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Diet-Induced Ketosis and Calorie Restriction in Mouse Models of Alzheimer's Pathology

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Diet-Induced Ketosis and Calorie Restriction in Mouse Models of Alzheimer’s Pathology

by

Milene Lara Brownlow

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy Department of Molecular Pharmacology and Physiology College of Medicine University of South Florida

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ABSTRACT

Dietary manipulations and their pharmacological outcomes have been increasingly studied in neurodegenerative diseases. However, a systematic comparison among different methods in validated animal models of Alzheimer’s disease is made necessary due to several different approaches applied in recent studies. Moreover, despite the large body of evidence on the effects of calorie restriction (CR) and ketogenic diets (KDs) on amyloid pathology, no consistent data is available on the effects of calorie restriction, ketogenic diet or ketone supplements on tau pathology in transgenic models of AD. Moreover, the ketogenic diet used in our studies was custom made with low carbohydrate content and rich in medium chain triglyceride (MCT) oils, known to be rapidly metabolized in the liver, resulting in sustained peripheral ketosis.

Chapter 2 tested the ability of KD to induce significant ketosis in a mouse model of amyloid deposition. We showed that, despite the mild ketosis induced, KD fed APP mice presented subtle behavioral improvement shown as faster learning in the radial arm water maze, making less errors than APP mice kept on a control diet. Additionally, we observed decreased Aβ immunoreactivity in the anterior cortex of KD fed versus control fed APP mice, despite the lack of changes in congophilic deposits. Due to the mild ketosis induced, a modified ketogenic diet was devised with decreased maltodextrin content and showed greater peripheral levels of β-hydroxybutyrate.

Chapter 3 investigated the effects of a ketogenic diet in two transgenic mouse models of Alzheimer’s pathology. Interestingly, we found that both transgenic lines, regardless of diet, weighed less than nontransgenic mice, despite their elevated food
intake. The reduced body weight may, in part, be explained by the increased locomotor activity shown by both transgenic lines in both the open field and y-maze. Moreover, KD fed mice performed significantly better on the rotarod compared to mice on the control diet independent of genotype. We did not observed KD-induced changes in spatial or associative memory in the radial arm water maze or contextual fear conditioning, respectively. Furthermore, immunohistochemical levels of amyloid, tau, astrocytic and microglial markers showed no differences between animals fed KD or the control diet.

Chapter 4 studied the effects of calorie restriction on a mouse model of tau deposition. We show here that 35% body weight reduction in Tg4510 mice did not prevent increased locomotor activity in the open field, previously reported in chapter 2. Similarly, CR did not affect motor performance or spatial memory assessed by the rotarod and radial arm water maze, respectively. Interestingly, CR Tg4510 mice showed improved short-term memory tested by the novel object recognition despite spending a minimal percentage of the trial time interacting with the objects presented. However, this improvement was not observed when the test was modified to replace the objects with mice. In this case, we noticed that nontransgenic mice spent most of the trial time interacting with the novel mouse whereas Tg4510 mice spent roughly the same amount of time at any of the areas in the test chamber. Moreover, no changes in histopathological or biochemical levels of tau, astrocytic, microglial or synaptic markers were observed.

Chapter 5 sought to investigate alternative approaches to inducing ketosis in the brain by either administering BHB intracerebroventricularly (i.c.v.) or by using the acetoacetate (AcAc) diester as a dietary supplement in mice. We observed that i.c.v administration of BHB in 20 months old APP mice did not affect body weight or food intake. Consistent with the lack of effects on behavioral performance, amyloid and congophilic load were not different between APP mice infused with either saline or BHB.
We also found that enteral administration of AcAc diester was well tolerated and induced peripheral ketosis for at least 3 hours. Acute ketosis, however, was not sufficient to attenuate behavioral deficits in old APP mice. Chronic dietary supplementation with AcAc was tested in control tet mice and was shown to effectively induce ketosis in mice fed a diet with normal contents of carbohydrates. Nonetheless, we observed that AcAc-induced ketosis was not significantly greater than levels induced by the ketogenic diet tested in our lab. Considering that KD did not rescue behavioral or histopathological features of either amyloid or tau depositing mouse models, we anticipated that dietary supplementation with AcAc would not likely modify the phenotype of the same mouse models tested previously.

Taken together, our findings show that our custom made ketogenic diet was effective in inducing and sustaining ketosis and may play an important role in enhancing motor performance in mice. However, the lack of changes on the cognitive and histopathological phenotype of the models studied suggests that KD may not be a disease modifying therapeutic approach to AD. Moreover, calorie restriction showed inconsistent effects on behavioral and histopathological outcomes of a mouse model of tauopathies. Furthermore, dietary supplementation with acetoacetate diester was successful in inducing peripheral ketosis to the same extend as a ketogenic diet even in the context of normal carbohydrate intake, suggesting that it may be of therapeutic interest for diseases of hypometabolism but not a disease modifying therapy in mouse models of Alzheimer’s pathology.
CHAPTER 1: INTRODUCTION

1.1 Alzheimer’s disease

Alzheimer’s disease (AD), first described in 1906 by Alois Alzheimer, is the most prevalent type of dementia. AD is a progressive and fatal disorder with as many as 5.2 million Americans and approximately 18 million people worldwide currently affected. It is the most common type of dementia, accounting for 60 to 80% of all cases, currently ranked as the sixth leading cause of death in the U.S. Because of the growing number of people age 65 and older, the annual incidence of AD and other dementias is projected to double by 2050 (Alzheimer’s Association Facts and Figures 2013).

AD can affect patients in different ways but the initial and most prominent symptom is the progressive decline in memory that disrupts daily activities. Later symptoms include impaired judgment, disorientation, confusion, behavioral changes and trouble speaking, swallowing and walking. The disease usually progresses until the patients lose basic reflexes such as bowel and bladder functions and, ultimately, the cough reflex. Individuals that reach this later stage tend to become acidotic, hypoxic and their plasma pH can reach levels as low as 6.0, leading to death.

In 2011, the National Institute on Aging (NIA) and the Alzheimer’s Association proposed new criteria and guidelines for diagnosing AD, recognizing that the disease begins well before the development of observable symptoms. Abnormal brain morphology may begin 20 years or more before symptoms manifest [for review see (Berti et al., 2010)]. Despite recent medical advances, the precise changes in the brain that trigger the disease progression and the sequence in which they occur are still
obscure. The decline in cognition observed in AD is believed to result from multiple factors rather than a single cause. However, the most prominent neuropathological changes currently associated with AD are dramatic brain shrinkage due to neuronal loss, the presence of extracellular amyloid plaques and intracellular neurofibrillary tangles.

Aging is the most prominent risk factor for AD. Most patients develop AD over the age of 65 whereas individuals that inherit mutations may develop symptoms as young as 30 years of age. Family history is also an important risk factor that may predispose to increased incidence of AD. Besides genetics, shared environmental and lifestyle factors may play a role, although the mechanisms involved are not clearly understood. Apolipoprotein E (APOE) is a glycoprotein that helps transport cholesterol, triglycerides and other lipids in the bloodstream. There are 3 common forms of the APOE gene, which are APOE2, 3 or 4 and they can combine in different ways. While the APOE3 genotype is the most common, individuals who inherit one APOE-4 gene have increased risk of developing Alzheimer’s disease (Strittmatter et al., 1993). Those who inherit two APOE-4 genes have an even higher risk. However, inheriting one or two copies of the gene does not guarantee that the individual will develop Alzheimer’s (Craft et al., 2003). Furthermore, because the APP gene is located on chromosome 21, individuals with Down’s syndrome, who possess an extra copy of chromosome 21, present increased APP expression and develop early plaques and tangles (Burger and Vogel, 1973). Moreover, educational levels and physical activities are notably known for their positive effects on cognition. Epidemiological studies confirm that AD’s incidence is inversely correlated to educational levels (Karp et al., 2004).

In the last decade, several studies have linked AD to type-2 diabetes mellitus (T2DM) [for a review see (Craft, 2009)]. They are both chronic, age-related diseases that present an important economical burden in our society. Evidence points to the existence of shared mechanisms between the disorders. Insulin resistance, hyperinsulinemia and
hyperglycemia, the main hallmarks of T2DM, are thought to act on different pathways that are important in the pathophysiology of AD; such as brain insulin uptake across the blood brain barrier (BBB), neuroinflammation and regulation of insulin-degrading enzyme (IDE), an important protease responsible for Aβ clearance (Craft, 2005).

In a mouse model of amyloid deposition, long-term consumption of sucrose-sweetened water was shown to induce glucose intolerance, insulin resistance, exacerbation of spatial learning and memory impairments and increased Aβ deposition (Cao et al., 2007). Diet-induced obesity increased APP expression in the brain of adult rats fed a high-fat diet for 4 months (Mohamed et al., 2010). Additionally, caloric restriction and exercise attenuated the adverse effects of diabetes on hippocampal neuronal plasticity (Stranahan et al., 2009), providing evidence that healthy lifestyle choices play an important role in brain health. Moreover, a recent study followed up 2067 cognitively normal participants (baseline age was 76 years) for 7 years and reported that higher glucose levels were related to increased incidence of dementia, even in patients without diabetes (Crane et al., 2013).

Craft (2009) suggested that peripheral hyperinsulinemia and insulin resistance could act indirectly, through increased peripheral free fatty acid (FFA) levels; this in turn would invoke elevations in inflammatory agents in the CNS. Through increased inflammation and insulin resistance-induced down regulation of insulin transporters across the BBB, the lack of insulin actions in the brain contributes to the excessive synthesis and deposition of the Aβ peptide into amyloid plaques and in the abnormal phosphorylation of tau protein leading to its intraneuronal accumulation. The cascade of events that leads to neuronal death by the formation of neurofibrillary tangles appears to be modulated by glycogen synthase kinase-3 (GSK-3) activity (Craft, 2009).

In an evolutionary approach, Samuel Henderson, based in a 1962 work published by James Neel, discussed the possibility of a conflict between our genetic
makeup and our recent nutritional habits. They proposed that pre-agricultural hunter-gatherers survived cycles of feast or famine which led to the selection of a metabolism that would readily store fat. Obesity and type II diabetes result when this genetic makeup is confronted with the modern excess of food. An alternative to this model is that the type, rather than quantities, of food changed throughout evolution. While protein and fat consumption was a significant part of the Paleolithic diet, the introduction of agriculture in the Neolithic revolution changed the macronutrient profile of their diets to higher contents of simple carbohydrates and lower contents of protein (Henderson, 2004).

1.2 Mouse models of AD

The molecular mechanisms involved in AD have been extensively investigated. Less than 1% of AD cases can be attributed to three previously described genetic autosomal mutations involving genes encoding amyloid precursor protein (APP), presenilin 1 (PS1) or presenilin 2 (PS2). The presenilin genes are located in chromosome 14 and presenilin proteins are part of the gamma secretase complex that processes APP (Levy et al., 1990). Individuals that inherit any of these dominant mutations develop symptoms before the age of 65 and these cases are often referred to as familial AD (FAD) or early onset AD (Fidani and Goate, 1992). These mutations result in alterations in the normal processing of APP by changing the proteolytic cleavage sites which result in the increased production and release of Aβ peptides in the brain. The released Aβ peptides can be 40 or 42 amino acids long. When there’s a failure at the clearance mechanism, the Aβ peptide forms fibrils which aggregate outside the cells, causing inflammation with astrocytic and microglial activation. This cascade of events results in the formation of the insoluble β-amyloid plaques, aggregated in a beta pleated sheet conformation (Frautschy et al., 1998). Because these mutations consistently
cause clinical and histopathological phenotypes consistent with AD, the amyloid cascade hypothesis was proposed suggesting that the accumulation of Aβ peptide in the brain is the initial trigger that results in neuronal dysfunction, synaptic loss and cognitive decline in AD (Hardy and Selkoe, 2002).

Neurofibrillary tangles (NFTs) result from the hyperphosphorylation of the protein tau. Tau is a microtubule associated protein, normally abundant in axons, that promotes microtubule assembly and stabilization. Post translational modifications of tau, notably hyperphosphorylation at specific sites, have been implicated in the formation of paired helical filaments (PHFs), which are components of NFTs (Bancher et al., 1989). Moreover, hyperphosphorylated tau loses its ability to bind to microtubules, leading to a disruption in microtubule assembly and deficits in neuronal transport (Terwel et al., 2002).

Transgenic mouse models can be used to evaluate the impact of manipulations on either the amyloid pathology or the tau pathology that are thought to be responsible for the development of AD. Transgenic expression of these human mutations led to the development of mouse models that express mutant Aβ peptides and form amyloid plaques in brain areas associated with cognitive functions, such as hippocampus, amygdala and entorhinal cortex. Mice that carry the human ‘Swedish’ mutation (K670N/M671) in the APP (Tg2576) gene have substantial increases brain levels of both Aβ_{1-40} and Aβ_{1-42} as well as deficits in spatial memory and spontaneous alternation in the y-maze that become evident at 10 months of age (Hsiao et al., 1996). Holcomb et al showed that AD-like pathology is greatly enhanced when a PS1 mutation (M146L) is added to the Swedish mutation, generating a double transgenic model (APP+PS1) that generates amyloid deposits as early as 6 months of age (Holcomb et al., 1998a). Deficits in spontaneous alternation, however, were detected as early as 3 months. The presence of deficits in alternation at 3 months and the lack of impairments in spatial memory at 6
and 9 months, despite the substantial amyloid burden suggest that earlier events other than amyloid pathology may be at least in part responsible for the phenotype present in these mice (Holcomb et al., 1999). However, APP transgenic mice are limited models of AD pathology due to their absence of NFTs and lack of neuronal loss (Hardy and Selkoe, 2002).

The Tg4510 mouse has a P301L mutation, but differs from other tau transgenic mice in that the major tau pathology is found in the forebrain rather than the spinal cord. This is because of the tet response element driven expression, with the tet activator regulated by the CaM kinase II promoter, resulting in expression largely in forebrain neurons (Ramsden et al., 2005). These mice develop progressive pathology with first discernible deposits being observed at 3 mo, progressing through a series of stages analogous to that found in AD patients until readily detectable neuron loss and cortical thinning are found by 6 mo of age (Santacruz et al., 2005, Dickey et al., 2009).

The triple transgenic mouse model of AD (3xTgAD) carries the 'Swedish' mutation in APP (K670N/M671L), the M146V mutation in PS1 and the human four repeats tau (P301L) mutation. It typically develops intracellular Aβ immunoreactivity and extracellular Aβ deposits in the neocortex by 6 months of age combined with detectable tau pathology by 12 months old, replicating two main hallmarks of AD, amyloid plaques and neurofibrillary tangles (Oddo et al., 2003a, Oddo et al., 2003b). An important feature of this model is that amyloid deposition into extracellular deposits precedes the formation of tangles, supporting the amyloid cascade theory, and spatial and contextual memory deficits are observed in these mice as early as 4 months old (Billings et al., 2005). Moreover, synaptic dysfunction was evident at 6 months coinciding with the presence of intracellular Aβ immunoreactivity. Importantly, reducing amyloid load, by either immunization or administration of a gamma secretase inhibitor, successfully resulted in clearance of tau, particularly early tau pathology that is not in its hyperphosphorylated
aggregate state (Oddo et al., 2004). Therefore, the triple transgenic model has many advantages to study interactions between amyloid deposition and tau pathology; however, this confounds interpretation of manipulations directly impacting tau pathology.

1.3 AD and brain hypometabolism

Cerebral hypometabolism has long been described in AD patients (Foster et al., 1983) and technological advances in functional imaging procedures have provided us with better insights in this pathological feature. Cerebral metabolic rate of glucose utilization, detected by functional MRI, has been reported to be substantially reduced in the brains of both AD and MCI patients and are more pronounced in areas that are most affected pathologically, such as the parieto-temporal and posterior cingulated cortices (Mosconi et al., 2008b). Lack of function or hypometabolism is indicative of less glucose action in these tissues, which could be caused by decreased insulin levels or insulin resistance. The impaired glucose metabolism in the brain may contribute or exacerbate the cognitive deficits observed during normal aging, suggesting that brain energy deficiency may be a primary player in the disease initiation (Blass, 2001, Cunnane et al., 2011).

Several studies have demonstrated abnormalities in mitochondrial enzymes in AD brain that are suggestive of impaired capacity to metabolize carbohydrates [for a review see (Blass et al., 2000)]. Deficiencies have been previously reported in three mitochondrial complexes: 1) the pyruvate dehydrogenase complex (PDH) (Perry et al., 1980), which catalyzes the entry of carbons derived from glucose into the Krebs tricarboxylic acid (TCA) cycle; 2) the α-ketoglutarate dehydrogenase complex (OGDH), which catalyzes a key step in the TCA cycle and is also an enzyme of glutamate metabolism (Gibson et al., 2000); and 3) cytochrome oxidase (COX, also referred to as complex IV), the component of the electron transport chain which uses molecular
oxygen as one of its substrates (Gibson et al., 1998). Impaired mitochondrial function can be detected in AD mouse models before amyloid deposition takes place (Yao et al., 2009). Accordingly, a shift in brain metabolism has been described in mouse models of AD, suggesting that increased use of ketone bodies as an alternative energy source occurs as a compensatory mechanism for the impaired glucose metabolism (Yao et al., 2010, Yao et al., 2011b).

Although the amyloid cascade hypothesis has been extrapolated to sporadic AD, these patients do not present mutations in neither APP nor PS genes and the etiology of the disease in these cases is largely unknown. As an alternative approach, the mitochondrial cascade hypothesis was first proposed in 2004 (Swerdlow and Khan, 2004) and it suggests that changes in mitochondrial function initiate the cascade that leads to AD, as opposed to beta-amyloid changes being the initiating event. Accordingly, clinical disability in AD patients correlated with reductions in mitochondrial DNA (mtDNA) but not with the density of neuritic plaques (Brown et al., 2001). Recently, maternally transmitted mtDNA was shown to accelerate aging and reduce fertility in mice with normal nuclear genomes (Ross et al., 2013). Furthermore, asymptomatic patients carrying the mutations in the PS1 gene and AD patients exhibited a significant decrease in mtDNA in the cerebrospinal fluid (CSF) and this reduction was detectable even before the appearance of AD related biomarkers in CSF. In agreement with this observation, cortical neurons from APP+PS1 transgenic mice also exhibited fewer mtDNA copies before the appearance of altered synaptic markers (Podlesniy et al., 2013).

Taken together, a large body of evidence indicates a strong link between impaired energy metabolism and AD pathogenesis. This metabolic deficit has been shown in both AD patients (Mosconi, 2005, Mosconi et al., 2008a) and transgenic models (Yao et al., 2009, Yao et al., 2010) to precede histopathological abnormalities and it appears to be highly predictive of cognitive decline in presymptomatic patients.
1.4 Ketogenic diet

In the early 1920s, Dr. Hugh Conklin thought that epilepsy was the result of the improper functioning of the bowel and suggested that fasting would reduce the systemic intoxication, therefore preventing seizures. He reported that fasting children for as long as 25 days, with access to water only, resulted in pronounced and long-lasting seizure control. The success of his approach led other physicians to note that the metabolic alterations of fasting could be replicated by restricting dietary carbohydrates and increasing the intake of fatty foods [for a review see (Hartman et al., 2007)].

Diets that are rich in fat and low in carbohydrates, known as ketogenic diets (KDs), induce a decrease in blood sugar levels. Consequently, the body is required to find an alternative fuel to provide energy, such as free fatty acids (FFA). Ketone bodies are a by-product of the incomplete breakdown of FFA in hepatocytes and glial cells and are released into the bloodstream to provide a supplement to glucose. The main endogenous ketone bodies are: acetone, acetoacetate (AcAc) and β-hydroxybutyrate (BHB). When ketone bodies accumulate in the bloodstream, they cause a metabolic state called ketosis and, at the same time, there is a decrease in glucose utilization (LaManna et al., 2009) and production leading to decreased insulin levels. Ketosis is a survival mechanism activated during prolonged fasting, starvation or reduced carbohydrate ingestion. Moreover, carbohydrate restriction improves insulin sensitivity, disinhibiting hormone-sensitive lipase and promoting breakdown of fat storages. Therefore, the lack of carbohydrates is a key factor in implementing a ketogenic diet [reviewed in (Hammami, 1997)]. The improved metabolic efficiency observed in animals fed a KD may be due to the fact that the KD enables cells to use excess body fats as a source of energy. This diet mimics the effects of fasting and the lack of glucose/insulin signaling promotes a metabolic shift toward fatty acids utilization (Hammami, 1997, Morris, 2005, Cahill, 2006).
Since its conception, ketogenic diets have been extensively used as an alternative to starvation for the treatment of refractive childhood epilepsy. However, other therapeutic applications of the diet have also been reported. GLUT1 is a constitutive glucose transporter known to mediate insulin-independent glucose transport across the blood brain barrier (BBB) and uptake into neurons (Brant et al., 1993). GLUT1 deficiency syndrome (GLUT1DS) is a developmental condition that results in low glucose levels in cerebrospinal fluid. This leads to infant seizures, delayed development and microencephaly. So far, administration of a ketogenic diet remains the most effective treatment in GLUT1DS, providing a means of immediate seizure control in most patients (Klepper, 2008). Thus, the increased circulating levels of ketone bodies are able to overcome the limitations in glucose uptake or use.

Besides its anticonvulsant properties, many studies in the last decade have reported beneficial effects of KDs in other models of neurodegenerative diseases, such as increased motor neuron number (Zhao et al., 2006) and motor performance in ALS (Zhao et al., 2012) and in APP+PS1 (Beckett et al., 2013b) models; reduced lesion volume after traumatic brain injury (Prins et al., 2005); increased cell survival and decreased seizure frequency in kainate-induced seizure models (Noh et al., 2003). AD transgenic mice fed a ketogenic diet for 43 days had decreased levels of Aβ40 and Aβ42, decreased body weight and increased blood levels of BHB (Van der Auwera et al., 2005). However, the absence of behavioral improvement observed was attributed to the modest lowering of Aβ levels and/or to the short period of treatment. Similarly, despite enhanced motor performance, Beckett et al (2013) reported that amyloid levels in young APP+PS1 mice were unaffected by KD. Moreover, clinical trials involving Parkinson’s and Alzheimer’s disease patients treated with diet-induced hyperketonemia resulted in improved motor function (Vanitallie et al., 2005) and enhanced cognition (Reger et al., 2004a), respectively.
There is evidence that some of the underlying mechanisms of ketogenic diets’ neuroprotective effects are similar to those activated by calorie restriction. The ketogenic diet has been associated with improved mitochondrial function, reduced levels of reactive oxygen species (ROS) (Maalouf et al., 2007), increased glutathione levels and activity in the hippocampus (Ziegler et al., 2003). Furthermore, the ketogenic diet has also been implicated in anti-apoptotic mechanisms, decreasing the expression or inhibiting the dissociation of pro-apoptotic factors as well as increasing the activity of an intracellular calcium buffer, calbindin (Noh et al., 2003, Noh et al., 2005, Noh et al., 2006a, Noh et al., 2006b).

The latest resurgence of the Atkins diet, the low glycemic index diet and the increasing popularity of coconut oil attest that the low carbohydrate, ketogenic diets have been recently become more amenable to individuals, providing greater food variability. Coconut oil is a rich source of medium chain triglycerides (MCT), known to be rapidly metabolized by the liver to produce a substantial increase in peripheral ketosis and decrease in glucose levels (Yeh and Zee, 1976).

1.5 Ketone Bodies

Under normal dietary conditions, glucose is the preferred fuel for most tissues in the body. However, under extreme conditions such as neonatal development, starvation or low carbohydrate intake, the body is forced to find an alternative fuel source, such as free fatty acids, which can be used by most cells. Although FFA can cross the blood brain barrier, the brain cannot use them. However, ketone bodies (by-products of FFA oxidation in hepatocytes or astrocytes) can be readily utilized by neurons as an alternative source of energy (Cahill, 2006). Ketone bodies cannot fully replace glucose but can account for a significant fraction of cerebral metabolism (LaManna et al., 2009), allowing the brain access to the body’s fat stores.
The BBB is relatively impermeable to most hydrophilic substances, such as ketone bodies. Therefore, its transport across the BBB is highly dependent on specific carrier-mediated facilitated transport by a family of proton-linked monocarboxylic acid transporters (MCTs). The MCT family has a total of 14 members, four of which (MCT1-4) have been extensively studied [reviewed on (Halestrap and Wilson, 2012)] and have been demonstrated to be present in neurons and glia as well as in endothelial cells of the BBB. Glial cells predominantly express MCT1, while neurons may express MCT1 or MCT2 (Pierre et al., 2000). Increased MCT1 expression was previously reported in studies of diet-induced ketosis in rats, as well as increased expression of GLUT1, suggestive of increased vascular density allowing for enhanced delivery of fuels to the brain (Leino et al., 2001, Puchowicz et al., 2007).

Therapeutic uses of ketone bodies have been extensively described (Veech, 2004, Henderson, 2008, Maalouf et al., 2009b). Ketone bodies can bypass the decreased activity of PDH complex, ensuring the activity of the TCA cycle regardless of insulin action (Kashiwaya et al., 1997). Treatment with beta-hydroxybutyrate (BHB), reduced cerebral infarct area in mice (Suzuki et al., 2002, Zou et al., 2002, Masuda et al., 2005). Other studies have shown increased cell survival and suppression of glutamate toxicity in vitro (Noh et al., 2006b). Consistent with the observed neuroprotective effects, 4mM BHB protected mesencephalic and hippocampal neurons from MPP and Aβ toxicity, respectively (Kashiwaya et al., 2000). In addition to improved neuronal survival, improved mitochondrial efficiency was also observed. Furthermore, treatment of cells with BHB increased lysosomal transport and degradation of proteins, suggesting that BHB could be involved in making damaged proteins better substrates for chaperone-induced degradation (Finn et al., 2005).

Due to the increasing popularity of low carbohydrate diets, many sources of information, meal planning and food varieties have been made available to individuals
on KD. However, long term maintenance is still a challenge and low compliance, despite its remarkable beneficial effects, is a major limitation. In an attempt to circumvent this obstacle, many researchers have been attempting to develop supplements that can be administered in the context of a normal diet and still induce pharmacological levels of ketone bodies [for a review see [(Rho and Sankar, 2008)]. AC-1202 (Accera, Inc.) is an orally administered MCT that is rapidly metabolized by the liver to produce a mild state of ketosis. In aged dogs, dietary supplementation with AC-1202 increased serum levels of ketone bodies, improved cognitive performance and blood brain barrier integrity (Costantini et al., 2008). Clinical trials in patients with AD or MCI have shown that AC-1202 can improve memory and attention in these individuals, particularly in those with higher BHB levels. Cognitive facilitation and memory improvements following elevation of plasma ketone body levels were greater in APOE4-negative adults with memory disorders (Reger et al., 2004a). Veech and colleagues have developed a ketone ester (D-β-hydroxybutyrate and (R)-1,3-butanediol) that, when added to the diet, induced peripheral ketone levels similar to those found in humans during prolonged fasting and were 3-5 times higher than those reported for mice in the KD (Srivastava et al., 2012).

1.6 Calorie Restriction (CR)

Calorie restriction is the only intervention capable of prolonging lifespan in several species. In addition to increasing longevity, the neuroprotective effects of calorie restriction have been implicated in several neurological disease models and recently reviewed by several research groups (Roth et al., 2005, Fontana et al., 2009, Maalouf et al., 2009b, a).

Calorie restriction delayed the onset and increased the threshold for seizures in mouse models of epilepsy (Greene et al., 2001, Eagles et al., 2003). In animal models of Parkinson’s disease, calorie restricted animals presented improved motor function and
less neuronal death in the substantia nigra (Duan and Mattson, 1999). Previous reports from our lab showed that calorie restriction significantly reduced the number and size of amyloid plaques in the brains of two transgenic models with early onset of Alzheimer’s disease (AD) (Patel et al., 2005). Although in the last decade several groups have been showing the effects of calorie restriction on Aβ accumulation, only recently researchers have studied tau and phospho-tau alterations.

A mouse model of conditional knockout for PS1 and PS2 resulted in severe degeneration, synaptic dysfunction, tau hyperphosphorylation and cognitive impairment. Interestingly, this neurodegenerative phenotype occurs in the absence of amyloid deposition (Saura et al., 2004). Besides improvement in cognitive performance, a significant reduction in phosphorylated tau in the cortex of double knockout of PS1 and PS2 transgenic mice was reported after these mice were submitted to 4 months of 30% calorie reduction (Wu et al., 2008). Furthermore, triple transgenic AD mice submitted to 40% calorie restriction showed lower levels of Aβ and phospho-tau in the hippocampus compared to a control group maintained on an ad libitum diet (Halogappa et al., 2007).

The neuroprotective effects of calorie restriction could be due to an increase in metabolic efficiency and regulation of gene expression. Improved mitochondrial function has been described with enhanced antioxidant effects (Gong et al., 1997) and decreased production of reactive oxygen species-ROS (Sohal et al., 1994, Merry, 2002, Lambert and Merry, 2004, Gredilla and Barja, 2005). Additional mechanisms suggested to play a role in calorie restriction-induced improvements are: decreased activity of pro-apoptotic and inflammatory factors (Daynes and Jones, 2002), increased neurogenesis (Lee et al, 2002) and increased levels of molecular chaperones in the brain (Guo and Mattson, 2000, Sharma and Kaur, 2005). Some of these actions are suggested to be mediated by a family of NAD+-dependent histone deacetylases, called the sirtuins (SIRT) (Cohen et al., 2004). Overexpression of SIRT1, one of the seven mammalian...
proteins belonging to the sirtuin family, has been shown to reverse both histopathological and behavioral deficits in APP/PS1 mice, possibly by shifting APP processing toward non-amyloidogenic pathway through increased transcription of ADAM-10. Interestingly, BHB also acts as an endogenous inhibitor of class I histone deacetylases and was recently reported to significantly suppress oxidative stress in human embryonic kidney (HEK) cells (Shimazu et al., 2013).

Other effects on peripheral metabolism could also be at play in calorie restriction. Extensive data show that CR results in decreased body weight, lowers blood insulin, glucose and cholesterol. Obesity, type 2 diabetes and hypertension are commonly associated with insulin resistance. Furthermore, abnormal plasma and CSF insulin levels were observed in AD patients (Craft et al., 1998) supporting the hypothesis that peripheral insulin resistance is a risk factor for age related cognitive decline. One of the major enzymes responsible for the clearance of Aβ is the insulin degrading enzyme (IDE) (Hoyer, 2002), which preferentially cleaves insulin. Thus, decreasing blood insulin may enhance clearance of brain Aβ by reducing levels of its competing substrate. One of the main actions of insulin is the inactivation of the glycogen synthase kinase 3 enzyme (GSK3). Indeed, lack of insulin’s proper function leads to an increase in GSK3 levels. Interestingly, this enzyme has also been extensively associated with abnormal phosphorylation of the microtubule associated protein tau (Martin et al., 2009). Furthermore, researches have focused on the low metabolic state imposed on the organism by calorie restriction. This suggests that its effects are mediated by the activation of cellular stress responses such as: activation of growth factors, energy-regulating enzymes and heat-shock proteins (Liu et al., 2002, Martin et al., 2006).

More recently, calorie restriction studies in non-human primates failed to show increased lifespan but they saw improvements in healthspan, with lower incidence of age-related diseases such as cancer, diabetes, cardiovascular disease (Mattison et al.,
2012). Although some of the rhesus macaques had been under calorie restriction for over 20 years, they survived no longer than animals eating regular lab chow. However, the authors point out that they may not live longer but they do appear to be healthier than controls. An important caveat is that the control macaques were not ad libitum fed; so this study was focused on comparing the effects of calorie restriction in healthy subjects, with normal body mass index. The Wisconsin study compared calorie restricted rhesus monkeys with ad libitum regimen. In this case, therefore, the comparison was between calorie restricted subjects to people who become overweight or obese with age (Colman et al., 2009). Differences between NIA and Wisconsin study make it difficult to compare the results. In addition, the initial age of the animals, the duration of the CR and the macronutrient composition of the diets could also play a role in the discrepancies reported, making it for a challenging interpretation. In addition, one possible explanation is that the quality of life benefits seen following CR could result simply from the maintenance of a healthier body weight. Given the predicted parallels between rhesus monkeys and humans, the beneficial effects of CR in delaying the onset of age-related pathology is suggested to occur in humans as well.
1.7 References


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CHAPTER 2. INDUCING KETOGENESIS IN MICE: TESTING A KETOGENIC DIET RICH IN MEDIUM CHAIN TRIGLYCERIDES

2.1 Abstract

Brains of Alzheimer’s disease (AD) patients display hypometabolism of glucose and mitochondrial deficits, features recapitulated by transgenic mouse models. Diets that are low in carbohydrates and rich in fats, known as ketogenic diets (KDs), may bypass deficits in glucose utilization present in AD brain by providing ketone bodies as an alternative source of fuel. Here we investigate the effects of a custom-made ketogenic diet, rich in medium chain triglyceride (MCT) oils on behavioral and histopathological outcomes in a mouse model of amyloid deposition. Nineteen month old amyloid precursor protein (APP) transgenic mice were offered either a ketogenic or a control (NIH-31) diet for 3 months. Age-matched nontransgenic mice on the NIH-31 diet were included throughout the experimental procedure for use as controls during behavioral testing. Body weight was monitored weekly; blood was collected at 2 weeks and at the endpoint of the study (4 months) for measurements of circulating levels of ketone bodies. The ketogenic diet induced mild ketosis and did not affect body weight in APP mice. In the open field and Y-maze tests, transgenic mice presented increased locomotor activity compared to nontransgenic controls and this effect was exacerbated by KD. Despite the deficits observed in all transgenic mice, early performance by KD-fed APP mice was improved relative to the control fed transgenic group in the radial arm water maze. Decreased Aβ immunoreactivity was observed in the anterior cortex of KD fed versus control fed APP mice with no changes in congophilic deposits. Even though
the KD tested in our experiments resulted in only a mild ketosis, we found positive trends towards behavioral and histopathological improvements in APP mice. Taken together, these preliminary data suggest that achieving greater ketosis could potentially have beneficial effects in reducing amyloid pathology and rescuing spatial behavioral deficits in mouse models of amyloid deposition.

2.2 Introduction

Alzheimer’s disease (AD) is a chronic disorder that was first described in 1906 by Alois Alzheimer. The disease is progressive and fatal with as many as 5.4 million Americans and approximately 18 million people worldwide currently affected. It is the most common type of dementia, accounting for 60 to 80% of all cases (Alzheimer’s Association, 2012).

The earliest and most commonly reported symptom is memory loss. Pathologically, AD is characterized by extracellular deposits of amyloid beta (Aβ), intracellular accumulation of neurofibrillary tangles and neuronal loss. Transgenic mouse models replicate some of the histopathological and cognitive features of the disease. These animal models carry mutations in genes associated with the incidence of the early onset or familial AD (FAD) cases. Mutations in these genes lead to the abnormal cleavage of the transmembrane APP protein, resulting in abnormal release of the Aβ peptide (Fidani and Goate, 1992).

More recently, functional imaging studies detected cerebral hypometabolism in several areas but especially in the hippocampus of AD patients (Foster et al., 1983, Mosconi, 2005, Mosconi et al., 2009). This is indicative of less glucose action in these tissues, which could be caused by decreased insulin levels or insulin resistance. The impaired glucose metabolism in the brain may contribute to or exacerbate the cognitive deficits observed during normal aging.
Consistent with a reduction in glucose metabolism, a shift in brain metabolism has been reported in mouse models of AD, going from the utilization of glucose to increased ketogenesis during aging (Yao et al., 2010, Yao et al., 2011b). Ketone bodies (KBs) are a by-product of fat metabolism in the liver. The main endogenous KBs are β-hydroxybutyrate (BHB), acetoacetate (AcAc) and acetone. When KBs accumulate in the blood stream, they cause a metabolic state called ketosis. Ketogenic diets are rich in fat and low in carbohydrates and have been successfully used in the treatment and prevention of seizures in epilepsy (Westman et al., 2003, Bough and Rho, 2007, Freeman et al., 2007, Bough, 2008, Caraballo and Vining, 2012).

Female transgenic mice carrying the London APP mutation fed a ketogenic diet for 43 days had decreased levels of Aβ40 and Aβ42, decreased body weight and increased blood levels of β-hydroxybutyrate (Van der Auwera et al., 2005). However, the lack of behavioral improvement observed was attributed to the modest lowering of Aβ levels and/or to the short period of treatment. Clinical trials involving Parkinson’s and Alzheimer’s disease patients treated with diet-induced hyperketonemia resulted in improved motor function (Vanitallie et al., 2005) and enhanced cognition (Reger et al., 2004a), respectively. Consistent with the observed neuroprotective effects in vivo, β-hydroxybutyrate protected mesencephalic and hippocampal neurons from MPP and Aβ toxicity, respectively (Kashiwaya et al., 2000).

Medium chain triglycerides (MCT) are fatty acids (6-12 carbons) that passively diffuse from the gastrointestinal tract and are metabolized in the liver without requirement for modifications, therefore, quickly producing a mild state of ketosis (Yeh and Zee, 1976, Liu, 2008). Oral administration of an emulsified solution enriched with MCT oils (AC1202, Accera, Inc.) in patients with AD or mild cognitive impairment showed improvements in the paragraph recall memory test. This effect was positively correlated with BHB plasma levels (Reger et al., 2004a).
Therefore, we sought to investigate the effects of a diet rich in medium chain triglycerides and low in carbohydrates in an amyloid depositing mouse model of Alzheimer’s pathology (Tg2576). Initially, we examined the effectiveness of this modified ketogenic diet in inducing a significant increase in peripheral levels of β-hydroxybutyrate. Subsequently, we examined the effects of this diet on behavioral and histopathological outcomes in the chosen amyloid depositing mouse model compared to age-matched nontransgenic littermates. Due to the low ketosis obtained, a modified version of the ketogenic diet was devised. We tested this new diet (KD2) in a subset of nontransgenic mice for 2 months and found it to be more effective at inducing and sustaining BHB levels throughout the experimental period.

2.3 Material and Methods

2.3.1 Mice

For this study, we tested mice carrying the human Swedish mutant APP transgene (Tg2576). This model has been extensively studied and it has been previously shown that they have elevated brain levels of soluble Aβ by 6-8 months of age and to develop Aβ-containing neuritic plaques in the cortex and hippocampus by 10-16 months of age (Hsiao et al., 1996, Frautschy et al., 1998). All mice were bred in our facility at the University of South Florida, as previously described (Holcomb et al., 1998a). Mice were 19 months old at the start of the experimental procedure and were individually housed for individual assessments of food intake and body weight. All animals were housed under a 12h light-dark cycle with free access to water and food. Testing procedures were approved by Institutional Animal Care and Use Committee of the University of South Florida and followed NIH guidelines for the care and use of laboratory animals.
2.3.2 Experimental Design

The ketogenic diet was devised by D. D'Agostino in consultation with a nutritionist at Teklad, Harlan (Madison, WI). Our aim was to create a low carbohydrate diet that induced ketosis in the absence of high amounts of hydrogenated fats. The control diet chosen was the commercially available NIH-31 (Teklad, Harlan), commonly used as a balanced standard vivarium diet. Table 2.1 shows the detailed macronutrient composition of diets used.

Mice were fed either a ketogenic (KD) or a control diet (NIH-31) for 3 months. Body weight was assessed once a week throughout the experiment. 2 weeks after the start of their respective diets, blood was collected by submandibular vein puncture in a microfuge tube with 50µl of EDTA. Plasma was obtained by centrifugation (1,000g at 4°C for 15 minutes) of the blood and non-fasting levels of BHB were measured using a commercially available assay kit (Cayman, MI). Following 3 months on their respective diets, mice were subjected to a battery of behavioral tests. At the end of the study, blood was collected by cardiac puncture and plasma was obtained for ketone assessment. Final plasma volumes were sufficient to measure both BHB values and protein concentration to normalize for differences in blood volumes diluted into the EDTA. Subsequently, mice were injected with euthanasia solution and were intracardially perfused with 25 ml of 0.9% normal saline solution. The right hemisphere was dissected for biochemical analysis while the left hemisphere was kept in 4% paraformaldehyde for immunohistochemical analysis.

2.3.3 Behavioral Testing

The open field was used as a standard test of general activity. Animals were monitored for 15 minutes in a 40 cm square open field with a video tracking software, under moderate lighting. General activity levels were evaluated by measurements of
horizontal and vertical activities. Subsequently, each animal was placed in a Y-maze for a single 5 minute trial, during which the sequence and total number of arm choices were recorded. Spontaneous alternation, expressed as a percentage, was calculated according to the method of (Anisman, 1975). Briefly, if an animal made the following sequence of arm selections (1,2,3,2,1,3,1,2), the total alternation opportunities will be 6 (total entries minus 2) and the percentage alternation would be 67% (4 out of 6).

Motor performance was assessed by placing the mice onto the round portion of an accelerating rotarod apparatus (Ugo Basile) with a 2cm diameter rod starting at an initial rotation of 4 rpm accelerating to 40 rpm over 5 minutes. Mice needed to walk at the speed of rod rotation to keep from falling. Mice were tested for the time spent on the rod during each of four trials per day, for two consecutive days. Latency to fall was recorded for each mouse.

Spatial memory was assessed by the radial arm water maze which contained 6 swim paths (arms) radiating out of an open central area, with a hidden escape platform located at the end of one of the arms. On each trial, the mouse was allowed to swim in the arms for up to 60 seconds to find the escape platform. The platform was located in the same arm on each trial. On day one, mice were given 15 trials alternating between a visible platform (above the water) and a hidden platform (below the water). The next day, they were given 15 trials using a hidden platform. The start arm was varied for each trial so that mice relied upon spatial cues to solve the task instead of learning motor rules (i.e. second arm on the right). The goal arm for each mouse was different to avoid odor cues revealing the goal arm (Alamed et al., 2006). We used the open pool test to confirm that mice can see and perform a platform task. The visible platform was elevated above the water surface and had an attached flag. All visual cues were removed from the room so the mice relied only on their ability to see and climb onto the platform. Latency to find and ascend the visible platform was recorded (60 second maximum).
2.3.4 Immunohistochemistry

Immunohistochemical procedural methods have been previously detailed (Gordon et al., 2002b). Briefly, sections from all animals were placed in a multi-sample staining tray and endogenous peroxidase was blocked (10% methanol, 3% H$_2$O$_2$ in PBS; 30 min). Tissue samples were permeabilized (with 0.2% lysine, 1% Triton X-100 in PBS solution) and incubated overnight in primary antibody. Rabbit polyclonal Aβ antibody (prepared by Paul Gottschall) was used at a 1:1000 concentration and Aβ immunoreactivity was assessed. Sections were washed in PBS, and then incubated in biotinylated secondary antibody (Vector Laboratories, Burlingame, CA). The tissue was again washed after 2h and incubated with Vectastain® Elite® ABC kit (Vector Laboratories, Burlingame, CA) during 1h for enzyme conjugation. Finally, sections were stained using 0.05% diaminobenzidine and 0.03% H$_2$O$_2$. Tissue sections were mounted onto slides, dehydrated, and cover slipped.

Congo red histology was performed using sections that were premounted on slides and air-dried for a minimum of 24 hours. The sections were rehydrated for 30 seconds before beginning staining protocol. NaOH (2.5mM) was added to a saturated sodium chloride-ethanol solution and slides were incubated for 20 min. Subsequently, slides were incubated in 0.2% Congo red in alkaline alcoholic saturated sodium chloride solution for 30 minutes. Slides were rinsed through three changes of 100% ethanol, cleared through three changes of xylene, and cover slipped with DPX. Aβ immunohistochemistry and Congo red staining were quantified with a digital scanning microscope and a purpose written program to perform HIS segmentation on either entire sections or user defined regions. Thresholds for object segmentation were established using a series of standard slides which have the extremes of intensity for the stain being measured and once set, remained constant throughout the analysis session. Sample numbers were randomized before the start of the tissue processing, and the code was
broken only after the analysis was complete. All values obtained from a single mouse were averaged together to represent a single value for that animal.

2.3.5 Statistics

Statistical analysis was performed using one-way ANOVA followed by Fisher's LSD post hoc means comparison test or Student's t test, when necessary (Statview software from SAS, Cary NC). Graphs were generated using Graph Pad Prism 5.01 (La Jolla, CA).

2.4 Results

2.4.1 KD did not affect body weight but induced mild ketosis in APP mice

Average body weight was the same for all groups at the start of the experiment (no group effect on body weight, FLSD, p=0.49). Although APP mice showed a trend for smaller body weights than nontransgenic mice, no statistically significant differences were observed in the percentage of body weight change throughout the 3 months of dietary intervention (ANOVA, p=0.58, figure 2.1).

After two weeks on their respective diets, plasma BHB levels were slightly increased in KD-fed APP mice compared to the control diet (NIH-31), although not statistically significant (FLSD, p=0.23, Figure 2.2A). At the end of the study, plasma BHB ratio to total protein was significantly elevated in APP mice fed a ketogenic diet (FLSD, p=0.038, figure 2.2B), compared to NIH-31 control diet (n=9/group).

Figure 2.3A shows that NIH-31-fed APP mice presented a trend for greater total distance traveled in the open field compared to nontransgenic controls (FLSD, p=0.057; n=8 each) and this increased locomotor activity was exacerbated in APP mice kept on KD for 3 months (APP KD: FLSD, p=0.003; n=9). Similarly, APP KD group made significant larger number of arm entries in the Y-maze when compared to both
nontransgenic mice on the control diet (NIH-31, FLSD, p=0.001) or APP mice on the control diet (APP NIH-31, FLSD, p=0.04), as shown in figure 2.3B. No differences between groups were observed in the % of alternation in the y-maze (figure 2.3C).

No differences in motor performance were observed in the rotarod test (figure 2.4A). Figure 2.4B shows that all groups presented motor learning on day 2 (ANOVA main effect of days, p<0.0001). KD did not affect latency to fall from the accelerating rotarod. However, KD-fed APP mice seemed to learn the task more efficiently than their NIH-31-fed controls. When all trials were combined, there was a non-significant trend for increased average latency to fall from the rod in the KD-fed APP group compared to NIH-31-fed APP group (APP KD mean: 141.2 ± 27.5, n=9 versus APP NIH-31 mean: 101.3 ± 19.2, FLSD, p= 0.24).

2.4.2 KD improved early performance on the radial arm water maze

Although mice on the KD performed slightly better on the radial arm water maze test, no overall effect of diet was observed when both days are combined. However, when the total number of errors on each day was analyzed separately, a significant reduction in number of errors was found between Days 1 and 2 (ANOVA main effect of days, p<0.001) and groups (ANOVA main effect of group, p=0.03, figure 2.5A). On day 1, KD-fed APP mice made significantly fewer errors in the RAWM than APP mice fed the control diet (FLSD, p=0.031), as shown in figure 2.5B. On day 2, APP mice fed NIH-31 made significantly more errors than nontransgenics on the same diet (FLSD, p=0.047), indicating an expected effect of the transgene on performance. However the APP mice fed KD had error rates similar to the nontransgenic mice and not significantly different from the other two groups. APP mice on either diet performed similarly on the open pool test showing no signs of physical or visual impairments (figure 2.5C).
2.4.3 KD-induced reduction in brain levels of beta amyloid but not congophilic plaques

Aβ immunoreactivity was assessed in 22 month-old transgenic APP mice fed either diet for 3 months. A trend for reduction in Aβ immunoreactivity was found in the ketogenic diet group (FLSD, p=0.061; n=9, figure 2.6), when entire brain sections were analyzed and compared to transgenic mice fed NIH-31 control diet (n=7).

When brain areas were outlined and analyzed separately (figure 2.7), a significant reduction in Aβ immunoreactivity was observed in the anterior cortex of animals fed a ketogenic diet for 3 months (panel C, t test, p=0.003). A non-significant trend for reduction was found in the entorhinal cortex of ketogenic diet fed mice (panel H, t test, p=0.097). No significant changes were observed in Congophilic dense core plaques when either whole sections or brain areas were analyzed (Figures 2.8 and 2.9).

2.4.4 Obtaining greater ketosis

As a result of the mild ketosis induced by the diet in this study, we reformulated the diet to exclude all maltodextrin and increased the cellulose content in an attempt to further boost ketosis in our animals. The resulting diet (KD2, macronutrient description listed in Table 2.2) was tested in 3 month-old nontransgenic mice (n=6 per group) and was shown to be more effective in increasing BHB levels at 2 weeks when compared to either NIH-31 (FLSD, p=0.04) or KD diets (FLSD, p=0.05, figure 2.10). At the end of the experimental period (2 months), we collected blood and measured final levels of peripheral BHB, which were still significantly increased compared to mice kept on KD (FLSD, p=0.02) and showed a trend for increased levels compared to mice fed NIH-31 (FLSD, p=0.06, figure 2.10). As previously seen with KD, no significant body weight changes were observed in either ketogenic diets throughout the 2 months (figure 2.11).
2.5 Discussion

Previous studies associate low carbohydrate, ketogenic diets with low palatability and, therefore, reduced food intake leading to weight loss. The diet we formulated for this study (KD) did not suffer from this potential confound. APP mice fed a ketogenic diet for approximately four months did not show changes in body weight in comparison to APP mice fed the control NIH-31 diet (figure 2.1). APP transgenic mice showed a non-significant trend for smaller body weight than their nontransgenic counterparts, regardless of the diet. Decreased body weight, despite increased food intake has been previously reported in amyloid depositing models (Morgan and Gordon, 2008), suggesting that APP mice may have increased metabolic demands.

Despite the lack of significant ketosis after two weeks on the diet, final plasma levels of β-hydroxybutyrate were found to be greater in mice fed KD (figure 2.2), showing that our diet induced a state of mild ketosis during the experimental procedure. Studies with dual-tracer positron emission tomography demonstrated that the brain uptake of the ketone body $^{11}$C-AcAc increased more than 2-fold when aged mice were submitted to either 48h of fasting or two weeks on the ketogenic diet. They also reported that the mild ketonemia induced was sufficient to increase the expression of the monocarboxilic transporter 1 (MCT1), responsible for increased permeability of the blood-brain-barrier to ketone bodies (Pifferi et al., 2011).

Behaviorally, we found an increase in locomotor activity in APP transgenic mice in both the open field and the y-maze tasks compared to nontransgenic mice. Interestingly, KD-fed APP mice displayed and even greater number of entries in the y-maze, when compared to NIH-31-fed APP group (figure 2.3). The increased locomotor activity could partly explain the lower body weight previously mentioned in the transgenic line. Despite this increase in locomotor activity, no differences in motor performance were observed in KD-fed mice tested in the rotarod. Figure 2.4B shows that the average
latency to fall from the rod on day 2 was increased for all groups, indicative of motor learning. Beckett et al. (2013) showed significantly improved latency to fall from the rotarod in APP+PS1 mice fed KD for one month with peripheral ketone levels of 1.2mM. Interestingly, no effects on levels of soluble amyloid in either brain or muscle were observed (Beckett et al., 2013a). In our study, although KD-fed APP mice seemed to perform better on day 1 of rotarod (the training component of the task), the mild ketosis achieved with our diet was possibly not enough to result in observable motor improvements (see chapter 3 below).

Subtle signs of improved spatial memory assessment were found in APP mice fed KD for 3 months during the 2-day radial arm water maze test. On day 1, KD-fed APP mice made significantly fewer errors than APP mice fed the control diet suggesting that they learned the task faster than their control-fed counterparts. On day 2, there was a trend, although not statistically significant, for APP mice on KD to make fewer errors than mice fed NIH-31. As expected, APP mice fed NIH-31 made significantly more errors than nontransgenic mice confirming their impaired cognitive phenotype.

In agreement with the reductions in brain levels of soluble Aβ40 and Aβ42 found by Van der Auwera et al. (Van der Auwera et al., 2005), we observed a trend for decreased Aβ immunoreactivity in whole brains of KD-fed APP mice that reached significance in the anterior cortex. However, the lack of effects on congophilic plaque load may reflect the need for more pronounced metabolic changes in order to obtain greater clearance of pre-existing pathology in the brain.

Brain hypometabolism and mitochondrial deficits were reported to appear even before cognitive and histopathological features in both AD patients and mouse models (Blass, 2001, Mosconi et al., 2008a, Yao et al., 2009). Therefore, developing a way to provide the brain with alternative nutrients could be a possible therapeutic approach to compensate for the energy imbalance present in AD patients. Accordingly, our modified
Ketogenic diet (KD2) was much more efficient in producing significant detectable ketosis as early as 2 weeks after the start of the study.

In summary, our results showed that a low-carbohydrate, medium chain triglyceride rich diet showed positive trends in reducing amyloid pathology and rescuing spatial behavioral deficits in old APP transgenic mice. Since ketosis induced by our first diet was modest, we hypothesize that achieving greater ketosis could be lead to more favorable outcomes. Another possible reason for failure to see a clear benefit is the age of the mice, and the long period of amyloid deposition present prior to offering the diet. Therefore, due to its better efficacy in increasing blood ketone levels and no effects on body weight, the modified ketogenic diet (KD2) was used in our subsequent ketogenic diet experiments. Moreover we opted to treat mice earlier in the phase of amyloid deposition in hopes of preventing deposition in the first place, rather than trying to remove already deposited material.
## Table 2.1 Diet Composition

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>NIH-31 grams/kg</th>
<th>KD grams/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>210</td>
<td>300</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>3</td>
<td>2.86</td>
</tr>
<tr>
<td>Sucrose</td>
<td>200</td>
<td>0</td>
</tr>
<tr>
<td>Maltodextrin</td>
<td>100</td>
<td>135.31</td>
</tr>
<tr>
<td>Corn Starch</td>
<td>369</td>
<td>0</td>
</tr>
<tr>
<td>Cellulose (Fiber)</td>
<td>40</td>
<td>110</td>
</tr>
<tr>
<td>MCT Oil (Medium Chain Triglycerides)</td>
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<td>270</td>
</tr>
<tr>
<td>Flaxseed Oil</td>
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<td>70</td>
</tr>
<tr>
<td>Canola Oil</td>
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<td>60</td>
</tr>
<tr>
<td>Mineral Mix Ca-P deficient (79055)</td>
<td>13.4</td>
<td>18.5</td>
</tr>
<tr>
<td>Calcium Phosphate Dibasic (CaHPO$_4$)</td>
<td>7</td>
<td>8.5</td>
</tr>
<tr>
<td>Calcium Carbonate (CaCO$_3$)</td>
<td>7.3</td>
<td>10.75</td>
</tr>
<tr>
<td>40060 VM Teklad</td>
<td>10</td>
<td>14</td>
</tr>
<tr>
<td>Ethoxyquin (Liquid)</td>
<td>0.01</td>
<td>0.08</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td>1000</td>
<td>1000</td>
</tr>
<tr>
<td>Protein, % by weight</td>
<td>17.9</td>
<td>26.4</td>
</tr>
<tr>
<td>Protein, % of Kcal</td>
<td>23.8</td>
<td>20.2</td>
</tr>
<tr>
<td>Carbohydrate, % by weight</td>
<td>46.8</td>
<td>13.4</td>
</tr>
<tr>
<td>Carbohydrate, % of Kcal</td>
<td>62.2</td>
<td>10.3</td>
</tr>
<tr>
<td>Fat, % by weight</td>
<td>4.7</td>
<td>40.3</td>
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<tr>
<td>Fat%, % by Kcal</td>
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<td>69.5</td>
</tr>
<tr>
<td>Kcal/g</td>
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<td>5.2</td>
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Table 2.2 Comparison between KD and KD2

<table>
<thead>
<tr>
<th></th>
<th>Control Diet- NIH-31</th>
<th>Ketogenic Diet (KD)</th>
<th>Ketogenic Diet (KD2)</th>
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<tbody>
<tr>
<td></td>
<td>grams/kg</td>
<td>grams/kg</td>
<td>grams/kg</td>
</tr>
<tr>
<td>Casein</td>
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<tr>
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<td>0</td>
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<td>Maltodextrin</td>
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<td>Corn Starch</td>
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<td>0</td>
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<tr>
<td>Cellulose (Fiber)</td>
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<td>MCT Oil (Medium Chain</td>
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<td>Canola Oil</td>
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<td>60</td>
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<tr>
<td>79055 MM Ca-P Deficient</td>
<td>13.4</td>
<td>18.5</td>
<td>18.5</td>
</tr>
<tr>
<td>Calcium Phosphate Dibasic</td>
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<td>8.5</td>
<td>8.5</td>
</tr>
<tr>
<td>CaHPO4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcium Carbonate CaCO3</td>
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<td>10.75</td>
</tr>
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<td>40060 VM, Teklad</td>
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<td>62.2</td>
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<tr>
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<td>40.3</td>
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<tr>
<td>Fat, % of kcal</td>
<td>14</td>
<td>69.5</td>
<td>77.1</td>
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<tr>
<td>kcal/g</td>
<td>3.0</td>
<td>5.2</td>
<td>4.7</td>
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</table>
Figure 2.1. Ketogenic diet did not affect body weight in APP mice. Percentage of body weight change shown every two weeks for nontransgenic (fed NIH-31, black open circles, n=6) and APP mice fed either a ketogenic diet (KD; red squares, n=10) or a control diet (NIH-31; gray open squares, n=8). Average body weight for all groups was not statistically different either at the start or the end of the experiment. No differences were observed throughout the study. Data shown as mean ± SEM.

Figure 2.2. Ketogenic diet-induced ketosis in APP mice. (A) Plasma β-hydroxybutyrate levels were measured after 2 weeks and (B) 4 months of the start of the study. 4 months ketone levels were corrected for total protein concentration and were significantly increased in comparison to NIH-31-fed mice. Data presented as mean ± SEM. *p<0.04 compared to NIH-31 control.
Figure 2.3. Increased locomotor activity in APP transgenic mice. Open field and Y-maze assessments of general activity in nontransgenic (NIH-31, white bars, n=6) and APP mice kept on either a control diet (NIH-31, gray bars, n=8) or a Ketogenic diet (KD, black bars, n=9). (A) Total distance traveled during 15 minutes in the open field, (B) KD-fed APP mice made significantly more entries in the Y-maze than both groups fed NIH-31 control diet (C) % alternation in the Y-maze was not different between groups. Bars represent mean ± S.E.M. * p<0.05. ** p<0.01.

Figure 2.4. Motor performance was not affected in APP mice fed either NIH-31 or KD. (A) Mice were tested in the accelerating rotarod for 4 trials each day for 2 consecutive days. No differences were observed between groups. (B) Average latency to fall from the rod shows that all groups displayed motor learning on day 2. Data represented as mean ± S.E.M. *p<0.001.
Figure 2.5. Radial arm water maze (RAWM) performance in nontransgenic controls and APP transgenic mice kept on a ketogenic or a control diet for 3 months. (A) Results shown as number of errors until escape platform was found. Each block represents an average of 3 consecutive trials. No significant differences were observed between the two diets studied when blocks were analyzed. (B) Total errors were analyzed by one-way ANOVA and main effects of Day (p=0.002) and Diet (p=0.029) were found. (C) Latency to find a visible platform in an open pool (arms and visual cues were removed) was measured. Mice on either diet did not differ in their ability to locate a visible platform. Data shown as mean ± S.E.M. *p<0.05.
Figure 2.6. (A) Micrograph representation of Aβ immunoreactivity in brains of APP transgenic mice fed either a control (NIH-31) or a Ketogenic diet (KD) for 3 months. (B) Quantification of Aβ immunoreactivity was digitally obtained by Mirax image analysis software. Bars represent mean ± S.E.M. Scale bar = 2000µm.
Figure 2.7. Micrograph representation of Aβ immunoreactivity in brain areas of APP transgenic mice fed either NIH-31 control (A, D, G) or a ketogenic diet (B, E, H) for 3 months. B) Quantification of Aβ immunoreactivity in the anterior cortex (Acx; C), hippocampus (Hp; F) and entorhinal cortex (Ecx; I) were digitally obtained by Mirax image analysis software. Scale bar = 500µm. Bars represent mean ± S.E.M. ***p=0.0003.
Figure 2.8. (A) Micrograph representation (1X) of Congophilic deposits in brains of APP transgenic mice fed either a control (NIH-31) or a Ketogenic diet (KD) for 3 months. (B) Quantification of Aβ immunoreactivity was digitally obtained by Mirax image analysis software. Bars represent mean ± S.E.M. Scale bar = 2000µm.
Figure 2.9. Micrograph representation (5X) of Congophilic deposits in the anterior cortex (Acx, panels A and B), hippocampus (Hpc, panels D and E) and entorhinal cortex (Ecx, panels G and H) of APP mice fed either a control (NIH-31, panels A, D, G) or a Ketogenic diet (KD, panels B, E, H) for 3 months. (C, F and I) Quantification of Congophilic immunoreactivity was digitally obtained by Mirax image analysis software. Bars represent mean ± S.E.M. Scale bar = 500µm.
**Figure 2.10. Comparison of ketogenic diets tested on nontransgenic mice for 2 months.** Blood was collected after 2 weeks and 2 months on respective diets by submandibular vein puncture. Plasma ketone bodies levels from nontransgenic mice kept on control diet (NIH-31, white bars), ketogenic diet (KD, gray bars) or the modified ketogenic diet (black bars, KD2) are graphed. Values were normalized to the total protein concentration in the samples. Bars represent mean ± S.E.M. *p<0.05 when compared to both NIH-31 and KD groups.

**Figure 2.11. Percentage of body weight change for nontransgenic mice fed a control (NIH-31), a ketogenic (KD) or modified ketogenic diet (KD2) for 8 weeks.** Average body weight for all groups was not significantly different either at the start or at the end of the experimental procedure. No group differences were observed throughout the study (n=6 per group). Data shown as mean ± S.E.M.
2.6 References


CHAPTER 3: KETOGENIC DIET IMPROVES MOTOR PERFORMANCE BUT NOT COGNITION IN TWO MOUSE MODELS OF ALZHEIMER’S PATHOLOGY

3.1 Abstract

Dietary manipulations are increasingly viewed as possible approaches to treating neurodegenerative diseases. Previous studies suggest that Alzheimer’s disease (AD) patients present an energy imbalance with brain hypometabolism and mitochondrial deficits. Ketogenic diets (KDs), widely investigated in the treatment and prevention of seizures, have been suggested to bypass metabolic deficits present in AD brain by providing ketone bodies as an alternative fuel to neurons. We investigated the effects of a ketogenic diet in two transgenic mouse lines. Five months old APP/PS1 (a model of amyloid deposition) and Tg4510 (a model of tau deposition) mice were offered either a ketogenic or a control (NIH-31) diet for 3 months. Body weight and food intake were monitored throughout the experiment, and blood was collected at 4 weeks and 4 months for ketone and glucose assessments. Both lines of transgenic mice weighed less than nontransgenic mice, yet, surprisingly, had elevated food intake. The ketogenic diet did not affect these differences in body weight or food consumption. Behavioral testing during the last two weeks of treatment found that mice offered KD performed significantly better on the rotarod compared to mice on the control diet independent of genotype.

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1 Portions of these results have been previously published (Brownlow ML et al, 2013) and are utilized with permission of the publisher.
In the open field test, both transgenic mouse lines presented increased locomotor activity compared to nontransgenic, age-matched controls, and this effect was not influenced by KD. The radial arm water maze identified learning deficits in both transgenic lines with no significant differences between diets. Tissue measures of amyloid, tau, astroglial and microglial markers in transgenic lines showed no differences between animals fed the control or the ketogenic diet. These data suggest that ketogenic diets may play an important role in enhancing motor performance in mice, but have minimal impact on the phenotype of murine models of amyloid or tau deposition.

3.2 Introduction

Alzheimer’s Disease (AD) is a chronic disorder that affects as many as 5.4 million people in the US and its incidence is expected to double by 2050 (Alzheimer’s Association, 2012). AD can affect people in different ways but the earliest and most prominent symptom is the progressive decline in memory. Pathologically, it is characterized by extracellular deposits of amyloid beta (Aβ), intracellular accumulation of neurofibrillary tangles, and neuronal loss. Several transgenic mouse models that express mutant genes demonstrated to cause dementia in humans replicate some of the histopathological and cognitive features of the disease. Some of these models express genes associated with familial Alzheimer’s disease (FAD), while others cause frontotemporal lobe dementia (FTD).

Other consistent features of dementias are hypometabolism in several brain areas but particularly in the hippocampus of AD patients (Foster et al., 1983, Costantini et al., 2008) and frontal lobes of FTD patients (Diehl et al., 2004, Diehl-Schmid et al., 2007), and impaired mitochondrial function (Blass et al., 2000). Decreased cerebral glucose utilization is an early event in AD pathology and may precede the neuropathological deposition of Aβ and/or clinical symptoms (Blass, 2001) (Mosconi et
al., 2008a) by decades. Similarly, mitochondrial deficits precede the deposition of amyloid and tau in the brains of a triple transgenic mouse model of AD (3xTgAD).

Furthermore, a shift in brain metabolism has been reported in mouse models of AD, changing from utilization of glucose to increased ketogenesis during aging (Yao et al., 2010, Yao et al., 2011b). Ketone bodies (KBs) are a by-product of the breakdown of fat in the liver. The main endogenous KBs are β-hydroxybutyrate (BHB), acetoacetate and acetone. When KBs accumulate in the blood stream, they cause a metabolic state called ketosis. Ketosis is a survival mechanism activated during prolonged fasting, starvation or lack of carbohydrate ingestion. During prolonged ketosis, the brain is capable of metabolizing ketone bodies as an alternate fuel, thus reducing its requirement for glucose (LaManna et al., 2009). Diets that are rich in fat and low in carbohydrate, known as ketogenic diets, mimic the effects of fasting and the lack of glucose/insulin signaling promotes a metabolic shift toward fatty acids utilization (Hammami, 1997). Blood ketone levels are typically modest, and well below those caused by the metabolic ketosis accompanying diabetes.

Ketogenic diets have been successfully used in the treatment and prevention of seizures in epilepsy (Westman et al., 2003, Bough and Rho, 2007, Freeman et al., 2007, Bough, 2008, Caraballo and Vining, 2012). The improved metabolic efficiency observed suggests that, besides its anticonvulsant properties, KDs may be useful for treating several other neurological disorders. Current models of neurodegenerative diseases showed positive outcomes induced by ketogenic diet such as increased motor neuron number in ALS transgenic models (Zhao et al., 2006, Zhao et al., 2012), reduced lesion volume after traumatic brain injury (Prins et al., 2005), increased cell survival and decreased seizure frequency in kainate-induced seizure models (Noh et al., 2003) and suppressed inflammatory cytokines and chemokines in an experimental model of multiple sclerosis (Kim do et al., 2012).
Medium chain triglycerides are fatty acids that are rapidly metabolized in the liver to produce a mild state of ketosis (Yeh and Zee, 1976, Liu, 2008). Recent clinical trials in patients with AD or mild cognitive impairment have shown that an orally administered mixture of medium chain triglycerides (AC-1202, Accera, Inc.) can improve memory and attention in these individuals, and better performance was associated with higher β-hydroxybutyrate plasma levels. Cognitive facilitation was greater in ApoE4-negative adults with memory disorders (Reger et al., 2004b).

Consequently, we investigated the effects of a diet rich in medium chain triglycerides and low in carbohydrate in two well-established mouse models with Alzheimer-like pathology, amyloid-depositing mice transgenic for mutant amyloid precursor protein and presenilin-1 (APP+PS1) and tau-depositing mice transgenic for tau (Tg4510). We used these two mouse models to dissociate dietary effects on the deposition of amyloid and tau proteins separately because our recent evidence suggests that these two main pathologies may be regulated differently (Lee et al., 2010b, Nash et al., 2013). We demonstrated that the ketogenic diet effectively elevated circulating ketone bodies while reducing glucose levels. We examined effects of this diet on behavioral and histopathological outcomes in transgenic models with Alzheimer-like pathology and age-matched nontransgenic littermates.

Because AD requires many years to fully manifest in patients, an effective diet treatment could delay the rate of symptom progression and delay or prevent institutionalization. Therefore, we sought to study the effects of the KD in two well-established mouse models of Alzheimer’s pathology, dissociating its effects on the deposition of amyloid and tau proteins. To our knowledge, this is the first report of the effects of a ketogenic diet on a mouse model of tauopathy in the absence of amyloid.
3.3 Materials and Methods

3.3.1 Ethics Statement

All animal testing procedures were approved by the Institutional Animal Care and Use Committee of the University of South Florida and followed NIH guidelines for the care and use of laboratory animals (Approval ID number: 0054R).

3.3.2 Ketogenic Diet

The ketogenic diet was devised by D. D’Agostino in consultation with a nutritionist at Teklad (Madison, WI). Briefly, the goal was to obtain a low carbohydrate, medium chain triglyceride-rich diet which induced ketosis, but did so without introducing high amounts of omega-6 or hydrogenated fats. We also wished to develop a diet which did not lead to weight loss, to avoid confounds of dietary or caloric restriction effects as a possible cause for any observed outcomes. A detailed list of macronutrient components of the diet used in this experiment is presented in Table 3.1.

3.3.3 Mice

APP+PS1 mice (Holcomb et al., 1998b) were acquired from our breeding colonies at the University of South Florida. These mice develop congophilic amyloid deposits by 3 months of age, with neuritic and glial involvement, which increase in number with aging, along with deficits in learning and memory (Gordon et al., 2002b). The Tg4510 mouse carries a regulatable tau transgene with the fronto-temporal dementia associated mutation P301L mutation. Mutant tau expression is restricted largely to forebrain neurons by CaM kinase II-driven expression of the tet trans activator. These mice develop progressive pathology with the first discernible abnormally phosphorylated tau observed at 3 months of age, leading to readily detectable neuron loss by 6 months of age (Santacruz et al., 2005, Dickey et al., 2009). In this experiment,
mice were given food and water *ad libitum* and maintained on a twelve-hour light/dark cycle and standard vivarium conditions. In order to assess the effects of a KD on mice with some pre-existing pathology, all animals were 5 months old at the start of the experiment. Animals were housed and treated according to institutional and National Institutes of Health standards. Nontransgenic mice used as a positive control for behavioral studies were littermates from the breeding of Tg4510 mice and are FVB/129S. APP+PS1 mice are on a mixed background of primarily C57/BL6 with smaller contributions from DBA/2, SW, SGL lines with the *rd1* mutation selected out of the breeding colony.

### 3.3.4 Experimental Procedure

Figure 3.1 depicts the experimental design and time course adopted in this experiment. Mice were randomly assigned to receive either ketogenic or NIH-31 control diet (n=10/group, *ad libitum*). Food was replaced three times and food consumption and body weight were monitored. The week before the commencement of the experiment, mice were transitioned to the new diet for a week by having gradually increasing quantities of KD offered in addition to their standard diet. Four weeks after the start of the diets, peripheral blood was collected, and non-fasting levels of β-hydroxybutyrate (BHB) and glucose were measured using a commercially available glucose/ketone meter (Nova Max Plus, Waltham, MA). After three months on their respective diets, the mice were submitted to a battery of behavioral testing. At the end of testing, mice were weighed, euthanized with a solution containing pentobarbital and transcardially perfused with 25 ml of 0.9% normal saline solution. Brains were collected immediately following perfusion. One hemisphere was dissected and immediately frozen on dry ice for biochemical analysis. The second hemisphere was immersion fixed in 4% phosphate-buffered paraformaldehyde for 24h. The fixed hemispheres were cryoprotected in
successive incubations of 10%, 20% and 30% solutions of sucrose for 24h each. Subsequently, brains were frozen on a cold stage and sectioned in the horizontal plane (25 µm thickness) on a sliding microtome and stored in Dulbecco’s phosphate buffered saline (DPBS) with 10 mM sodium azide solution at 4C. Every 8th section was cut at 50 µm thickness for stereological counts of neurons and measurement of hippocampal volume in Nissl-stained sections.

3.3.5 Behavioral Testing

The open field was used as a standard test of general activity. Animals were monitored for 15 minutes in a 40 cm square open field with a video tracking software, under moderate lighting. General activity levels were evaluated by measurements of horizontal and vertical activity.

Each animal was placed in a walled Y-maze for a single 5 minute trial. The sequence of arm entries and total number of arm choices were recorded. Spontaneous alternation (entering all three arms sequentially without repetition) was expressed as a percentage, as calculated according to the method of (Anisman, 1975).

Motor performance was evaluated by an accelerating rotarod apparatus with a 3cm diameter rod starting at an initial rotation of 4 RPM slowly accelerating to 40 RPM over 5 minutes. Mice were expected to walk at the speed of rod rotation to keep from falling. The time spent on the rod during each of four trials per day for two consecutive days was measured. Testing was completed when the mouse fell off the rod (distance of 12 cm) onto a spring-cushioned lever, and rarely attained the maximal time allotted. Motor performance in an endurance version of the rotarod was assessed in a single trial on the third day. For this trial, the rod was accelerated to a lower maximum speed of 25
RPM and held constant, and the mice were allowed to stay on the rod for a maximum trial length of 1000s (the longest duration allowed by the software).

3.3.6 Radial Arm Water Maze (RAWM)

A detailed description of this test has been previously published, complete with goal arm assignments and scoring sheets (Alamed et al., 2006). Briefly, the radial arm water maze contains 6 swim paths (arms) radiating out of an open central area with a hidden escape platform located at the end of one of the arms. The pool is surrounded by several extra-maze cues to allow spatial navigation. On each trial, the mouse was allowed to swim for up to 60 seconds to find the escape platform. The platform was located in the same arm on each trial. On day one mice were given 15 trials alternating between a visible platform (above the water) and a hidden platform (below the water). On day two, mice were given 15 additional trials with all the trials using a hidden platform. The start arm was varied for each trial so that mice relied upon spatial cues to solve the task instead of learning motor rules (i.e. second arm on the right). The goal arm for each mouse was different to avoid odor cues revealing the goal arm. Entry into an incorrect arm (all four limbs within the arm) was scored as an error. Failure to make an arm entry within 15 seconds was also scored as an error. The errors for blocks of 3 consecutive trials were averaged for data analysis. Mice averaging of 1 error or less by the end of day two are considered to have reached the learning criterion. On the third day, a reversal trial was performed with the goal platform placed in the arm 180° from the original location. Mice were given 15 trials all with a hidden platform. The open pool with a visible platform test was performed on the day following the reversal trial to confirm that all mice were capable of seeing and ascending the platform. The visible platform was elevated above the water surface and had an attached flag. For the open pool test, all visual cues were removed from the room so the mice relied only on their
sight to find the platform. Latency to find and ascend the platform was recorded (60 seconds maximum).

3.3.7 Fear Conditioning

Fear conditioning was used to assess memory formation that is especially sensitive to proper hippocampal function. For these experiments, an aversive stimulus (in this case a mild foot shock, 0.5mA) was paired with an auditory conditioned stimulus (white noise) within a novel environment. Freezing on the training day in response to the foot shock was used as an estimate of learning during the acquisition trial. Animals were placed in the fear conditioning apparatus for 3 min, then a 30 s acoustic conditioned stimulus (white noise, 70 dB) was delivered with a 0.5-mA shock (unconditioned stimulus) applied to the floor grid during the last 2 s of the CS. Training consisted of two mild shocks paired with two conditioned stimuli with a 2-min interval between each shock. For contextual memory, the mice were placed in the chamber and monitored for freezing to the context approximately 24 hour after training (no shocks or auditory cue given) and tested for 3 min. Learning was assessed by measuring freezing behavior (i.e. motionless position) every 1 s and % of time spent freezing was calculated (Bolognani et al., 2007).

3.3.8 Histopathology

Immunohistochemical procedural methods were described by Gordon et al. (Gordon et al., 2002a). For each marker, sections from all animals were placed in a multi-sample staining tray and endogenous peroxidase was blocked (10% methanol, 3% H₂O₂ in PBS; 30 min). Tissue samples were permeabilized (with 0.2% lysine, 1% Triton X-100 in PBS solution) and incubated overnight in appropriate primary antibody. Anti-NeuN (Millipore); anti-Aβ (prepared by Paul Gottschall), anti-GFAP (Dako), anti-Iba1
total tau H150 (rabbit polyclonal, Santa Cruz Biotechnology), anti-pSer199/202 (rabbit polyclonal, Anaspec) and anti-pS396 tau (rabbit polyclonal, Anaspec) antibodies were used in this experiment. Sections were washed in PBS, and then incubated in corresponding biotinylated secondary antibody (Vector Laboratories, Burlingame, CA). The tissue was again washed after 2h and incubated with Vectastain® Elite® ABC kit (Vector Laboratories, Burlingame, CA) for enzyme conjugation. Finally, sections were stained using 0.05% diaminobenzidine and 0.03% H₂O₂. Tissue sections were mounted onto slides, dehydrated, and cover slipped.

Congo red and Gallyas histology were performed using sections that were premounted on slides and air-dried for a minimum of 24 hours. The sections were rehydrated for 30 seconds before beginning staining protocol. For Congo red, 2.5mM NaOH was added to a saturated sodium chloride-ethanol solution and slides were incubated for 20 min. Subsequently, slides were incubated in 0.2% congo red in alkaline alcoholic saturated sodium chloride solution for 30 minutes. Slides were rinsed through three changes of 100% ethanol, cleared through three changes of xylene, and cover slipped with DPX. Gallyas staining was performed as described in (Lee et al., 2010a). Slides were treated with 5% periodic acid for 5 min, washed with water, and incubated sequentially in silver iodide (1 min) and 0.5% acetic acid (10 min) solutions prior to being placed in developer solution (2.5% sodium carbonate, 0.1% ammonium nitrate, 0.1% silver nitrate, 1% tungstosilicic acid, 0.7% formaldehyde). Slides were treated with 0.5% acetic acid to stop the reaction, then incubated with 0.1% gold chloride and placed in 1% sodium thiosulphate. Following a final wash in water, slides were dehydrated and cover slipped.

Stained sections were imaged using a Zeiss Mirax-150 digital scanning microscope and Image Analysis software. Area of positive stain for the entire brain region in each section (no sampling) was analyzed using 200X magnification. The software used hue,
saturation and intensity (HSI) to segment the image fields. Thresholds for object segmentation were established with images of high and low levels of staining to identify positive staining over any background levels. These limits were held constant for the analysis of every section in each study (Gordon et al., 2002a).

3.3.9 Statistics

Statistical analysis was performed using two-way ANOVA followed by Fisher's LSD post hoc means comparison test using Stat View software version 5.0 (SAS Institute Inc, Cary NC). Graphs were generated using Graph Pad Prism 5.01 (La Jolla, CA).

3.4 Results

3.4.1 KD increased ketosis and reduced glucose levels

KD diet efficiently increased blood BHB levels when compared to NIH-31 control diet at 4 weeks (ANOVA main effect of diet, p<0.0001, figure 3.2A). However, this increase was more pronounced in the nontransgenic genotype than in either APP+PS1 or Tg4510 transgenic lines (FLSD, p=0.013 and p=0.033, respectively), and there was a significant genotype and diet interaction (p=0.021). In addition, blood glucose levels were significantly decreased in KD-fed mice (ANOVA main effect of diet, p=0.002). A main effect of genotype was observed (p<0.0001) with APP+PS1 and Tg4510 mice presenting higher glucose levels compared to nontransgenic mice (FLSD, p<0.0001 and p=0.036, respectively; figure 3.2B). Ketosis was shown to be maintained for the duration of the experiment, and blood BHB levels were also at higher levels in mice fed a KD at the 16 week time point (ANOVA main effect of diet, p<0.0001, figure 3.2C).
3.4.2 Genotype, but not diet, affected body weight, food intake and locomotor activity

Average body weight was the same for all groups at the start of the experiment. An overall main effect of genotype on body weight was observed throughout the course of the study (ANOVA, p=0.018). At 16 weeks, both APP+PS1 and Tg4510 mice had smaller body weights, not affected by diet, relative to the nontransgenic mice (FLSD, p=0.018 and p=0.005, respectively; Fig 3.3A). Interestingly, the transgenic lines presented significantly greater food intake than nontransgenic control mice throughout the experiment, regardless of their diets (ANOVA main effect of genotype, p<0.0001, figure 3.3B).

Figure 3.4 shows that total distance travelled in the open field was significantly greater for both Tg4510 and APP+PS1 mice than for nontransgenic mice (ANOVA main effect of genotype, p<0.0001; FLSD, p<0.0001 and p=0.0043, respectively). Tg4510 showed even greater activity than APP+PS1 mice (FLSD: p=0.002), suggesting a possible hyperactive phenotype. In addition, Tg4510 mice made significantly more arm entries in the Y-maze test than nontransgenic controls (ANOVA main effect of genotype, p=0.012, Table 3.2). Furthermore, Tg4510 showed higher percentage of alternation in the Y-maze as well (ANOVA main effect of genotype, p=0.02, Table 3.2), when compared to APP+PS1 genotype (FLSD, p=0.009). No diet effects were observed in any genotype in either the open field or the Y-maze tests.

3.4.3 KD enhanced motor performance but did not rescue memory deficits

Mice fed a ketogenic diet spent significantly more time on the accelerating rotarod (figure 3.5). Both genotype and diet affected their performance (ANOVA main effects, p=0.01 and p=0.005, respectively, figure 3.5A). APP+PS1 mice performed poorly in comparison to nontransgenics (FLSD, p=0.05) or Tg4510 (FLSD, p=0.006). Figure 3.5B represents the averaged latency to fall for all trials (days 1 and 2) of the rotarod and
highlights the KD-induced improvement in motor performance. In addition, in a variation of the rotarod paradigm where mice were tested for a maximum trial length of 1000 s while the rod was held constant at 25 rpm (lower maximum speed, Endurance trial), KD greatly enhanced motor activity and both genotype (p=0.002) and diet (p=0.006) main effects were observed (figure 3.5C). Latency to fall in nontransgenic mice was significantly greater than APP+PS1 (FLSD, p=0.002) and Tg4510 (FLSD, p=0.003) mice (figures 3.5B and 3.5C).

Spatial memory deficits were assessed by the 2-day radial arm water maze. ANOVA main effect of genotype (p=0.001) showed that both APP+PS1 and Tg4510 made significantly more errors in the attempt to find the platform compared to nontransgenic control groups (FLSD, p=0.01 and p<0.001, respectively), regardless of the diet (figure 3.6A). Neither transgenic line performed as well on the reversal trial as the nontransgenic controls (ANOVA main effect of genotype, p<0.0001; FLSD APP+PS1, p<0.001 and Tg4510, p<0.0001, compared to Ntg). The open pool test showed that all mice were capable of performing a visual platform test (Table 3.2).

Diet did not affect the freezing response during the training session of the CFC (data not shown). Memory for contextual fear tested 24 hours after training session was not affected by diet but a genotype effect was observed (p=0.0004) with APP+PS1 and Tg4510 mice freezing significantly less than the nontransgenic groups (Table 3.2; FLSD, p=0.005 and p=0.0002, respectively).

3.4.4 Neuronal loss in Tg4510 mice was not rescued by KD and was associated with increased astrocytosis and microglial activation

Tg4510 mice overexpress human tau with a P301L mutation and have been reported to exhibit significant neuronal loss by 6 months of age (Dickey et al., 2009). Mice in this experiment were 9 months old at the time of euthanasia. To investigate whether our
dietary manipulation affected total neuronal loss in our mouse lines, representative sections were stained with a biotinylated antibody against neuron-specific protein NeuN. Indeed, we found estimated reductions of 23% and 27% in area immunoreactive for NeuN in the brains of Tg4510 mice, when compared to APP+PS1 or nontransgenic mice, respectively (FLSD, p=0.001 and p<0.0001, figure 3.7G). This difference, indicative of the significant neuronal loss observed in this model, was not prevented by a KD. As expected, APP+PS1 mice did not show any differences in the percentage of NeuN positive cells when compared to nontransgenic groups, regardless of the diet. Accordingly, the hippocampal volume (expressed in mm$^3$) of Tg4510 mice was significantly smaller than either APP+PS1 or nontransgenic genotypes (FLSD, p<0.0001).

In addition, Tg4510 mice showed prominent GFAP-positive reactive astrocytes in comparison to either age-matched nontransgenic controls or APP+PS1 mice (FLSD, p<0.0001), figure 3.8 (panels A-G). Microglial activation was similarly elevated in the Tg4510 transgenic mice, compared to nontransgenic controls or APP+PS1 mice (FLSD, p<0.0001 and p=0.001, respectively), as shown in figure 3.8 (panels H-N). These changes are consistent with the presence of degenerating neurons. No diet effects were observed for either astrocyte or microglial markers.

3.4.5 No changes in amyloid or tau deposition were observed

Results from histological assessments are detailed in Table 3.3. As expected, no Aβ or tau immunoreactivity was observed in nontransgenic control mice, regardless of diet. Whole sections were analyzed and no changes in Aβ immunoreactivity or congophilic compact plaques were observed in APP+PS1 mice fed a ketogenic diet for 4 months. Additionally, no changes in the percentage of area immunoreactive for total tau or phosphorylated tau were observed in Tg4510 mice on the ketogenic diet, compared to
the control diet. Total tau immunoreactivity was assessed by staining with H150 antibody (against aa 1-150), which recognizes both human and mouse tau. Antibodies against tau phosphorylated at epitopes Ser 199/202 and Ser 396 were also used in this experiment. Moreover, Gallyas silver staining against deposited fibrillar tau was also performed. KD did not change any of the tau pathology markers used in our study. Moreover, cortical and hippocampal brain regions were analyzed separately and no differences were found between diets for either tau or amyloid deposition in these selected areas (data not shown).

3.5 Discussion

In our experiment, we were able to effectively induce ketosis reaching 1 mM ketone body levels in adult transgenic and nontransgenic mice with a specially designed MCT-rich, low carbohydrate ketogenic diet. Accordingly, plasma glucose levels were significantly diminished in KD-fed mice. Four months later, at the end of the study, plasma BHB levels were still increased when compared to mice fed a standard, NIH-31 diet (figure 3.2). Therefore, we showed that our diet was successful in inducing and maintaining therapeutic ketosis throughout the course of the experiment.

Many previous reports associate KDs with weight loss in both humans and rodents (Yancy et al., 2004, Mohamed et al., 2010, Yao et al., 2011a, Kashiwaya et al., 2012, Krikorian et al., 2012, Paoli et al., 2012, Srivastava et al., 2012). Additionally, KDs are often associated with low palatability and, therefore, voluntary caloric restriction (Van der Auwera et al., 2005). Previously we demonstrated that caloric restriction slows amyloid deposition in APP+PS1 mice (Patel et al., 2005). However, in our experiment, KD-fed mice did not reduce food intake or display decreased body weight (figure 3.3). Therefore, caloric restriction was not a confound in this experiment. The failure of the ketogenic diet to slow amyloid deposition would imply that the ketosis associated with
Caloric restriction is unlikely to mediate the effects of caloric restriction on the APP+PS1 mouse phenotype.

Genotype, but not diet, was found to affect body weight, with transgenic mouse lines presenting smaller body weights. However, most surprising in this regard was the significantly greater food intake than nontransgenic age-matched controls. One possible explanation for this could be increased locomotor activity associated with the Tg4510 mouse line, and to a lesser extent with the APP+PS1 mice, in both the open field (Fig 3.4) and y-maze arm entries (table 3.2). It is also feasible that the Tg4510 mice have a difference in basal metabolism. In agreement with this observation, Morris et al. (Morris et al., 2013b) found that the ablation of tau (Tau^-) caused weight gain in middle-aged mice. This is suggestive of tau playing a possible role in the hypermetabolic phenotype encountered in this study. Indeed, hypermetabolism in amyloid models has been previously reported (Morgan and Gordon, 2008, Knight et al., 2012). Anecdotally, it has been reported that dementia patients sometimes exhibit significant weight loss, associated with increased intake of calories per kg body weight (Wang et al., 2004). We are planning calorimetry studies in the future to evaluate these options more completely.

In our study, KD-fed mice presented significantly enhanced motor performance, regardless of genotype (figure 3.5). Our findings suggest that, despite the fact that all genotypes showed motor learning after one day of training (figure 3.5A), mice that were fed a low carbohydrate diet presented significantly greater latency to fall from the rotarod in both the high and low maximum rotation speed versions of the task (figures 3.5B and 3.5C, respectively). There is a possibility that the enhanced performance observed could be due to motor learning, which permitted them to stay longer on the rod. Alternatively, we suggest that this improved motor performance could be due to the KD-induced increase in metabolic efficiency in muscles. Several recent studies reported the beneficial effects of KDs on motor performance in different mouse models. A medium
chain triglyceride diet improved rotarod performance in a mouse model of amyotrophic lateral sclerosis (ALS) (Zhao et al., 2012), despite the lack of an increase in survival. The authors attributed this effect to the preservation of motor neurons in the spinal cord at the end stage of the disease. This enhanced motor performance was recently shown to be duplicated by supplementing typical rodent diets with 10% caprylic acid, a medium chain triglyceride that elevated plasma ketones to 0.5 mM. Using a mouse model of experimental autoimmune encephalomyelitis (EAE), Kim et al (Kim do et al., 2012) showed that feeding a KD diet starting 7d before induction diminished all components of the EAE phenotype. In addition to improving motor disability scores, KD-fed EAE mice showed significantly shorter latencies to find the platform in a water maze. This effect possibly resulted from their increased swim speed, compared to EAE mice fed a control diet. Rescue of deficits in long term potentiation, reduced lesion volumes, fewer infiltrating T cells and reduced inflammatory cytokine levels were reported in mice offered KD. In humans, Paoli et al (Paoli et al., 2012) demonstrated that artistic gymnasts on a very low carbohydrate KD for 30 days decreased body weight and fat without negative effects on strength performance in athletes.

In agreement with our results, a recent study reported that KD improved rotarod performance in both nontransgenic and APP+PS1 mice when fed KD for one month (Beckett et al., 2013a). This diet also elevated ketones to 1 mM, as in the present study. The mice in this study were quite young (1-2 mo) and not yet depositing amyloid in the brain, but no effects on the soluble amyloid in brain or muscle could be detected by ELISA, consistent with the present results. Another recent study (Kashiwaya et al., 2012) described effects of supplementing the diets of 3xTg mice with a ketone ester, providing 20% of calories and reaching 0.7 mM BHB. These mice were treated for 4-7 mo and the authors observed subtle differences in learning, decreased anxiety and increased locomotor activity in the ketone-supplemented mice. Unfortunately, no nontransgenic
mice were included to aide in determining if these differences represented a) a worsening or rescue of the transgenic phenotype or b) a general effect observed in all mice or a specific interaction with the transgenic mouse phenotype. Although these mice did not deposit amyloid, they did exhibit intracellular staining with an anti-Aβ antiserum. The number of cells exhibiting this staining was reduced by the ketone ester supplement. This contrasts with our inability to discern improvements in spatial or associative memory deficits or reductions in amyloid deposits in APP+PS1 mice offered the KD.

KD did not rescue neither global neuronal loss nor hippocampal atrophy in Tg4510 mice. These are hallmarks of AD and are replicated in the Tg4510 mouse model, with widespread development of neurons bearing hyperphosphorylated tau and later cell death (Ramsden et al., 2005, Santacruz et al., 2005, Spires et al., 2006, Dickey et al., 2009). Indeed, we did observe a significant decrease in the fractional area occupied by NeuN staining in the Tg4510 genotype, but the ketogenic diet did not prevent it. It is possible that small differences in select neuronal populations were not detected using this analysis. We did not observe a decrease in NeuN staining in the APP+PS1 genotype, an amyloid overexpression model. As previously observed (Ramsden et al., 2005), neuronal loss in Tg4510 mice was accompanied by an increase in GFAP-positive reactive astrocytes and microglial activation (Lee et al., 2010b) (figure 3.8). Markers for tau pathologies were also analyzed in Tg4510 mice. Significant numbers of neurons were positive for tau, phosphorylated tau epitopes and argyrophilic tau, but no differences in the amount of staining were observed after 4 months of KD treatment (Table 3.3).

Yao et al (Yao et al., 2011a) reported that treatment with 2-deoxyglucose significantly reduced both mitochondrial APP and the 16kD Abeta oligomer levels. Because 2-DG competitively blocks glucose metabolism, it induces a compensatory rise
in alternative substrates, primarily ketone bodies by the liver, suggesting that other mechanisms activated by CR may underlie its neuroprotective effects observed in reducing amyloid pathology (Patel et al., 2005). Despite significant reductions in amyloid markers in their study, tau hyperphosphorylation remained unchanged, suggesting that different mechanisms are possibly at play in tau pathology. This lack of effect on tau pathology contrasts with the finding using ketone ester supplementation in 3xTg mice (Kashiwaya et al., 2012), where treatment decreased the number of cells staining for PHF-tau by roughly one third.

In summary, our results suggest that a MCT-rich, low carbohydrate ketogenic diet, significantly induced ketosis and improved motor performance in all genotypes tested in our study. In the past few years, increasingly anecdotal evidence of the benefits of KD has been reported in AD or MCI patients. Clinical trials involving Parkinson’s and Alzheimer’s disease patients treated with diet-induced hyperketonemia resulted in improved motor function [22] and enhanced cognition [23], respectively. These benefits tend to appear in a relatively short time frame (weeks to several months). Our data testing the hypothesis that these dietary manipulations would impact the deposition of amyloid or tau and slow the underlying disease process is not supported by the data we obtained in this study. Instead, the behavioral improvements observed are likely due to a metabolic effect, enhancing the performance of remaining neurons, while the underlying disease proceeds unabated. Therefore, KD may play an important role in improving motor performance and providing symptomatic relief to individuals with Alzheimer’s and/or other dementias, but would seem unlikely to modify the rate of accumulation of the neuropathology in these disorders.
Table 3.1 Diet Composition

<table>
<thead>
<tr>
<th>Ingredient composition</th>
<th>NIH-31 grams/kg</th>
<th>Ketogenic Diet grams/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>210</td>
<td>300</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>3</td>
<td>2.86</td>
</tr>
<tr>
<td>Sucrose</td>
<td>200</td>
<td>0</td>
</tr>
<tr>
<td>Maltodextrin</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Corn Starch</td>
<td>369</td>
<td>0</td>
</tr>
<tr>
<td>Cellulose (Fiber)</td>
<td>40</td>
<td>245.31</td>
</tr>
<tr>
<td>MCT Oil (Medium Chain Triglycerides)</td>
<td>0</td>
<td>270</td>
</tr>
<tr>
<td>Flaxseed Oil</td>
<td>21</td>
<td>70</td>
</tr>
<tr>
<td>Canola Oil</td>
<td>19</td>
<td>60</td>
</tr>
<tr>
<td>Mineral Mix Ca-P Deficient (79055)</td>
<td>13.4</td>
<td>18.5</td>
</tr>
<tr>
<td>Calcium Phosphate Dibasic CaHPO4</td>
<td>7</td>
<td>8.5</td>
</tr>
<tr>
<td>Calcium Carbonate CaCO3</td>
<td>7.3</td>
<td>10.75</td>
</tr>
<tr>
<td>40060 Vitamin Mix, Teklad</td>
<td>10</td>
<td>14</td>
</tr>
<tr>
<td>Ethoxyquin (Liquid)</td>
<td>0.1</td>
<td>0.08</td>
</tr>
<tr>
<td>TOTAL</td>
<td>1000</td>
<td>1000</td>
</tr>
</tbody>
</table>

- Protein, % of kcal: NIH-31 23.8, Ketogenic Diet 22.4
- Carbohydrate, % of kcal: NIH-31 62.2, Ketogenic Diet 0.5
- Fat, % by kcal: NIH-31 14, Ketogenic Diet 77.1
- Vitamin Mix, % of kcal: NIH-31 1.3, Ketogenic Diet 1.2
- kcal/g: NIH-31 3.0, Ketogenic Diet 4.7
Table 3.2 Behavioral tests results

<table>
<thead>
<tr>
<th>Test</th>
<th>Ntg</th>
<th>APP+PS1</th>
<th>Tg4510</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NIH-31</td>
<td>KD</td>
<td>NIH-31</td>
</tr>
<tr>
<td>Y-Maze</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Entries</td>
<td>42.4 ± 1.7</td>
<td>43.1 ± 2.0</td>
<td>49.7 ± 4.5</td>
</tr>
<tr>
<td>% Alternation</td>
<td>62.9 ± 3.0</td>
<td>62.3 ± 3.1</td>
<td>51.6 ± 4.0</td>
</tr>
<tr>
<td>Open Pool (s)</td>
<td>7.5 ± 1.1</td>
<td>8.3 ± 2.9</td>
<td>13.9 ± 0.7</td>
</tr>
<tr>
<td>Context (% Freezing)</td>
<td>98.5 ± 0.8</td>
<td>94.3 ± 2.1</td>
<td>80.4 ± 9.1**</td>
</tr>
</tbody>
</table>

Data presented as mean ± SEM. FLSD, *p< 0.05, **p<0.006 and ***p=0.0002 compared to Ntg genotype. *p<0.01 compared to APP+PS1 genotype. Open pool results shown as seconds to reach a visible platform. Context and Cued results shown as % of freezing.

Table 3.3 Immunohistochemical markers

<table>
<thead>
<tr>
<th>Markers (% Area)</th>
<th>APP+PS1</th>
<th>Tg4510</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NIH-31</td>
<td>KD</td>
</tr>
<tr>
<td>Abeta</td>
<td>2.41 ± 0.89</td>
<td>2.83 ± 0.68</td>
</tr>
<tr>
<td>Congo</td>
<td>0.23 ± 0.02</td>
<td>0.25 ± 0.01</td>
</tr>
<tr>
<td>H150</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Ser 199/202</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Ser 396</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Gallyas</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

% Positive Area of staining shown. Data presented as mean ± SEM. nd: not detected
Figure 3.1. *Experimental design for the study of a ketogenic diet using two mouse models of Alzheimer's pathology.* APP+PS1, Tg4510 and nontransgenic littermates received either a control (NIH-31) or a low carbohydrate, medium-chain triglyceride rich, ketogenic diet (KD) for 16 weeks. Blood was collected at 4 weeks and 16 weeks for measurement of circulating ketone and glucose levels. Near the end of the administration period, mice received a variety of behavioral tests. Tissue was collected after a 16 week diet administration.
Figure 3.2. KD increased ketosis and reduced glucose levels. (A, C) KD (black bars) successfully increased peripheral β-hydroxybutyrate (BHB) levels after 4 weeks or 4 months, compared to a control diet (NIH-31, open bars). (B) Accordingly, circulating glucose levels were found to be decreased in KD-fed mice, in all genotypes. Glucose and BHB levels were measured utilizing a commercially available glucose/ketone monitoring system (Nova Max© Plus). Data are presented as mean ± SEM (n=10). **p<0.0004, ***p<0.0001.
Figure 3.3. Changes in body weight and food intake throughout experiment. (A) Assessments of body weight and (B) food intake in APP+PS1, Tg4510 and nontransgenic mice on either control diet (NIH-31, open symbols) or ketogenic diet (KD, solid symbols) for 4 months. Both transgenic mouse lines weighed significantly less than nontransgenic mice. (B) Smaller body weights did not result from reductions in food intake. The Tg4510 mice ate significantly more food than did nontransgenic mice. Data are presented as mean ± SEM. *p<0.02, ***p<0.0001.

Figure 3.4. AD transgenic mouse models had increased locomotor activity in the open field. Both APP+PS1 and Tg4510 mice displayed greater total distance travelled in the open field, compared to nontransgenic control mice, regardless of the diet. Locomotor activity was assessed during a single 15min trial in the open field. Data are presented as mean ± SEM. **p<0.005 and ***p<0.0001.
Figure 3.5. KD enhanced motor performance in all genotypes. Motor performance was assessed using two variations of the rotarod test. (A) Both genotype and diet effects were found in the standard accelerating rotarod test and (B) Average latency to fall in the accelerating rotarod was significantly greater in mice fed a KD. (C) Similarly, the ketogenic diet significantly enhanced motor performance in the non-accelerating variation of the test in all genotypes. Overall, latency to fall was greater in nontransgenic mice than in either APP+PS1 or Tg4510 mice. Data are presented as mean ± SEM. **p<0.007.
**Figure 3.6. KD did not rescue spatial memory deficits.** Spatial memory was assessed by the 2-day radial arm water maze (RAWM). (A) Both APP+PS1 and Tg4510 (tau) mice made more errors on the 2-day RAWM compared to Ntg control mice, regardless of the diet. (B) APP+PS1 and Tg4510 mice consistently made more entries into the wrong arms, suggesting that neither were able to learn a new platform location on the reversal trial. Data are presented as mean ± SEM. **p<0.01 and ***p<0.0001.
Figure 3.7. KD did not rescue neuronal loss in Tg4510 mice. Micrograph representation (2X) of neuronal staining (NeuN) in Ntg (A, D), APP+PS1 (B, E) and Tg4510 (C, F) mice kept on either NIH-31 (A, B, C) or KD diets (D, E, F). (G) Immunoreactivity for the neuronal marker NeuN was significantly reduced in Tg4510 mice compared to both nontransgenic and APP+PS1 mice. (H) Hippocampal volume (expressed in mm³) was calculated in Nissl stained sections. In agreement with the neuronal loss observed, hippocampal volume was significantly smaller in Tg4510 line, compared to the other genotypes. No diet effects were observed. Immunostaining was digitally quantified by Mirax image analysis. Data are presented as mean ± SEM. 2X Scale bar = 1000 μm; 20X inset scale bar = 100 μm. **p<0.01 and ***p<0.0001.
Figure 3.8. Astrocytosis and microglial activation were observed in Tg4510 mice. Micrograph representation (5X) of hippocampi stained for GFAP positive astrocytes (panels A-G) or Iba-1 positive microglia (panels H-N) in Ntg (A, D, H, K), APP+PS1 (B, E, I, L) and Tg4510 (C, F, J, M) mice kept on either NIH-31 (A, B, C, H, I, J) or KD diets (D, E, F, K, L, M). (G, N) Immunostaining was digitally quantified by Mirax image analysis. Data are presented as mean ± SEM. Scale bar = 500μm; inset scale bar = 100 μm ***p<0.0001.
3.6 References


CHAPTER 4: APPARENT RESCUE OF SHORT-TERM MEMORY DEFICITS INDUCED BY CALORIE RESTRICTION IN A MOUSE MODEL OF TAU DEPOSITION

4.1 Abstract

Calorie restriction (CR) has been previously shown to efficiently improve cognition and decrease pathology in amyloid depositing mouse models of Alzheimer’s disease (AD). In the present study, we investigated the effects of a CR on a tau (Tg4510) depositing model of AD. Mice in the CR group had food intake gradually decreased until they reached an average of 35% body weight reduction. Body weight and food intake were carefully monitored throughout the study. After being on their respective diets for 3 months, all animals were submitted to a battery of behavioral tests. Interestingly, we found that Tg4510 mice fed ad libitum showed lower body weight despite their increased food intake in comparison to ad libitum fed nontransgenic littermates. Additionally, Tg4510 showed increased locomotor activity in the open field test, regardless of diet. No differences in motor performance or spatial memory were observed in the rotarod and radial arm water maze tests, respectively. Tg4510 mice submitted to a 35% body weight reduction over 3 months performed significantly better in the novel object recognition test, suggesting improved short-term memory, compared to transgenic mice fed ad libitum. However, in a modified version of the test that allows for interaction with other mice instead of inanimate objects, genotype only differences were found, with Tg4510 mice spending the same amount of time interacting with either familiar or novel mouse. CR Tg4510 mice did not perform better in this modified, more ethologically relevant task. Histopathological and biochemical assessments showed no diet-induced changes in total or phospho-tau levels. Moreover, increased activation of
both astrocytes and microglia in Tg4510 mice was not rescued by calorie restriction. Taken together, our data suggests that, despite an apparent rescue of short-term memory, CR had no consistent effects on behavioral and histopathological outcomes of a mouse model of tau deposition.

4.2 Introduction

Calorie restriction (CR) is the most robust intervention capable of prolonging lifespan in several species from yeast to smaller mammals [reviewed in (Fontana, 2009)]. In addition to increasing longevity, it displays robust anti-aging effects in many different physiological systems. The neuroprotective effects of calorie restriction have been implicated in several neurological disease models and have been extensively reviewed by many research groups (Roth, 2005, Fontana, 2009, Maalouf et al., 2009a).

Reducing caloric intake by 30% in rhesus monkeys for up to 20 years lowered the incidence of age-related diseases, predominantly cancer, diabetes and cardiovascular disease. Age-related diseases were three times more prevalent in the control group compared to calorie-restricted subjects. In addition to the subjective more youthful appearance, CR animals displayed attenuated brain atrophy, a characteristic aging hallmark in both humans and non-human primates (Colman et al., 2009).

Alzheimer’s disease (AD) is the most prevalent type of dementia and a common age-related condition clinically described by progressive cognitive decline. Pathologically, it is characterized by post-mortem findings of widespread deposition of amyloid-beta peptide (Aβ) forming plaques and neurofibrillary tangles, which are aggregates of hyperphosphorylated forms of tau protein. Given that many epidemiological studies of obesity, diabetes, cardiovascular disease, hypertension and hypercholesterolemia support the hypothesis that dietary factors play an important role in the risk and prevalence of dementia (Luchsinger et al., 2002, Brubacher et al., 2004,
Luchsinger and Mayeux, 2004, Kivipelto and Solomon, 2006, Luchsinger et al., 2007, Reitz et al., 2007, Acharya et al., 2013), dietary approaches have increasingly become attractive in treating and preventing neurodegenerative diseases.

Our lab showed that short-term calorie restriction in early adulthood significantly reduced the number and size of amyloid plaques in the brains of two transgenic mouse models of amyloid deposition that express mutations associated with familial or early onset AD (APP and APP+PS1) (Patel et al., 2005). These mice presented increased GFAP immunoreactivity (a known marker of increased astrocytic activation) that was attenuated in the CR group in comparison to wild type littermates.

Recent advances in AD diagnosis consistently show that morphological changes in the brain occur decades before symptoms begin. Therefore, in most cases, diagnosis and treatment start late in the course of the disease when patients already display substantial deposition of amyloid plaque, neurofibrillary tangles and hypometabolism. Mouton et al submitted middle-aged APP+PS1 mice (13-14 months old) to a 40% CR for 18 weeks to address the question of whether CR would be beneficial in decreasing amyloid load after heavy accumulation has taken place. Stereological analysis of amyloid deposits showed reductions of 33% in the neocortex and 32% in the hippocampus for the CR group compared to the same brain regions in the ad libitum fed mice (Mouton et al., 2009). However, this reduction in amyloid load was not replicated in studies of non-human primates included in the longitudinal “Dietary Restriction and Aging Study” at the Wisconsin National Primate Research Center (WNPRC). Post-mortem histological analysis confirmed CR-induced attenuated astrogliosis but lack of changes in amyloid load compared to controls (Sridharan et al., 2013).

In the last decade, several studies have focused on the impact of dietary changes on neurodegenerative diseases (Henderson, 2004, Roth et al., 2005, Noh et al., 2006b, Henderson, 2008, Maalouf et al., 2009a, Mehta and Roth, 2009). Most of the
evidence to date has focused on amyloid pathology in rodent models. Although several groups have shown the effects of calorie restriction on Aβ accumulation, only recently researchers have studied its effects on tau and phospho-tau level alterations. Besides improvement in cognitive performance, another group showed significant reduction in phosphorylated tau in the cortex of conditional double knockout of PS1 and PS2 transgenic mice (Wu et al., 2008) after 4 months of 30% calorie reduction. Furthermore, when the triple transgenic mouse model of AD (3xTgAD) was submitted to 40% calorie restriction for 7 or 10 months, lower levels of both Aβ and phospho-tau in the hippocampus were observed compared to a control group maintained on an ad libitum diet (Halogappa et al., 2007).

Calorie restriction has been reported to increase metabolic efficiency which is often associated with enhanced cellular stress resistance mechanisms such as: improved mitochondrial function, enhanced antioxidant effects (Gong et al., 1997) and decreased production of reactive oxygen species (ROS) (Sohal et al., 1994, Merry, 2002, Lambert and Merry, 2004, Gredilla and Barja, 2005). Other possible mechanisms at play are: decreased activity of pro-apoptotic and inflammatory factors (Daynes and Jones, 2002), increased neurogenesis (Lee et al., 2002) and increased levels of molecular chaperone in the brain (Guo and Mattson, 2000, Sharma and Kaur, 2005).

Furthermore, abnormal plasma and CSF insulin levels were observed in AD patients (Craft et al., 1998) supporting the hypothesis that peripheral insulin resistance is a risk factor for age related cognitive decline. One of the major enzymes responsible for the clearance of Aβ is the insulin degrading enzyme (IDE) (Hoyer, 2002), which preferentially cleaves insulin. Thus, decreasing blood insulin may enhance clearance of brain Aβ by reducing levels of its competing substrate. One of the main actions of insulin is the inactivation of the glycogen synthase kinase 3 enzyme (GSK3). Indeed, lack of insulin’s proper function leads to an increase in GSK3 levels. Interestingly, this enzyme
has also been extensively associated with abnormal phosphorylation of the microtubule associated protein tau (Martin et al., 2009).

Recent studies in non-human primates carried out at National Institute of Aging (NIA) showed that CR did not affect longevity but significantly impacted health span in those animals (Mattison et al., 2012). They found that primates submitted to moderate CR for up to 20 years were less afflicted with cancer or other age-related diseases in agreement with their previous findings. Based on the above mentioned, we hypothesized that CR would prevent tau-associated pathology and behavioral deficits in a well established mouse model of tau deposition.

4.3 Materials and Methods

4.3.1 Mice

Tg4510 mice and parental mutant tau and tetracycline-controlled transactivator protein lines were generated and maintained as described previously (Santacruz, 2005). Briefly, the Tg4510 mouse has a P301L mutation, but differs from other tau transgenic mice in that the major tau pathology is found in the forebrain rather than the spinal cord. This is due to the tet response element driven expression, with the tet activator regulated by the CaM kinase II promoter, resulting in expression predominantly in forebrain neurons. These mice develop progressive pathology with first discernible deposits being observed at 3 mo, progressing through a series of stages analogous to that found in AD patients until a readily detectable neuron loss is found by 6 mo of age (Santacruz et al., 2005, Dickey et al., 2009). All animals were 3 months old at the start of the study and nontransgenic littermates were used as positive control groups (FVB/129S background). Mice were maintained in a specific pathogen-free environment (NIH Guidelines for the care and use of laboratory animals) and kept on a twelve-hour light/dark cycle. Water was provided ad libitum throughout the experiment.
4.3.2 Calorie Restriction

All animals were individually caged before the commencement of the study for accurate assessments of food intake and body weight. Measurements of daily food consumption started when the animals were 3 months old and were carried out for 4 weeks before the start of the calorie restriction procedure. The CR group received a diet identical to the ad libitum diet except that it is supplemented with micronutrients to maintain normal vitamin and mineral intake (diet devised by Dr. Robert Engelman) and manufactured at Harlan Teklad (Madison, WI). Thus, the CR diet provided all necessary nutrients in a smaller quantity of food. During the first week, mice were slowly transitioned into either the fortified CR or to NIH-31 ad libitum (AL) control diet (n=10 per group). A detailed list of macronutrient components of each diet used in this experiment is presented in Table 4.1. CR was introduced gradually with 10% food reduction in the first week. Body weight was assessed 3 times a week for careful observation of body weight loss so adjustments could be made to food offered. The goal was to achieve and maintain body weight at 35-40% less than initial individual values. Control groups were fed ad libitum but consumption was monitored three times a week for assessment of individual food intake throughout the experimental period.

4.3.3 Experimental Procedure

Figure 4.2 details the experimental design and time course adopted in this study. After three months on their respective diets, the mice were submitted to a battery of behavioral testing. At the end of testing, mice were weighed, euthanized with a solution containing pentobarbital (100mg/kg) and transcardially perfused with 25ml of 0.9% normal saline solution. Brains were collected immediately following perfusion. One hemisphere was collected for mitochondrial assays and the second hemisphere was immersion fixed in 4% phosphate-buffered paraformaldehyde for 24h. The fixed
hemispheres were cryoprotected in successive incubations of 10%, 20% and 30% sucrose solutions for 24h each. Subsequently, brains were frozen on a cold stage and sectioned in the horizontal plane (25 µm thickness) on a sliding microtome and stored in Dulbecco’s phosphate buffered saline (DPBS) with 10mM sodium azide solution at 4°C. Every 8th section was cut at 50 µm thickness for stereological counts of neurons and measurement of hippocampal volume in Nissl-stained sections.

4.3.4 Behavioral Testing

All behavioral testing was carried out by an observer blind to the genotypes of the mice. The open field was used as a standard test of general activity. Animals were monitored for 15 minutes in a 40 cm square open field with a video tracking software (ANY-Maze, Stoelting, IL), under moderate lighting. General activity levels were evaluated by measurements of horizontal and vertical activity.

Each animal was placed in a walled Y-maze for a single 5 minute trial. The sequence of arm entries and total number of arm choices were recorded. Spontaneous alternation (entering all three arms sequentially without repetition) was expressed as a percentage, as calculated according to the method of (Anisman, 1975).

Motor performance was evaluated by an accelerating rotarod apparatus with a 3cm diameter rod starting at an initial rotation of 4 RPM slowly accelerating to 40 RPM over 5 minutes. Mice were expected to walk at the speed of rod rotation to keep from falling. The time spent on the rod during each of four trials per day for two consecutive days was measured. Testing was completed when the mouse fell off the rod (distance of 12 cm) onto a spring-cushioned lever.

Short term memory was evaluated by the novel object recognition test (NOR) which consists of a 40 x 40 cm arena monitored and quantified by video tracking (ANY-Maze, Stoelting, IL). Two objects similar in scale to the mouse were placed along the
center line of the arena approximately 3-5 cm from the outside wall. Each animal was
given three acclimation trials of 5 minutes each with a 5 minute inter-trial interval. After
each trial the arena and object cues were cleaned with 70% ethanol to minimize
olfactory cues. After the acclimation trials, one of the acclimated objects was replaced
with a novel object. Animals were given a 5 minute exploratory trial during which object
exploration is monitored by video recording. Working memory was evaluated by the
percentage of exploration index, which is defined as the time exploring the novel object
divided by combined time spent exploring both novel and familiar objects multiplied by
100.

The novel mouse recognition test was conducted as an alternative to the NOR and was performed in a novel cage with a center partition. The experimental mouse was
placed in the test cage and, after a 10-min habituation period (trial 1); one mouse was
added to each side of the partition. The interaction between the test mouse and the
other mice was videotaped for 10 min (trial 2). For trial 3, one of the mice was replaced
with a novel. The cage was thoroughly cleaned between trials with 70% ethanol to
minimize olfactory cues. Active interaction was initiated by the experimental mouse
toward the partner and includes sniffing, direct aggressive attacks, lateral threats, tail
rattling, chasing, aggressive grooming, and wrestling/boxing. The amount of time spent
in the mouse's own territory and in each partner's territories during the 10-min test was
monitored and quantified by video recording (ANY-Maze, Stoelting, IL).

The hesitation or tendency to withdrawal from novel situations is an instinctive
survival mechanism. In order to control for the neophobia effect possibly present in the
NOR test, we deprived all mice of water for 5h and subsequently offered them a known
volume of either water or a 0.1% solution of saccharin. Importantly, saccharin was
chosen for its lack of caloric content so it wouldn’t interfere with the calorie restriction
protocol. After 10 minutes, the bottles were retrieved and the remaining volume of
solutions was measured. Intake was calculated by subtracting the final and remaining volumes.

4.3.5 Radial Arm Water Maze (RAWM)

A detailed description of this test has been previously published, complete with goal arm assignments and scoring sheets (Alamed et al., 2006). Briefly, the radial arm water maze contains 6 swim paths (arms) radiating out of an open central area with a hidden escape platform located at the end of one of the arms. The pool is surrounded by several extra-maze cues to allow spatial navigation. On each trial, the mouse was allowed to swim for up to 60 seconds to find the escape platform. The platform was located in the same arm on each trial. On day one mice were given 15 trials alternating between a visible platform (above the water) and a hidden platform (below the water). On day two, mice were given 15 additional trials with all the trials using a hidden platform. The start arm was varied for each trial so that mice relied upon spatial cues to solve the task instead of learning motor rules (i.e. second arm on the right). The goal arm for each mouse was different to avoid odor cues revealing the goal arm. Entry into an incorrect arm (all four limbs within the arm) was scored as an error. Failure to make an arm entry within 15 seconds was also scored as an error. The errors for blocks of 3 consecutive trials were averaged for data analysis. Mice averaging of 1 error or less by the end of day two are considered to have reached the learning criterion. On the third day, a reversal trial was performed with the goal platform placed in the arm 180° from the original location. Mice were given 15 trials all with a hidden platform. The open pool with a visible platform test was performed on the day following the reversal trial to confirm that all mice were capable of seeing and ascending the platform. The visible platform was elevated above the water surface and had an attached flag. For the open pool test, all visual cues were removed from the room so the mice relied only on their
sight to find the platform. Latency to find and ascend the platform was recorded (60 seconds maximum).

4.3.6 Fear Conditioning

Fear conditioning was used to assess memory formation that is especially sensitive to proper hippocampal function. For these experiments, an aversive stimulus (in this case a mild foot shock, 0.5mA) was paired with an auditory conditioned stimulus (white noise) within a novel environment. Freezing on the training day in response to the foot shock was used as an estimate of learning during the acquisition trial. Animals were placed in the fear conditioning apparatus for 3 minutes, and then a 30 seconds acoustic conditioned stimulus (white noise, 70 dB) was delivered with a 0.5-mA shock applied to the floor grid during the last 2 seconds of the conditioned stimulus. Training consisted of two mild shocks paired with two conditioned stimuli with a 2 minute interval between each shock. For contextual memory, the mice were placed in the chamber and monitored for freezing to the context approximately 24 hours after training (no shocks or auditory cue given) and tested for 3 minutes. Immediately after the contextual test, mice were placed in a novel environment, consisting of a chamber with different shape, floor and olfactory cues from the training chamber. Mice were allowed to explore it for 3 minutes (cued no tone) and exposed to the conditioned stimulus for 3 minutes (cued tone). Learning was assessed by measuring freezing behavior (i.e. motionless position) every second and % of time spent freezing was calculated (Bolognani et al., 2007).
4.3.7 Histopathology

Immunohistochemical procedural methods were described by Gordon et al. (Gordon et al., 2002a). For each marker, sections from all animals were placed in a multi-sample staining tray and endogenous peroxidase was blocked (10% methanol, 3% H₂O₂ in PBS; 30 min). Tissue samples were permeabilized (with 0.2% lysine, 1% Triton X-100 in PBS solution) and incubated overnight in appropriate primary antibody. Anti-NeuN (Millipore); anti-GFAP (Dako), anti-Iba1 (Wako), total tau H150 (rabbit polyclonal, Santa Cruz Biotechnology), anti-pSer199/202 (rabbit polyclonal, Anaspec) and anti-pS396 tau (rabbit polyclonal, Anaspec) antibodies were used in this experiment. Additionally, rabbit polyclonal anti-GSK3 α/β antibody (Phosphorylated tyrosine 216 + 279, Abcam) was used. Sections were washed in PBS, and then incubated in corresponding biotinylated secondary antibody (Vector Laboratories, Burlingame, CA). The tissue was again washed after 2h and incubated with Vectastain® Elite® ABC kit (Vector Laboratories, Burlingame, CA) for enzyme conjugation. Finally, sections were stained using 0.05% diaminobenzidine and 0.03% H₂O₂. Tissue sections were mounted onto slides, dehydrated, and cover slipped.

Nissl and Gallyas silver staining were performed using sections that were premounted on slides and air-dried for a minimum of 24 hours. The sections were rehydrated for 30 seconds before beginning staining protocol. Gallyas staining was performed as described in (Lee et al., 2010a). Briefly, slides were treated with 5% periodic acid for 5 min, washed with water, and incubated sequentially in silver iodide (1 min) and 0.5% acetic acid (10 min) solutions prior to being placed in developer solution (2.5% sodium carbonate, 0.1% ammonium nitrate, 0.1% silver nitrate, 1% tungstosilicic acid, 0.7% formaldehyde). Slides were treated with 0.5% acetic acid to stop the reaction, then incubated with 0.1% gold chloride and placed in 1% sodium thiosulphate. Following a final wash in water, slides were dehydrated and cover slipped. Cresyl violet (Nissl)
stained 50 µm sections every 400 µm were used for hippocampal volume assessment. Hippocampal volume (expressed in mm³) was calculated by measuring hippocampal area of 8 sections (50 µm thickness) utilizing Mirax software, Zeiss Inc. and applying the formula: \( V = \Sigma A \times T \), where \( \Sigma A \) = sum of the hippocampal areas on the sections analyzed, and \( T \) = section interval \( \times t \) (section thickness after tissue processing).

Stained sections were imaged using Zeiss Mirax-150 digital scanning microscope and Image Analysis software. Area of positive stain for the entire brain in each section (no sampling) was analyzed using 200X magnification. The software used hue, saturation and intensity (HSI) to segment the image fields. Thresholds for object segmentation were established with images of high and low levels of staining to identify positive staining over any background levels. These limits were held constant for the analysis of every section in each study (Gordon et al., 2002a).

4.3.8 Western Blotting

Tissues for Western blot analysis were prepared as previously described (Yao et al., 2011a). Briefly, brains were quickly minced in mitochondrial isolation buffer (MIB, pH 7.2) containing 1M sucrose, 10% bovine serum albumin, 1M HEPES, 10mM EGTA, 500mM mannitol and centrifuged at 1300 X g for 5 min. The supernatant (soluble fraction) was collected and the pellet was resuspended in MIB further processed to obtain the mitochondrial fraction (mitochondrial data not shown here). Protease inhibitor cocktail (Sigma) and phosphatase inhibitor cocktails I and II were added to the soluble fraction and protein concentrations were determined by the BCA protein assay kit (Pierce, Rockford, IL). Equal amounts of proteins (10µg/well) were loaded in each well of a SDS-PAGE gel and transferred to a 0.2µm pore size nitrocellulose membrane and immunoblotted with different antibodies. In addition to the antibodies against total tau and phosphorylated tau mentioned above, PHF-1 and MC1 antibodies were kindly donated.
from Peter Davies; PSD95 and synaptophysin were obtained from Cell Signaling Technology. Bands intensities were quantified by densitometric analysis and normalized to levels of β-actin antibody (Sigma) used as loading control.

4.3.9 Statistics

Statistical analysis was performed using two-way ANOVA with genotype and diet as variables, followed by Fisher’s LSD post hoc means comparison using Stat View software version 5.0 (SAS Institute Inc, Cary NC). Repeated measures ANOVA or unpaired Student’s t-tests were performed when appropriate. Graphs were generated using Graph Pad Prism 5.01 (La Jolla, CA).

4.4 Results

4.4.1 Tg4510 mice show slower body weight gain despite increased food intake

Average body weight was the same for all groups at the start of the experiment. Calorie restriction was implemented gradually so that 35% body weight reduction was achieved approximately 6 weeks after the start of the study. Figure 4.3A shows the body weight progression of nontransgenic and Tg4510 mice kept on either calorie restricted (CR) or ad libitum (AL) diet. AL-fed mice from either genotype gained weight throughout the experimental procedure, however, nontransgenic mice gained significantly more weight than Tg4510 mice fed ad lib (ANOVA main effect of genotype, p=0.01). Although AL-fed Tg4510 mice displayed lower body weights, two-way ANOVA revealed that they consumed significantly more food than the AL-fed nontransgenics (Main effect of genotype, p=0.004). We estimated that Tg4510 mice were consuming 44% more food (in grams) than nontransgenic controls at the end of the study (figure 4.3B). Metabolic rate was estimated as grams of food consumed divided by grams of
body weight). Figure 4.4 shows that metabolic rate in Tg4510 mice was 77% higher than in nontransgenic controls (ANOVA main effect of genotype, p=0.0003).

4.4.2 Locomotor activity and motor performance of Tg4510 mice were not affected by calorie restriction

Total distance travelled in the open field during a single 15 minute trial was found to be significantly greater in Tg4510 mice (ANOVA main effect of genotype, p=0.02, figure 4.5), compared to nontransgenic groups. Despite the fact that calorie restricted Tg4510 showed slightly lesser activity toward the end of the testing period, no diet effect was observed (p=0.43). Two-way ANOVA with repeated measures found no differences between genotypes or diets in motor performance tested by the accelerating rotarod test.

4.4.3 Novel mouse recognition test requires greater attention than novel object recognition test

Short-term memory was assessed by the novel object recognition test. Two-way ANOVA revealed main effects of genotype (p=0.01), diet (p=0.0003) and a significant interaction between genotype and diet (p=0.003). Figure 4.6A highlights the post hoc comparisons showing that Tg4510 mice presented significantly lower percentage of exploration compared to nontransgenic mice (FLSD, p=0.01, n =10/group). In addition, CR resulted in significantly greater percentage of exploration than AL diet (FLSD, p=0.0003, n=10/group). However, when comparing time spent in each area of the testing chamber, we observed that the vast majority of the testing period was spent in the open area (center), where no object was placed (ANOVA main effect of area: p<0.0001, figure 4.6C).
When the paradigm was tested with mice instead of inanimate objects, two-way ANOVA showed a main effect of genotype (p=0.03, figure 4.6B) and no diet effects, confirming the presence of short-term memory deficits in the Tg4510 mouse line. Importantly, most of the testing time was spent equally distributed among different sides of the chamber instead of predominantly spent in the center. When compared to the nontransgenic controls, Tg4510 mice spent significantly greater time in the center (FLSD, p=0.002) and lesser on the side where the novel mouse was placed (FLSD, p=0.001, figure 4.6D).

As a control for neophobic behavior, we offered all groups new bottles with two choices of solutions and observed that all mice consumed more saccharin solution than water (ANOVA main effect of solution, p=0.0005, figure 4.7). This increase was even more pronounced in calorie restricted mice (ANOVA main effect of diet, p<0.0001).

**4.4.4 Calorie restriction had no consistent effects on behavior or tau pathology in Tg4510 mice**

Spatial memory deficits were assessed by the 2-day radial arm water maze. ANOVA main effect of genotype (p=0.001) revealed that Tg4510 mice made significantly more errors in the attempt to find the platform compared to the nontransgenic groups (figure 4.8A). The learning deficit in the transgenic line was even more pronounced when the goal platform was placed in the opposite arm for the reversal trial (ANOVA main effect of genotype, p<0.0001, figure 4.8B).

Associative memory was tested by the fear conditioning test, shown in figure 4.9. Although Tg4510 AL group presented lower freezing rates than nontransgenic AL, no main effects of genotype or diet were observed (ANOVA, p=0.45 and p=0.42, respectively). There was, however, a significant interaction between diet and genotype (p=0.036), showing that Tg4510 mice submitted to calorie restriction had higher freezing
rates than their AL-fed counterparts (figure 4.9A). Figure 4.9B shows that freezing rates during either the no tone or tone components of the cued test were no different between groups.

Tg4510 mice presented severe hippocampal atrophy that was not prevented by CR. Nissl-stained sections were used for measurements of hippocampal volume and figure 4.10 highlights the 40% reduction in hippocampal volume (volume expressed in mm$^3$) observed in Tg4510 mice when compared to nontransgenic mice (ANOVA main effect of genotype, $p<0.0001$, figure 4.10). Additionally, to investigate the effects of calorie restriction on total neuronal loss, we stained representative sections with a biotinylated antibody against neuron-specific protein NeuN. We found an estimated 35% reduction in area immunoreactive for NeuN in the brains of Tg4510 mice, when compared to nontransgenic mice (ANOVA main effect of genotype, $p<0.0001$), confirming the significant neuronal loss previously reported in this model. No main effect of diet was found, suggesting that CR did not prevent neuronal loss. Furthermore, Tg4510 mice showed pronounced activation of both GFAP-positive reactive astrocytes (ANOVA main effect of genotype, $p<0.0001$) and Iba-1 positive microglia (ANOVA main effect of genotype, $p<0.0001$), as shown in table 4.2. No diet effects were observed for either astrocyte or microglial markers. Increased immunoreactivity to phosphorylated GSK3 $\alpha/\beta$ was present in Tg4510 mice, regardless of the dietary treatment (ANOVA main effect of genotype, $p<0.0001$, table 4.2).

Table 4.3 summarizes results from immunohistochemical and biochemical analysis of tau pathology markers. As expected, no tau immunoreactivity was observed in nontransgenic control mice. Therefore, results shown here refer to Tg4510 only and were analyzed by unpaired Student’s t-test. Whole sections were analyzed and no changes in the percentage of area immunoreactive for total tau or phosphorylated tau were observed in calorie restricted Tg4510 mice, compared to the AL diet. Total tau
immunoreactivity was measured by staining with H150 antibody (against aa 1-150), which recognizes both human and mouse tau. Antibodies against conformational changes in tau (MC1) or tau phosphorylated at epitopes Ser 199/202, Ser 262 and Ser 396 were also used in this experiment. Gallyas silver staining against neurofibrillary tangles was also performed. CR did not change any of the tau pathology markers analyzed. Additionally, cortical and hippocampal brain regions were analyzed separately and no differences were found between diets in these selected areas (data not shown). Quantification of western blotting confirmed that CR did not affect biochemical levels of total or phosphorylated tau protein. Moreover, the levels of pre- and post-synaptic proteins (synaptophysin and PSD95, respectively) were not affected by diet (table 4.3).

4.5 Discussion

In our study, we found that Tg4510 mice showed a lower rate of body weight gain when offered food ad libitum, despite eating almost twice as much as their nontransgenic counterparts. In humans, lower BMI later in life can be a predictor of poor health outcomes. Previous studies reported an association between weight loss and higher risk for dementia among elderly individuals (Nourhashemi et al., 2003, Luchsinger et al., 2008, Nourhashemi and Vellas, 2008). Previous reports from our group and others suggested a possible hypermetabolic profile in mouse models of Alzheimer’s pathology (Morgan and Gordon, 2008, Knight et al., 2012). One possible explanation is that tau pathology is involved in the metabolic changes reported. Accordingly, tau ablation caused weight gain in middle-aged mice (Morris et al., 2013a), indicating that tau may play a role in the hypermetabolism reported. Further metabolic and calorimetric studies are currently ongoing in our lab to address these questions.

Despite the smaller body weights, these mice showed significantly higher levels of activity in the open field measured by the increased total distance travelled, when
compared to nontransgenic mice. Increased locomotor activity or wandering behavior has been previously described in AD patients (Wang et al., 2004, Rolland et al., 2007).

In animal models of Parkinson's disease, calorie restricted animals presented improved motor function and less neuronal death in the substantia nigra (Duan and Mattson, 1999). In contrast to previous reports, we did not see significant improvements in motor performance in the rotarod.

To our knowledge only two studies to date have investigated the impact of calorie restriction on tau pathology in mouse models. Wu et al. reported higher exploratory preference for the novel object in a conditional double knockout (cDKO) model of both PS1 and PS2. These mice develop severe tau pathology in the absence of amyloid deposition. Improved behavioral outcomes in the novel object recognition and contextual memory tasks were described in the CR group. Moreover, CR attenuated ventricle enlargement, hippocampal atrophy and astrogliosis in addition to reducing hyperphosphorylated forms of tau. One noteworthy aspect of their study, however, is that reduced caloric intake was accomplished using a low carbohydrate diet (Wu et al., 2008). The second study evaluated the impact of CR or intermittent fasting (IF) in the 3xTgAD model. Improved performance in the Morris water maze test was reported in mice submitted to either CR or IF. In addition, Aβ levels were significantly decreased in CR, but not IF, mice. However, both CR and IF were effective in decreasing phospho tau (AT8) levels in the 3xTgAD model (Halogappa et al., 2007).

Importantly, both studies applied CR by decreasing food intake according to the averaged food intake from the AL counterparts. In our study, CR was applied according to each mouse's individual intake that had been previously measured and averaged over the course of four weeks. We found considerable differences in daily food intake among individual subjects with a wide range varying from 3 g to 6.5 g. Therefore, restricting food
offered according to their own individual averaged consumption ensured that all mice were subjected to the same extent of caloric restriction.

A genotype effect was observed in the novel object recognition test, showing that Tg4510 mice presented short-term memory deficits and spent significantly less time interacting with the novel object than the familiar. However, CR Tg4510 mice showed increased percentage of exploration in the novel object recognition test compared to the AL Tg4510 group, indicative of a rescue in short-term memory (figure 4.6A). Nonetheless, we observed that mice from all groups spent less than 40% of trial time interacting with either object, while the majority of time was spent in the center (figure 4.6C). Therefore, in an attempt to optimize this paradigm, we chose to use a more ethological approach assuming that social interaction with other mice would be more enticing to our test subjects. In this version of the test, we clearly saw that nontransgenic mice spent the majority of trial time interacting with the novel mouse and spent very little time in the center area, whereas Tg4510 mice, regardless of the diet, spent equal amounts of time in every area of the test chamber (figure 4.6D). This is indicative of short-term memory impairment and/or the lack of social aptitude in these mice that was not ameliorated by calorie restriction.

One intrinsic confounder in the novel object recognition test is neophobic behavior. Mice that are not familiar with the objects presented may choose not to approach them. To control for this possible variable, we measured differences in consumption of water or saccharin in a single 10 minutes test. All mice consumed more saccharin solution than water indicating that they were not frightened by the new bottle introduced (figure 4.7). The fact that this increase was even more pronounced in the CR groups suggests that these animals are likely trying to compensate for the caloric deficit present.
Tg4510 mice made significantly more errors in the attempt to find a hidden platform in the 2-day radial arm water maze and reversal trial tests, consistently with the severe cognitive impairment associated with this model of tau deposition. 4 months of calorie restriction did not prevent this hippocampal associated deficit. This is in contrast to the improved performance in the water maze reported in the 3xTgAD mice submitted to either CR or IF for either 7 or 14 months (Halogappa et al., 2007).

On the other hand, a small trend for improved contextual memory was observed in CR Tg4510 mice. However, the lack of a genotype effect was surprising and may account for the lack of statistical significance between the CR and AL Tg4510 groups. Freezing response to the conditioned stimulus (sound) was observed since there was significant increase in the freezing rate during the cued tone portion of the test, compared to the no tone.

Neuronal loss and hippocampal atrophy are hallmarks of Alzheimer’s pathology that are widely present in patients and consistently replicated in the Tg4510 mouse model (Santacruz et al., 2005, Dickey et al., 2009). In the present study, no beneficial effects of CR were detected in either total area of NeuN immunoreactivity or hippocampal atrophy measured in Nissl-stained sections (table 4.2). Increased astrocytic and microglial activation are often associated with neuronal loss (Ramsden et al., 2005, Lee et al., 2010b) and were indeed observed in the Tg4510 transgenic mice (table 4.2), with no significant effects of diet. Our results are in contrast with recent studies showing that CR aged rhesus macaques showed significantly lower GFAP immunoreactivity in the hippocampus and entorhinal cortex when submitted to 30% reduced intake from 7 to 20 years (Wisconsin National Primate Research Center) (Sridharan et al., 2013).

To determine the effects of dietary restriction on the development of tau pathology, we measured early, middle and late stages of tau phosphorylation and tangle formation. No differences were detected between AL and CR fed Tg4510 mice. Results from
immunohistochemical and immunoblotting assessments are listed in table 4.3. CR-induced decreases in biochemical levels of hyperphosphorylated tau have been previously described in the cDKO of PS1 and PS2 model (Wu et al., 2008) with the use of a carbohydrate restricted diet. This is in contrast with previous work from our lab where a low carbohydrate, ketogenic diet did not affect tau accumulation in the Tg4510 model (chapter 3).

We further looked at a few possible mechanisms involved in the potential benefits of the CR, such as synaptic signaling and GSK3 phosphorylation. Despite previous reports in the literature of the beneficial effects of CR on synaptic function (Stranahan et al., 2009), no changes in either pre- or post-synaptic markers (synaptophysin and PSD95, respectively) were observed in our study. Based on the direct effect of GSK3 on tau pathology (Selenica et al., 2007), we sought to determine whether CR would affect GSK3 activation. Despite the marked increase in pGSK3 (activated GSK3) levels in Tg4510 mice compared to nontransgenic controls, no differences were observed between dietary treatments. This finding is consistent with the lack of reductions in phospho-tau levels taken into account that co-localization of activated GSK3 and phospho-tau has been previously demonstrated (Selenica et al., 2013).

According to our results, CR did not prevent tau pathology (total and phospho tau levels), astrocytic and microglial activation (GFAP and Iba-1), synaptic signaling (synaptophysin and PSD95) and other associated mechanisms (activation of GSK3) in the Tg4510 mouse model of tau deposition. Furthermore, we report here that CR showed inconsistent effects on behavioral performance. We observed improved short memory outcomes in the novel object recognition test and contextual fear conditioning but lack of effects on spatial memory tested by the radial arm water maze. Therefore,
CR may be effective in attenuating subtle cognitive deficits in short term memory by its effects on peripheral metabolism and on the maintenance of a healthier body weight.
Table 4.1 Composition of diets used in the study

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<th>Ad Lib Diet - NIH-31</th>
<th>Calorie Restriction Diet</th>
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<td>grams/kg</td>
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Table 4.2 Immunohistochemical assessments

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Immunohistochemistry values shown as % of immunoreactive area. Data presented as mean ± SEM. *p<0.0001 when compared to nontransgenic (ANOVA main effect of genotype).

Table 4.3 Immunohistochemical and biochemical assessments of tau markers

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Western blotting

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Immunohistochemistry values shown as % of positive area. Western blotting values shown as ratio to actin. Data presented as mean ± SEM.
Figure 4.1. Body weight progression in Tg4510 (transgenic, open symbols) and nontransgenic (Wild type, closed symbols) mice. Tg4510 mice clearly display smaller body weights compared to nontransgenic control mice and the dissociation starts at 3 months old concomitantly with early tau pathology.
Figure 4.2. *Experimental design for the study of Calorie restriction (CR) in the Tg4510 model of tau deposition.* Individual food intake was daily monitored for 4 weeks prior to the start of the restriction procedure. After being restricted for 12 weeks, mice were submitted to behavioral testing, which lasted 5 weeks. All animals were then euthanatized and tissue was collected for posterior analysis.
Figure 4.3. **Body weight and food intake following 17 weeks of calorie restriction.**

(A) Nontransgenic and Tg4510 mice were submitted to 35% reduction in body weight.

(B) Food intake was carefully monitored throughout the experiment. Tg4510 mice on an *ad libitum* (AL, grey open circles) diet consumed an average of 44% more food (g) compared to nontransgenics AL (black open squares), n=10/group. Data presented as mean ± S.E.M.
Figure 4.4. *Tg4510 mice showed significantly greater estimated metabolic rate than nontransgenic mice.* Metabolic rate was calculated as grams of food consumed divided by grams of body weight. Tg4510 mice AL (grey open circles) showed an average 77% increase in metabolic rate compared to nontransgenic AL controls (black open squares), n=10/group. Data presented as mean ± S.E.M.
Figure 4.5. *Calorie restriction did not affect activity or motor performance in the open field and rotarod tests in Tg4510 mice.* (A) Total distance traveled during 15 minutes of testing in the open field. Tg4510 mice (grey circles) showed increased locomotor activity than nontransgenic mice (black squares), regardless of diet, n=10/group. (B) No differences between genotype or diet were observed in motor performance tested by the accelerating rotarod test. Data presented as mean ± S.E.M. *p=0.02.
Figure 4.6. **Novel mouse recognition (NMR) test commands greater attention than novel object recognition test (NOR).** (A) Calorie restricted Tg4510 mice showed significantly greater percentage of exploration (calculated as the time spent with the novel object divided by the sum of times spent with novel and familiar, multiplied by 100) of the novel object presented in the last trial of the NOR test, when compared to Tg4510 mice fed *ad libitum*. The dotted line represents 50% exploratory preference. (B) No differences were observed in the percentage of exploration of the novel mouse in a modified version of the NOR, here denominated Novel Mouse Recognition Test (NMR). This test consists of allowing the mice to interact with other mice, instead of objects. (C) (D) Time spent in each area during the testing period was monitored and showed for all four groups for both NOR and NMR, respectively. Mice that were allowed to interact with other mice spent significantly less time in the center area, whereas mice that were exposed to different inanimate objects spent the majority of their time in the center area of the testing chamber, where no stimulus was presented. Data presented as mean ± S.E.M, n=10/group. **p=0.003.
Figure 4.7. Neophobia was not observed in either genotype. Mice were offered solutions of water and 0.1% saccharin for 10 minutes to assess the possible effects of neophobia. No genotype differences were observed in the consumption of either solution. All groups consumed more saccharin than water and this increase was exacerbated in calorie restricted mice. Data presented as mean ± S.E.M, n=10/group.
Figure 4.8. Tg4510 mice presented spatial memory deficits in the radial arm water maze (RAWM) and reversal trial that were not rescued by calorie restriction. (A) Tg4510 mice made significantly more errors attempting to locate a hidden platform in the 2-day RAWM test, compared to Ntg mice and regardless of the diet. (B) Tg4510 mice were not able to learn a new platform location on the reversal trial. Data are presented as mean ± SEM, n=10/group. *p =0.001 and ***p<0.001.
Figure 4.9. Associative memory was tested in Tg4510 mice submitted to calorie restriction. (A) No genotype or diet effects were observed in the contextual fear conditioning. (B) Cued fear conditioning memory was not affected and % of freezing response was the same in Tg4510 and nontransgenics in both no tone and tone portions of the test, n=10/group. Data are presented as mean ± SEM.
Figure 4.10. *Tg4510* mice present severe hippocampal atrophy that was not prevented by calorie restriction. Micrographic representation (5X) of cresyl violet staining (Nissl) in Ntg (A, B) and Tg4510 mice (C, D) fed AL (A,C) or CR (B,D) for 17 weeks. Percentage area of Nissl-positive cells was significantly reduced in Tg4510 mice compared to nontransgenics. (E) Hippocampal volume (expressed in mm$^3$) was calculated by measuring hippocampal area of 8 sections (50 µm thickness) utilizing Mirax software, Zeiss Inc. and applying the formula: $V = \Sigma A \times T$. Two way ANOVA showed that a significant decrease in hippocampal volume in Tg4510 mice that was not prevented by calorie restriction, n=10/group. Data are presented as mean ± SEM. ***p<0.001. Scale bar = 500 µm.
4.6 References


CHAPTER 5: ALTERNATIVE APPROACHES TO INDUCING KETOSIS: 
INTRACEREBROVENTRICULAR ADMINISTRATION OF β-HYDROXYBUTYRATE 
AND TESTING KETONE ESTER SUPPLEMENTS

5.1 Abstract

Ketone bodies have been increasingly examined as a possible therapeutic approach to neurodegenerative diseases. Previous studies in our lab showed that a ketogenic diet was effective in inducing high peripheral levels of ketone bodies but did not improve cognition or histopathology in mouse models of amyloid or tau deposition. We thus sought to investigate alternative approaches to increasing ketosis in the central nervous system. In this study, we tested the hypotheses that: 1) administering β-hydroxybutyrate (BHB), the main endogenous ketone body, intracranially could be a more efficient way to deliver ketone bodies to brain cells, bypassing peripheral metabolism and reaching higher brain levels of ketosis; and 2) the use of a custom made ketone ester (acetoacetate diester, AcAc) as a dietary supplement could be a suitable replacement for the ketogenic diet, inducing peripheral ketosis and improvements in behavioral performance in mice. We found that intracerebroventricular administration of BHB in 20 months old APP mice did not affect body weight or food intake. Behavioral performance was equally unaffected, although we did not observe significant impairment in the APP transgenic mice. Similarly, infusion of BHB for 28 days did not affect amyloid or congophilic load in the brains of old APP mice. AcAc diester was found to be effective in acutely elevating peripheral levels of BHB that remained elevated 3 hours after enteral administration. However, no effects were observed when we acutely gavaged AcAc into
22 months old APP mice immediately before behavioral testing. Chronic dietary supplementation with ketone esters was effective in inducing ketosis in mice carrying the tetracycline-operon responsive element (tet) when compared to tet mice fed the control NIH-31 diet. However, we observed that AcAc-induced ketosis was not significantly greater than levels induced by KD2. Taken into consideration that KD2 was ineffective in rescuing behavioral and histopathological features of both amyloid and tau pathologies, we concluded that the ketosis induced by supplementation with the AcAc diester would not likely result in physiologically relevant outcomes.

5.2 Introduction

In the normal state, glucose is the main energy source for the brain. However, under extreme conditions such as starvation and neonatal development, ketone bodies can be readily utilized by neurons as an alternative fuel. Ketone bodies cannot fully replace glucose but can account for a significant fraction of cerebral metabolism, acting as efficient substrates for the mitochondrial ATP production (LaManna et al., 2009). The main endogenous ketone bodies are acetone, acetoacetate and β-hydroxybutyrate (BHB) (Hammami, 1997).

Therapeutic uses of ketone bodies have been extensively reviewed (Veech, 2004, Henderson, 2008, Maalouf et al., 2009b). In studies of perfused hearts, ketones decreased the need for glycolysis (Kashiwaya et al., 1994). Ketone bodies can bypass the mitochondrial pyruvate dehydrogenase complex, ensuring the activity of the TCA cycle regardless of insulin action (Kashiwaya et al., 1997). Several studies showed that treatment with BHB reduced cerebral infarct area in mice (Suzuki et al., 2002, Zou et al., 2002, Masuda et al., 2005). Other studies have shown increased cell survival and suppression of glutamate toxicity in vitro (Noh et al., 2006b). Consistent with the
observed neuroprotective effects, 4mM BHB protected mesencephalic and hippocampal neurons from MPP and Aβ toxicity in vitro, respectively (Kashiwaya et al., 2000).

The beneficial effects of the ketogenic diet (KD) have been vastly described on a variety of neurological disorders such as drug-resistant epilepsy (Freeman and Kossoff, 2010), glucose transporter deficiency syndrome (Klepper, 2008), multiple sclerosis (Kim do et al., 2012), Parkinson’s disease (Gasior et al., 2006), amyotrophic lateral sclerosis (Zhao et al., 2006, Zhao et al., 2012), Alzheimer’s disease (Reger et al., 2004a, Henderson et al., 2009) and traumatic brain injury (Prins, 2008). However, its chronic use is challenging due to limitations such as severe restriction of food choices, unbalanced macronutrient profile and gastrointestinal side effects. The low compliance to this diet led to the study and development of supplements that produce therapeutic levels of ketone bodies. AC-1202 (Accera, Inc.) is an orally administered medium-chain triglyceride (MCT) that is rapidly metabolized by the liver to produce a mild state of ketosis. In aged dogs, dietary supplementation with AC-1202 increased serum levels of ketone bodies, improved cognitive performance and blood brain barrier integrity (Costantini et al., 2008). Clinical trials in patients with AD and mild cognitive impairment (MCI) have shown that AC-1202 can improve memory and attention in ApoE4-negative individuals, particularly in those with higher BHB levels. However, when all participants are included the trend failed to reach statistical significance (Reger et al., 2004a). These results are consistent with previous findings that i.v. insulin administered to older adults with AD significantly improved memory recall and this effect was more pronounced in non ApoE4 genotype carriers (Craft et al., 2003).

The search for a “ketogenic diet in a pill” (Rho and Sankar, 2008) has led to the development of alternative approaches to inducing clinically relevant ketosis such as the use of synthetic ketone esters (KE) that mimic the sustained ketosis achieved with KD or prolonged starvation without the need for strict dietary restrictions. Dietary
supplementation with a ketone ester (D-β-hydroxybutyrate and (R)-1,3-butanediol) has been previously shown to result in subtle differences in learning, decreased anxiety and increased locomotor activity in 12 and 15 months old 3xTgAD mice (Kashiwaya et al., 2012). In another publication from the same group, the KE diet reduced food intake, increased insulin sensitivity, increased resting energy expenditure and increased brown adipose tissue mitochondrial bioenergetics in C57BL6 mice (Srivastava et al., 2012). One noteworthy aspect, however, is that the ester of D-3-hydroxybutyrate and (R)-1,3 butanediol used in the aforementioned studies was added to the diet as an equicaloric replacement for maltodextrin (an easily digestible oligosaccharide) (Srivastava et al., 2012), or corn starch and sucrose (Kashiwaya et al., 2012), therefore, the KE diet invariably presented a moderate carbohydrate restriction. Moreover, body weight of KE-fed mice was approximately 12% lower than that of mice fed the control diet and, since no food intake data was shown, a possible calorie restriction effect cannot be ruled out in the study mentioned (Kashiwaya et al., 2012).

In our study, we used the R,S-1,3-butanediol acetoacetate diester (AcAc) that has been previously shown to significantly elevate and sustain blood levels of BHB and acetoacetate levels in adults Sprague-Dawley rats after a single oral administration (D’Agostino et al., 2013). Furthermore, AcAc administration delayed seizures induced by central nervous system oxygen toxicity. Importantly, administration of the AcAc diester was performed without any changes in the animals’ standard diet and/or feeding schedule. Another advantage of using ketone esters is that, due to their rapid absorption, elevated blood levels of ketones can be achieved promptly whereas ketosis achieved by ketogenic diets is dependent upon strict carbohydrate restriction and metabolic adaptation to fat utilization.

Previous studies from our lab showed that a low carbohydrate, ketogenic diet rich in medium chain triglycerides was able to successfully induce peripheral ketosis in two
mouse models of AD pathology without any significant effects on cognitive or histopathological outcomes (see chapter 2). One possible explanation could be that the dietary approach used in our study was not sufficient to shift brain metabolism to ketogenesis. Sun et al (1997) showed that intracerebroventricular (i.c.v.) infusion of BHB was efficient in inducing metabolic changes such as lower body weight change in BHB-infused animals compared to aCSF-infused rats, despite the lack of differences in food intake. Moreover, BHB-infused rats showed higher preference for a high fat/low carbohydrate diet (Sun et al., 1997).

In order to test if increasing ketosis in the brains of older APP mice would be effective in reducing pathology and behavioral deficits, we administered β-hydroxybutyrate (BHB) intracranially using osmotic minipumps that continuously infused either saline or BHB for 28 days. On the other hand, using ketone esters as dietary supplements is a potential therapeutic approach to induce metabolic changes toward achieving ketosis without the need for a very restrictive diet, such as the ketogenic diet. We hypothesized that a ketone ester given orally could successfully induce peripheral ketosis and potentially mimic the suggested beneficial effects of the ketogenic diet. Therefore, we tested the effects of the custom-made AcAc diester used as a dietary supplement and investigated its effects on peripheral ketosis and behavioral outcomes of mice.
5.3 Materials and Methods

5.3.1 Mice

To determine the effects of the intracerebroventricular administration of BHB in amyloid pathology, we tested mice carrying the human Swedish mutant APP transgene (Tg2576). The Tg2576 mice derive from a C57BL6 background. This model has been extensively studied and it has been previously shown that they have elevated brain levels of soluble Aβ by 6-8 months of age and to develop Aβ-containing neuritic plaques in the cortex and hippocampus by 10-16 months of age (Hsiao et al., 1996, Frautschy et al., 1998). All mice were bred in our facility at the University of South Florida, as previously described (Holcomb et al., 1998a). Mice were 20 months old at the start of the experimental procedure and age-matched littermate nontransgenic controls were used.

Peripheral effects of single gavage of AcAc diester on BHB levels were tested in 9-month-old mice carrying the tetracycline-operon responsive element (tet). These mice lack the tau responder; therefore, they do not express the pathology associated with the mutant human tau transgene (P301L). Mice were fasted overnight (n=5/group) prior to the gavage of different volumes of AcAc diester. Twenty two-month-old APP and nontransgenic mice (n=21 and 9, respectively) were used to test the effects of acute enteral administration of AcAc diester on behavioral performance.

The effects of ketone esters as possible dietary supplements were tested in 8 months old tet mice (n=6/group). For 9 weeks, food was offered and weighed three times a week and body weight was monitored weekly.

All animals were singly housed for individual assessments of food intake and body weight and were maintained under a 12h light-dark cycle with free access to water and food. Testing procedures were approved by Institutional Animal Care and Use Committee of the University of South Florida and followed NIH guidelines for the care and use of laboratory animals.
5.3.2 Subcutaneous implantation of osmotic minipumps

Chronic infusion (28 days) of BHB into 20 months old APP or nontransgenic mice was performed as described previously (Sun et al., 1997, Selenica et al., 2012). Briefly, mice were anesthetized with isoflurane and a cannula was stereotaxically implanted into the right lateral ventricle (coordinates from bregma: -0.4 mm anteroposterior; 1.1 mm lateral; 2.5 mm vertical). An osmotic pump (Alzet No 1004; Durect, Cupertino, CA) was attached and implanted subcutaneously near the scapula. Pumps contained 100 µl of either 0.9% sterile saline or a 0.5M solution of (R)-(-)-3-β-hydroxybutyrate (Sigma Aldrich, St Louis, MO) dissolved in saline. After the surgical procedure, the animals recovered on a warm heating pad until they were upright and moving around. Pumps remained in place for BHB delivery for 28 days.

5.3.3 Experimental Procedures

Figure 5.1 shows the experimental design for the study of the intracerebroventricular infusion of either BHB or saline into 19-month-old APP mice or littermate nontransgenic controls. Alzet minipumps infused for 28 days at the rate of 0.11µl per hour. Mice were allowed to recover for two weeks after surgery. Body weight and food intake were monitored three times a week. After two weeks, all animals were submitted to a battery of behavioral tests. At the end of the 28 days period, mice were overdosed with pentobarbital (100mg/kg) and decapitated. The left hemisphere was used for mitochondrial studies (performed in collaboration with Patrick Bradshaw’s lab, data not shown here) and the right hemisphere was kept in 4% paraformaldehyde for 24 h for immunohistochemical analysis.

The R,S-1,3-butanediol acetoacetate diester (AcAc) is a nonionized sodium-free and pH-neutral precursor of acetoacetate that is converted to the ketone bodies beta-hydroxybutyrate and acetoacetate by metabolism after ingestion. Briefly, it was
synthesized by transesterification of t-butylacetoacetate with $R,S$-1,3 butanediol (Savind Inc., Seymour, IL) as described by (D’Agostino et al., 2013).

Figure 5.2 shows the experimental design for the blood collection 1 or 3 hours following administration of either water or AcAc diester. Nine-month-old mice were fasted overnight and gavaged with different volumes of AcAc diester; 1 or 3 hours after gavage, blood was collected by submandibular vein puncture and collected in ependorf tubes without an anticoagulant. Serum was collected by centrifugation at 2,000g for 15 minutes at 4°C. Serum BHB levels were measured using a commercially available kit (Cayman Chemical, MI) and values were normalized to total protein levels.

In order to test the acute effects of AcAc diester on behavioral performance of 22-month-old APP mice, baseline tests were performed. In order to do so, we chose behavioral tests that were likely to be sensitive to acute changes. Spatial memory was assessed by the 2-day RAWM test. The ability to learn a new strategy, tested in the reversal trial, was evaluated after APP and nontransgenic mice were subdivided and gavaged with either AcAc diester or water. Similarly, baseline motor performance in the accelerating rotarod was obtained in 2 days of testing (4 trials per day). On the third day of testing, mice were gavaged with either AcAc diester or water and tested for another 4 trials.

For the ketone ester supplementation experiment, the commercially available NIH-31 diet was used as a control (Teklad Harlan, Madison, WI) and was purchased in powdered format. Ketone ester supplementation was tested in 8-month-old tet mice by adding the ketone esters at different concentrations (10 and 20%, w/v) into powdered NIH-31 diet (1:1 mix of NIH-31 and water). The low carbohydrate, medium-chain triglyceride rich, ketogenic diet shown in chapter 3 was used as a positive control, since it was shown to be effective in inducing significant ketosis in mice. AcAc diester and butanediol (BD, Sigma Aldrich, St Louis, MO) were used as ketone ester supplements.
Six groups were included in this study: NIH-31 control diet group, KD2 and 2 different concentrations (10 and 20%) of either the ketone diester AcAc (1,3 butanediol acetoacetate diester) or butanediol control group (a commercially available hypoglycemic agent previously shown to increase beta-hydroxybutyrate levels). Sample sizes were of 6 mice per group.

All mice were slowly transitioned into their respective diets over the course of a week during which their previous chow was still available. Body weight and food intake were monitored throughout the experimental period. Two weeks after the start of the study, blood was collected by tail clipping for glucose and ketone measurements (Nova Max Plus, Waltham, MA). After 6 weeks, mice were behaviorally tested for general activity and motor performance assessments. At the end of 9 weeks, mice were fasted for 5 hours after which food was made available for 2h. Subsequently, mice were overdosed with pentobarbital (100mg/kg) and blood was collected by cardiac puncture and plasma was obtained by centrifugation (1,000g @ 4°C for 15 minutes) for final measurements of diet-induced changes in peripheral levels of ketone bodies. Samples were processed for the quantification of BHB and acetoacetate levels at Case Western University by mass spectrometry. Brown adipose tissue was quickly dissected, weighed and kept at -80°C for further biochemical analysis. Brains were dissected and kept at 4°C for posterior biochemical analysis.

5.3.4 Behavioral Testing

General activity was assessed by the open field test. Animals were monitored for 15 minutes in a 40 cm square open field with a video tracking software, under moderate lighting. General activity levels were evaluated by measurements of horizontal and vertical activities. Subsequently, each animal was placed in a Y-maze for a single 5 minute trial, during which the sequence and total number of arm choices were recorded.
Spontaneous alternation, expressed as a percentage, was calculated according to the method of (Anisman, 1975). Briefly, if an animal made the following sequence of arm selections (1,2,3,2,1,3,1,2), the total alternation opportunities will be 6 (total entries minus 2) and the percentage alternation would be 67% (4 out of 6).

Motor performance was assessed by placing the mice onto the round portion of an accelerating rotarod apparatus (Ugo Basile) with a 2cm diameter rod starting at an initial rotation of 4 rpm accelerating to 40 rpm over 5 minutes. Mice needed to walk at the speed of rod rotation to keep from falling. Mice were tested for the time spent on the rod during each of four trials per day, for three consecutive days. Latency to fall was recorded for each mouse.

Spatial memory was assessed by the radial arm water maze (RAWM) which contained 6 swim paths (arms) radiating out of an open central area, with a hidden escape platform located at the end of one of the arms. On each trial, the mouse was allowed to swim in the arms for up to 60 seconds to find the escape platform. The platform was located in the same arm on each trial. On day one, mice were given 15 trials alternating between a visible platform (above the water) and a hidden platform (below the water). The next day, they were given 15 trials using a hidden platform. The start arm was varied for each trial so that mice relied upon spatial cues to solve the task instead of learning motor rules (i.e. second arm on the right). The goal arm for each mouse was different to avoid odor cues revealing the goal arm (Alamed et al., 2006). We used the open pool test to confirm that mice can see and perform a platform task. The visible platform was elevated above the water surface and had an attached flag. All visual cues were removed from the room so the mice relied only on their ability to see and climb onto the platform. Latency to find and ascend the visible platform was recorded (60 second maximum).
5.3.5 Immunohistochemistry

Immunohistochemical procedural methods have been previously detailed (Gordon et al., 2002b). Briefly, sections from all animals were placed in a multi-sample staining tray and endogenous peroxidase was blocked (10% methanol, 3% H₂O₂ in PBS; 30 min). Tissue samples were permeabilized (with 0.2% lysine, 1% Triton X-100 in PBS solution) and incubated overnight in primary antibody. Rabbit polyclonal Aβ antibody (prepared by Paul Gottschall) was used at a 1:1000 concentration. Sections were washed in PBS, and then incubated in corresponding biotinylated secondary antibody (Vector Laboratories, Burlingame, CA). The tissue was again washed after 2h and incubated with Vectastain® Elite® ABC kit (Vector Laboratories, Burlingame, CA) during 1h for enzyme conjugation. Finally, sections were stained using 0.05% diaminobenzidine and 0.03% H₂O₂. Tissue sections were mounted onto slides, dehydrated, and cover slipped.

Congo red histology was performed using sections obtained from 20 months old APP mice that were infused with either saline or BHB for 28 days. Pre-mounted sections (air-dried for a minimum of 24 hours) were rehydrated for 30 seconds before beginning staining protocol. 2.5mM NaOH was added to a saturated sodium chloride-ethanol solution and slides were incubated for 20 min. Subsequently, slides were incubated in 0.2% Congo red in alkaline alcoholic saturated sodium chloride solution for 30 minutes. Slides were rinsed through three changes of 100% ethanol, cleared through three changes of xylene, and cover slipped with DPX. Aβ immunohistochemistry and Congo red staining were quantified with a digital scanning microscope and a purpose written program to perform HIS segmentation on either entire sections or user defined regions. Thresholds for object segmentation were established using a series of standard slides which have the extremes of intensity for the stain being measured and once set, remained constant throughout the analysis session. Sample numbers were randomized.
before the start of the tissue processing, and the code was broken only after the analysis was complete. All values obtained from a single mouse were averaged together to represent a single value for that animal.

5.3.6 Statistics

Statistical analysis was performed using analysis of variance (ANOVA) followed by Fischer's LSD post hoc means comparison test. Repeated measured ANOVA and Student's t test were performed when appropriate (Statview software from SAS, Cary NC). Graphs were generated using Graph Pad Prism 5.01 (La Jolla, CA).

5.4 Results and Discussion

5.4.1 Intracerebroventricular infusion of β-hydroxybutyrate for 28 days did not reduce amyloid load or cognitive deficits in old APP mice.

Average body weight was the same for all groups at the start (body weights values at day 0 were: APP BHB: 32.6 ± 2.1, APP Saline: 33.7 ± 2.2, Ntg BHB: 35.0 ± 1.3, Ntg Saline: 36.2 ± 2.5, no effect of group, p=0.67, n=7-11) and at the end of the experiment (body weight values at day 28 were: APP BHB: 32.8 ± 1.6, APP Saline: 32.1 ± 1.6, Ntg BHB: 31.6 ± 1.0, Ntg Saline: 34 ± 2.4, p=0.80). Figure 5.3 shows the percent body weight change during the 28 days of infusion of saline or BHB in either APP or nontransgenic mice. Repeated measures ANOVA showed that, while nontransgenic mice significantly lost weight, APP transgenic mice maintained body weight around their initial values (main effect of genotype, p=0.04, figure 5.3A). This is an interesting finding since, despite the fact that all mice lost weight in the few days following surgery, transgenic mice (irrespective of treatment group) recovered and maintained initial body weight a week later while nontransgenic mice continuously lost weight until reaching and maintaining a new lower value. Moreover, this body weight maintenance occurred in the
absence of significant differences in food intake (data not shown). The lack of changes in food intake we observed was in agreement with a previous study of BHB infusion in rats (Sun et al., 1997). Metabolic rate was estimated by calculating grams of food intake divided by grams of body weight. No genotype or treatment effects were found in metabolic rate by repeated measures ANOVA (figure 5.3B).

Increased locomotor activity in the open field was observed in the APP genotype (ANOVA main effect of genotype, p<0.0001, figure 5.4A), regardless of treatment. This finding was corroborated by the increased number of arm entries made in the y-maze by APP transgenic mice compared to nontransgenics (ANOVA main effect of genotype, p=0.03, figure 5.4B). No differences in the percentage of alternation in the y-maze were observed (figure 5.4C).

APP mice showed motor impairment in the accelerating rotarod test (figure 5.5). Latency to fall from the rod was significantly smaller in the transgenic mice (ANOVA main effect of genotype, p=0.02). Interestingly, there was a non-significant trend for APP mice infused with BHB to show increased average latency to fall from the rod, when compared to APP mice in the saline control group (APP BHB: 169.2 versus APP Saline: 139.0, n=10 and n=11, respectively, p=0.3). We have previously reported that a ketogenic diet improved motor performance in AD mouse models (see chapter 3) and this finding is corroborated by enhanced motor function described in mouse models of amyotrophic lateral sclerosis (Zhao et al., 2012) and multiple sclerosis (Kim do et al., 2012). Despite the non-significant trend for higher latency to fall in the BHB-infused mice, no treatment differences were observed. This could possibly be due to the shorter treatment period of infusion (28 days) in comparison to the longer treatment (4 months) in our previous study.

Figure 5.6A shows that no spatial memory impairment was observed in the 2-day RAWM test in the APP mice. When tested on the reversal trial, there was a non-
significant trend for APP mice to make more errors in the attempt to find the platform (p=0.09, figure 5.6B). The lack of a genotype effect was surprising considering that these mice were 20 months old and showed significant deposits of brain amyloid and congophilic load. This could possibly be suggestive of dissociation between brain amyloid load and cognitive symptoms in these mice after reaching plateau levels of amyloid deposition. Performance in the open pool test with a visible platform showed that all mice were capable of performing a platform test and visual acuity was not a variable in the test (figure 5.6C). Immunohistochemical analysis of amyloid load showed that infusion of BHB for 28 days did not reduce amyloid or congophilic deposits in the brains of 20 mo APP mice, compared to APP mice infused with saline (figure 5.7). These findings corroborate our previous results obtained from a ketogenic diet, showing that directly (intracerebroventricular) or indirectly (ketogenic diet) inducing brain ketosis did not affect pathological or behavioral outcomes in mouse models of amyloid deposition.

5.4.2 Acute enteral administration of AcAc diester induced rapid elevations in peripheral BHB levels.

Significant increases in the serum levels of BHB normalized to total protein were observed in mice gavaged with either 0.125 or 0.5ml of AcAc diester (ANOVA main effect of volume, p=0.04). All mice injected with 1ml of AcAc diester were found dead within 15 minutes of the gavage. Similarly, all mice gavaged with 0.50 ml of AcAc diester were found dead within three hours of the administration. However, we found that mice gavaged with 0.25 ml of AcAc diester showed a slight increase 1h after administration but significant ketosis after 3h (ANOVA main effect of volume, p=0.006, figure 5.8), when compared to both 0.125 ml or control (non gavaged) mice (FLSD, p=0.02 and p=0.002, respectively). Therefore, we were able to show that enteral administration of AcAc diester (with a volume of up to 0.25 ml) was not only well tolerated but successfully
induced and sustained ketosis in mice. This finding is supported by recent studies from our collaborators showing that enteral administration of AcAc diester increased peripheral levels of both BHB and AcAc for up to 4 hours in Sprague-Dawley rats (D'Agostino et al., 2013).

5.4.3 Enteral administration of AcAc diester prior to behavioral testing did not affect performance.

Preliminary data collected by our lab (described above) showed that acute enteral administration of the AcAc diester was capable of inducing peripheral ketosis for at least 3h. Therefore, we sought to investigate the effects of acute administration of the ketone diester on behavioral outcomes of 22 months old APP mice. Baseline assessment of general activity showed that APP mice presented increased locomotor activity in the y-maze. As shown in figure 5.9A, APP transgenic mice made significantly more arm entries compared to nontransgenic mice (ANOVA main effect of genotype, p=0.003, n=21 and 9, respectively). No differences in the percentage of spontaneous alternations were observed between genotypes. These results are consistent with previous reports from our lab (see figure 5.4 above and chapter 3), confirming the increased locomotor activity associated with AD mouse models.

Motor performance was assessed by the accelerating rotarod. Mice were submitted to 4 trials a day for 2 days and no baseline differences were observed between genotypes (figure 5.10A). Figure 5.10B shows latency to fall from the rod for APP mice after gavage. No effects of the acute administration of AcAc were observed in APP mice. Interestingly, no motor impairment was observed in the 22 months old APP mice tested in this experiment. This is in contrast to our abovementioned reports shown in figure 5.5 where 20 months old APP mice showed smaller latency to fall from the rotarod, in comparison to nontransgenic controls.
Similarly, baseline performance in hippocampal-dependent spatial memory test was assessed by the 2-day RAWM test. Similarly to the experiment where i.c.v. BHB was administered in 20 month old APP mice, no spatial memory impairment was observed in 22 months old APP mice (figure 5.11A). When the total number of errors was calculated, there was a non-significant trend for APP mice making more errors in the attempt to find the escape platform (p=0.07, figure 5.11B). Acute administration of AcAc diester did not affect performance of APP mice in the reversal trial. The lack of a genotype effect makes it difficult to accurately assess the effects of the acute administration of the ketone ester on behavioral performance. Data from chronic intragastric administration of a hydroxybutyrate methyl ester (HBME) into 10 months old APP+PS1 mice showed beneficial effects in the Morris water maze task. APP+PS1 mice that were daily treated with 40mg/kg of HBME for 2.5 months had significantly shorter latency to locate the platform compared to non treated controls (Zhang et al., 2013). This suggests that longer periods of administration may be necessary in order to observe improved behavioral performance in animals.

5.4.4 Dietary supplementation with ketone esters efficiently reduced glucose and increased BHB peripheral levels but did not affect performance in the open field or rotarod tests.

Two weeks after the start of the study, blood glucose levels were significantly decreased in all groups of mice fed KD2 or NIH-31 diets supplemented with either ketone ester (AcAc or BD) when compared to mice fed NIH-31 only (ANOVA main effect of group, p=0.02, n=6/group, figure 5.12A). This reduction in glucose levels is physiologically relevant since it occurs in the presence of normal carbohydrate intake and, therefore, insulin levels. Ketone ester supplementation (in the context of reduced carbohydrate content) was unable to decrease glucose levels in the 3xTgAD model in
previous studies (Kashiwaya et al., 2012, Srivastava et al., 2012). Moreover, blood BHB levels were significantly increased in all groups compared to NIH-31 group (ANOVA main effect of group, p=0.003). Interestingly, KD2-fed group had the highest BHB levels and values were significantly different than 20% AcAc or 10% BD (FLSD, p=0.05 and p=0.03, respectively, figure 5.12B). Importantly, the increased ketosis obtained with ketone ester supplementation is also physiologically relevant since it occurred in the presence of normal dietary carbohydrate content.

Initial and final average body weight values were the same for all groups (p=0.99 and p=0.97, respectively). None of the diets tested affected the percentage of body weight change throughout the experimental procedure (figure 5.13A). Furthermore, brown adipose tissue (BAT) was collected and weighed at sacrifice. Despite the lack of a main effect of diet in BAT weight relative to body weight, Fisher’s post hoc analysis showed a significant reduction in KD2 fed group when compared to mice fed NIH-31, 10% AcAc and 20% BD (FLSD, p=0.03, 0.04 and 0.007, respectively, figure 5.13B). BAT reduction has been previously shown to occur in mice fed either a commercially available ketogenic diet (Srivastava et al., 2013) or a diet supplemented with a BHB ester (Srivastava et al., 2012). One possible explanation in the overall reduction of BAT could be due to the observed reduction in lipid content of the tissue, which is consistent with the increased sympathetic activity in the BAT reported in the study mentioned.

General activity in the open field and motor performance in the accelerating rotarod were unaffected by the different dietary supplementation with ketone esters tested (figure 5.14A and 5.14B, respectively).

Figure 5.15 shows final plasma levels of acetoacetate and BHB, the main ketone bodies, from all groups. All groups showed AcAc levels greater than NIH-31 fed mice (ANOVA main effect of group, p<0.0001, figure 5.15A). This increase was even greater in mice fed the control diet supplemented with 20% BD and this increased reached
significance when compared to the 10 or 20% AcAc and the 10% BD groups (FLSD, p=0.002, p=0.005 and p=0.01, respectively). Similarly, BHB levels were increased in all groups compared to NIH-31 fed mice (ANOVA main effect of group, p<0.0001, figure 5.15B). Interestingly, 20% BD-fed group showed an even higher magnitude of BHB levels compared to any of the other groups (FLSD, p<0.0001 for each comparison). Figure 5.15C shows total ketone levels, calculated as the sum of AcAc and BHB values. All groups displayed elevated total ketone levels when compared to control NIH-31-fed mice (ANOVA main effect of group, p<0.0001).

Brain hippocampal levels of the main mitochondrial enzymes involved in ketogenesis pathways are shown in figure 5.16A and a schematic representation of mitochondrial bioenergetics is illustrated in figure 16B [extracted from (Yao et al., 2011b)]. Succinyl-CoA:3-ketoacid coenzyme A transferase (SCOT), the key enzyme that converts ketone bodies into acetyl-CoA, was found to be significantly increased in all groups compared to NIH-31 fed mice (ANOVA main effect of diet, p<0.001, figure 5.16C). Moreover, post hoc comparisons showed that SCOT expression was even more pronounced in mice fed 20% BD when compared to mice fed either KD2 or 20% AcAc. Acetyl-CoA acetyltransferase (ACAT 1) catalyzes the reversible formation of acetoacetyl-CoA from two molecules of acetyl-CoA was shown to be unaffected by any of the diets tested (figure 5.16D). Figure 5.16E shows biochemical expression of α-ketoglutarate dehydrogenase (OGDH), a key enzyme in the tricarboxilic acid (TCA) cycle that generates NADH required for ATP generation. ANOVA revealed a main effect of diet (p=0.001), and post hoc analysis showed that 10% AcAc and both concentrations of BD were successful in inducing greater expression of OGDH in the hippocampus, when compared to NIH-31 fed mice. Interestingly, mice fed 20% BD showed even greater levels of OGDH expression, in comparison to the groups fed either KD2 or 20% AcAc. This is an important finding since OGDH expression is documented to be significantly
decreased in AD brain (Gibson et al., 2000). Biochemical expression of mitochondrial enzymes involved in ketogenesis suggests that metabolic adaptations occurred in the brain of those mice. Increased expression of SCOT and OGDH have been previously associated with increased ketogenesis and with a shift in metabolism from the utilization of predominantly glucose to the alternative use of ketone bodies in the 3xTgAD model (Yao et al., 2011b).

Because our aim was to study the effects of the AcAc diester on generating prominent peripheral ketosis in rodents, we did not test these mice for cognitive changes. Investigating general activity and motor performance allowed us to accurately report that the compounds tested were safe and did not affect overall activity in these animals. To our knowledge, only a few studies to date have tested the effects of ketone ester supplements in animal models of neurodegenerative diseases. Mostly, they have focused on the effects of ketone esters on mitochondrial bioenergetics and peripheral metabolic effects. Importantly, these studies investigated the effects of ketone ester diets in the context of reduced carbohydrate content. Our intention was to test the possibility of a ketone ester acting as therapeutic supplement to a normal diet, in an attempt to minimize the challenges imposed by adhering to a strict ketogenic diet for longer periods of time. Despite the elevations in peripheral levels of ketone bodies observed after dietary supplementation, neither concentration of the AcAc diester tested was efficient in generating higher ketosis than KD2. As previously mentioned (see chapter 3), KD2 was unable to rescue behavioral or histopathological hallmarks of AD in both amyloid and tau deposition models. Therefore, we inferred that it would be unlikely to observe beneficial outcomes from a dietary supplementation with the AcAc diester tested in the same mouse models.
Figure 5.1. Experimental design for the study of intracerebroventricular delivery of β-hydroxybutyrate or saline for 28 days in either 20 months old APP or nontransgenic mice. Osmotic minipumps were surgically implanted and the mice were allowed two weeks of recovery before behavioral testing commenced. After 28 days, mice were sacrificed and blood and tissue were collected for analysis.

Figure 5.2. Enteral administration of a ketone ester (acetoacetate diester) by gavage into 9-month-old mice carrying the tetracycline-operon responsive element. Blood was collected by submandibular vein puncture 1 and 3 hours after gavage. β-hydroxybutyrate levels were measured using an enzymatic assay for the assessment of peripheral levels of ketone bodies.
Figure 5.3. Metabolic assessments for up to 28 days after intracerebroventricular administration of either β-hydroxybutyrate or saline in APP or nontransgenic mice. (A) Initial and final body weights from all groups were not statistically different. Weekly measurements of body weight showed that APP mice, regardless of the treatment, maintained initial body weight whereas nontransgenic mice showed significant weight loss during the experimental procedure. (B) Estimated metabolic rate was calculated as grams of food intake divided by grams of body weight. No differences were observed between groups. Data presented as mean ± SEM. *p=0.04.
Figure 5.4. Intracerebroventricular β-hydroxybutyrate did not rescue increased locomotor activity observed in APP mice tested in the open field and y-maze. (A) Total distance traveled in the open field is significantly greater in APP mice. (B) This increased activity is consistent with the increased number of entries made in the y-maze by 20-month-old APP mice. (C) Percentage of alternation in the y-maze was calculated as the number of alternations divided by the number of entries -2 (Alt (entries-2)*100). No treatment or genotype effects were observed in the percentage of spontaneous alternation. Data presented as means ± SEM. *p = 0.03 and ***p<0.001.
**Figure 5.5. Impaired motor performance observed in 20 months old APP mice.**
Motor performance was assessed in the accelerating rotarod test. Motor learning is assessed by their performance on the second day of testing. Latency to fall from the rod on day 2 was significantly shorter in APP mice. However, there was a non-significant trend for improved motor performance in BHB infused APP mice. Data presented as means ± SEM. * p=0.02.
Figure 5.6. Spatial memory deficits tested by the radial arm water maze test. (A) No genotype effect was observed when 20 months old APP were compared to age-matched nontransgenic mice in the 2-day radial arm water maze test. (B) A non-significant trend for deficits in the reversal trial was found. APP mice made slightly more errors in the attempt to find the escape platform. (C) Visual acuity was not a variable in the testing procedure as all mice were able to find and ascend a visible platform. Data presented as mean ± SEM.
Figure 5.7. Micrographs showing that amyloid deposition was not decreased after i.c.v. infusion of BHB for 28 days. No differences in Aβ Immunoreactivity were found between mice infused with either saline (A) or BHB (B). Micrographs show that congophilic deposits were similarly unaffected by infusion of saline (D) or BHB (E). (C, F) Quantification of percentage of positive area of Aβ and Congo red staining, respectively. Data presented as mean ± SEM. Scale bar = 200µm for all panels.
Figure 5.8. **Increased peripheral levels of β-hydroxybutyrate induced by a single administration of acetoacetate diester by gavage.** (A) Serum levels of BHB were significantly increased 1h after gavage of 0.125 and 0.50 ml of the AcAc diester. (B) 3h after gavage BHB levels of mice gavaged 0.25ml of AcAc were found to be significantly elevated. Sample sizes indicated in the bars. Data presented as mean ± SEM.

Figure 5.9. 22 months old APP mice showed increased activity in the number of entries in the y-maze. (A) APP mice made significantly more entries in the y-maze arms, compared to age-matched nontransgenic mice. (B) No differences in the percentage of alternation were observed. Data presented as mean ± SEM. **p=0.003.
Figure 5.10. No changes in rotarod performance after gavage of water or AcAc in 22 months old APP mice. (A) Baseline motor performance was not different between APP and nontransgenic control mice. (B) APP mice previously trained in the rotarod were subdivided and gavaged with 0.25ml of either AcAc or water prior to additional rotarod testing. Acute enteral administration of AcAc did not affect motor performance in APP mice. Data presented as mean ± SEM.
Figure 5.11. Subtle spatial memory deficits were not affected by acute gavage of AcAc. (A) Baseline performance in the 2-day radial arm water maze was compared between 22 months old APP or nontransgenic mice. (B) A non significant trend for APP mice making more errors in the attempt to find the escape platform was found when total errors were analyzed. (C) APP mice previously tested on the 2-day RAWM were subdivided and gavaged with 0.25ml of either water or AcAc prior to the reversal trial. No differences were observed between treatments. Data presented as mean ± SEM.
Figure 5.12. Blood levels of glucose and BHB in nontransgenic mice fed NIH-31 diets supplemented with ketone esters for two weeks. Blood glucose and β-hydroxybutyrate levels were measured using a commercially available glucose/ketone meter (Nova Max Plus). (A) Peripheral glucose levels were significantly decreased in all groups, when compared to NIH-31 fed mice. (B) BHB values were significantly greater in all groups compared to NIH-31. This increase was more pronounced in the KD2-fed group. Data presented as means ± SEM. *p<0.05 compared to NIH-31; +p<0.05 compared to KD2.

Figure 5.13. Body weight and brown adipose tissue (BAT) measurements after 9 weeks of dietary supplementation with ketone esters. (A) Percentage of body weight change was not different between groups throughout the experimental procedure. Average body weight values at the start were the same for all groups. (B) Brown adipose tissue weight relative to body weight was smaller in mice fed KD2. Data presented as means ± SEM. *p<0.05 compared to KD2.
Figure 5.14. Activity and motor performance were unaffected by dietary supplementation with ketone esters. (A) Total distance traveled in the open field was unaffected by different dietary supplementations with ketone esters. (B) All groups showed similar motor performance in the rotarod. Data presented as mean ± SEM.
Figure 5.15. Peripheral levels of main endogenous ketone bodies after 9 weeks of dietary supplementation with ketone esters. Ketone bodies were measured from plasma by our collaborators in Case Western University using mass spectrometry. (A) Acetoacetate levels were significantly increased in all groups compared to NIH-31. (B) β-hydroxybutyrate levels were increased in all groups compared to NIH-31 fed mice. (C) Total ketones values were obtained by adding AcAc and BHB values. All groups presented elevated total ketones in comparison to NIH-31 group. Mice fed 20% BD also showed increased levels of BHB (B) and total ketones (C) compared to NIH-31, KD2, 10% AcAc, 20% AcAc and 10% BD. Note the that plasma values for AcAc levels are smaller than for BHB, hence the different magnitude in the y-axis. Data presented as means ± SEM. *p<0.01, **p<0.005, ***p<0.001 compared to NIH-31 and +p<0.02 and ++p<0.001 compared to 20% BD.
Figure 5.16. Biochemical levels of mitochondrial enzymes involved in ketogenesis in nontransgenic mice fed NIH-31 supplemented with ketone esters. (A) Western blot showing hippocampal homogenate samples of mice fed different ketone esters. (B) Schematic illustration extracted from Yao et al (2011) showing mitochondrial bioenergetics involved in the utilization of glucose and ketone bodies. (C) Succinyl-CoA:3-ketoacid coenzyme A transferase (SCOT) expression was significantly greater in mice fed KD or different concentrations of either AcAc diester or BD in comparison to NIH-31 fed mice. Moreover, SCOT expression levels in 20% BD fed mice were significantly greater than those presented by mice fed either KD2 or 20% AcAc. (D) Acetyl-CoA acetyltransferase (ACAT1) levels were not changed by any of the dietary treatments tested. (E) α-ketoglutarate dehydrogenase levels were significantly greater in all groups compared to NIH-31 and expression levels were even more pronounced in mice fed 20% BD compared to KD2 and 20% AcAc groups. * p<0.05 compared to NIH-31. + p<0.05 compared to 20% BD. Western blot data was normalized to actin levels and are presented as means ± SEM.
5.5 References


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CHAPTER 6: FINAL CONSIDERATIONS

In summary, our major findings indicate that: a) a ketogenic diet rich in medium chain triglycerides and low in carbohydrates can effectively elevate and sustain peripheral ketosis in mice while decreasing glucose levels; b) chronic ketosis, in mice, significantly enhanced motor performance but did not affect cognitive or histopathological outcomes in mouse models of amyloid or tau pathologies; c) long-term calorie restriction did not prevent tau deposition and its related pathological features, such as activation of microglia and astrocytes. Moreover, behavioral outcomes were inconsistent with subtle improvements in short term memory, assessed by both the novel object recognition and contextual fear memory tests but no rescue in spatial memory deficits; d) intracerebroventricular administration of β-hydroxybutyrate in old APP mice was not capable of reverting the phenotype associated with abundant amyloid burden; e) dietary supplementation with acetoacetate diester resulted in elevated peripheral ketosis to the same extent as the ketogenic diet despite normal carbohydrate intake.

Importantly, none of the diets tested resulted in weight loss. Although weight loss is a consistent finding in studies of low carbohydrate diets, the reduced food intake associated with the low palatability would have introduced a confounding variable, suggesting that calorie restriction could have, at least in part, played a role in the results observed. Furthermore, our studies have provided further evidence for a metabolic dysfunction in mouse models of Alzheimer’s pathology. The metabolic abnormalities have been previously described by a few groups including ours (Morgan and Gordon, 2008, Brownlow et al., 2013), and agree with clinical reports (Wang et al., 2004, White et al., 2004). In the triple transgenic model, increased food intake and body weight were
reported at 2 months of age despite lack of differences in metabolic rate, however, at 12 months of age, while the increased food consumption persisted, body weight was significantly lower than nontransgenic counterparts. These changes were accompanied by greater oxygen consumption and carbon dioxide production, indicative of increased metabolic rate (Knight et al., 2012). Another possibility is that the decreased body weight can be a result of the hyperactivity reported here in the mouse models of Alzheimer's pathology. This outcome was consistently observed throughout our studies and is in agreement with clinical reports of increased pacing or wandering behavior commonly observed in AD patients. Furthermore, elderly subjects present deficits in thermoregulation and are prone to hypothermia (Whittington et al., 2010). Planel et al (2004) showed that lower body temperature resulted in increased tau hyperphosphorylation in the brains of wild-type mice and this was likely due to the inhibition of phosphatase activity observed (Planel et al., 2004). These non-cognitive behavioral symptoms are clinically relevant and could account for differences in basal metabolism in AD patients. Further metabolic and calorimetric studies are currently ongoing in our lab to evaluate this phenomenon more thoroughly.

Our lab has, in the past, initiated studies on the impact of caloric restriction on amyloid pathology in an APP+PS1 transgenic mouse model (Patel et al., 2005). We observed reductions in amyloid pathology that did not involve reductions in transgene expression in young mice. One of our colleagues, Mary Newport, a physician in the Tampa region has found that a diet high in MCT and coconut oils has dramatically modified her husband’s dementia. As one of the effects of caloric restriction is the development of ketosis (Maalouf et al., 2009a), this appeared to be a possible explanation of the caloric restriction effects we identified previously. The positive outcomes described by Dr. Newport occurred in a patient with a previously established diagnosis of AD and repeated low scores on neuropsychological tests, therefore, we
chose to test animals that already had started depositing tau and amyloid (chapter 3) or that had substantial amyloid pathology (chapter 2) to investigate the effects of diet-induced ketosis on pre-existing pathology and behavioral deficits. Considering that by the time most patients are diagnosed with MCI or AD changes in the brain are already advanced, this approach addressed the question of whether diet-induced ketosis would be able to rescue histopathological alterations in the brain and impairments in cognition associated with AD.

The fact that KD improved motor performance on the rotarod despite the absence of motor deficits suggests that this is likely a metabolic effect, possibly enhancing cellular efficiency in muscle. Several recent studies have observed similar effects of KD in motor performance in other neurodegenerative diseases such as multiple sclerosis and ALS (Zhao et al., 2006, Kim do et al., 2012, Zhao et al., 2012). Our data is in accordance with previous findings of motor improvements in APP+PS1 mice kept on a KD for a month in the absence of reductions of abeta load (Beckett et al., 2013b). The lack of changes in underlying disease pathology suggests that the observed enhanced motor performance likely results from symptomatic effects on cerebral energy metabolism.

The absence of cognitive improvements induced by the ketogenic diet led us to question whether peripheral ketosis was sufficient to elevate brain ketone levels and, therefore, effective in addressing the energy deficits commonly associated with AD. Western blot analysis showed increased expression of the monocarboxylic transporter 1 (MCT1) protein in KD fed mice, in all genotypes (data now shown), confirming that there was increased uptake of ketone bodies across the BBB. Nonetheless, in order to ensure ketosis in the brain, we infused old APP mice with BHB for 28 days using osmotic minipumps. We chose to infuse BHB since it's the main ketone body found in peripheral circulation during a long term ketogenic diet. In agreements with the results obtained
after chronic dietary procedure, infusion of BHB for 28 days did not rescue behavioral or histopathological feature of older APP mice. The inconsistent behavioral phenotype displayed by the APP mice, however, suggests that this model of amyloid deposition may not be the most appropriate for cognitive inferences. Lack of concordance between behavioral deficits and amyloid deposition has been previously demonstrated in the APP+PS1 transgenic model (Holcomb et al., 1999). Indeed, many subjects are reported to show amyloid deposits in the brain and are cognitively normal (Aizenstein et al., 2008, Mathis et al., 2013).

Furthermore, ketogenic diets may cause unwanted side effects (mostly gastrointestinal disturbances); therefore, many groups are seeking to develop other options that could elevate ketone bodies sufficiently with fewer adverse events. Given that the vast majority of AD patients do not carry the dominant mutations replicated in the models studied, one can speculate that using genetically-driven mouse models may not be the most appropriate approach to testing metabolic hypothesis of cognitive impairments and or dementia. One possible alternative approach would be to investigate the metabolic effects of nutritional ketosis (either KD or ketone ester supplementation) in diet-induced models of cognitive impairments.

AD mouse models present numerous advantages and each model has its strengths and flaws. However, they do not produce the full spectrum of AD pathology, for instance amyloid depositing models do not display brain atrophy, a major hallmark of AD. There are now over 100 treatments reported to modify Aβ deposition in amyloid precursor protein transgenic mouse models of amyloid deposition (Blennow et al., 2006). Clinical trials have been targeting abeta with no clinically relevant outcomes so far. One possible reason for the failure is that all trials simply started too late in the disease course. According to the present view, derived mainly from the recent advances in PET imaging with an amyloid-binding ligand, amyloid deposition may start up to 20 years
before the symptomatic phase of the disease (Villemagne et al., 2013). More recently, tau transgenic mouse models have been developed that replicate much of the tangle pathology found in AD patients, including neuron loss (Santacruz et al., 2005). An emerging view is that amyloid is an initiating factor and tau is a neurodegenerative factor in the progression of AD. Our previous work indicates that at least some treatments that successfully reduce amyloid in APP mice might conversely exacerbate pathology in tau transgenic mice (Lee et al., 2010b, Lee et al., 2013). Such an effect might be difficult to identify in, for example, the triple transgenic mouse as the tau pathology appears to be largely driven by the presence of amyloid. Thus, agents reducing amyloid may block tau pathology irrespective of a direct effect on tau. Using two distinct mouse strains permits discrimination of effects on amyloid from those on tau. The goal in these studies is to understand the impact of these manipulations on each form of AD-like pathology in isolation. This may be particularly relevant when treating AD patients, where the amyloid pathology appears to be complete at the onset of symptoms, and the progression of the disease and disease severity are better correlated (although still not satisfyingly so) with the tau pathology (DeKosky et al., 1992, Nagy et al., 1995, Morris and Price, 2001, Giannakopoulos et al., 2003). To our knowledge, this was the first study to examine the effects of ketogenic dietary manipulations on tau and phospho-tau levels.

Furthermore, calorie restriction did not affect tau deposition, astrocytic or microglial activation and the expression of pre- and post-synaptic markers in a mouse model of tau deposition. The inconsistent behavioral results observed suggest that mechanisms other than clearance of tau pathology may be at play. Subtle improvements in short term memory may reflect changes in mechanisms other than those investigated in this study, such as improved glucose tolerance and insulin sensitivity for instance. The subtle improvement observed in the novel object recognition test was abolished when
the task was adapted to reflect a more ethological approach. Additionally, CR did not prevent spatial memory deficits in the radial arm water maze.

Caloric restriction may be too severe a regimen to impose on most AD patients, considering that weight loss and frailty are commonly observed in these subjects (Luchsinger et al., 2008). Therefore, the widely described effects of calorie restriction on preventing age-related diseases are more likely to be relevant as a preventive approach, prior to disease onset or possibly even at the very early stages of AD. Accordingly, in a study of elderly people, greater BMI in middle age was highly predictive of poor cognitive outcomes later in life whereas lower BMI in elderly was indicative of poorer cognition (Nourhashemi and Vellas, 2008). The recent publications of the effects of CR in non-human primates support the notion that, when started early in life and sustained throughout adulthood, CR has a significant impact on quality of life, despite the lack of effects on longevity (Mattison et al., 2012).

In humans, most people would agree that maintaining a CR regimen would be challenging, hence the growing interest in finding compounds that can mimic the effects of CR without the need for a strict diet. These compounds, known as calorie restriction mimetics (CRM) are believed to mimic metabolic, hormonal and physiological effects of CR and activate stress response pathways observed in CR without reducing food intake [reviewed in (Ingram and Roth, 2011)]. The main candidates are: resveratrol, the antidiabetic drug metformin, 2-deoxy-glucose (2-dg), lipoic acid, rapamycin and sirtuins among others. One possible mechanism at play is the activation of interoceptive cues (such as hunger, for instance) rather than reducing caloric intake per se. Accordingly, APP+PS1 mice treated with a hunger-inducing ghrelin agonist performed significantly better in the water maze task than control mice and showed reductions of hippocampal Aβ similar to calorie-restricted mice (Dhurandhar et al., 2013). Importantly, these outcomes were not accompanied by weight loss or reductions in body fat composition, in
contrast to the CR group. Moreover, treatment with 2-dg was effective in decreasing levels of both mitochondrial APP and Aβ oligomers in 3xTgAD female mice (Yao et al., 2011a). Acting as a competitive inhibitor of glucose uptake, 2-dg induces a compensatory increase in the use of alternative substrates, primarily ketone bodies.

Similarly, dietary supplementation with ketone esters has been shown to induce significant ketosis in mice (Kashiwaya et al., 2012, Srivastava et al., 2012) and rats (D’Agostino et al., 2013). The current search for the ‘ketogenic diet in a pill’ addresses some of the pitfalls encountered by researchers and clinicians trying to study the long term effects of nutritional ketosis (Rho and Sankar, 2008). The feasibility of a ketone supplement would allow the widespread investigation of the effects of nutritional ketosis in diseases of hypometabolism, such as AD. In agreement with previously published data, we report here that supplementing a standard diet with acetoacetate diester was capable of inducing and sustaining ketosis in mice for at least 3 hours. However, in the context of normal carbohydrate intake, the peripheral levels of BHB found were in the same range as dietary ketosis induced by the ketogenic diet. Therefore, we predict that the acetoacetate ester used in our study would be likely to affect amyloid and tau pathologies to the same extent and similar outcomes would be obtained. Importantly, the beneficial effects reported by other groups studying the use of ketone esters as a dietary supplement cannot be completely separated from the effects of calorie restriction and/or carbohydrate reduction.

Altogether, our findings suggest that ketogenic dietary manipulation may alleviate some symptoms in AD patients, such as improved motor activity and possibly slight improvements in short term memory. Enhanced motor performance may lead to subtle improvements in quality of life, allowing patients to perform small tasks without the need of assistance. These changes, however, are likely to result from improved
metabolic functions and increased availability of ketones as alternative fuels to cells and not directly from biochemical changes in underlying pathology in the brain.

6.1 References


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