behaviors, namely grooming including in the three-chamber test. KD treatment has been shown to reduce these behaviors; therefore, it is possible to go back and reanalyze current video scores for grooming behavior. For this measure, we score for cumulative time spent grooming all body regions to analyze repetitive patterns. In terms of self-directed repetitive behavior, BTBR mice

fed the KD spent significantly less time grooming in both phase 2 of the three-chamber which suggested an improvement of one of the core symptoms of ASD (Ruskin et al., 2013).

In the future, research regarding the diet reversal and persistent effects of the KD on autistic symptoms of BTBR mouse models may use a modified experiment timeline. Ours was modeled after the experiment conducted by Lusardi et al. (2105) that demonstrated rodents fed the KD for fifteen weeks showed a prolonged reduction of seizures which continued after a 4-day reversal to control diet. Ongoing research may increase length of diet administration prior to reversal which may allow for increase epigenetic changes to take place. It would be interesting to use methods of detecting epigenetic markers, such as DNA methylation assay, to gather total genomic DNA from frozen tissue and assess global DNA methylation of KD and Reversal treatment groups compared to the CD group (Williams-Karnesky et al., 2013). This would provide evidence for changes that may be induced by the diet but do not affect behavior. Subtle, yet effective changes to study design may include a larger sample size and including additional measures of sociability including frontal contact and self-directed repetitive behaviors.

CONCLUSION

This study focused on the BTBR mouse model to determine if improvement in ASD-associated behaviors persists when animals are returned to a CD. Despite ketone, glucose, and weight changes data that represent hallmark characteristics of an effective KD treatment, we did not find significant improvements in sociability in either KD or Reversal groups. Further analysis of social animals in behavioral tests (after three weeks of diet administration) and Re-Test (after six weeks of diet administration) reveals a trend of pro-social effects of the diet in KD-fed group, however results do not provide support that increased sociability persists after diet administration. Ongoing research will focus on additional measures of sociability including frontal contact and self-directed repetitive behaviors.

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