Efficacy of Diet Therapies in the Treatment of Neurological and Neurodegenerative Diseases

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EFFICACY OF DIET THERAPIES IN THE TREATMENT OF NEUROLOGICAL AND NEURODEGENERATIVE DISEASES

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ABSTRACT

Epilepsy is a prevalent disabling chronic and socially isolating neurological disorder that involves recurrent abnormal discharges of neurons. Despite seizures afflicting about 10% of people worldwide, antiepileptic drugs (AEDs) are largely unable to manage seizures in many persons with epilepsy. As an alternative to AEDs, dietary therapies possess a broad therapeutic potential in both humans and animals models of various neurological and neurodegenerative disease etiologies. My research focus was to identify the therapeutic efficacy and potential mechanism(s) of action of calorie restriction (CR) and the ketogenic diet (KD) in both the epileptic EL mouse model and the Mecp2 $^{308/y}$ mouse model of Rett syndrome. My findings indicate that both the KD and CR can reduce seizure susceptibility in EL mice, a natural model for multifactorial idiopathic generalized epilepsy. CR and circulating glucose and ketone levels significantly influence the therapeutic efficacy of the KD. A concurrent reduction in circulating plasma glucose levels and elevation in circulating plasma β-hydroxybutyrate levels was predicted to associate with the anticonvulsant effect of these diets in

EL mice. For the first time, I was able to show that a KD fed in unrestricted amount is able to reduce seizure threshold in EL mice. Interestingly, supplementation of calories in the form of carbohydrate in the water of calorie-restricted EL mice results in a diminished anticonvulsant efficacy of the KD. In my effort to elucidate the neuroprotective mechanism(s) associated with these changes in metabolite availability, I started investigating the complex alterations occurring in multiple integrated neural and metabolic processes. Furthermore, I showed that a restricted KD diet improves aspects of the behavioral abnormalities seen in Rett mice, in particular with respect to anxiety. Finally, for the first time, I provide a standardized protocol for the implementation of diet therapies in the management of an array of neurological and neurodegenerative diseases, which ultimately may help elucidate the complex neuroprotective mechanism(s) of CR and the KD. This research overall has provided a new understanding in the therapeutic efficacy of diets in epilepsy and Rett Syndrome.

DEDICATION

To my loving parents, Georgios and Stavroula Mantis, who have always supported me and believed in me throughout my life. They have sacrificed so much of themselves to give me the opportunities they never had. I thank them for helping me become the individual I am today --- the person, the student, the scientist, and most recently the new proud father of Georgios I. Mantis. I miss you every day during these past 14 years that we have lived apart. I deeply appreciate and thank you for all your love.

To my sister, Amilia Mantis, and my brother, Nikolaos Mantis. You are both not only my siblings, but also my best friends. I look up to both of you for help and encouragement. I thank you both for your support, understanding, for checking up on me when I "had not call for a few days", and for always caring for me.

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To my newborn son, Georgio I. Mantis. Words can't describe how excited I am that you are part of my life, and sharing with you and your mother memories of endless happiness. Your giggles and cute smile make me forget all the bad days. I cannot wait to see what the future may hold for us!

To the Sanxaridis family, thank you all for making me feel part of your family and being there for me. Your support has been invaluable!

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ABBREVIATIONS

AED	Antiepileptic Drug
ALS	Amyotrophic Lateral Sclerosis
β-OHB	beta-hydroxybutyrate
CR	Calorie Restriction
DER	Dietary Energy Restriction
DR	Dietary Restriction
ECS	Electroconvulsive Shock
EL	Epileptic “EL” mouse
FAA	Free Fatty Acids
FI	Food Intake
ddH2O	Double Distilled Water
GABA	gamma-aminobutyric acid
GAD	Glutamic Acid Decarboxylase
GAPDH	Glyceraldehyde Phosphate Dehydrogenase
GluR4	Glutamate Receptor subunit 4
IF	Intermittent Fasting
IGE	Idiopathic Generalized Epilepsy
IGF	Insulin Growth Factor
KC	KetoCal®
KD	Ketogenic Diet
MCAO	Middle Cerebral Artery Occlusion
MES	Maximal Electroshock
NPY	Neuropeptide Y
PTZ	Pentylenetetrazol
QTL	Quantitative Trait Loci
R	Restricted
RTT	Rett Syndrome
SD	Standard Diet
THC	Tetrahydrocannabinol

TZD	Thiazolidiedione	
UCP1	Uncoupling	Protein 1
UR	Unrestricted	

CHAPTER ONE

INTRODUCTION

Epilepsy

Dating back to the ancient times, Greeks thought that epileptic seizures were caused by a supernatural power. Hippocrates later suggested that epilepsy could be a hereditary disease and not connected to witchcraft. Epilepsy is a disabling chronic and socially isolating neurological disorder involving recurrent abnormal discharges of neurons that produce epileptic seizures (Engel and Pedley, 1997; Johnston and Smith, 2008). With the exception of stroke, epilepsy is one of the most prevalent human neurological afflictions affecting about 1% of the US population (Hauser, 1997; Seyfried and Todorova, 1999). According to CDC data, worldwide, about 10% of people will experience a seizure sometime during their lifetime and about 3% will have had a diagnosis of epilepsy by age 80. Many persons with epilepsy manifest partial or generalized seizures without symptoms of brain abnormality or obvious structural pathology, such as idiopathic epilepsy (Baumann, 1982; Wolf, 1994; Engel and Pedley, 1997; Marini et al., 2004; Wolf, 2005). In idiopathic epilepsies, the genetic defect responsible for the spontaneous recurrence of epileptic seizures presumably produces a disturbance in neuronal response, transmission, or wiring that is continuously present between seizures (Wolf, 1994; Engel and Pedley, 1997; Wolf, 2005).

Interestingly enough only for a few idiopathic epilepsies have the genes been identified (Marini et al., 2004). Genetic heterogeneity, variable age of onset, and multifactorial inheritance has hindered progress in identifying the genetic and biochemical components responsible for the most common forms of human idiopathic generalized epilepsies (Tan et al., 2004; Todorova et al., 2006). While some idiopathic epilepsies are inherited as simple Mendelian traits, most are multifactorial where more than one gene together with environmental factors contribute to the disease phenotype (Berkovic, 1998; Todorova et al., 1999a; Todorova et al., 2006). In contrast to idiopathic epilepsy, symptomatic or acquired epilepsy often accompanies brain trauma, injury, or neurostructural defects. Generalized seizures tend to involve both cerebral hemispheres, whereas partial (also called local or focal) seizures are localized in one cerebral hemisphere (Hauser, 1982; Hauser, 1992). Furthermore, symptomatic epilepsies result from a variety of underlying pathological processes that might be localized or diffuse, unilateral or bilateral, static or progressive (Engel and Pedley, 1997; Johnston and Smith, 2008).

Epileptic Animal Models

The epileptic EL mouse: A natural model of human idiopathic generalized epilepsy.

Since the early half of the 20th century, a great array of animal models for seizures and epilepsy have played a fundamental role in our understanding of the physiology and behavioral changes associated with human epilepsy (Sarkisian, 2001). Many of the animal models of epilepsy range in diversity from drosophila to nonhuman primates, which can provide insight on the influence of environmental and genetic factors on the mechanisms of seizure onset (epileptogenicity), and anticonvulsant therapies (Putnam and Merritt, 1937; Engel, 1992; Engel and Pedley, 1997; Noebels, 1999, 2001; Sarkisian, 2001). Natural occurring models of epilepsy are especially important, since many of the non-natural models (e.g. PTZ-induced, kainic acid) draw mechanistic conclusions about epilepsy based on studies performed in normal and non epileptic brain, and the behavioral manifestations associated with each model can differ and look nothing like a human's behavior (Stafstrom, 1999; Sarkisian, 2001). The epileptic EL mouse is a natural model for human multifactorial idiopathic epilepsy, and it was first discovered in 1954 in an outbred DDY mouse colony (Naruse and Kurokawa, 1992; Frankel et al., 1995a; Seyfried et al., 1999; Suzuki, 2004). EL mice experience complex partial seizures, seizures limited to one cerebral hemisphere, causing impairment of awareness or responsiveness, with secondary generalization similar to those seen in humans (Seyfried et al., 1999; Todorova et al., 1999a; Suzuki, 2004). Seizures in EL mice originate in or near the parietal lobe, quickly spread to the hippocampus and to other brain regions, and commence with the onset of sexual maturity (50-60 days of age) (Suzuki et

al., 1991; Kasamo et al., 1992; Ishida et al., 1993; Todorova et al., 1999a; Uchibori et al., 2002). The seizures are accompanied by electroencephalographic (Di Pasquale et al.) abnormalities, vocalization, incontinence, loss of postural equilibrium, excessive salivation, and head, limb, and chewing automatisms (Suzuki, 1976; Suzuki and Nakamoto, 1977; Sato, 1985; Ishida et al., 1993; Nakano et al., 1994; Seyfried et al., 1999; Uchibori et al., 2002; Suzuki, 2004) (Figure 1). Epileptic seizures in EL mice also model Gowers' dictum, where each seizure increases the likelihood and severity of recurrent seizures (Gowers, 1901; Todorova et al., 1999a; Pitkanen and Sutula, 2002; Stafstrom and Sutula, 2005). Indeed, the EL mouse is the only known model of this hallmark of progressive epilepsy. Adult EL male mice also experience a sexual dysfunction similar to that described in men with temporal lobe epilepsy (Todorova et al., 1999b). EL mice express abnormalities of excitatory and inhibitory neurotransmission and develop a hippocampal gliosis with seizure progression (Flavin and Seyfried, 1994; Lambert et al., 1996; Fueta et al., 1998; Seyfried et al., 1999). A reactive gliosis that accompanies seizure progression in adult EL mice, and involves both astrocytes and microglia, is not associated with obvious hippocampal neuronal loss or synaptic rearrangements (e.g., mossy fiber sprouting) (Brigande et al., 1992; Drage et al., 2002; Murashima et al., 2005). Seizure susceptibility in EL mice can be controlled with antiepileptic drugs (Phenytoin and Phenobarbital) as well as with diet therapies to include the ketogenic diet and calorie restriction (Nagatomo et al., 1996; Todorova et al.,

2000; Greene et al., 2001). The inheritance of seizure susceptibility in EL mice is complex and gene-environmental interactions play a significant role in the determination of seizure frequency and onset in EL mice (Frankel et al., 1995a; Frankel et al., 1995b; Poderycki et al., 1998; Todorova et al., 1999a; Marini et al., 2004; Tan et al., 2004; Todorova et al., 2006), as also seen in many persons with multifactorial idiopathic generalized epilepsy. Most recently, a novel Quantitative Trait Loci (QTL) analysis identified *El-N* as a potential QTL for age-dependent predisposition to seizures (Todorova et al., 2006). *EL-N* was found on proximal Chromosome 9 in naïve EL mice that were tested for seizures once at 150 days of age (Todorova et al., 2006). All aforementioned findings suggest that the EL mouse is a good model for evaluating not only gene-environmental associations but also the effects of dietary therapies in the management of generalized idiopathic epilepsies.

Diet Therapies for the Management of Epilepsy

Despite intensive antiepileptic drug (AED) research and development, seizures remain unmanageable or refractory in many persons with epilepsy (Jallon, 1997; Freeman et al., 2000; Browne and Holmes, 2001). Epidemiological data indicate that 20-40% of the patients with newly diagnosed epilepsy will become refractory to treatment, due to both environmental (e.g. trauma, prior drug exposure) and genetic factors that predetermine the rate of

drug absorption, uptake, and metabolism (French, 2007). In addition, while many available AEDs provide seizure control to about 60-70% of all persons with epilepsy, their use is often associated with unanticipated and adverse side effects that diminish quality of life (Vermeulen and Aldenkamp, 1995; Porter et al., 1997; Gates, 2000; Browne and Holmes, 2001; Mattson, 2001; Wheless et al., 2001; Ortinski and Meador, 2004; Sheth, 2004; French, 2007; Kossoff et al., 2008). As an alternative to AEDs diet therapies have been shown to be effective in the management or control of epilepsy. Diet therapies for the control of epilepsy are as old as the disease itself, with references of their usage dating back to the time of the ancient Greeks and Romans (Temkin, 1971; Eadie and Bladin, 2001). Although, these early diet therapies were designed to rid the brain of toxic agents that were believed to underlie the development of epileptic seizures, the type or composition of antiepileptic diets (e.g. fasting, calorie restriction, ketogenic diet) have been adapted over time to reflect new perspectives on the etiology and management of epilepsy (Eadie and Bladin, 2001).

Fasting and the Ketogenic Diet

Fasting has long been recognized as an effective antiepileptic therapy for a broad range of seizure disorders (Lennox and Cobb, 1928; Lennox, 1960; Freeman et al., 2000; Greene et al., 2003; Seyfried et al., 2009b). Interestingly,

reference to fasting as a cure for epileptic seizures can be found as back as the biblical times (Mark 9:14-29) (Seyfried et al., 2009b). Using Conklin's water-only diet, Lennox and colleagues, in 1928, demonstrated that although seizure incidence, or onset, actually increased over the first couple days of fasting, seizure susceptibility, or seizure frequency, was significantly decreased after three days in most patients (Lennox and Cobb, 1928; Lennox, 1960; Seyfried et al., 2004; Seyfried et al., 2009b) (Figure 2). This latter finding is of great importance in regard to brain energy metabolism and trying to decode the antiepileptic mechanism of action of fasting. Under normal physiological conditions brain cells derive most of their energy from glucose or glucose-derived metabolites (e.g. lactate, glycogen) (Clarke and Sokoloff, 1999; Bouzier-Sore et al., 2002; Kasischke et al., 2004). However, during fasting or other forms of dietary energy restriction (DER) cerebral energy metabolism gradually transitions from glucose to ketone utilization in about 3-4 days (Bhagavan, 2002; Seyfried et al., 2004; Seyfried et al., 2008a). This metabolic transition was also observed in Lennox's patients, where blood glucose levels were reduced and blood ketone levels were increased (ketosis) upon fasting (Lennox, 1960). Amazingly, after fasting was terminated through food intake some of Lennox's patients remained seizure free for extended periods, indicating individual variability for the anticonvulsant (a phenomenon where only seizures are reduced) and antiepileptic (a phenomenon where all or some aspects of epilepsy are affected) effects of fasting (Lennox, 1960; Seyfried et al., 2004; Seyfried et al., 2009b).

The return of seizures was often associated with a rise in blood glucose levels and a subsequent fall in blood ketone levels.

Although clinically effective in managing seizure disorders, fasting is impractical for the long-term seizure management due largely to issues of compliance (Seyfried et al., 2004). As mentioned earlier, fasting produces ketosis. It was originally thought that ketone bodies (β-hydroxybutyrate and acetoacetate) might play an important role in the antiepileptic effects of fasting (Wilder, 1921; Lennox, 1960). Consequently, high fat, low protein, low carbohydrate ketogenic diets (KD) were developed to mimic the physiological effects of fasting without causing severe food restriction or starvation (Peterman, 1928; Lennox, 1960; Freeman et al., 2000; Stafstrom and Bough, 2003). Although the KD significantly elevates circulating ketone body levels, subsequent studies have shown that ketone bodies alone were unable to account for the antiepileptic and anticonvulsant effects of the KD in humans or in animal epilepsy models (Appleton and DeVivo, 1974; Bough et al., 1999a; Likhodii et al., 2000; Thio et al., 2000; Todorova et al., 2000; Harney et al., 2002; Stafstrom and Bough, 2003; Mantis et al., 2004; Seyfried et al., 2004). An explanation for this may stem from the fact that brain ketone utilization depends on the plasma levels of ketones, glucose, and other metabolites (Nehlig and Pereira de Vasconcelos, 1993). Thus associations between plasma ketone levels and seizure protection may be masked (Seyfried et al., 2004).

Currently, although the KD is widely used clinically as an effective therapy in managing refractory seizures in children, recent findings suggest that the KD has therapeutic efficacy across a wide variety of ages (including adults), seizure types and severities, as well as different etiologies (Kossoff et al., 2002; Mady et al., 2003; Kossoff and McGrogan, 2005; Bodenant et al., 2008; Mosek et al., 2009). Additional evidence also supports the view that the KD improves the long-term outcome in children with refractory epilepsy (Freeman, 2001; Hemingway et al., 2001; Marsh et al., 2006). Moreover, from the reports of the diet's efficacy worldwide in recent years, it appears that approximately half of patients receiving the KD will have ~50% reduction in their seizures, and ~33% of patients receiving the KD will have 90% reductions in their seizures (Hassan et al., 1999; Kankirawatana et al., 2001; Coppola et al., 2002; Klepper et al., 2002; Francois et al., 2003; Kim et al., 2004; Vaisleib et al., 2004).

Based on all aforementioned findings, a great interest is developing in the natural therapeutic potential of the ketogenic diet in the treatment of neurological disorders other than epilepsy, including Alzheimer's and Parkinson's disease (Gasior et al., 2006). Studies in these neurodegenerative disorders have led to the hypothesis that the ketogenic diet may not only provide symptomatic benefit, but could have beneficial disease-modifying activity applicable to a broad range of brain disorders characterized by the death of neurons (Gasior et al., 2006; Gasior et al., 2007). Interestingly, the KD is most effective in reducing seizure susceptibility in children when administered with fasting or under restricted

caloric intake (Freeman and Vining, 1999; Freeman et al., 2000; Freeman et al., 2007). Clinical studies have also shown that the anticonvulsant efficacy of the KD is associated with body weight and blood glucose reductions of about 10% (Livingston, 1972). Furthermore, patients who experience a rise in blood glucose levels, as in the case of those who gain weight on the KD or those who consume carbohydrates, the neuroprotective effect of the KD has have been shown to be ameliorated (Freeman et al., 2000; Freeman et al., 2007).

Adverse effects of the KD occur only when the diet is given in *ad libitum* or unrestricted amounts for a prolonged period of time (usually occurring after 4 weeks) (e.g. weight gain, hypercholesterolemia, diabetes, kidney stones, and cardiovascular disease) (Kang et al., 2004). Although these adverse effects are important for neurologists and pediatricians to recognize, only infrequently do they cause a discontinuation of the KD treatment in patients (Freeman et al., 2007). Early-onset adverse effects associated with the initiation of the KD are transient and include acidosis, hypoglycemia, gastrointestinal distress, dehydration, and lethargy (Ballaban-Gil, 2004; Freeman et al., 2007). Later adverse effects include dyslipidemia, kidney stones, and slowing of growth. Although cholesterol and lipids are shown to be affected by the diet, it is interesting to note that the lipid profiles of children maintained on the KD for greater than 6 years returned toward baseline (Kwiterovich et al., 2003). Kidney stones occur in 5% of children on the KD and are thought to be secondary to a combination of acidosis, urine acidification, hypercalciuria, and hypocitraturia.

From all these findings, it is clear that the success and safety of the KD are best achieved when administered to patients in restricted amounts by close supervision of an experienced medical team (the physician, dietician, or nurse).

Previous findings have shown the KD to have both an antiepileptic/anticonvulsant and antiepileptogenic effect in various animal models of epilepsy (Bough et al., 2000; Todorova et al., 2000; Mantis et al., 2004). Table 1, illustrates some of the correlatives that have been observed in mice and humans when treated with the KD by Stafstrom et al., 2004 (Stafstrom, 2004). While the mechanisms by which the KD inhibits seizure susceptibility remain unresolved, alterations in brain energy metabolism are likely involved (Mantis et al., 2004; Maalouf et al., 2009). Since the KD manages epilepsy best when administered in restricted amounts and since fasting lowers blood glucose levels, it is our contention that calorie restriction might contribute to the antiepileptic and anticonvulsant effects of the KD (Greene et al., 2001; Greene et al., 2003; Mantis et al., 2004). Furthermore, administration of the KD in restricted amounts would also reduce the adverse effects of the diet's high fat content (e.g. weight gain, hypercholesterolemia, diabetes, and cardiovascular disease) if the diet were to be administered *ad libitum* for extended periods of time. Based on all presented findings, we consider that an energy restricted high fat ketogenic diet, or calorie restriction alone, would be more therapeutic for the management of seizures in both humans and animal models of epilepsy (such as the EL mouse), by transitioning brain energy metabolism from glucose to ketone bodies.

My dissertation research sought to examine the therapeutic efficacy of different dietary regimes, such as calorie restriction (CR) and the KD, in the management of both neurological and neurodegenerative diseases, including epilepsy and the $Mecp2^{308/y}$ mouse model of Rett Syndrome. Very few studies have investigated the relationship among ketones, glucose, and seizure susceptibility under long-term antiepileptic diet therapies. This thesis provides new evidence in the antiepileptic and neuroprotective mechanism of both calorie restriction and the KD. Investigation of blood metabolite changes in calorie-restricted mice further supports the role of ketone bodies (β-hydroxybutyrate) and glucose in the neuroprotective effect of CR and the KD. The molecular biomarkers that were investigated might provide additional evidence in the therapeutic efficacy of CR and the KD. Finally, my thesis provides the first guidelines for standardizing the implementation of diet therapies in the management of an array of neurodegenerative, neurological, or other types of diseases.

Figure 1. The Epileptic EL Mouse. The EL mouse is a model for multifactorial human idiopathic epilepsy. The mouse expresses excessive salivation, and head, limb, swallowing, and chewing automatisms. The arching Straub tail is indicative of seizure spread to spinal cord.

Figure 2. Influence of Fasting on Human Epileptic Seizures. The Y-axis represents the percentage of seizures taking the prefasting number as 100%. The X-axis represents days of fasting. The heavy solid line is the average of the five curves. The initials of Lennox's patients are shown within the various data curves. Figure is reprinted with permission from Lennox and Cobb, *Arch. Neurol. Psychiat.* 20:771-779, 1928.

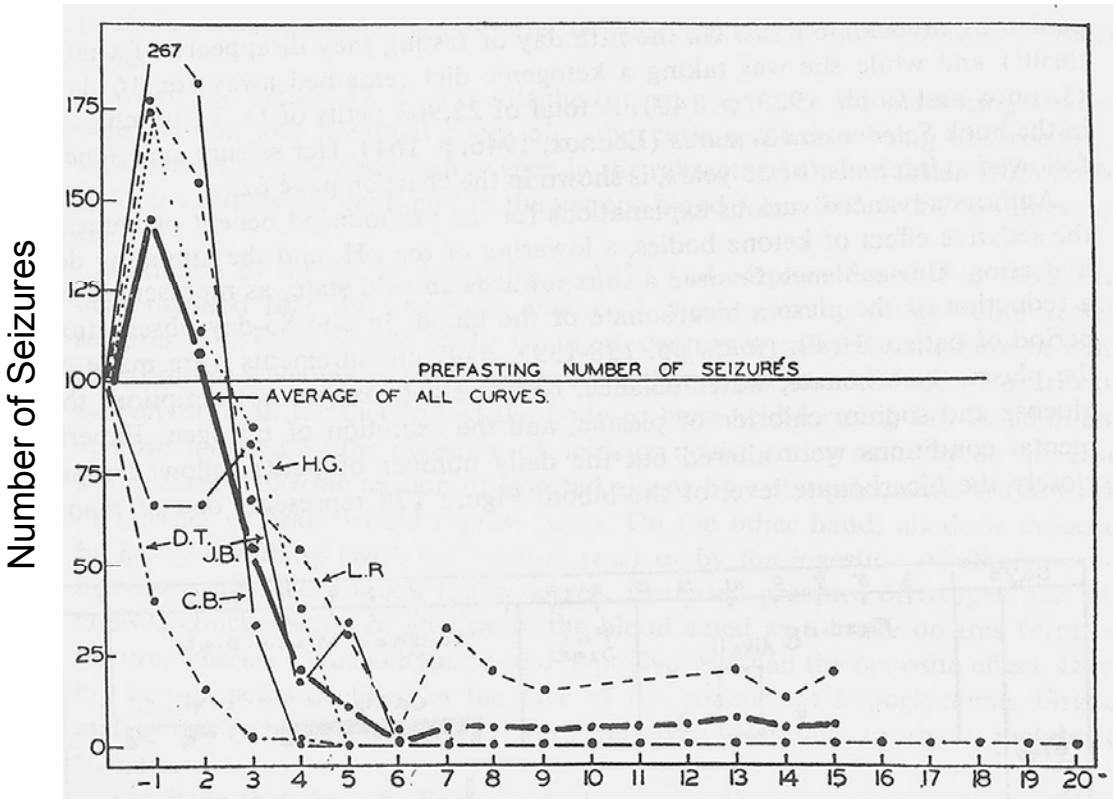

Number of Seizures
267
175
150
125
100
75
50
25
0
PREFASTING NUMBER OF SEIZURES
AVERAGE OF ALL CURVES.
H.G.
D.T.
J.B.
L.R
C.B.
-1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Fasting Days

Table 1: Animal Models of the Ketogenic Diet. Observations and Clinical Correlates[1]

	Observations in Animal Model	Clinical Correlates
Seizure type	KD is effective in a wide variety of seizure	KD is effective in a wide variety of seizure types and
Age range	Younger animals respond better to KD	Children utilitize ketones more efficiently than adults
Calorie or energy restriction	Anticonvulsant properties (increases seizure threshold)	Anticonvulsant properties (reduces seizures)
Ketosis	A threshold level of ketosis is necessary but not sufficient for the anticonvulsant	Ketosis is necessary but not sufficient
Reduced glycolysis (reduction in circulating glucose)	A reduction in circulating glucose is necessary for the anticonvulsant properties of	A reduction in circulating glucose is necessary for the anticonvulsant properties of the
Latency to KD effectiveness	Several days	Several days to weeks
Reversal of the anticonvulsant effects	Rapid (hours)	Rapid (hours)

[1]This table is modified from Stafstrom et al., 2004.

CHAPTER TWO

Management of Multifactorial Idiopathic Epilepsy in Adult EL Mice with Calorie Restriction and the Ketogenic Diet: Role of Glucose and Ketone Bodies

INTRODUCTION

The Influence of Calorie Restriction in the Management of Idiopathic Epilepsy in Adult EL Mice

CR is a natural dietary therapy that improves health, extends longevity, and reduces the effects of neuroinflammatory diseases in rodents and humans (Weindruch and Walford, 1988; Greene et al., 2001; Duan et al., 2003; Greene et al., 2003). CR is produced from a total dietary restriction and differs from acute fasting or starvation in that CR reduces total caloric energy intake without causing anorexia or deficiencies of any specific nutrients (Mantis et al., 2004; Seyfried et al., 2004). In other words, CR extends the health benefits of fasting while avoiding starvation. Recently, we showed that a 40% CR in the inbred control C57BL/6J mice produced changes in serum lipids similar to those seen in humans following therapeutic fasting or very low calorie dieting (below 500 kcal/day) (Mahoney et al., 2006). Besides improving health, CR has both antiepileptic and anticonvulsant effects in EL mice and in other animal epilepsy

models (Bough et al., 1999b; Todorova et al., 2000; Greene et al., 2001). Although the mechanisms underlying the neuroprotective effects of CR are unknown, it is believed that neuroprotection is associated with reduced circulating glucose levels and elevated ketone body levels. With regard to epilepsy, the metabolic transition from glucose to ketone bodies as the primary cerebral energy source under CR conditions has been shown to reduce seizure frequency in epileptic rodents and humans by inducing synaptic changes that ultimately attenuate neuronal hyperexcitability thus increasing the extent to which these hyperexcitable foci are inhibited (Greene et al., 2003; Mantis et al., 2004; Seyfried et al., 2009b).

Glucose uptake and metabolism increases more during epileptic seizures than during most other brain activities (McIlwain, 1969; Meldrum and Chapman, 1999; Cornford et al., 2002). Also, blood glucose levels positively correlate with flurothyl-induced seizures in rats and high levels of glucose may exacerbate human seizure disorders (Schwechter et al., 2003). Neuronal excitability and epileptic seizures are directly related to rapid glucose utilization and glycolysis (McIlwain, 1969; Ackermann and Lear, 1989; Meric et al., 1994; Clarke and Sokoloff, 1999; Meldrum and Chapman, 1999; Cornford et al., 2000; Knowlton et al., 2002; Ikemoto et al., 2003; Schwechter et al., 2003). It is not yet clear, however, to what extent enhanced glycolysis is related to the cause or effects of seizure activity (Greene et al., 2003). Nevertheless, a transition in brain energy metabolism from glucose utilization to ketone body utilization reduces neural

excitation and increases neural inhibition through multiple integrated systems (Greene et al., 2003; Seyfried et al., 2004). Based on these observations (Ting and Degani, 1993; Li et al., 2000; Knowlton et al., 2002; Vielhaber et al., 2003), we proposed that most epilepsies, regardless of etiology or causality, might ultimately involve altered brain energy homeostasis (Greene et al., 2003).

In this study, we compared the antiepileptic and anticonvulsant effects of both the KD and CR in adult EL mice that experienced at least 15 recurrent complex partial seizures. The results show that seizure control in EL mice is more associated with the amount than with the origin of dietary calories, and that CR underlies the antiepileptic and anticonvulsant action of the KD in EL mice. A report of these findings has been presented (Mantis et al., 2003; Mantis et al., 2004).

MATERIALS AND METHODS

Mice

The inbred EL/Suz (EL) mice were originally obtained from J. Suzuki (Tokyo Institute of Psychiatry). The mice were maintained in the Boston College Animal Care Facility as an inbred strain by brother x sister mating. The mice were group housed (prior to initiation of study) in plastic cages with Sani-chip

bedding (P.J. Murphy Forest Products Corp., Montville, N.J.) and kept on a 12-hr light/dark cycle at approximately 22^{o}C. Cotton nesting pads were provided for warmth when animals were individually housed. All cages and water bottles were changed once per week. Only females were used for these studies as adult males die sporadically with age from acute uremia poisoning due to urinary retention (Todorova et al., 2003). The procedures for animal use were in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care Committee.

Seizure Susceptibility and Seizure Testing

Seizure onset in EL mice (Figure 1) is generally between 60-70 days of age as previously described (Todorova et al., 1999a). These seizures occur occasionally during routine cage changing. Our recently developed seizure handling protocol was used to regularly induce seizure susceptibility in EL mice (Todorova et al., 1999a; Greene et al., 2001). Briefly, the testing procedure included repetitive handling and simulated the stress normally associated with weekly cage changing, i.e., picking the mouse up by the tail for short intervals and transferring it to a clean cage with fresh bedding. The test included two trials that were separated by 30 min. In each trial, a single mouse was held by the tail for 30 sec at approximately 10-15 cm above the bedding of its home cage. After 30 sec, the mouse was placed into a clean cage with fresh bedding for 2 min.

The mouse was then held again for 15 sec before being returned to its home cage. Trial 2 was performed even if the mouse experienced a seizure in trial 1. The epileptic seizures commenced during holding or soon after the mice were placed on the clean bedding. Mice that developed an epileptic seizure while handled were placed immediately in either the clean cage or their home cage depending on the testing stage. Mice were tested each week for a total of 13 measurements over a 12-week period using this method. Mice were undisturbed between testing phases (no cage changing) and testing was performed between 12 to 3 pm.

Seizure Phenotype

Mice were designated seizure susceptible if they experienced a generalized seizure during seizure testing. Generalized seizures in EL mice involve loss of postural equilibrium and consciousness, together with excessive salivation, head, limb, and chewing/swallowing automatisms. An erect forward-arching Straub tail, indicative of spinal cord activation, was also seen in most mice having generalized seizures. Mice that displayed only vocalization and twitching without progression to generalized seizure were not considered seizure susceptible (Todorova et al., 1999a; Greene et al., 2001). Seizure susceptibility scores were generated for each mouse according to the seizure severity scores previously described (Table 2) (Todorova et al., 1999a). Mice having a score of

4 or 5 were assigned a susceptibility score of 1.0, whereas mice having a seizure severity score less than 4 were given a susceptibility score of 0. The seizure susceptibility for each mouse was then averaged over multiple tests and the mean seizure susceptibility for a mouse dietary group was determined.

Diets

All mice received PROLAB RMH3000 chow diet (SD) prior to experimentation (LabDiet®). This is the standard food pellet diet (SD) and contained a balance of mouse nutritional ingredients. According to the manufacturer's specification, this diet delivers 4.4 Kcal/g gross energy, where fat, carbohydrate, protein, and fiber comprised 55 g, 520 g, 225 g, and 45 g/Kg of the diet, respectively. The ketogenic diet was obtained from the Zeigler Bros., Inc. (Gardners, PA, USA) in butter-like form and also contained a balance of mouse nutritional ingredients. According to the manufacturer's specification, the KD delivers 7.8 Kcal/g gross energy, where fat, carbohydrate, protein, and fiber comprised 700 g, 0 g, 128 g, and 109 g/Kg of the diet, respectively. The fat in this diet was derived from lard and the diet had a ketogenic ratio (fats: proteins + carbohydrates) of 5.48:1. The individual % composition of each dietary energy component for the SD and KD diets used in our studies is shown on Table 3.

Pre-Trial Period

Seizure susceptibility, body weight, and food intake were measured four times over a three-week period in 24 singly caged female EL mice (approximately 210 days of age). All mice received the SD during the pre-trial period and food intake was determined by subtracting the weight of food pellets remaining in the food hopper after one week from the initial amount given (200 g). The difference was then divided by seven to estimate the average daily food intake. Thus, all mice were highly seizure susceptible at the initiation of the diet therapy.

Dietary Treatment

After the three-week pre-trial period, the mice were placed into four groups (n = 6 mice/group) where the average body weight of each group was similar (about 31.0 ± 1.5 g) (Figure 3). All mice were then fasted for 14 hr to establish a similar metabolic set point at the start of the experiment (arrow, Figure 3). The mice in each group were then given one of four diets to include: 1) the standard diet fed *ad libitum* or unrestricted (SD-UR), 2) the KD fed *ad libitum* or unrestricted (KD-UR), 3) the SD restricted to achieve a 20-23% body weight reduction from the pre-trial weight (SD-R), and 4) the KD restricted to achieve a 20-23% body weight reduction from the pre-trial weight (KD-R). Each mouse in

the two R groups served as its own control for body weight reduction. Based on food intake and body weight during the pre-trial period, food in the R-fed mouse groups was reduced until each mouse achieved the target weight reduction of 20-23%. In other words, the daily amount of food given to each R mouse was reduced gradually until it reached 77-80% of its initial (pre-trial) body weight.

The mice in the SD-UR group received 200 g of food in the hopper/week as in the pre-trial period. For mice in the SD-R group, weighed food pellets were dropped directly inside each cage for easy access. The KD was administered to the mice in a modified plastic Falcon tissue culture dish (60 mm x 15 mm). The dish edges were shaved to reduce the height from 15 mm to about 6 mm. After placing about 5 g of KD in the dish for the KD-UR mice, the dish with the weighed KD was inverted and placed on the top of the food hopper. An empty water bottle was placed on top of the dish to prevent dish movement during animal feeding. The butter-like consistency adhered the KD to the inverted dish. This feeding apparatus allowed the mice easy access to the KD and prevented KD contact with bedding material. After about 24 hr, the amount of KD consumed was determined by measuring the left over KD in the dish and another 5 grams of fresh KD were added to the dish. The KD was therefore given fresh every day without moving or disturbing the mice. The total amount of KD consumed per day was summed each week and divided by 7 to obtain the average weekly food intake of each mouse. For the KD- R mice, a calculated restricted amount of KD was placed directly on top of the food hopper bars for easy access. The R-fed

mice licked the bars clean of the KD. The dietary treatment was implemented for nine weeks.

Measurement of Plasma Glucose and β-Hydroxybutyrate

Blood was collected approximately 1 hr after seizure testing except for the pre-trial period where blood was not collected. Blood was first collected from all mice about 24 hr prior to the initiation of the 14 hr fast (arrow on Figure 3). Mice were anesthetized with isoflurane, USP (Halocarbon, River Edge, NJ, USA) and blood was collected in heparinized tubes by puncture of the retro-orbital sinus using a borosilated capillary tube (FHC, Bowdoinham, ME, USA). The blood was centrifuged at 6,000 x g for 10 min, the plasma was collected, and aliquots were stored at –80^{o}C until analysis. Plasma glucose concentration was measured spectrophotometrically using the Trinder Assay (Sigma-Aldrich, St. Louis, MO, USA). Plasma β-hydroxybutyrate concentration was measured using either the Stanbio β-Hydroxybutyrate LiquiColor® procedure (Stanbio, Boerne, TX, USA), or a modification of the Williamson et al. procedure (Williamson et al., 1962).

Briefly, for measuring β-Hydroxybutyrate using Williamson's modified assay, 50 μl of a substrate containing cocktail buffer, containing: 0.5 ml of 1 M of 2-Amino-2-methylpropanol (AMP) (pH 9.9), 3 ml of 50 mM NAD^{+}, 0.2 ml of 100 mM EDTA, and 1.3 ml ddH_2O was pipetted in each well of a half-area clear microplate. Pipet in triplicate 50 μl of ddH_2O (Lin et al.), 50 μl of β-

hydroxybutyrate standards (0.25 mM, 0.165 mM, 0.0825 mM, 0.04125 mM, 0.033 mM, 0.0165 mM), and finally 50 μl of your plasma/serum sample in each corresponding well. Initial absorbance for all samples is read at 340 nm, using a 5 min kinetic absorbance mode on a SpectaMax M5 spectrophotometer. Absorbance is then corrected using the pathcheck function (normalize the well absorbance to a cuvette of an equivalent 1 cm pathlength) on the plate reader. 5 μl of a 2.6-fold diluted 1.33% (w/v) β-hydroxybutyrate dehydrogenase enzyme dissolved in 3.2 M $(NH_4)_2SO_4$ is then pipetetd in each well. After absorbance is corrected once more using the pathcheck function, and the final absorbance for all samples is read at 340 nm, using a 40 min kinetic absorbance mode. After making a standard curve by plotting the corrected change of absorbance (Abs_{final}-$Abs_{initial}$) for each standard, the β-hydroxybutyrate concentration for each sample was calculated. An analytical version of this assay is shown on Appendix C.

Statistical Analysis

Both ANOVA and a two-tailed *t*-test were used to evaluate the significance of differences of body weight, seizure susceptibility, plasma glucose levels, and plasma β-hydroxybutyrate levels between unrestricted and restricted groups. Chi-square analysis was performed on the association between glucose and seizures. Pearson bivariate correlation analysis (SPSS software) was used to determine the relationship between body weight, food intake, plasma glucose

levels, plasma β-hydroxybutyrate levels, and seizure susceptibility. Binary logistic regression (SPSS) was used to determine the relationship between seizure susceptibility, plasma glucose, and β-hydroxybutyrate levels on mice fed either the SD or the KD. Differences were considered significant at $P \leq 0.01$. All values are expressed as mean ± SEM. All statistical data were presented according to the recommendations of Lang et al., (Lang and Secic, 1997).

RESULTS

Diet Composition and Tolerance

The composition of each diet is shown in Table 3 and in the Methods. No adverse effects of the diets were observed in either R-fed mouse group. Despite the 20-23% body weight reduction, mice in both R-fed groups appeared healthy and were more active than the mice in the UR-fed groups as assessed by ambulatory and grooming behavior. With the exception of oily fur, the KD-fed mice appeared active and healthy throughout the study as previously found (Todorova et al., 2000). No signs of vitamin or mineral deficiency (e.g. reduced life span, kidney and eye abnormalities) were observed in the R-fed mice according to standard criteria for mice (Hoag and Dickie, 1968). These findings are consistent with the well-recognized health benefits of mild to moderate caloric

restriction in rodents (Keenan et al., 1999), and support our previous findings that both the KD and a moderate CR are well tolerated by EL mice (Todorova et al., 2000; Greene et al., 2001).

Influence of Calorie Restriction on Body Weight

All mice were matched for age (approximately 210 days) and body weight (approximately 31.0 ± 1.5 g) before the start of the dietary treatment (Figure 3). All mice lost approximately 7-9% of their body weight during the 14 hr fast. Body weight remained relatively stable over the nine-week treatment period in both UR-fed mouse groups (Figure 3). The 20-23% body weight reduction was achieved in the R-fed groups after about two weeks of gradual food restriction. However, more difficulty was encountered initially in maintaining a stable body weight reduction for the KD-R group than for the SD-R group. This difficulty may result from the high caloric content of the KD that produces greater body weight changes per calorie adjustment than the SD. We also estimated that the degree of CR necessary to maintain the 20-23% body weight reduction was about 38-45% for the SD and about 45-52% for the KD.

Influence of Diets on Seizure Susceptibility in Adult EL Mice

All mice had at least 15 recurrent seizures before the start of dietary treatment (arrow, Figure 3). The seizures occurred occasionally during routine cage changing prior to the pre-trial period and regularly from handling during the pre-trial test period. Seizure susceptibility was analyzed in all mouse groups after the R-fed mice achieved a stable body weight reduction, i.e., week five of treatment (Figures 3 and 4). Seizure susceptibility was high for both UR-fed groups throughout the study. In both R-fed groups, seizure susceptibility decreased from 1.0 to about 0.3 after two weeks and remained significantly lower than that of the UR-fed control groups from treatment weeks 5-12 (Figure 4). Only a single mouse in the KD-R group had a break-through seizure on week 8. Taken together, our findings show that seizure management in EL mice is more associated with the amount than with the origin of dietary calories.

Influence of Diets on Plasma Glucose and β-Hydroxybutyrate Levels

Plasma glucose levels were analyzed in all mouse groups after the R-fed mice achieved a stable body weight reduction (Figures 3 and 5). Glucose levels remained high for both UR-fed groups throughout the study and were stable over treatment weeks 5-12. However, plasma glucose levels were somewhat lower (about 8 mM) in both UR-fed groups between treatment weeks 3-5 compared to the pre-trial glucose levels (about 10 mM). This reduction might result from a combination of repetitive handling, seizures, blood collection, and the initial fast

(Figure 5). In both R-fed mouse groups, the plasma glucose levels decreased from about 10 mM to about 5.0 mM after three weeks and remained significantly lower than those of their respective UR-fed control groups.

Plasma β-hydroxybutyrate levels were also analyzed in all mouse groups after the R-fed mice achieved a stable body weight reduction (Figures 3 and 6). These levels remained low in the SD-UR group throughout the study and were stable for treatment weeks 5-12 (Figure 6). β-hydroxybutyrate levels were significantly higher in the R-fed groups than in their respective UR-fed control groups. These levels were also significantly higher in the KD-UR group than in the SD-UR group. The levels increased from about 0.4 mM to about 1.7 mM in the SD-R group and to about 3.0 mM in the KD-R group. These findings demonstrate that circulating β-hydroxybutyrate levels were inversely related to circulating glucose levels and that elevated β-hydroxybutyrate levels alone are not associated with seizure susceptibility.

Statistical Relationships Among Variables

The relationship between body weight, food intake, plasma glucose levels, plasma β-hydroxybutyrate levels, and seizure susceptibility was determined using Pearson bivariate correlation analysis (Table 4). All variables were significantly ($P < 0.01$) correlated with each other. Positive correlations were found among body weight, food intake, glucose, and seizure susceptibility. On

the other hand, β-hydroxybutyrate was negatively correlated with all variables. The correlations among glucose, β-hydroxybutyrate, and seizure susceptibility were also apparent from the data in Figures 4-6. Plasma glucose was significantly ($P < 0.001$) associated with seizure susceptibility in the EL mouse, as determined by Chi-square analysis (Figure 7). These results support our previous findings that glucose levels are predictive of seizure susceptibility in adult EL mice (Greene et al., 2001; Greene et al., 2003).

Binary logistic regression was also used to determine the relationship between seizure susceptibility, plasma glucose, and plasma β-hydroxybutyrate levels when mice were fed either the SD and/or the KD. The data indicate that regardless of diet, glucose could predict seizure susceptibility with an approximate 75 to 78 % accuracy (Table 5). Although β-hydroxybutyrate could also predict seizure susceptibility, we previously showed that β-hydroxybutyrate levels were dependent on and were inversely related to plasma glucose levels (Greene et al., 2001).

DISCUSSION

We found that restriction of either a high carbohydrate low fat standard diet or a high fat low carbohydrate KD was equally effective in reducing seizure susceptibility in adult EL mice with active epilepsy. Moreover, seizure

susceptibility remained similarly high in these mice when either diet was fed *ad libitum* or unrestricted. These findings indicate that the KD, when fed unrestricted, is unable to reduce seizure susceptibility in adult EL mice. Although the KD delays epileptogenesis in young seizure naïve EL mice when fed *ad libitum*, the effect is transient (Todorova et al., 2000). These findings are interesting since previous observations with children suggest that the antiepileptic and anticonvulsant effects of the KD are best when the diet is administered in restricted amounts (Freeman et al., 2000; Stafstrom and Bough, 2003). Indeed, seizure protection is often less in children that gain weight than in those who maintain or reduce body weight on the KD (Freeman, personal communication). Previous studies also indicate that restriction of high carbohydrate diets elevate seizure threshold (Eagles et al., 2003). Our findings in EL mice support these observations and suggest that CR may be necessary for the antiepileptic and anticonvulsant effects of the KD.

We previously showed that mild to moderate CR delayed epileptogenesis and reduced seizure susceptibility in seizure naïve juvenile and adult EL mice by reducing blood glucose and elevating ketone bodies (Greene et al., 2001). Although our data show that circulating β-hydroxybutyrate levels are inversely related to circulating plasma glucose levels, elevated ketone body levels are not directly associated with reduced seizure susceptibility in EL mice. This conclusion derives from the finding that seizure susceptibility is high in the KD-UR mice despite elevated β-hydroxybutyrate levels and from finding that seizure

protection was similar in the SD-R and KD-R groups despite significantly higher β-hydroxybutyrate levels in the KD-R than in the SD-R group. These results are consistent with previous studies in EL mice and in non-genetic seizure models that elevated ketone bodies alone are unable to account for the antiepileptic or anticonvulsant action of the KD (Appleton and DeVivo, 1974; Bough et al., 1999a; Likhodii et al., 2000; Thio et al., 2000; Todorova et al., 2000; Harney et al., 2002; Stafstrom and Bough, 2003; Seyfried et al., 2004).

Under normal physiological conditions brain cells derive most of their energy from glucose or glucose-derived metabolites, e.g., lactate (Clarke and Sokoloff, 1999; Bouzier-Sore et al., 2002; Kasischke et al., 2004). Also, brain glucose uptake is greater during epileptic seizures than during most other brain activities (Meldrum and Chapman, 1999). During fasting or calorie restriction, however, circulating glucose levels fall causing brain cells to rely more heavily for energy on ketone bodies that gradually increase with food restriction (Owen et al., 1967; Greene et al., 2003). It is the transition from glucose to ketone bodies for brain energy that is thought to underlie the antiepileptic and anticonvulsant effects of calorie restriction (Greene et al., 2003). Although the KD we used contained no carbohydrates, the mice eating this diet maintained high glucose levels and seizure susceptibility. The persistence of high glucose levels in the KD-UR group would prevent the transition to ketones for energy despite high levels of circulating ketone bodies. Our results show that circulating glucose

levels accurately predict seizure susceptibility in EL mice regardless of diet composition or circulating ketone body levels.

We used a new experimental design for calorie restriction in this study (Chapter 5 addresses this rationale further). Briefly, instead of restricting calories in the R-fed mice based on the average food consumption of the UR control mice as previously done (Greene et al., 2001), each R-fed mouse served as its own control to achieve and maintain a 20-23% body weight reduction. The new experimental design reduces variability in body weights and in caloric intake among mice fed diets widely different in nutritional composition and caloric content. In using body weight, rather than caloric intake, as an independent variable we were able to more accurately measure the statistical associations among circulating energy metabolites and seizure susceptibility. Thus, this type of experimental design is recommended for those studies attempting to evaluate the relationships among nutrition, metabolism, and disease phenotype.

We conclude that seizure susceptibility in EL mice is dependent on plasma glucose levels and that seizure control depends more on the amount than on the origin of dietary calories. A reduction of glucose and a subsequent increase in ketone bodies results in the zone of seizure management in the EL mice (Figure 8). Also, we found that CR underlies the antiepileptic action of the KD in EL mice. A transition from glucose to ketone bodies for energy is predicted to manage EL epileptic seizures through multiple integrated changes of inhibitory and excitatory neural systems. A detail biochemical and molecular analysis of the

anticonvulsant mechanism of calorie restriction and the ketogenic diet is shown in Chapter 6.

Table 2. Severity Scores for Handling Induced Seizures in EL Mice[a]

Scores	Response to handling stimulation
1	Squeaking
2	Immobility, blinking, mild facial clonus
3	Catatonic posture with erect tail
4	Forelimb clonus
5	Generalized tonic convulsion

[a]Reprinted with permission from Todorova et al., 2000.

Table 3. Composition (%) of the Standard Diet, and Various Ketogenic Diets[1]

Components	Standard Diet (SD)	Ketogenic Diet (KD)	KetoCal(R) Diet (KC)
Carbohydrate	62	0	3,3
Fat	6	75	80
Protein	27	14	16,7
Fiber	5	12	0
Energy (Kcal/gr)	4,1	7,8	7,2

[1] According to manufacturer's specifications (see Methods).

Figure 3. Influence of Diet on Body Weight in Adult EL Mice Fed the SD (A) or the KD (B). Asterisks indicate that the body weight of the R-fed mice was significantly different from their respective UR-fed groups ($P < 0.01$) during weeks 5-12. Squares represent the pre-trial period when all mice were fed the SD-UR. Circles and triangles represent the UR-fed and R-fed groups, respectively. Values are expressed as the mean ± SEM (n = 6 mice per group). Arrow indicates initiation of CR.

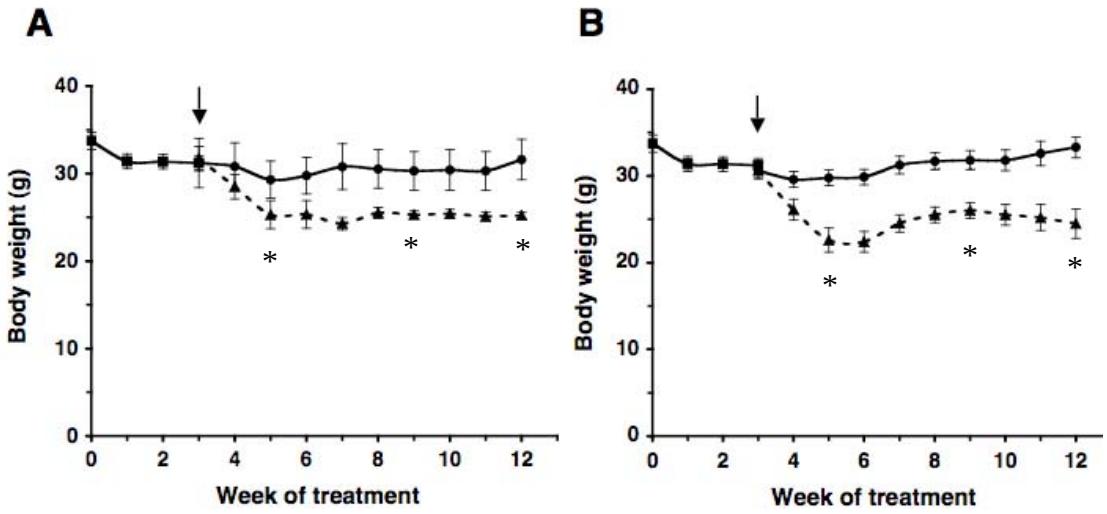

A
B
Body weight (g)
Week of treatment
0
10
20
30
40
0
2
4
6
8
10
12
*

Figure 4. Influence of Diet on Seizure Susceptibility in Adult EL Mice. Seizure susceptibility was significantly lower (*$P < 0.001$) in the R-fed groups than in their respective UR-fed groups. Values were pooled from treatment weeks 5-12 (see Figure 1) and are expressed as the mean ± SEM (n = 6 mice per group).

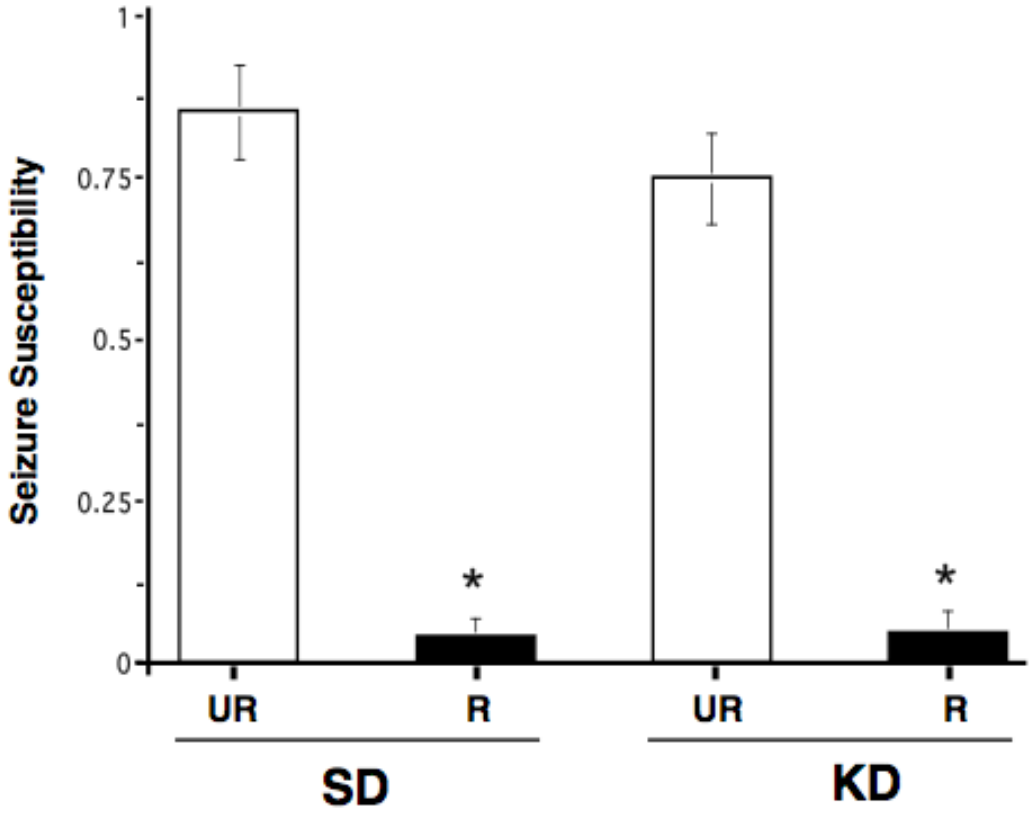

Seizure Susceptibility
1
0.75
0.5
0.25
0
*
*
UR
R
UR
R
SD
KD

Figure 5. Influence of Diet on Plasma Glucose Levels in Adult EL Mice. Plasma glucose levels were significantly lower (*$P < 0.001$) in the R-fed groups than in their respective UR-fed groups. Other conditions are as in Figures 1 and 2.

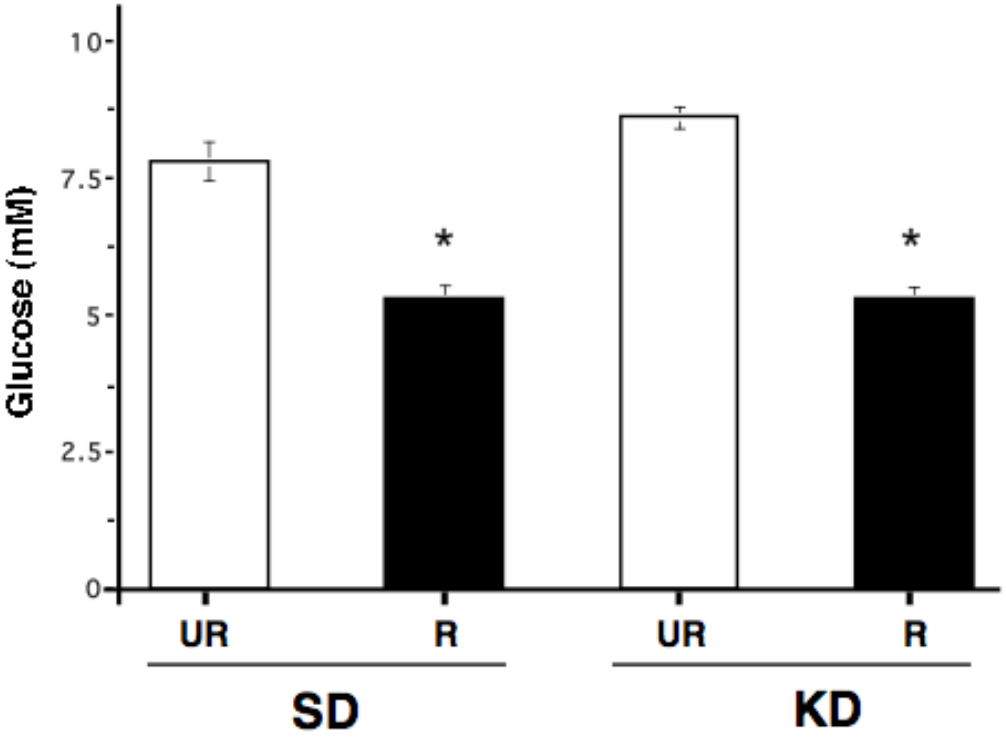

Glucose (mM)
10
7.5
5
2.5
0
*
*
UR
R
UR
R
SD
KD

Figure 6. Influence of Diet on Plasma β-Hydroxybutyrate Levels in Adult EL Mice. Plasma β-hydroxybutyrate levels were significantly higher (*$P < 0.001$) in the R-fed groups than in their respective UR-fed groups. In addition plasma β-hydroxybutyrate levels were significantly higher (†$P < 0.001$) in the KD-UR group than in the SD-UR group. Other conditions are as in Figures 3 and 4.

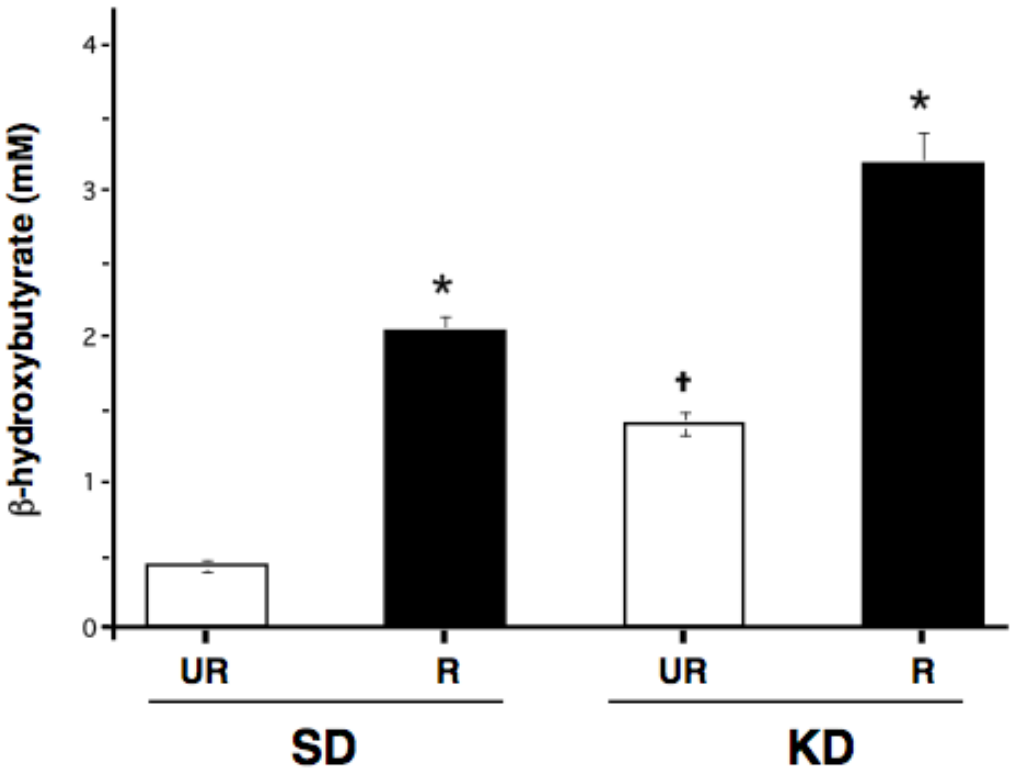

β-hydroxybutyrate (mM)
4
3
2
1
0
*
*
†
UR
R
UR
R
SD
KD

Figure 7. Association of Plasma Glucose and Seizure Susceptibility in Adult EL Mice. Data were obtained from all four dietary groups over treatment weeks 3-12 for a total of 234 seizure and glucose measurements. Seizure frequency in the three plasma glucose groups (< 6.5 mmol, 6.5-8.5 mmol, and > 8.5 mmol/L) was 8/234, 44/234, and 70/234, respectively. The association between glucose and seizure susceptibility was highly significant as determined by Chi-square analysis ($P < 0.001$).

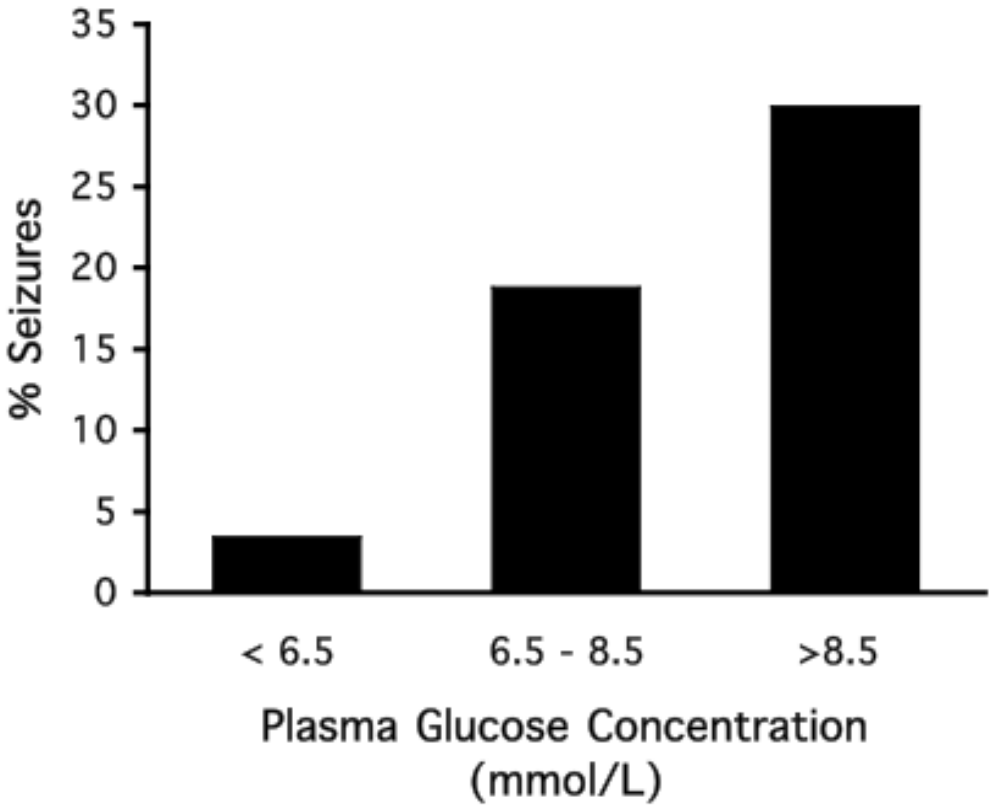

35
30
25
20
15
10
5
0
% Seizures
< 6.5
6.5 - 8.5
>8.5
Plasma Glucose Concentration
(mmol/L)

Figure 8. Relationship of Circulating Glucose and Ketone Levels to Seizure Management in Epileptic EL Mice.

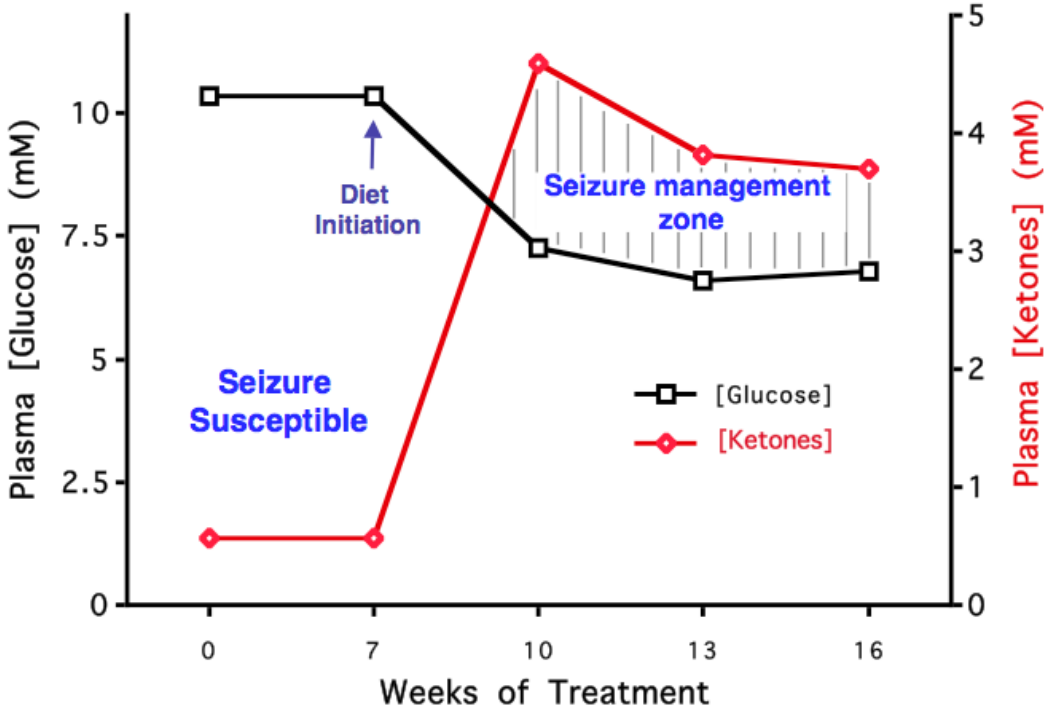

Plasma [Glucose] (mM)
10
7.5
5
2.5
0
Plasma [Ketones] (mM)
5
4
3
2
1
0
Diet
Initiation
Seizure management
zone
Seizure
Susceptible
[Glucose]
[Ketones]
0
7
10
13
16
Weeks of Treatment

Table 4 - Pearson Bivariate Correlation of Body Weight, Food Intake, Plasma Glucose Levels, Plasma β-hydroxybutyrate Levels, and Seizure Susceptibility in Adult EL Mice[1]

Parameter	Body weight (g)	Food Intake (Kcal)	Glucose (mM)	Ketones (mM)	Seizure Susceptibility
Body weight (g)	1.000				
Food Intake (Kcal)	0.488*	1.000			
Glucose (mM)	0.509*	0.382*	1.000		
Ketones (mM)	-0.379*	-0.379*	-0.429*	1.000	
Seizure Susceptibility	0.512*	0.464*	0.616*	-0.510*	1.000

[1] Data were obtained from all four dietary groups over the treatment weeks 3-12 for a total number of 210 seizure and glucose measurements (see figure 1).

* All correlations were significant at the 0.01 level (2-tailed).

Table 5 - Binary Logistic Regression Analysis of the Maximum Likelihood Estimates Between Plasma Glucose, and Seizure Susceptibility in Adult EL Mice Fed Either the SD or KD[1]

Dietary groups	Parameter	Df[2]	B[3]	SEM[4]	Wald x^{2} [5]	p value[6]
SD	Glucose	1	0.774	0.139	30.962	0.01
	Constant	1	-5.484	1.013	29.292	0.01
KD	Glucose	1	0.787	0.157	25.033	0.01
	Constant	1	-5.801	1.180	24.177	0.01
Both Diets	Glucose	1	0.752	0.102	54.682	0.01
	Constant	1	-5.507	0.759	52.625	0.01

[1] Data were obtained from all four dietary groups over the treatment weeks 3-12 for a total number of 210 individual measurements of plasma glucose and seizure susceptibility.

[2] Df, degrees of freedom.

[3] B, Estimate of the association between glucose and seizure susceptibility.

[4] The estimated error of the mathematical weighting, indicating the precision of the estimated coefficient.

[5] The Wald test statistic was computed from the data compared by using x^{2} distribution with 1 degree of freedom. The test statistic is used to determine the p value.

[6] The probability of Type I error.

CHAPTER THREE

Glucose Reduces the Antiepileptic and Anticonvulsant Effects of the Ketogenic Diet in EL mice

INTRODUCTION

The influence of KetoCal® in the Management of Idiopathic Epilepsy in Young Adult EL Mice

Our research focuses on the use of diets as a therapy for neurological and neurodegenerative diseases. We previously found that CR underlies the anticonvulsant and antiepileptic effect of the KD in reducing seizure susceptibility in adult EL mice (Greene et al., 2001; Mantis et al., 2004; Seyfried et al., 2009b). Also as previously found, this anticonvulsant effect of CR and the KD was associated with a significant reduction in circulating plasma glucose levels and a subsequent elevation of ketone body levels (Mantis et al., 2004; Seyfried et al., 2009b). Furthermore, we have shown that the KD when given in unrestricted or *ad libitum* amounts has a transient effect in delaying the epileptogenesis (seizure onset) in young EL mice (Todorova et al., 2000). Interestingly, viewing together all our previous findings indicate that the seizure control in the EL mice using CR or the KD is associated more with the amount rather than the origin of dietary calories.

Currently, several types of ketogenic diets are being employed for epilepsy treatment (Gasior et al., 2006). The most frequently used therapeutic KD is the traditional ketogenic diet, developed by Wilder in 1921, which is based on long-chain fatty acids (Wilder, 1921). A medium-chain triglyceride diet was introduced in the 1950's, which startlingly produces greater ketosis, due to a faster rate of fatty acid oxidation (Huttenlocher et al., 1971; Huttenlocher, 1976). This stems from the fact that ketosis was originally believed to underlie the anticonvulsant effects of fasting (see Chapter 1 for additional information) (Wilder, 1921; Lennox, 1960). This modification has not been widely accepted because it is associated with bloating and abdominal discomfort and is no more efficacious than the traditional ketogenic diet (Gasior et al., 2006). A third variation on the diet, known as the Radcliffe Infirmary diet, represents a combination of the traditional and medium-chain triglyceride diets (Schwartz et al., 1989; Gasior et al., 2006). Its efficacy is also similar to the traditional ketogenic diet.

In contrast to other ketogenic diet formulations (lard-based or medium chain triglyceride diets), which are not standardized or commercially available, KetoCal®, (KC), is a nutritionally balanced soy oil-based KD that has been approved by the FDA for the management of seizures in children with intractable epilepsy (Zhou et al., 2007; Mantis et al., 2009). According to the manufacturer's (Nutricia North America) recommendation for the management of seizures, KC is administered in restricted amounts. This involves a 65–70%

recommended daily allowance of calories or an approximate 30–35% calorie restriction. Hence, as seen with a restricted KD this recommended administration of KC should not only reduce the adverse effects of the diet's high fat content (Kang et al., 2004; Sampath et al., 2007; Marsh et al., 2008a), but also provide maximum therapeutic antiepileptic and anticonvulsant efficacy (Mantis et al., 2004; Zhou et al., 2007; Raffo et al., 2008; Coppola et al., 2009; Seyfried et al., 2009b). This suggests that the neuroprotective effects of both the KD and that of KC could only be achieved under CR conditions (Zhou et al., 2007). Interestingly, in clinical settings, it has been shown that the antiepileptic efficacy of the KD or that of fasting is usually lost as a result of administration of excess calories (Lennox and Cobb, 1928; Huttenlocher, 1976; Freeman et al., 2000; Freeman et al., 2007; Seyfried et al., 2009b). Similar observations are seen in energy-restricted animals weaned away from the KD to an unrestricted high carbohydrate diet, which has resulted in a loss of the anticonvulsant effects of the diet (Bough and Rho, 2007).

As mentioned previously, since epileptic seizures depend on glucose uptake and metabolism (McIlwain, 1969; Meldrum and Chapman, 1999; Cornford et al., 2002), it will be interesting to know whether supplementation of the free form of D-glucose has a similar effect in abolishing the antiepileptic efficacy of the KD in calorically restricted EL mice. In accordance to our previous findings where an unrestricted KD was shown to transiently delay epileptogenesis in young naïve (not yet seizure susceptible) EL mice (30 days-old), we propose that

KC could also have a positive antiepileptic and anticonvulsant effect in young sexually mature EL mice with active epilepsy, and that glucose supplementation in dietary restricted EL mice may result in marked change of the therapeutic efficacy of CR.

In this study, we evaluated the antiepileptic and anticonvulsant efficacy of KC in young adult EL mice. Our results are consistent with our previous findings that seizure control in the EL mice is associated more with the amount rather than the origin of dietary calories. We also confirmed that CR underlies the anticonvulsant action of the KD in EL mice, and that the neuroprotective effect of CR was associated with a significant reduction in circulating plasma glucose levels and a subsequent elevation of ketone body levels. Although a restricted KC was able to reduce seizure susceptibility in young adult EL mice, supplementation of glucose in the drinking water of restricted mice resulted in a reduced anticonvulsant efficacy of CR. Finally, for the first time we were able to show that KC fed in unrestricted amounts was able to reduce the severity and frequency of seizures in young EL mice.

MATERIALS AND METHODS

Mice

The EL mouse model used in this study is previously described in the Materials and Methods section of Chapter 2.

Seizure Susceptibility, Seizure Testing, and Seizure Phenotype

The seizure susceptibility paradigm for testing EL mice, along with the seizure phenotype of these mice is previously described in the Materials and Methods section of Chapter 2.

Diets

Similarly to what was previously described for adult EL mice (see Chapter 2), all mice received the SD prior to experimentation. The KetoCal® ketogenic diet was obtained as a gift from Nutricia North America (Rockville, MD, formally SHS International, Inc.). The KetoCal® diet (KC) is a nutrient balanced soy oil-based high fat, low carbohydrate KD diet that delivers 7.2 kcal/g of gross energy where fat, carbohydrate, protein, and fiber comprised 720 g, 30 g, 150 g, and 0 g/Kg of the diet, respectively (Zhou et al., 2007; Mantis et al., 2009). There are also minor differences between the SD and KC for the content (g/kg of diet) of amino acids, vitamins, minerals and trace elements. KC has a ketogenic ratio (fats: proteins + carbohydrates) of 4:1 and the fat was derived from soybean-oil. KC was fed to the mice in paste form (water: KC; 1:2) within the cage as

previously described (Zhou et al., 2007; Mantis et al., 2009). The specific feeding regimen for the SD-R and the KC-R mice was performed accordingly to what was described previously (Mantis et al., 2004; Zhou et al., 2007). Briefly both the SD and KC diets are calorie restricted to reduce mouse body weights by 15-18% compared to their pre-trial body weight. Water was provided *ad libitum* to all mice throughout the study. The energy composition of the SD and the KC diets is shown in Table 2.

Pre-Trial Period

Seizure susceptibility, body weight, and food intake was measured 5 times over a 6-week period in 34 singly housed young adult female EL mice (about 40 days old) fed the SD unrestricted as previously described (Chapter 2) (Mantis et al., 2004). Mice were approximately 80 days of age at the end of the pre-trial period. All mice were highly seizure susceptible at the initiation of the diet therapy, and had experienced at least 3 recurrent complex partial seizures prior to diet initiation.

Dietary Treatment

After the 6-week pre-trial period, the mice were placed into five groups (n = 6-8 mice/group) where the average body weight of each group was similar

(about 26.2 ± 0.5 g) (Figure 8). All mice were then fasted for 14 hr to establish a similar metabolic set point at the start of the experiment. The mice in each group were then given one of five diets: 1) the standard diet fed *ad libitum* or unrestricted (SD-UR), 2) the KC fed *ad libitum* or unrestricted (KC-UR), 3) the KC restricted to achieve a 15-18% body weight reduction from the pre-trial weight (KC-R), 4) the KC restricted to achieve a 15-18% body weight reduction from the pre-trial weight, with the supplementation of D-glucose (25 mM) in the drinking water 0.5hr before seizure testing (KC-R + 0.5hr [Glu]), and 5) the KC restricted to achieve a 15-18% body weight reduction from the pre-trial weight, with the supplementation of D-glucose (25 mM) in the drinking water 2.5hr before seizure testing (KC-R + 2.5hr [Glu]). Each mouse in the three R groups served as its own control for body weight reduction. Based on the food intake and body weight during the pre-trial period, food in the R-fed mouse groups was reduced until each mouse achieved the target weight reduction of a 15-18%. In other words, the daily amount of food given to each R mouse was reduced gradually until it reached 82-85% of its initial (pre-trial) body weight. The dietary treatment period lasted for nine weeks.

The feeding paradigm for the SD-UR and KC mouse groups is similar to that of the SD-UR and KD mouse groups described in the Materials and Methods section of Chapter 2. The two KC-R mouse groups that were supplemented with glucose in their drinking water also were fed similarly to KD-R group described previously in the Materials and Methods section of Chapter 2, with the exception

that 25 mM of D-glucose was provided *ad libitum* in their drinking water 0.5 hr or 2.5 hr prior to seizure testing. For the KC-R mouse group that no had no glucose on its drinking water, KC was administered as previously described in the Materials and Methods section of Chapter 2. For all KC-R mice, a calculated restricted amount of KC was placed directly on top of the food hopper bars for easy access.

Measurement of Plasma Glucose and β-Hydroxybutyrate

Blood was collected approximately 1 hr after seizure testing every three weeks, as described in the Materials and Methods section of Chapter 2. In various animals it has been shown that exposure to stressors, such as eye bleeding, can cause a varying effect of physiological changes in the animal body weight, growth, and food intake regulation (Armario et al., 1988; Marti et al., 1993; Marti et al., 1994; Valles et al., 2000). Plasma glucose and β-hydroxybutyrate concentrations were measured spectrophotometrically, using the StanBio® Enzymatic Glucose Assay (1075-102) (StanBio Laboratory, Boerne, TX, USA) and a modification of the Williamson *et al*., enzymatic procedure (Williamson et al., 1962), respectively. Detailed description of the β-hydroxybutyrate assay is shown in the methods and materials of Chapter 1 and also in Appendix C.

Statistical Analysis

Both ANOVA and a two-tailed *t*-test were used to evaluate any significant differences in body weight, seizure susceptibility, plasma glucose levels, and plasma β-hydroxybutyrate levels between unrestricted and restricted groups. Differences were considered significant at $P \leq 0.01$. All values are expressed as mean ± SEM. All statistical data were presented according to the recommendations of Lang et al., (Lang and Secic, 1997).

RESULTS

Influence of KC on Body Weight and Diet Tolerance

Similarly to what we have observed in brain cancer management using KC, no adverse effects, or signs of vitamin and mineral deficiency were observed in the KC-R fed mouse groups. Despite the 15-18% body weight reduction, KC-R fed mice appeared healthy and more active than mice in the UR-fed groups. With the exception of oily fur, the KC-fed mice appeared active and healthy throughout the study as previously found (Zhou et al., 2007). These findings indicate that KC was well tolerated by EL mice and moderate calorie restriction has the well-recognized health benefits in rodents (Keenan et al., 1999).

Throughout the study, the body weights remained relative similar (e.g. about 30 g at the end of the study) in the two unrestricted mouse groups (Figures 9A and B), despite major differences in calorie and compositional content of these diets (Table 3). In the R-fed groups, a significant loss of body weight was noticed within the first week of the treatment (Figure 9A). The suggestive 15-18% body weight reduction was achieved and maintained in all R-fed groups by week three of the dietary treatment. Supplementation of D-glucose prior to seizure testing had no effect in body weight. These findings indicate that KC when given in restricted amounts produced noticeable improvement in health and vitality, in concurrence with the body weight reduction.

Influence of KC on Seizure Susceptibility in Young Adult EL Mice

All mice had experienced at least three complex partial seizures with secondary generalization prior to the initiation of the diet. Feeding young EL mice with unrestricted amounts of either the KC or the SD had a varying effect on seizure susceptibility. Although seizure remained relative high for the duration of the study in both UR-fed groups, the seizure susceptibility in the SD-UR group increased significantly compared to the seizure susceptibility in the KC-UR mice at the end of the study (Figures 10 and 11). On the other hand, mean seizure frequency was reduced by almost 50% in all KC-R fed groups by week three of treatment (data not shown). In addition, the mean seizure susceptibility in the

KC-R group remained significantly lower than that of the two UR-fed groups for the duration of the dietary treatment (Figure 10). Moreover, supplementation of D-glucose 0.5 hr or 2.5 hr prior to seizure testing in the drinking water of restricted KC mice resulted in a marked increase in the seizure susceptibility of these mice compared to the seizure susceptibility of the KC-R mice (Figure 10). More importantly, supplementation of glucose 2.5 hr prior to seizure testing increased seizure susceptibility to the same levels as that of the KC-UR group (Figure 10). Interestingly, supplementation of 50 mM β-hydroxybutyrate (given *ad libitum* daily) in the drinking water of another group of mice fed the KC-R diet did not result in a better anticonvulsant efficacy of the restricted KC diet alone (data not shown). These findings support our previous notion that the antiepileptic efficacy of the high fat ketogenic diet is best when given in restricted amounts and that seizure management in EL mice is dependent on a reduction of body weight and thus glucose levels. Furthermore, whereas glucose supplementation resulted in an increase of the seizure susceptibility, administration of KC in unrestricted amounts reduced seizure susceptibility in young EL mice. This latter finding might suggest that unrestricted KC reduces the influence of Gower's dictum, that "seizures beget seizures", in the seizure susceptibility of EL mice.

Influence of KC on Plasma Glucose and β-Hydroxybutyrate Levels

Plasma and ketone body levels were analyzed every three weeks during the diet treatment period. Plasma glucose levels were similar in both UR-fed mouse groups and remained relative high throughout the study (Figure 12). Contrary to that, glucose levels were significantly lower in the KC-R group compared to either unrestricted group (Figure 12). Supplementation of glucose had no effect on the glucose levels of the KC-R mice (Figure 12). In contrast to glucose, circulating β-hydroxybutyrate levels were significantly different in the KC-UR group compared to the SD-UR mice throughout the study (Figure 13). Interestingly, this marked increase of ketone levels in the KC-UR was evident by the third week of dietary treatment (data not shown). As previously shown, ketone levels in the SD-UR group remained low and were stable for the duration of the experiment. Similar to the effect in glucose levels, supplementation of glucose had no effect on the ketone levels of the KC-R mice (Figure 13). Interestingly, *ad libitum* supplementation of 50 mM β-hydroxybutyrate in the drinking water of another group of mice fed the KC-R diet resulted in similar glucose and ketone levels as in the three KC-R groups (data not shown). These findings are consistent with our previous studies in mice showing that the high fat KD does not lower plasma glucose levels when administered in unrestricted amounts and that a transition from the glucose to ketone bodies for energy underlies the anticonvulsant and antiepileptic effects of the KD and that of calorie restriction.

DISCUSSION

Our current findings demonstrate that KetoCal ® (KC), a new nutritionally balanced soy oil-based high fat, low carbohydrate KD, has both antiepileptic and anticonvulsant properties in reducing seizure susceptibility in young adult EL mice. This neuroprotection was associated more with the amount rather than the origin of dietary calories. These observations are consistent with the already known neuroprotective properties of the traditional KD (Gasior et al., 2006; Freeman et al., 2007; Seyfried et al., 2009b). Despite recent findings indicating a distinct increase of seizure threshold in a pentylenetetrazol (PTZ)-induced seizure rat model by KC (Raffo et al., 2008), no prior studies have evaluated the therapeutic efficacy of KC in a natural model of epilepsy, such as the EL mouse. Most of the studies involving the study of the antiepileptic mechanism(s) of the KD have been based on acute seizure models (e.g. PTZ and kainic acid), and not on developmental natural epilepsy models (Bough and Rho, 2007). Since, those models may not recapitulate all essential features of the human epileptic condition (Stafstrom, 1999), the need of studying the therapeutic efficacy of the KD in natural model of epilepsy is further validated.

Consistent with our previous findings that CR is important in the seizure control of EL mice, our current results show that the anticonvulsant efficacy of KC is best when given in restricted amounts (Mantis et al., 2004; Seyfried et al., 2009b). Also as previously found, this anticonvulsant effect of KC was associated with a significant reduction in circulating plasma glucose levels and a subsequent elevation of ketone body levels (Mantis et al., 2004; Seyfried et al., 2009b). The aforementioned findings are in accordance with clinical studies, which indicate that the antiepileptic and anticonvulsant efficacy of the traditional KD is best when the diet is administered in restricted amounts and is associated with body weight and blood glucose reductions of about 10% (Livingston, 1972; Freeman et al., 2000; Freeman et al., 2007; Seyfried et al., 2009b). Recent findings have also shown a restricted KC to have both anti-tumor and anti-angiogenic effects in experimental mouse and human brain tumors mainly due to a reduction of total caloric content and circulating glucose (Zhou et al., 2007; Seyfried et al., 2008a).

Interestingly we show for the first time that an unrestricted KD is able to reduce seizure severity and frequency without a corresponding reduction in body weight or circulating glucose levels. Nevertheless, this reduced epileptogenicity in the KC-UR group was correlated with an elevation in plasma ketone (β-hydroxybutyrate) levels compared to the levels of the SD-UR mice. This is important, since it was initially thought that the antiepileptic action of the KD was largely due to ketosis, a phenomenon also observed both in fasted humans

(Wilder, 1921; Peterman, 1928; Lennox, 1960; Freeman et al., 2000) and in animal models fed the KD (DeVivo et al., 1978; Bough and Eagles, 1999; Eagles et al., 2003). During states of reduced glucose availability brain cells can transition from glucose to ketone bodies for energy (Owen et al., 1983; Greene et al., 2003; Mantis et al., 2004; Seyfried et al., 2009b). However, ketone utilization by the brain is dependent not only on plasma ketone levels, but also the levels of circulating glucose and other metabolites (Nehlig and Pereira de Vasconcelos, 1993). Hence, although the persisted high glucose levels observed in the KC-UR group may prevent the complete transition to ketone metabolism, it is clear that some degree of sustained ketosis is needed for the therapeutic efficacy of the KD. Furthermore, ketone bodies alone, especially acetoacetate and acetone, have also been shown to be anticonvulsant in both humans and various animal models (Helmholz and Keith, 1930; Yamashita et al., 1976; Rho et al., 2002; Likhodii et al., 2003; Bough and Rho, 2007).

The reduced seizure threshold of the KC-UR group can also be explained in part by a similar transient antiepileptogenic effect as seen in young (32 days old) seizure naïve EL mice fed with the lard-based KD in unrestricted amounts (Todorova et al., 2000). Specifically, we showed that the KD delayed epileptogenesis in young EL mice without affecting glucose levels (Todorova et al., 2000). It is known that seizures in the EL mice commence with the onset of sexual maturity (60-75 days of age) and progressively get worse with age (model Gower's dictum) (Suzuki et al., 1991; Kasamo et al., 1992; Ishida et al., 1993;

Todorova et al., 1999a; Uchibori et al., 2002). While Gowers' dictum, "seizures beget seizures", stipulates that the incidence and severity of future seizures depends on the incidence and severity of any previous seizure (Gowers, 1901; Todorova et al., 1999a; Pitkanen and Sutula, 2002; Stafstrom and Sutula, 2005), administration of KC in this critical stage of seizure development and progression (mice were fed KC starting at about 80 days of age) reduces the influence of Gower's dictum on EL seizure susceptibility. Furthermore, with the emergence of recent clinical evidence suggesting that the KD has both short- and long-term efficacy (Freeman, 2001; Hemingway et al., 2001; Marsh et al., 2006; Kossoff and Rho, 2009), it becomes even more apparent that although the KD has a dual anticonvulsant and antiepileptogenic effect, its potential mechanism(s) of action may vary.

Glucose supplementation, prior to seizure testing, resulted in an increase of seizure susceptibility in young adult EL mice fed a calorically restricted regimen. Although, the reduction in seizure susceptibility was independent of any changes in glucose or ketone levels, this finding is consistent with previous reports. Specifically, supplementation of glucosamine, a carbohydrate analog, resulted in no net change in fasted blood glucose levels (Tannis et al., 2004). Also as seen with the influence of glucose ingestion after prolonged exercise in glucose absorption kinetics (Jeukendrup et al., 1999b; Jeukendrup et al., 1999a; Vannucci and Vannucci, 2000), supplementing glucose in R-fed EL mice will too result in a rapid clearance of circulating glucose for energy metabolism or seizure

induction. Neuronal excitability and epileptic seizures are directly related to rapid glucose utilization and glycolysis (McIlwain, 1969; Ackermann and Lear, 1989; Meric et al., 1994; Clarke and Sokoloff, 1999; Meldrum and Chapman, 1999; Cornford et al., 2000; Knowlton et al., 2002; Ikemoto et al., 2003; Schwechter et al., 2003).

Furthermore, supplementation of calories in the form of carbohydrate or protein in energy-restricted KD animals usually translates in diminished anticonvulsant efficacy due to an increase in the pool of metabolic substrates for gluconeogenesis (Appleton and DeVivo, 1974; Huttenlocher, 1976; Bough and Rho, 2007; Freeman et al., 2007). Thus to confirm the role of glucose in seizure susceptibility, in a follow up study, previously seizure controlled R-fed EL mice were reverted back to *ad libitum* conditions. Our findings indicate that re-feeding of either the SD or the KD in previously R-fed EL mice resulted in a progressive reduction of the anticonvulsant effects of CR (data not shown). Specifically, we showed that seizure susceptibility in the re-fed restricted mice returned by the fourth week of *ad libitum* feeding. This finding is in accordance with our previous findings in C57BL/6J mice, suggesting that CR was able to establish a new homeostatic state for mice (Mahoney et al., 2006). Surprisingly, although body weight returned to pre-restricted levels within a week of re-feeding, glucose and ketone levels returned to pre-restricted levels more gradually (data not shown). This latter finding is also consistent with the findings of Lennox et al., 1928, where the return of seizures after termination of fasting were often associated

with rising blood glucose levels and falling blood ketone levels (Lennox and Cobb, 1928; Seyfried et al., 2009b). Interestingly, in a clinical setting breakthrough seizures are believed to also result from a rise in blood glucose levels, as evident with the loss of the anticonvulsant effect of the KD in patients who gain weight on the KD or those who consume excess carbohydrates (e.g. “sneak” a cookie) (Huttenlocher, 1976; Freeman et al., 2000; Freeman et al., 2007).

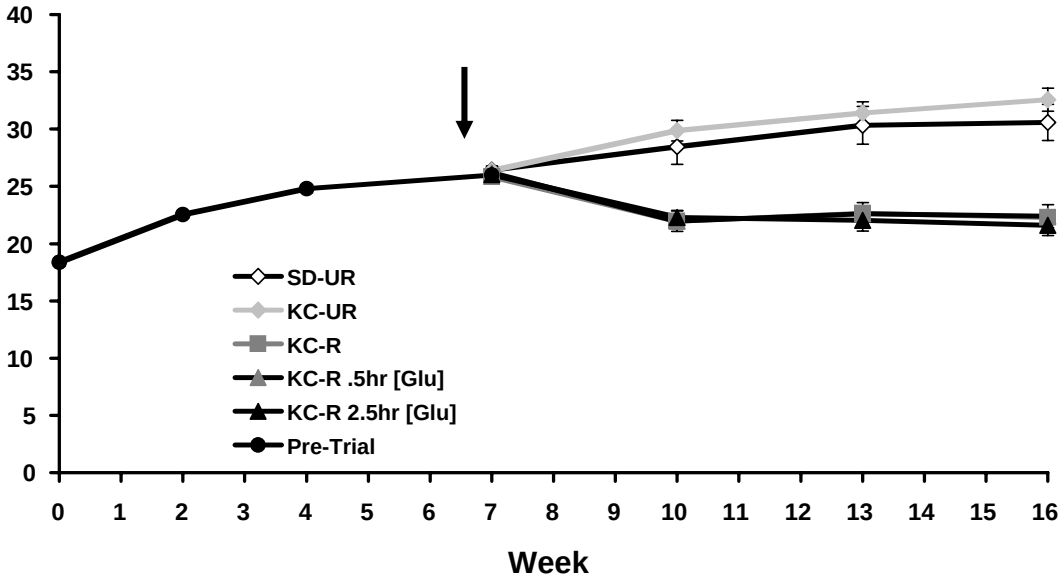
Although the exact neuroprotective mechanism of the KD still eludes us, it is thought to result from a combination of the reduction in metabolite availability, oxidative damage, the increase in glutathione antioxidant properties, mitochondrial biogenesis, increased cerebral ATP and phosphocreatine levels, as well as an increase in GABA levels (Cheng et al., 2003; Ziegler et al., 2003; Cheng et al., 2004; Sullivan et al., 2004; Dahlin et al., 2005; Yudkoff et al., 2005; Bough et al., 2006; Seyfried et al., 2009b). Hence, the neuroprotective effect of CR or the KD may be in large due to adaptations to ketosis rather than ketosis directly influencing the therapeutic efficacy of those diets (Bough and Rho, 2007).

In conclusion, our results indicate that KC alone has both anticonvulsant and antiepileptic properties and that CR underlies the neuroprotective action of KC in EL mice. Interestingly, supplementation of glucose decreases the anticonvulsant action of the KD, without affecting restricted glucose and ketone levels. A further detailed biochemical and molecular analysis of the

anticonvulsant and antiepileptogenic mechanism of KetoCal® is shown in Chapter 6.

Figure 9. Influence of the High Fat Soybean-oil KC Diet on Body Weight in Young Adult EL Mice. Asterisks indicate that the body weight of all KC-R groups was significantly lower compared to the body of the KC-UR mouse group ($P < 0.001$). Body weight was similar between the two UR groups. A. Values are expressed as the mean ± SEM for the duration of the study including the pre-trial period (weeks 0-9) (n = 8-10 mice per group). Arrow indicates initiation of the dietary treatment; B. Values are expressed as the mean ± SEM for the duration of weeks 3-9 of the dietary treatment period (n = 8-10 mice per group).

A.


40
35
30
25
20
15
10
5
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Week
SD-UR
KC-UR
KC-R
KC-R .5hr [Glu]
KC-R 2.5hr [Glu]
Pre-Trial

B.

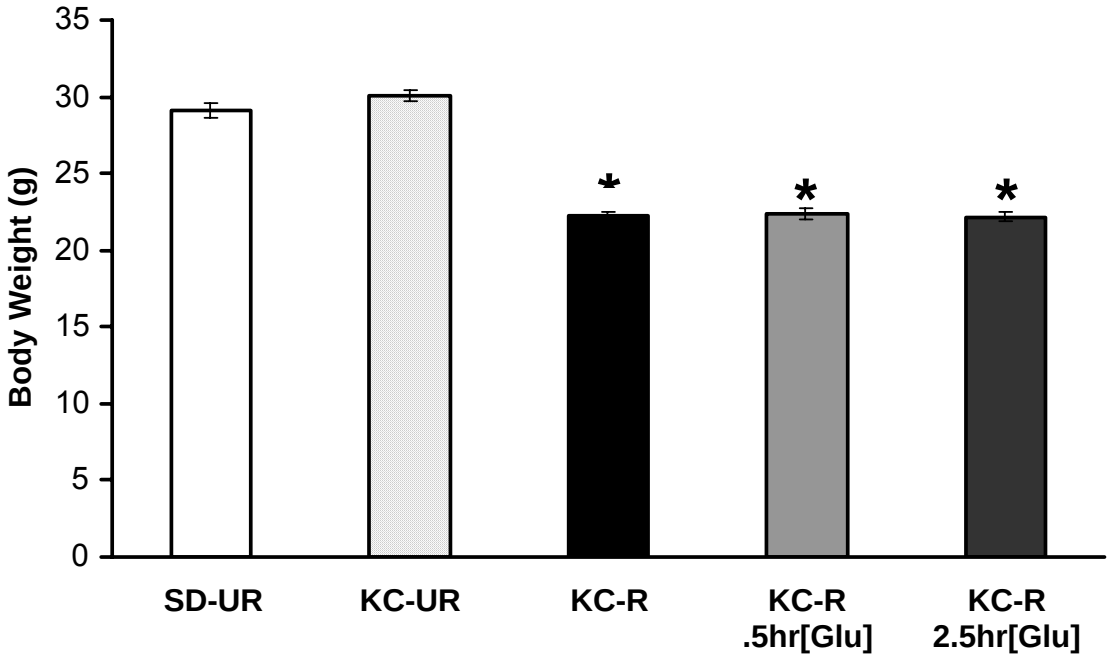

Body Weight (g)
35
30
25
20
15
10
5
0
*
*
*
SD-UR
KC-UR
KC-R
KC-R .5hr[Glu]
KC-R 2.5hr[Glu]

Figure 10. Influence of the KC Diet on Seizure Susceptibility in Young Adult EL Mice. Double asterisks indicate that seizure susceptibility in the KC-R and KC-R + 0.5hr [Glu] groups were significantly lower compared to the KC-UR mouse group ($P < 0.001$). Double cross indicates that seizure susceptibility in the KC-UR group was significantly lower compared to the SD-UR group ($P < 0.001$). Asterisks indicate that the seizure susceptibility of the KC-R + 0.5hr [Glu] and KC-R + 2.5hr [Glu] groups were significantly higher compared to the KC-R group ($P < 0.02$). Other conditions are as in Figure 9.

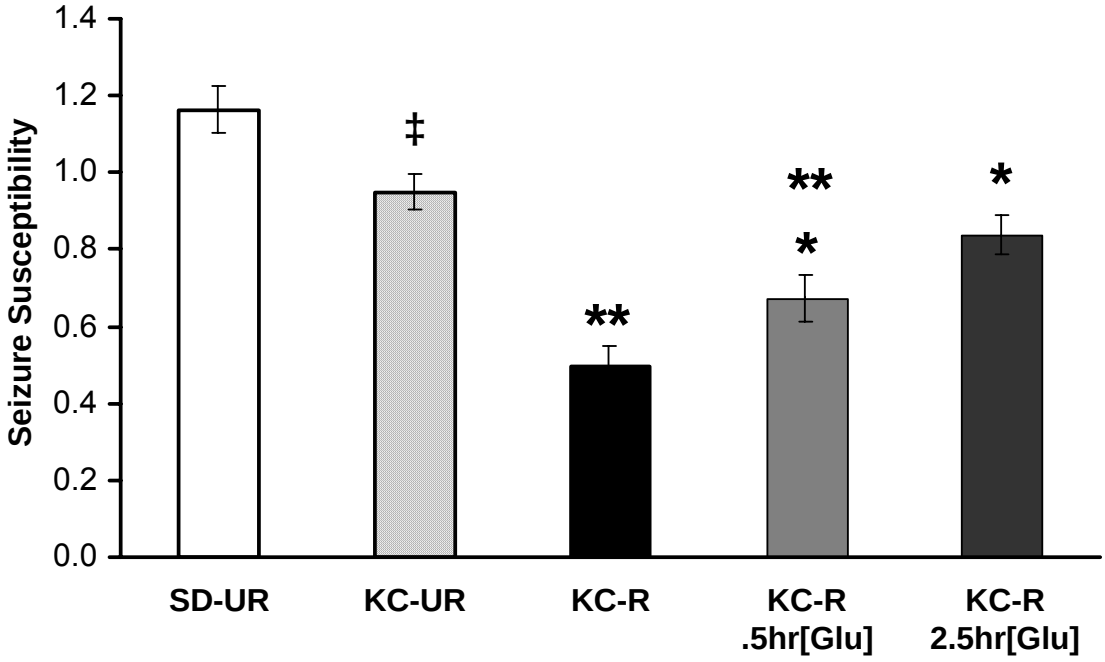

Seizure Susceptibility
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
‡
**
**
*
*
SD-UR
KC-UR
KC-R
KC-R .5hr[Glu]
KC-R 2.5hr[Glu]

Figure 11. Comparative Analysis of the Influence of the KC Diet on the Seizure Susceptibility in Young Adult EL Mice on Weeks 0 and 9 of the Study. Asterisk indicates that at week 9 of the study seizure susceptibility of the SD-UR group was significantly higher than that at the beginning of the diet treatment ($P < 0.05$). No increase in seizure frequency was observed in the KC-UR group at the end of the study. Other conditions are as in Figure 9.

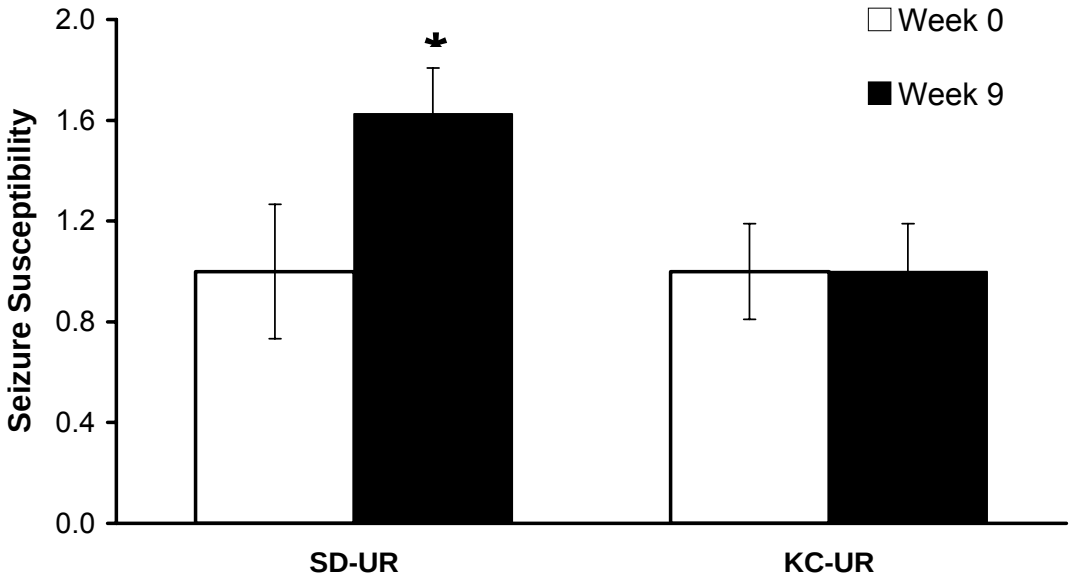

2.0
1.6
1.2
0.8
0.4
0.0
Seizure Susceptibility
*
Week 0
Week 9
SD-UR
KC-UR

Figure 12. Influence of the KC Diet on Plasma Glucose Levels in Young Adult EL Mice. Asterisks indicate that plasma glucose levels in all KC-R groups were significantly lower compared to the KC-UR mouse group ($P < 0.001$). Other conditions are as in Figure 9.

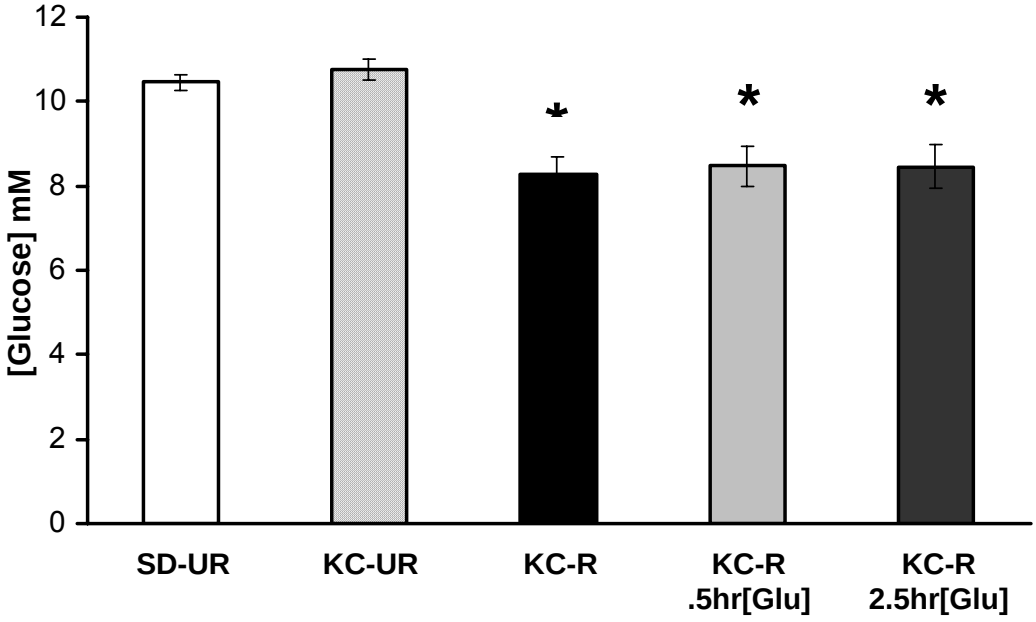
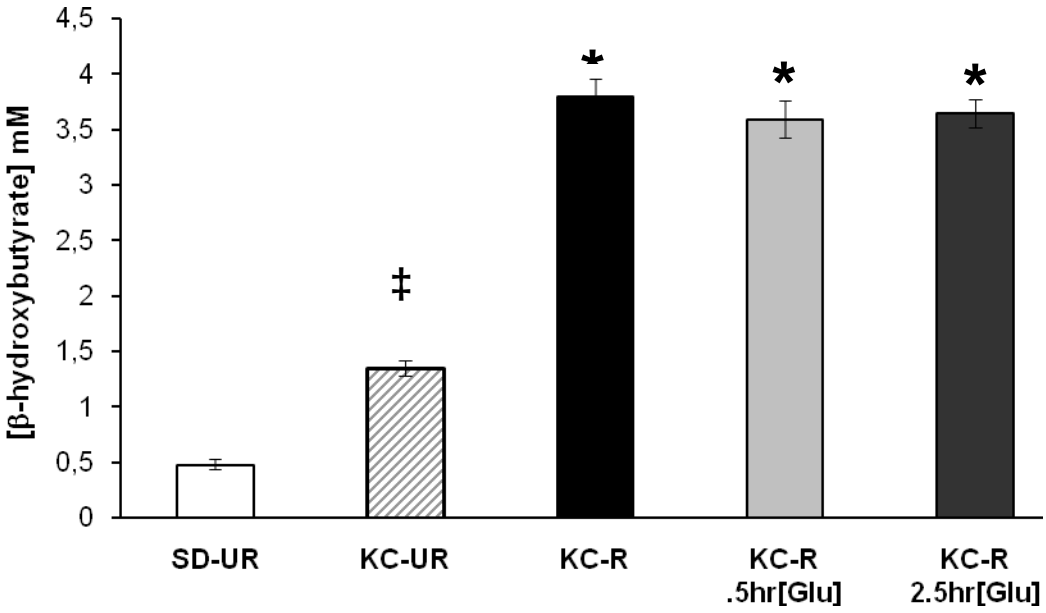

12
10
8
6
4
2
0
[Glucose] mM
*
*
*
SD-UR
KC-UR
KC-R
KC-R .5hr[Glu]
KC-R 2.5hr[Glu]

Figure 13. Influence of the KC Diet on Plasma β-Hydroxybutyrate Levels in Young Adult EL Mice. Asterisks indicate that plasma β-hydroxybutyrate levels in all KC-R groups were significantly higher compared to the KC-UR mouse group ($P < 0.001$). Double cross indicates that plasma β-hydroxybutyrate levels in the KC-UR group was significantly higher than that of the SD-UR mouse group ($P < 0.001$). Other conditions are as in Figure 9.


[β-hydroxybutyrate] mM
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
‡
*
*
*
SD-UR
KC-UR
KC-R
KC-R .5hr[Glu]
KC-R 2.5hr[Glu]

CHAPTER FOUR

Improvement of Motor and Exploratory Behavior in Rett Syndrome Mice with Restricted Ketogenic and Standard Diets

INTRODUCTION

Rett Syndrome

Rett syndrome (RTT) is an X-linked dominant neurodevelopmental disorder that affects about 1 in 9,000 girls (Bebbington et al., 2008). Girls with RTT develop normally for about 6-18 months after birth before exhibiting signs of speech and behavioral regression in addition to progressive motor impairment (Guy et al., 2001; Zoghbi, 2002; Williamson and Christodoulou, 2006). Many symptoms of RTT are age dependent and include hand wringing, reduced muscle tonicity, anxiety, microencephaly, indications of mental retardation, and seizures, among other autistic-like behaviors (Witt Engerstrom, 1992; Mount et al., 2001; Mount et al., 2003; Jian et al., 2007). Although RTT patients show abnormal neuronal morphology, no neuronal loss is evident. The relatively low incidence of RTT in humans often results from misdiagnosis of the disorder as autism or, to a lesser extent, as Angelman syndrome (Jedele, 2007).

About 80% of girls with RTT have a mutation in the *Mecp2* (Methyl-CpG-binding protein 2) gene (Renieri et al., 2003; Percy and Lane, 2005; Zoghbi,

2005), which encodes a protein involved with transcriptional regulation, and more specifically with histone deacetylation and methylation-dependent gene silencing (Amir et al., 1999; Percy, 2002; Shahbazian et al., 2002b). Males with mutations in the *Mecp2* gene often die before birth or in infancy due to severe neonatal encephalopathy (Wan et al., 1999). A small number of males with a *Mecp2* mutation, however, have developed signs and symptoms similar to those of classic Rett syndrome (Villard et al., 2000; Dayer et al., 2007; Villard, 2007). Some of these boys have an extra X chromosome in many or all of the body's cells. Several mouse *Mecp2* gene mutants have been generated including a partially truncated form of the MeCP2 protein (*Mecp2*$^{308/y}$) that is commonly found in girls with RTT (Chen et al., 2001; Guy et al., 2001; Shahbazian et al., 2002a). In contrast to humans, male Rett mice exhibit the classical RTT phenotype much earlier in life than female mice, and thus are predominately used for animal studies. Importantly, *Mecp2*$^{308/y}$ mice exhibit several symptoms associated with RTT in humans, to include behavioral abnormalities and impaired social interactions (Chen et al., 2001; Guy et al., 2001; Shahbazian et al., 2002a; Moretti et al., 2005; Moretti et al., 2006). More specifically, around 6 weeks of age *Mecp2*$^{308/y}$ mice begin to display learning and memory deficits that are indicative of synaptic dysfunction as well as other symptoms of RTT progression. The RTT phenotype in the female *Mecp2* mice is milder and shows greater variability, presumable due to differences in the pattern of X chromosome inactivation (Young and Zoghbi, 2004; Metcalf et al., 2006). Skewed X-

inactivation is also believed to be responsible for the mild and hardly recognizable RTT phenotype in girls with RTT (Huppke et al., 2006; Takahashi et al., 2008).

RTT children are generally smaller than normal children and these differences become increasingly exaggerated over time (Oddy et al., 2007). Girls with RTT tend to be disinterested in social interactions and are often emotionally withdrawn (Thommessen et al., 1992). They also have elevated circulating levels of pyruvate, lactate, and glucose, which could be indicative of an abnormal metabolic phenotype (Haas et al., 1986; Haas et al., 1995a; Haas et al., 1995b). Interestingly, *Mecp2*-null mice also have reduced levels of brain glutamine, glutamate, choline, N-acetyl aspartate, and ATP, further indicating that RTT could be associated with abnormal neuronal and glial cell metabolism (Saywell et al., 2006; Ward et al., 2009). These findings, viewed together, indicate that abnormal energy metabolism may contribute to the growth failure associated with RTT, and also suggest that diet therapies, and in particular restricted diet could help delay the onset or at least mitigate severity of the RTT phenotype (Rice and Haas, 1988; Motil et al., 1994; Reilly and Cass, 2001; Oddy et al., 2007). This hypothesis has been confirmed in (some) girls with RTT who demonstrated modest improvements in behavior and motor performance when maintained on a ketogenic diet (Haas et al., 1986; Liebhaber et al., 2003).

Diet Therapies in the Treatment of Autism

The ketogenic diet (KD), as previously described, is a high fat, low carbohydrate diet that has been shown to have antiepileptic, anticonvulsant, and other neuroprotective effects in both rodents and humans (Mantis et al., 2004; Seyfried et al., 2004; Freeman et al., 2007; Hartman et al., 2007; Hartman and Vining, 2007; Baranano and Hartman, 2008; Maalouf and Rho, 2008). We previously showed that although an unrestricted KD could delay the onset of seizures in EL mice with a genetic predisposition to epileptic seizures (Todorova et al., 2000), greater seizure control could be achieved in these mice when fed a calorically restricted KD (KD-R) (Mantis et al., 2004) (see also Chapter 2 for additional information). Interestingly, the KD has been shown to positively influence the behavior of autistic children (Evangeliou et al., 2003), and produce metabolic alterations in the brain and in the body that enhance energy expenditure and ultimately reduce body weight (Kennedy et al., 2007). Similarly to the KD, calorie restriction (CR) is a natural dietary therapy that too has long been recognized to improve health, promote longevity, and to reduce the incidence as well as delay the onset and/or severity of symptoms associated with a variety of neurochemical and neurobehavioral disorders, including epilepsy (Weindruch and Walford, 1988; Greene et al., 2001; Greene et al., 2003; Mantis et al., 2004; Maswood et al., 2004; Halagappa et al., 2007; Seyfried et al., 2009b). These latter findings suggest that a KD-R has a greater neuroprotective

effect than an unrestricted KD, at least in rodent models of epilepsy (Mantis et al., 2004).

Thus, in light of the evidence described above, in this study we evaluated whether CR could have a positive influence on the anxiety behavior and motor characteristics of a mouse model of RTT. Our preliminary results indicate that calorically restricted diets can be of clinical importance since CR improved symptoms of behavioral abnormalities in Rett mice, particularly with respect to reduced anxiety involving exploratory activity within an unfamiliar environment. A report of these findings was recently presented (Mantis et al., 2009).

MATERIALS AND METHODS

Mice

The inbred B6.129S-*Mecp2*^{tm1Hzo}/J (*Mecp2*$^{308/y}$) Rett mice were originally obtained from JAX laboratories (Maine). Mice were generated as previously described by Shahbazian et al., 2002 (Shahbazian et al., 2002a). The mice were maintained through brother-sister inbreeding and kept in the Animal Care Facility of Boston College with all procedures in strict adherence with the NIH Guide for the Care and Use of Laboratory animals and approved by the Institutional Animal Care Committee. The mice were group housed (prior to initiation of study) in

plastic cages with Sani-chip bedding (P.J. Murphy Forest Products Corp., Montville, N.J.) and kept on a 12-hr light/dark cycle at approximately 22°C. Cotton nesting pads were provided for warmth when animals were individually housed. All cages and water bottles were changed once per week. Only males were used for these studies since female Rett mice have a less severe disease phenotype (Shahbazian et al., 2002a).

Genotyping Rett Mice

DNA from 30-day old Rett mice was isolated from ~3 mm of tail using the Qiagen DNeasy tail tissue protocol. The PCR reaction was set up similar to that of the JAX genotype protocol for the *Mecp2*$^{308/y}$ mice with the following modifications as previously described (Seyfried et al., 2008b). Briefly, 1 µL of DNA (~50-100 ng) was amplified with 5 µL of 5X Buffer, 0.5 µL dNTPs, 5 µL Forward primer (10 mM), 2.5 µL AR Primer (10 mM), 2.5 µL BR primer (10 mM), 0.25 µL GoTaq DNA Polymerase (Promega) and 8.25 µL water for a 25 µL total reaction volume. The DNA PCR amplification protocol used was: 94°C for 2 min, followed by 31 cycles of 94°C for 45 sec; 62˚C for 45 sec; and 72°C for 45 sec, with a final extension at 72°C for 5 min following the last cycle. The forward and AR primer set amplified a 396 bp fragment from the wild-type allele, whereas the forward and BR primer set amplified a 318 bp fragment from the knockout allele.

PCR products (5–15 μL) were separated on 1% agarose gels containing ethidium bromide, visualized with UV light.

Diets

All mice were fed SD prior to experimentation (see Chapters 2 and 3 for additional information on diet composition). Briefly, the SD is a nutrient balanced low fat, high carbohydrate diet that delivers 4.1 kcal/g of gross energy (Mantis et al., 2004), whereas the KetoCal® diet (KC) is a nutrient balanced soy oil-based high fat, low carbohydrate KD diet that delivers 7.2 kcal/g of gross energy and has a ketogenic ratio (fats: proteins + carbohydrates) of 4:1 (Zhou et al., 2007). KC was used in this current study because it is a more palatable form of the KD. The feeding regime for the SD-R and the KC-R mice was previously described (Mantis et al., 2004; Zhou et al., 2007). Briefly both the SD and KC diets are calorie restricted to reduce mouse body weights by 20-23%. Water was provided *ad libitum* to all mice throughout the study. The energetic composition of the SD and the KC diets is shown in Table 3.

Pre-Trial Testing Period for Rett Mice

12 wild-type *Mecp2*$^{+/y}$ (control) and 18 *Mecp2*$^{308/y}$ (Rett) mice (188 days of age) were selected for the study and were individually housed for an 11-day pre-

trial period. Young adult symptomatic male *Mecp2* mice were selected in contrast to female *Mecp2* mice because of the greater degree of RTT phenotypic similarities of the male *Mecp2* mice to RTT children. All mice were fed the SD *ad libitum* during the pre-trial period and the daily food intake of each mouse was determined (Mantis et al., 2004). This pre-trial period was used to establish baseline physiological (metabolism) and behavioral (motor coordination, proprioception, and exploration) parameters for each mouse. The experimental protocol for each behavioral test used is summarized below.

Testing Battery

All behavioral testing was conducted before body weights or food/water intakes were determined for each mouse. Only one behavioral test was performed on a given mouse in a given day. The following behavioral tests sensitive to motor and sensory function were employed: 1) grip strength, 2) incline latency, 3) righting reflex, 4) visual placing, 5) light-dark compartment, 6) rotorod, and 7) open-field.

1) The grip strength test examined defects in motor neurodevelopment related to muscle strength (Meyer et al., 1979). The test was performed in triplicate with 60 sec being the maximum allowable time for mice to grab/hold with their forelimbs and/or hindlimbs onto a wire suspended two

feet above a soft, padded surface. Only the maximum grab/hold time for a mouse to accomplish the task was considered for statistical analysis.

2) The incline latency test (negative geotaxis) was performed to examine proprioceptive neurodevelopment and the ability to sense gravitational forces (Pryor et al., 1983). The test was performed in triplicate with 60 sec being the maximum allowable time for mice to reorient themselves 180^{o} (head facing upward) after being placed head facing downward on a soft, high friction surface with a negative 40^{o} from horizontal slope. Only the maximum time for a mouse to accomplish the task was considered for statistical analysis.

3) The righting latency test was also performed to examine proprioceptive neurodevelopment necessary to restore the body to an upright spatial position (Fox, 1965). The test was performed one time unless a mouse demonstrated a reduced ability to turn over onto its belly (position itself in an upright position - all 4 limbs) after being placed gently on its back atop a flat padded surface. Only the maximum time for a mouse to accomplish the task (60 sec trial) was considered for statistical analysis.

4) The placing latency test examined neurodevelopmental defects in visual proprioception necessary to see and grasp an approaching solid

surface. Mice were lifted gently by the tail, suspended briefly in mid-air, and then lowered slowly towards the edge of a table/mouse cage rack that mice were able to reach by extending their forelimbs. The test was performed one time unless a mouse demonstrated a reduced ability to grab/extend forelimbs towards an edge 2-3 cm away. Only the maximum time for a mouse to accomplish the task (60 sec trial) was considered for statistical analysis.

5) The light-dark latency test examined anxiety and the propensity of a mouse to explore a novel environment (Crawley et al., 1997; Crawley, 1999; Bourin and Hascoet, 2003). The test was performed one time for each mouse. The testing apparatus consisted of two compartments: a dark compartment and a light compartment. The dark compartment, a standard mouse cage covered with a solid box, served as the control environment and the light compartment, an uncovered mouse cage, served as the novel environment. The mouse was initially placed in the dark compartment and was allowed to move freely between the light and dark compartments. The length of time that it took for a mouse to completely enter the lighted compartment, the amount of time that the mouse spent in the lighted compartment, and the total number of times that the mouse entered and exited this compartment were considered for statistical analysis. Each test lasted for 5 min.

6) The rotorod test examined defects in mouse motor neurodevelopment related to coordination and balance (Crawley, 2007). The test was performed in duplicate at four different speeds (20, 30, 40, and 60 rpm) with 60 sec being the maximum allowable time for mice to stay on a rotating bar/rough edge cylinder positioned over mouse bedding. Mice were allowed to rest for 30 sec between trials at the same speed, and for 2 min between trials at different speeds. The average length of time that a mouse remained on the bar for a given speed was considered for statistical analysis.

7) The open field test examined defects in mouse locomotor/exploratory activity, anxiety, and rearing events using the SmartFrame Cage Rack System (Kinder Scientific, San Diego, CA) (Crawley and Paylor, 1997; Paylor et al., 1998). Photobeams along the frame of the system track mouse movement within the cage and register mouse location, distance, and rearing capabilities. A mouse was placed in the center of the open-field apparatus and behavior was measured for 15 min. The data were analyzed using the MotorMonitor software (Kinder Scientific, San Diego, CA). Locomotor activity was measured as the total distance traveled in either the center or in the periphery (in cm), as well as the basic (all horizontal beam breaks) and fine (a change in a single beam while all

other beams remain unchanged – e.g. grooming and/or head activities) movements the mouse performed during the 15-min period. Rearing events were measured as the number of times the mouse stood on its hind legs. Anxiety was measured as the degree of avoidance the mouse showed in exploring the center of the apparatus (number of entries in the center).

Dietary Regimen

On the 11th day of the pre-trial period, the mice were separated into the following diet groups: 1) a wild-type (*Mecp2*$^{+/y}$) mouse group fed the standard diet *ad libitum* or unrestricted (SD-UR), 2) a wild-type mouse group fed the KetoCal® diet restricted (KC-R), 3) a wild-type mouse group fed the standard diet restricted (SD-R), 4) a Rett (*Mecp2*$^{308/y}$) mouse group fed the SD-UR diet, 5) a Rett mouse group fed the KC-R diet, and 6) a Rett mouse group fed the SD-R diet. Mice in each of the wild-type mouse groups were matched for body weight (29.5 ± 1.7 g), as were the mice in each of the Rett mouse groups (30.5 ± 1.5 g). All mice were then fasted for 17 hours before the diets were initiated in order to establish a similar metabolic starting point. The feeding regime for all KC-R and SD-R group mice was designed to reduce mouse body weights by 20-23% compared to each mouse's individual pre-trial body weight as we previously described (Mantis et al., 2004; Zhou et al., 2007). The recommended body weight reduction was

achieved and maintained during the dietary treatment period by adjusting the food intake of the R-fed mice every three days. Mice in the SD-UR groups were provided with ~200 g of fresh SD food pellets on a weekly basis. The body weights and food intakes of all mice were measured every three days. No KC-UR groups were included in these studies because this particular feeding regimen was not found to be neuroprotective in mouse models of brain cancer (Zhou et al., 2007), nor was the KD-UR found as effective as the KD-R in reducing seizure frequency in a mouse model of epilepsy (Mantis et al., 2004). At the end of the dietary treatment period the same battery of behavioral tests was performed for each mouse to evaluate the effect of the diet on the behavior of these mice.

Statistical Analysis

Both ANOVA and a two-tailed *t* test were used to evaluate the significance of differences of body weight, and each behavioral parameter between unrestricted and restricted groups (SPSS software). Differences were considered significant at $P \leq 0.05$. The three-way mixed factor ANOVA statistical analysis was also performed to verify any significant effect between the diet, the mice, and the performance of the mice on the various rotorod speeds and the open-field. All values are expressed as mean ± SEM. All statistical data were

presented according to the recommendations of Lang et al. (Lang and Secic, 1997).

RESULTS

Influence of Diet on Behavior

All mice were tested prior to the initiation of the dietary treatment period (see Methods) in order to establish baseline information pertaining to their behavioral features. At the end of the one-month dietary treatment period, all mice from each of the six groups were subjected to the same battery of behavioral tests to evaluate the effects of the calorically restricted KC diet or the restricted SD on their behavior. Consistent with the well-recognized health benefits of mild to moderate calorie restriction in rodents, no adverse effects were observed in either mouse group fed a calorically restricted diet. Despite a 20–23% body weight reduction, all R-fed mice appeared healthy and more active than mice in the groups fed *ad libitum*, as assessed by ambulatory and grooming behavior. Furthermore, nesting behavior was similar for all dietary groups (empirical observation). It is important to mention that no epileptic seizures were observed throughout this study in the Rett mice.

Influence of Diet on Body Weight and Food Intake

All Rett (*Mecp2*$^{308/y}$) and wild-type (*Mecp2*$^{+/y}$) mouse groups were matched for age (~ 199 days) and body weight at the beginning of the dietary treatment period. The average daily food intakes for the wild-type and Rett groups over the pre-trial period were 4.2 and 4.6 g, respectively. All mice lost approximately 8-13% body weight over the course of the 17-hour fast at the beginning of the treatment period. Mice in both the KC-R and the SD-R groups achieved the desired 20-23% reduction in body weight within 2-3 weeks of the initiation of dietary treatment (Figure 14). The degree of CR needed to maintain the 20-23% body weight reduction was approximately 50%. No significant differences in body weight were observed between the wild-type (29.3 g) and the Rett (30.0 g) SD-UR mouse groups (Figure 14).

Influence of Diet on Grip Strength

The grip strength test was used to distinguish motor neurodevelopment deficits between the Rett mice and the wild-type control mice. The suspension time was significantly less in the Rett SD-UR mice (16.7 sec) than in the control SD-UR mice (47.5 sec) (Figure 15). Restriction of the KC diet or the SD did not improve the performance of the Rett mice on the wire (21.9 and 23.7 sec, respectively) compared to the Rett mice fed SD-UR (Figure 15). Moreover, CR

of either diet had no effect in improving performance of wild-type mice on the grip strength test. These findings indicate that the Rett mice have a motor deficit and that restriction of either the KC or the SD diet does not improve this Rett phenotype.

Influence of Diet on Incline Latency

The incline latency test measured the ability of a mouse to orient (face upward) itself against gravitational forces when placed facing downward on a negative 40^{o} slope. No significant differences in the incline latency to face upwards were observed between the wild-type (40.2 sec) and the Rett (41.4 sec) SD-UR mouse groups (Figure 16). The incline latency of both KC-R mouse groups was significantly reduced relative to their respective SD-UR groups (Figure 16). In addition, the latency of the wild-type SD-R mouse group was significantly reduced compared to the Rett SD-UR mouse group. Although, CR of the SD had no significant effect (P = 0.058) in improving the incline latency in the Rett mice (Figure 16), a definite trend of improved behavior is evident in these mice as well. These findings suggest that restriction of either the KC or the SD diet improves overall reorientation (face upwards) to negative geotaxis.

Influence of Diet on Righting Reflex

The righting reflex test was used to measure each mouse's proprioception and reflex response to revert back on its four limbs after being placed on its back. No differences were found between the Rett and wild-type mice (all mice performed the task within 0-2 sec). In addition, restriction of KC or the SD had no effect on this behavior (data not shown).

Influence of Diet on Visual Placing

The visual placing test was used to measure the visual proprioception of the Rett mice. No differences were found between the Rett and wild-type mice (all mice performed the task within 0-2 sec). In addition, restriction of KC or the SD had no effect on this behavior (data not shown).

Influence of Diet on the Light-Dark Compartment Test

The light-dark compartment test was used to measure the ability or tendency of the mice to explore a novel environment. Rett mice significantly underperformed in their ability to emerge from the dark or stay in the lighted compartment (Figures 17 and 18). The emergence from dark to the light of the wild-type SD-UR mouse group (98.0 sec) was significantly earlier than in the Rett SD-UR mouse group (289.6 sec) (Figure 17). In addition, the time spent in the light was significantly longer in the wild-type SD-UR mice (64.2 sec) than in the

Rett SD-UR mice (2.6 sec) (Figure 18). Restriction of either KC or SD significantly improved the emergence time into the light and the total time in the light of the Rett mice compared to their respective Rett SD-UR mice (Figures 17 and 18). Furthermore, CR of the SD increased the total time the wild-type mice spent in the light compared to their respective wild-type SD-UR mice (Figure 18). The transition frequency between the two compartments was significantly greater in the wild-type SD-UR mice than in the Rett mice fed the SD-UR (Table 6). Restriction of the SD significantly increased the transition frequency between the light and dark compartments for both the Rett and the wild-type mice (Table 6). It is important to mention that although the restricted KC diet moderately increased the number of transitions between the two compartments in both the wild-type and Rett mouse groups, these differences were statistically significant only for the Rett mice (Table 6). Overall, these findings suggest that the Rett mice have an exploratory deficit and that restriction of either the KC or the SD diet reduces that deficit by increasing the activity of the Rett mice compared to Rett mice fed the SD unrestricted.

Influence of Diet on Rotorod Performance

The rotorod was used to measure motor development, coordination, and balance in the Rett mice. At the three lower speeds (20, 30, and 40 rpm), the performance in the Rett SD-UR mice was significantly worse than in the wild-type

SD-UR mice (Table 7). Restriction of KC did not increase the time the mice spent on the bar (Table 7). On the other hand, restriction of the SD significantly increased the time that the Rett mice spent on the bar compared to the Rett SD-UR mice (Table 7). No significant differences were found between the four groups in the rotorod performance at 60 rpm. The three-way mixed ANOVA test further verified that the Rett SD-UR mice significantly unperformed on the rotorod compared to the wild-type SD-UR mice; however, the restricted KC diet had no significant effect on the rotorod performance. These findings indicate that the Rett mice have a motor coordination/balance deficit. Although, the restricted KC diet had no effect on improving this deficit, the restricted SD was able to do improve the time the Rett mice spent on the bar.

Influence of Diet on Open-Field Performance

The open-field test was performed to measure motor defects in locomotor activity and rearing events in the Rett mice during a 15 min testing period. No significant differences were observed between the Rett SD-UR mice and the wild-type SD-UR mice in all the behavioral parameters we measured (Table 8). Total time and rest time in each zone (center and periphery) was also similar between Rett and the wild-type mice (data not shown). The restricted KC diet significantly increased the number of entries in the center and the periphery of the open-field apparatus as well as the number of rearing events in the Rett mice

compared to the Rett mice fed the SD-UR (Table 8). Furthermore, the restricted KC diet significantly increased all behavioral parameters measured in the wild-type mice compared to the wild-type mice fed the SD-UR (Table 8). Restriction of the SD also improved both the number of entries in the center and in the periphery of the open-field apparatus as well as the number of rearing events of the wild-type mice compared to the wild-type mice fed the SD-UR (Table 8). Although the average total distance traveled in the center of the open-field apparatus by the wild-type SD-R mice was greater than that traveled by the wild-type SD-UR mice, this difference did not reach a statistical significance (P = 0.058) (Table 8). Furthermore, although restriction of either diet significantly increased the basic movement in all R-fed groups compared to respective UR-fed mice, fine movement was not significant different (data not shown). These findings indicate that the locomotor activity is similar in normal and Rett mice and that restriction of either the KC or the SD diet increases the locomotor activity in mice.

DISCUSSION

Therapeutic diets, such as the KD, have been shown to have a wide range of neuroprotective effects (e.g. antiepileptic, anticonvulsant, antitumorigenic) in both humans and in rodent disease models (Seyfried et al., 2003; Mantis et al.,

2004; Veech, 2004; Gasior et al., 2006; Freeman et al., 2007; Hartman et al., 2007; Prins, 2008), as well as improve the behavior of some girls with RTT (Haas et al., 1986; Liebhaber et al., 2003). The KD can also positively influence the behavior of autistic children (Evangeliou et al., 2003), and can produce metabolic alterations in the brain and in the body that enhance energy expenditure and ultimately reduce body weight (Kennedy et al., 2007). A reduction of circulating glucose levels coupled with an elevation of circulating ketone body levels is thought to underlie the therapeutic effects of the KD (Greene et al., 2003; Mantis et al., 2004; Zhou et al., 2007). These neuroprotective effects of the KD suggest that a restricted KD could improve behavioral abnormalities and motor dysfunction in mouse models of RTT (Haas et al., 1986; Evangeliou et al., 2003; Liebhaber et al., 2003; Mantis et al., 2004; Seyfried et al., 2004). Our current findings support our prior evidence that the neuroprotective effects of either the KD or that of CR stem primarily from a reduction in total calorie intake rather than caloric origin (e.g. from carbohydrates, protein, or fats) (Mantis et al., 2004; Denny et al., 2006). Administration of the KD in restricted amounts also reduces the adverse effects of the diet's high fat content (e.g. weight gain, hypercholesterolemia, diabetes, kidney stones, and cardiovascular disease) if the diet were to be administered *ad libitum* for extended periods of time (Kang et al., 2004; Sampath et al., 2007; Marsh et al., 2008a). Hence, we considered a restricted KD to be more therapeutic for the management of behavioral

abnormalities in RTT mice than the unrestricted KD. This latter observation further supports our rational for omitting an unrestricted KetoCal® mouse group.

In agreement with the findings previously reported by Shahbazian et al., our results show that adult Rett (*Mecp2*$^{308/y}$) mice are deficient in their ability to hang on a suspended wire or perform adequately on the rotorod motor test compared to control wild-type (*Mecp2*$^{+/y}$) mice (Shahbazian et al., 2002a). In contrast, the performance of the Rett mice on the open-field test was not significantly different from that of the wild-type mice. Although this particular finding is not consistent with that previously observed (Shahbazian et al., 2002a), it is important to mention that the length of the testing period between the two open-field tests in the two studies was different, and could hence explain this discrepancy in our findings. More specifically, our test was performed for a total of 15 min, whereas Shahbazian et al. performed the test at 10 min intervals for a total of 30 min. In the aforementioned study, the performance of the Rett mice was not significantly different from the wild-type mice after 10 min, but differences were observed at the 20 and 30 min time points (Shahbazian et al., 2002a). Consistent with prior evidence that the *Mecp2*$^{308/y}$ mice display interaction deficiencies (Shahbazian et al., 2002a; Moretti et al., 2005), our findings from the light-dark latency test also suggest that *Mecp2*$^{308/y}$ mice express deficits in the exploration of a novel environment. The failure of the Rett mice to explore novel environments may reflect a heightened level of anxiety (Paylor et al., 1998). It is important to mention that the performance of the Rett SD-UR

mice on the light-dark compartment test is consistent with previous findings in rodents (Li and Quock, 2001, 2002; Blanco et al., 2009).

Previous studies showed that both CR and the KD increase the activity and exploratory behavior of rodents (Ziegler et al., 2005; Faulks et al., 2006). Our current findings in the Rett mice support these observations and suggest that CR underlies the mechanism of the increased activity observed in mice fed either the restricted KC or SD diets. Interestingly, we observed that calorie restricted Rett mice exhibited not only an increased ability or tendency to explore a novel environment (i.e. the light-dark paradigm test) but also an increased number of entries in the center of the open-field apparatus (i.e. the open-field test) as compared with Rett mice fed a SD-UR. These findings are consistent with prior evidence in rodents that CR increases the number of entries, and the total amount of time spent, in the center of the open-field apparatus (Geng et al., 2007; Levay et al., 2007). It is important to mention that both the light-dark test (the emergence time to the light and the total time spent in the lit environment) and the open-field test (the entry into the center of the open-field apparatus) are measures of anxiety (Paylor et al., 1998). Hence, these data suggest that restriction of either the KC, or the SD could reduce anxiety associated with the RTT phenotype. Furthermore, CR of either diet enhanced the performance of Rett mice on both the incline latency test (proprioception) as well as on the number of rearing events on open-field test (motor function) relative to Rett mice fed the SD-UR.

Although seizure susceptibility was assessed using our established handling induced seizure susceptibility protocol for the epileptic EL mice (Greene et al., 2001; Mantis et al., 2004); no myoclonic episodes were observed in the *Mecp2*$^{308/y}$ mice that we studied. Consequently, we are unable to determine if the restricted KD could reduce seizure susceptibility in Rett mice as was shown previously in girls with RTT (Haas et al., 1986; Liebhaber et al., 2003). As an aside, although nesting is a measure of home-cage activity related to both social behavior and motor function (Moretti et al., 2005), it is important to point out that neither the restricted KC diet nor the restricted SD was able to improve nesting behavior in the Rett mice (empirical observation). In general, R-fed mice spent significantly less time interacting with their nesting material compared to mice fed an unrestricted diet due to a persistent search for food. Thus, our data, viewed together, suggest the possibility that the increased activity observed in the Rett mice may be associated with increased hunger resulting from CR. Nevertheless, the restriction of either the KD or SD diet can be of clinical importance since the diet improved symptoms of certain behavioral abnormalities in Rett mice, particularly those with respect to reduced anxiety involving exploratory activity within a novel environment and the number of entries in the center of the open-field apparatus.

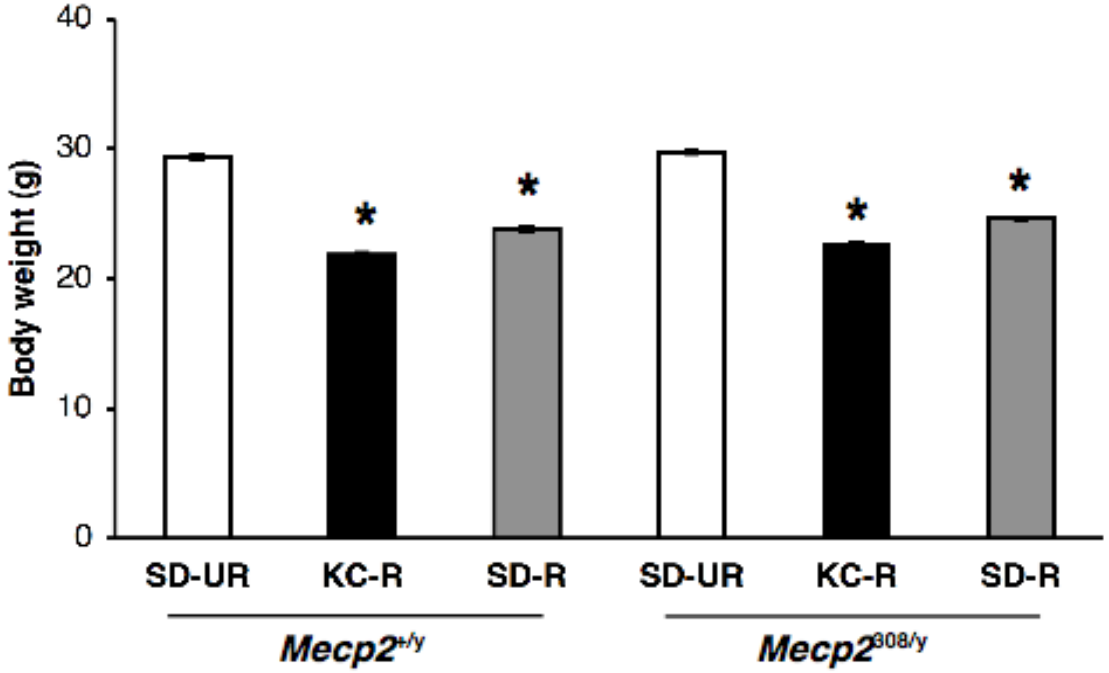
It seems likely that the beneficial effects of the KD in human patients with RTT are similar to those observed in Rett mice in the present study. Because girls with RTT are withdrawn emotionally and hesitant, and because the

restricted KD appeared to reduce some of the cautious tendencies in Rett mice, our results suggest that a restricted KD could be administered to help mitigate anxiety or fearfulness in females with RTT. Our results also suggest that the KD cannot, by itself, correct neurodevelopmental deficits associated with the RTT phenotype, but that it may help to confer emotional stability in RTT patients, thus leading to reduced anxiety, and increased activity and exploratory behavior. Furthermore, it is important to mention that since girls with Rett are smaller and have reduced body weights (Thommessen et al., 1992; Oddy et al., 2007); CR of any diet should be implemented with caution under a careful clinical supervision (Bhagavan, 2002; Crowe, 2005; Dirks and Leeuwenburgh, 2006).

Interestingly, supplementation of dietary choline increased N-acetyl aspartate levels; a marker of neuronal integrity, in young Mecp2-null mice (*Mecp2*^{1lox}) further suggests that nutritional supplementation may be therapeutic in improving neuronal function in girls with RTT (Nag and Berger-Sweeney, 2007; Ward et al., 2009). Finally, it is important to mention that alternative dietary therapies to the KD or CR, such as the Atkins diet, the Low-Glycemic-Index diet, or even a diet enriched with Omega-3 fatty acids, could also have a positive influence in the behavior of girls with Rett. Previous studies showed that both the Atkins and the Low-Glycemic-Index diet have similar effects to the KD and CR in reducing seizures in human patients with epilepsy (Pfeifer and Thiele, 2005; Kossoff et al., 2007), so it would be of interest to know whether those diets show similar results to what we found with the Rett mice.

In conclusion, our results demonstrate that CR of either the ketogenic diet or the standard diet could improve behavioral abnormalities in a mouse model of RTT, particularly by reducing anxiety associated with the exploration of an unfamiliar environment. Considering that we used adult Rett mice, which displayed the full spectrum of symptoms associated with RTT, it would be interesting to determine whether a restricted KD or SD diet could help to delay the onset or, at least, reduce the severity of motor and exploratory neurodevelopment deficits in juvenile Rett mice that have not yet become fully symptomatic.

Figure 14. Influence of Diet on Body Weight in *Mecp2*$^{+/y}$ and *Mecp2*$^{308/y}$ Mice. Asterisks indicate that the body weight of the R group mice was significantly lower ($P < 0.001$) than their respective SD-UR groups; n = 4 for all *Mecp2*$^{+/y}$ mouse groups, and for the *Mecp2*$^{308/y}$ SD-R group, whereas n = 7 for both *Mecp2*$^{308/y}$ (Rett) SD-UR and KC-R mouse groups.


40
30
20
10
0
Body weight (g)
*
*
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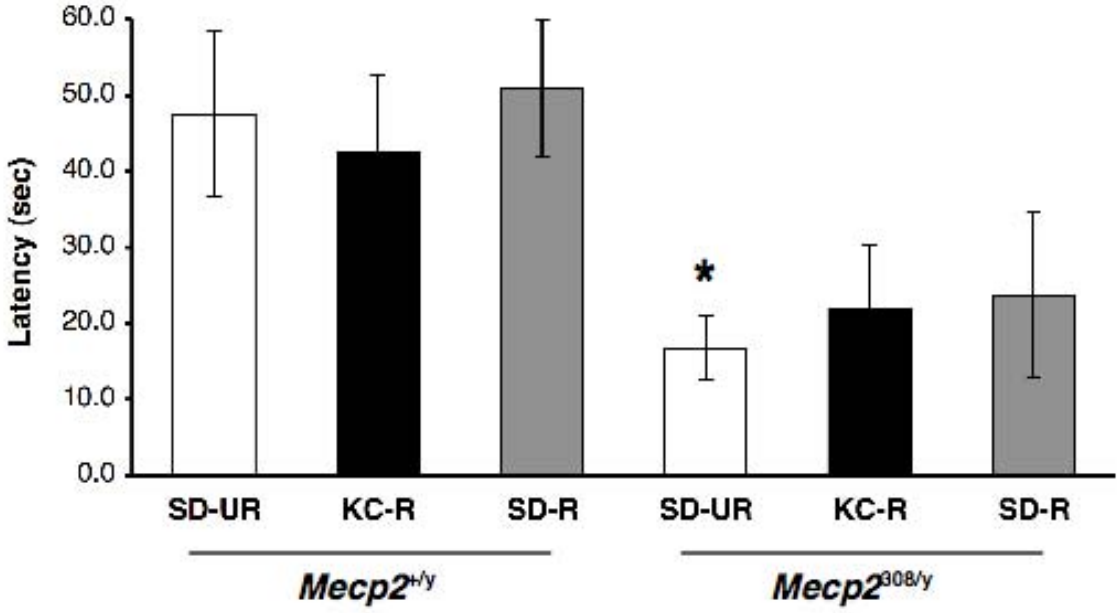
*
SD-UR
KC-R
SD-R
SD-UR
KC-R
SD-R
Mecp2+/y
Mecp2308/y

Figure 15. Influence of Diet on Wire Suspension Latency in *Mecp2*$^{+/y}$ and *Mecp2*$^{308/y}$ Mice. Asterisks indicate that the grip strength of the Rett mouse groups was significantly lower ($P < 0.05$) than the wild-type SD-UR group. Diet had no effect on improving the ability of the Rett mice to suspend from the wire. Other conditions are similar as those shown in Figure 14.


60.0
50.0
40.0
30.0
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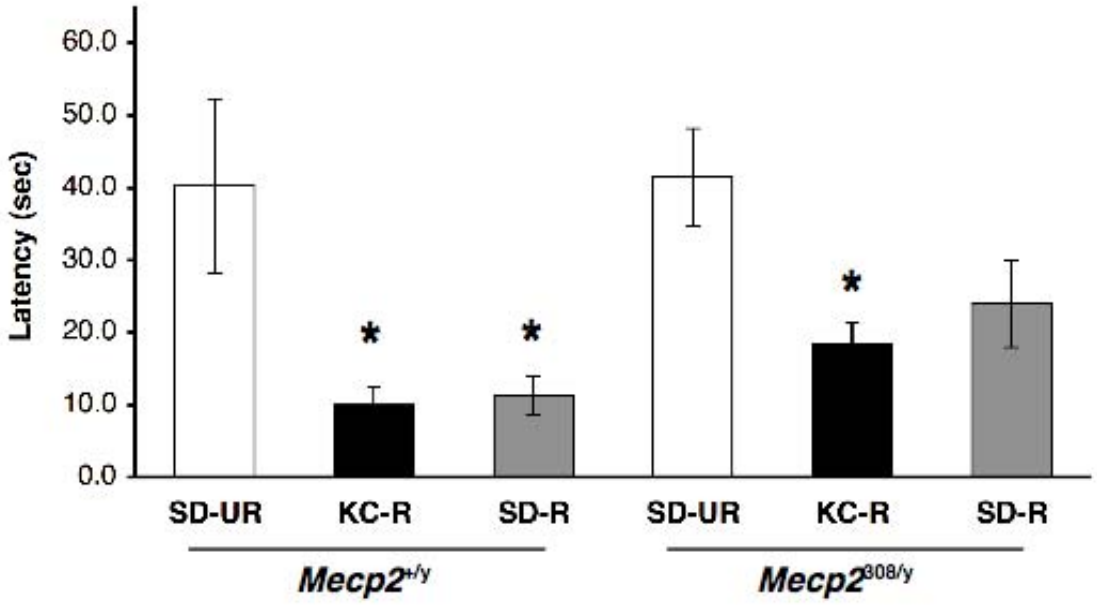
Latency (sec)
*
SD-UR
KC-R
SD-R
SD-UR
KC-R
SD-R
Mecp2+/y
Mecp2308/y

Figure 16. Influence of Diet on Incline Latency (Negative Geotaxis) in *Mecp2*$^{+/y}$ and *Mecp2*$^{308/y}$ Mice. Asterisk indicates that the performance of the mice in both KC-R mouse groups and in the *Mecp2*$^{+/y}$ SD-R was significantly improved in orientating themselves against negative geotaxis ($P < 0.01$) compared to the mice in their respective SD-UR groups. The average incline latency by the *Mecp2*$^{+/y}$ SD-R mice was greater than that of the *Mecp2*$^{+/y}$ SD-UR mice, but this difference did not reach a statistical significance ($P = 0.058$). Other conditions are similar as those shown in Figure 14.


Latency (sec)
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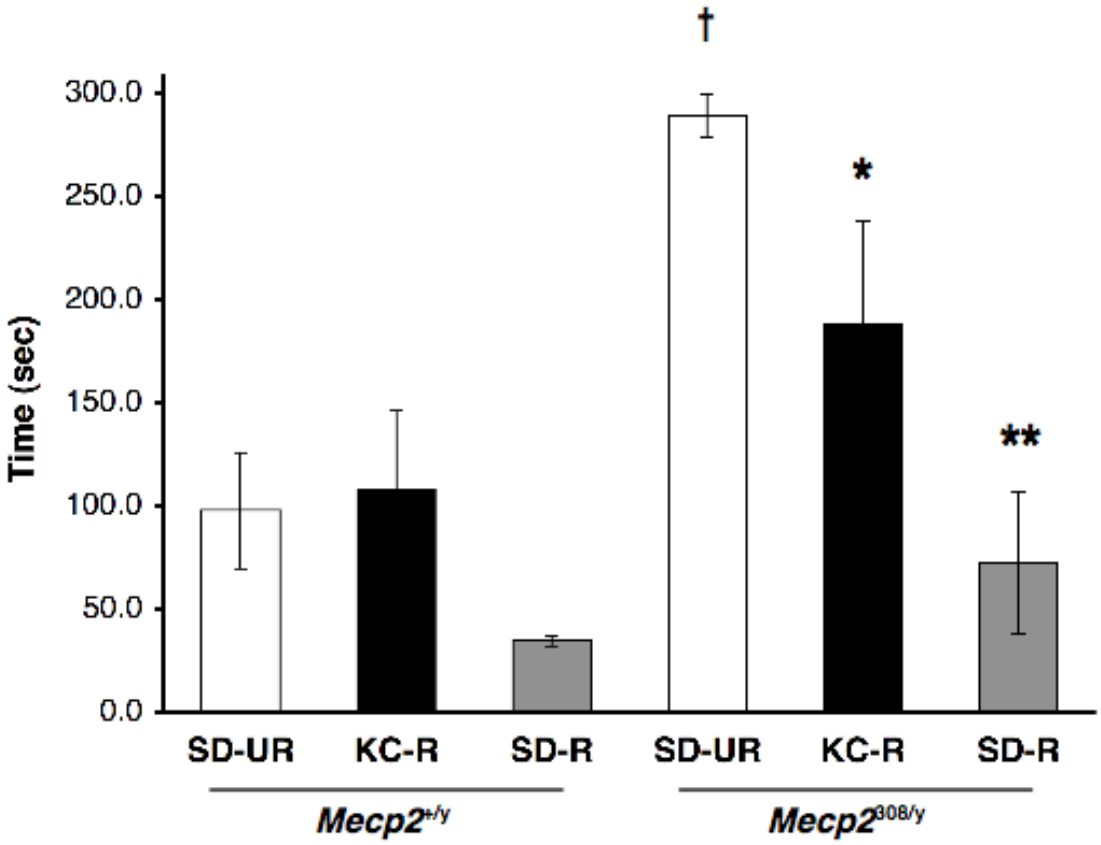
SD-UR
KC-R
SD-R
SD-UR
KC-R
SD-R
Mecp2+/y
Mecp2308/y

Figure 17. Influence of Diet on Dark to Light Emergence in *Mecp2*$^{+/y}$ and *Mecp2*$^{308/y}$ Mice. Cross indicates that the *Mecp2*$^{308/y}$ SD-UR mice emerged significantly later into the light compared to the *Mecp2*$^{+/y}$ control SD-UR (P < 0.01). Asterisk indicates that the *Mecp2*$^{308/y}$ KC-R mice emerged to the light significantly earlier than the *Mecp2*$^{308/y}$ SD-UR (P < 0.05). Double asterisk indicates that the *Mecp2*$^{308/y}$ SD-R mice emerged to the light significantly earlier than the *Mecp2*$^{308/y}$ SD-UR (P < 0.01). Other conditions are similar as those shown in Figure 14.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300.0
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Time (sec)
SD-UR
KC-R
SD-R
SD-UR
KC-R
SD-R
Mecp2+/y
Mecp2308/y

Figure 18. Influence of Diet on Total Time in the Light in *Mecp2*$^{+/y}$ and *Mecp2*$^{308/y}$ Mice. Cross indicates that the *Mecp2*$^{308/y}$ SD-UR mice spent significantly less time in the light compared to the *Mecp2*$^{+/y}$ control SD-UR ($P < 0.05$). Asterisk indicates that the *Mecp2*$^{308/y}$ KC-R mice spent significantly more time in the light than the *Mecp2*$^{308/y}$ SD-UR ($P < 0.05$). Double asterisk indicate that the *Mecp2*$^{308/y}$ SD-R mice spent significantly more time in the light than the *Mecp2*$^{308/y}$ SD-UR ($P < 0.01$). Double cross indicates that the *Mecp2*$^{+/y}$ SD-R mice spent significantly more time in the light than the *Mecp2*$^{+/y}$ SD-UR ($P < 0.05$). Other conditions are similar as those shown in Figure 14.

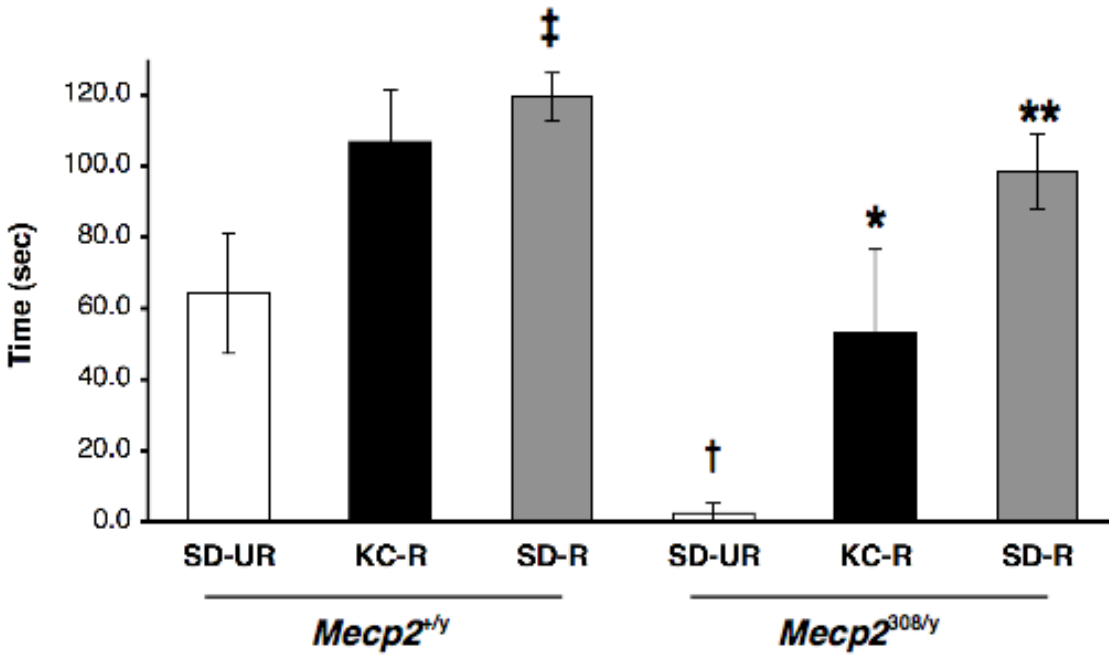

‡
120.0
100.0
80.0
60.0
40.0
20.0
0.0
Time (sec)
**
*
†
SD-UR
KC-R
SD-R
SD-UR
KC-R
SD-R
Mecp2+/y
Mecp2308/y

Table 6: Mean light-dark compartment transitions[1]

Group	Light-dark transitions
$Mecp2^{+/y}$ SD-UR	4.50 ± 1.32
$Mecp2^{+/y}$ KC-R	6.00 ± 1.73
$Mecp2^{+/y}$ SD-R	10.25 ± 1.18[a]
$Mecp2^{308/y}$ SD-UR	0.29 ± 0.29[b]
$Mecp2^{308/y}$ KC-R	3.71 ± 1.67[c]
$Mecp2^{308/y}$ SD-R	5.25 ± 1.65[d]

[1] Data are expressed as Means ± SEM for all six groups of mice.

[a] $Mecp2^{+/y}$ SD-R significantly different from $Mecp2^{+/y}$ SD-UR group at $P < 0.05$ (as determined from the Anova analysis).

[b] $Mecp2^{308/y}$ SD-UR significantly different from control $Mecp2^{+/y}$ SD-UR group at $P < 0.05$ (as determined from the Anova analysis).

[c] $Mecp2^{308/y}$ KC-R significantly different from $Mecp2^{308/y}$ SD-UR group at $P < 0.05$ (as determined from the Anova analysis).

[d] $Mecp2^{308/y}$ SD-R significantly different from $Mecp2^{308/y}$ SD-UR group at $P < 0.05$ (as determined from the Anova analysis).

Table 7: Mean walking time on the bar[1]

Group	Time on bar (20 rpm)	Time on bar (30 rpm)	Time on bar (40 rpm)
$Mecp2^{+/y}$ SD-UR	41.38 ± 10.52	52.50 ± 3.97	44.88 ± 7.56
$Mecp2^{+/y}$ KC-R	52.25 ± 5.42	60.00 ± 0.00	59.63 ± 0.38
$Mecp2^{+/y}$ SD-R	59.62 ± 0.37	59.62 ± 0.37	57.37 ± 2.62
$Mecp2^{308/y}$ SD-UR	18.14 ± 3.58[a]	27.64 ± 7.18[a]	19.14 ± 3.61[b]
$Mecp2^{308/y}$ KC-R	27.50 ± 9.05	38.36 ± 8.44	30.07 ± 6.06
$Mecp2^{308/y}$ SD-R	43.00 ± 11.68[c]	53.75 ± 6.25[c]	24.5 ± 7.64

[1] Data are expressed as Means ± SEM for all six groups of mice.

[a] $Mecp2^{308/y}$ significantly different from control $Mecp2^{+/y}$ SD-UR group at $P < 0.05$ (as determined from the Anova analysis).

[b] $Mecp2^{308/y}$ significantly different from control $Mecp2^{+/y}$ SD-UR group at $P < 0.01$ (as determined from the Anova analysis).

[c] $Mecp2^{308/y}$ SD-R significantly different from $Mecp2^{308/y}$ SD-UR group at $P < 0.05$ (as determined from the Anova analysis).

Table 8: Performance of Mecp2 mice in the open-field task[1]

Group	Total distance in periphery (cm)	Total distance in center (cm)	Number of entries in periphery	Number of entries in center	Rearing events
$Mecp2^{+/y}$ SD-UR	859.15 ± 86.80	410.21 ± 61.43	36.00 ± 3.70	35.25 ± 3.70	68.00 ± 10.45
$Mecp2^{+/y}$ KC-R	1225.55 ± 96.17[a]	737.23 ± 149.31[b]	74.25 ± 8.57[a]	73.75 ± 8.82[a]	159.75 ± 23.88[a]
$Mecp2^{+/y}$ SD-R	954.40 ± 120.56	646.43 ± 65.57[c]	64.50 ± 11.03[a]	64.00 ± 11.30[a]	128.50 ± 16.76[a]
$Mecp2^{308/y}$ SD-UR	812.80 ± 64.02	402.77 ± 81.46	36.29 ± 5.76	35.71 ± 5.84	58.86 ± 10.72
$Mecp2^{308/y}$ KC-R	953.95 ± 45.16	528.32 ± 28.93	54.29 ± 2.56[d]	53.71 ± 2.51[d]	93.71 ± 11.66[d]
$Mecp2^{308/y}$ SD-R	835.66 ± 51.22	725.17 ± 27.79[e]	63.25 ± 3.33[e]	62.75 ± 2.78[e]	55.25 ± 12.00

[1] Data are expressed as Means ± SEM for all six groups of mice (N = 4 - 7 mice per group).

[a] $Mecp2^{+/y}$ KC-R or $Mecp2^{+/y}$ SD-R group mice are significantly different from control $Mecp2^{+/y}$ SD-UR group mice at $P < 0.01$ (as determined from the Anova analysis).

[b] $Mecp2^{+/y}$ KC-R group mice are significantly different from control $Mecp2^{+/y}$ SD-UR group mice at $P < 0.05$ (as determined from the Anova analysis).

[c] $Mecp2^{+/y}$ SD-R group mice are approaching significance level compared to control $Mecp2^{+/y}$ SD-UR group mice at $P = 0.058$ (as determined from the Anova analysis).

[d] $Mecp2^{308/y}$ KC-R group mice are significantly different from $Mecp2^{308/y}$ SD-UR group mice at $P < 0.05$ (as determined from the Anova analysis).

[e] $Mecp2^{308/y}$ SD-R group mice are significantly different from $Mecp2^{308/y}$ SD-UR group mice at $P < 0.01$ (as determined from the Anova analysis).

CHAPTER FIVE

Implementation of Calorically Restricted Diets for the Management of Neurological and Neurodegenerative Disease in Murine Models

INTRODUCTION

Influence of Diet Therapies on Disease Phenotype

Diet therapies, especially calorie restriction (CR) and the ketogenic diet (KD), possess therapeutic potential in clinical settings and also delay or reduce symptoms associated with a range of age-associated pathologies in laboratory rodents (Tables 9 and 10). CR is a natural dietary therapy that has long been recognized to improve health, promote longevity, and to reduce the incidence and symptoms of a broad spectrum of neurological and neurodegenerative diseases (Tables 9 and 10). Those diseases include epilepsy (Azarbar et al., ; Bough et al., 1999b; Greene et al., 2001; Mantis et al., 2004; Seyfried et al., 2009b), aging (Weindruch and Walford, 1988; Forster et al., 2003; Smith et al., 2004; Everitt and Le Couteur, 2007; Barzilai and Bartke, 2009; Sohal et al., 2009), Sandoff's disease (Denny et al., 2006), Alzheimer's disease (Qin et al., 2006; Halagappa et al., 2007; Qin et al., 2008), Parkinson's disease (Maswood et al., 2004), Huntington's disease (Duan et al., 2003), ALS (Hamadeh et al., 2005),

neuronal damage (Anson et al., 2003), and brain cancer (Seyfried et al., 2003 ; Mukherjee et al., 2004; Zhou et al., 2007; Marsh et al., 2008b). CR also reduces some of the symptoms associated with diabetes (Kelley et al., 1993; Pedersen et al., 1999; Minamiyama et al., 2007 ; Ugochukwu and Figgers, 2007; Jazet et al., 2008), cardiovascular diseases (Ahmet et al., 2005; Mager et al., 2006; Fontana, 2008; Hammer et al., 2008), hypertension (Zimmerman and Wylie-Rosett, 2003; Fontana, 2009), other types of cancer (Mukherjee et al., 1999; Jiang et al., 2008; Bonorden et al., 2009; Mavropoulos et al., 2009), and non-neurological diseases (Fenton et al., 2009; Fontana, 2009). While the exact neuroprotective mechanism(s) of CR are not completely understood, we suggest that the protection conferred by the diet in the majority of disorders is associated (i) with a reduction in circulating glucose levels and (ii), from a concomitant and indeed compensatory elevation of ketone body levels (Tables 9 and 10) (Mantis et al., 2004; Seyfried et al., 2009b). This transition could be thought as a “normal” physiological cerebral/somatic adaptation to the implementation of CR. It has been postulated, that the therapeutic effects of CR stem from caloric restriction *per se* and not from the pre-defined restriction of any specific dietary component such as proteins, vitamins, mineral, fats, and/or carbohydrates (Tannenbaum, 1959; Seyfried et al., 2003; Mantis et al., 2004; Barzilai and Bartke, 2009; Seyfried et al., 2009a). Although CR in mice mimics therapeutic fast in humans (Mahoney et al., 2006), it is important to emphasize that CR differs from acute fasting or starvation in that CR reduces total caloric intake without producing

anorexia or malnutrition (Tannenbaum, 1959; Cahill, 1970; Weindruch and Walford, 1988; Mantis et al., 2004; Seyfried et al., 2009a).

Historically, the high fat, low carbohydrate ketogenic diet (KD) has been reported to produce antiepileptic, anticonvulsant, and other neuroprotective effects similarly to that seen in CR in both human and animal models of seizures/epilepsy (Table 9) (Appleton and De Vivo, 1973; Todorova et al., 2000; Greene et al., 2003; Mantis et al., 2004; Nylen et al., 2005; Yamada et al., 2005; Freeman et al., 2007; Hartman et al., 2007; Bough, 2008; Samala et al., 2008; Fenoglio-Simeone et al., 2009). Hence, the KD has garnered increasing interest as a novel alternative therapy for a variety of neurological disorders (Table 10). Those include Rett Syndrome (Liebhaber et al., 2003; Mantis et al., 2009), Alzheimer's disease (Van der Auwera et al., 2005; Gasior et al., 2006), Parkinson's disease (Maswood et al., 2004), brain cancer (Zhou et al., 2007; Marsh et al., 2008a), and Amyotrophic Lateral Sclerosis (Zhao et al., 2006; Mattson et al., 2007). In addition, CR has been reported to reduce the acute loss of neural parenchyma during ischemic and/or hemorrhagic stroke, as well as following traumatic brain injury or insulin-induced hypoglycemia (Table 10) (Yamada et al., 2005; Gasior et al., 2006; Prins, 2008; Puchowicz et al., 2008). This neuroprotective effect of CR most likely stems from the ability of the brain to utilize ketones during low glycolytic conditions (Vannucci and Simpson, 2003; Pierre and Pellerin, 2005). Furthermore, the KD has been shown to positively influence the behavior of autistic children (Evangeliou et al., 2003), as well as

produce cerebral and somatic metabolic alterations that enhance energy expenditure and ultimately reduce body weight (Kennedy et al., 2007). This latter phenomenon serves as the marketing platform of the high fat, low carbohydrate Atkins diet as a weight loss paradigm (Kossoff et al., 2007; Wylie-Rosett and Davis, 2009). As illustrated in Figure 22, the therapeutic efficacy of the KD is best when the diet is administered in restricted amounts (Mantis et al., 2004; Freeman et al., 2007; Seyfried et al., 2009b). Although the role glucose and ketone bodies (β-hydroxybutyrate [β-OHB] and acetoacetate) in potentiating the protective effects of the KD in various disease processes remains unclear, it is our contention that alterations in brain/parenchyma cell energy metabolism are likely involved, as seen with CR (Tables 9 and 10) (Mantis et al., 2004; Marsh et al., 2008a; Maalouf et al., 2009; Seyfried et al., 2009b).

ISSUES WITH DIET IMPLEMENTATION

One of the most promising advances in delineating the mechanism(s) by which CR extends survival and regresses multiple disease processes was the observation that the physiological adaptations incurred by the onset of CR to improve health are evolutionary conserved (Klass, 1977; Weindruch and Walford, 1988; Jiang et al., 2000; Bross et al., 2005; Goldberg et al., 2009). Organisms have evolved to sense and adapt to environmental cues for their survival when

compromising conditions such as food depletion or various forms of energy stress are present (Vaquero and Reinberg, 2009). The sirtuin family of proteins has been implicated as such a potential evolutionarily conserved mechanism for life span regulation and aging retardation (Blander and Guarente, 2004; Guarente and Picard, 2005; Bishop and Guarente, 2007; Feige et al., 2008b; Hipkiss, 2008). Specifically in mammals, SIRT1 deacetylates many key transcription factors and co-factors, thereby affecting crucial cellular pathways involved in stress resistance and metabolism (Westphal et al., 2007).

Nevertheless, previous studies in rodents have shown that the effects of CR on extending lifespan are strongly correlated and dose dependent to the degree of caloric intake reduction (Weindruch et al., 1986; Merry, 2002). Furthermore, in drosophila D. *melanogaster* the neuroprotective effects of CR are very rapidly, given the short lifespan of the organism, suggesting that the measurements of the rate of age-specific mortality may require large numbers of animals (Mair et al., 2005). Although comparisons of a variety of age/disease-related changes have been made between energy-restricted and unrestricted rodents in an effort to identify the underlying physiological/biochemical neuroprotective process(es) of those diets many studies fail to provide a standardized regimen for diet implementation. Thus, the complexity of the physiology involved in determining the beneficial effects of CR as well as technical issues in experimental design, the therapeutic mechanism(s) of CR remain still elusive (Masoro, 2009). As briefly indicated in Tables 9 and 10, in the

literature there is considerable evidence that the health beneficiary effects of CR or the KD are not consistent between various rodent models, thus resulting in discrepancies in data interpretation regarding the neuroprotective effects of those diets. Therefore, the goal of this review is address issues of diet implementation in disease management and to provide proper implementation guidelines that may ultimately aid in the elucidation of the CR neuroprotective mechanism(s). We suggest that these diet implementation guidelines, on a working "standard" calorically restricted paradigm for disease animal models, should not only maximize the therapeutic diet efficacy but also normalize data interpretation for comparison within different animal studies by reducing laboratory artifacts.

A. A Comparative Analysis of CR to Other Dietary Energy Restriction Regimens

In the literature, CR is also referred to as dietary restriction (DR), dietary energy restriction (DER), and food restriction. However, these terms are not synonymous (Table 11), thus one needs to be cautious when studying them (Thompson et al., 2003). As illustrated in Table 11, CR specifically refers to a dietary regimen formulated so that animals are fed different number of calories while still receiving the same facet of all other nutrients (Thompson et al., 2003). Although several forms of DR have been reported to extend survival and increase life span in animal disease models (Piper and Bartke, 2008; Anderson

et al., 2009; Colman et al., 2009; Masoro, 2009), many studies inappropriately describe “intermittent fasting, (IF),” as a prototypical variant of CR (Anson et al., 2003; Duan et al., 2003; Halagappa et al., 2007; Armentero et al., 2008). Recent studies suggest that the daily food consumption and underlying therapeutic mechanism(s) of CR and IF may vary considerably (Goodrick et al., 1990; Martin et al., 2006; Masoro, 2009).

In a typical IF regimen, food is provided *ad libitum* to animals on an “every-other-day” basis accompanied with a 24-hr fast in between feeding days (Figure 19). However, each IF regimen usually results in varying degrees of body weight loss (Anson et al., 2003; Mattson and Wan, 2005; Bates et al., 2007; Marsh et al., 2008b; Froy et al., 2009; Katare et al., 2009; Madorsky et al., 2009). This probably is due to the influence of both the animal genotype and the age of the animal during diet initiation (Goodrick et al., 1990). In contrast to the aforementioned IF regimen, animals fed a calorie-restricted diet usually consume enough food to maintain a stable body weight loss relative to their initial body weight (Figure 22) (Mantis et al., 2004). It is possible that the complex relationship of body weight to life span both between and within the various dietary groups makes it difficult to predict that lower body weight increases survival or that is neuroprotective (Goodrick et al., 1990). Furthermore, IF-treated mice eat roughly twice as much food compared to the mice eating *ad libitum* on the days they have access to food, a phenomenon also seen in rats during an IF regimen (Lueker et al., 1956; Anson et al., 2003; Descamps et al.,

2005; Froy et al., 2009). This finding suggests that rodents exhibit increased food consumption on the fed days in order to maintain similar body weights as those seen in rodents fed *ad libitum*.

Interestingly, this binge-eating phenomenon is also observed upon re-feeding of normal mice calorie restricted for 30 days (Mahoney et al., 2006). As shown in Figure 20, re-feeding of restricted mice results in an approximate two-fold increase in food intake compared to either their *ad-libitum* pre-restricted period food intake or the food intake of *ad libitum* fed mice (Grand and Millar, 1990; Hagan and Moss, 1997; Hagan et al., 2002; Mahoney et al., 2006). Similarly, re-feeding adult EL previously restricted to 20-24% of their pre-trial body weight resulted in similar body weights at the end of the re-feeding period (Figure 21). However, while the body weight and plasma lipids returned to unrestricted levels, plasma glucose and food intake remained significantly lower in the re-fed CR mice (Mahoney et al., 2006). Contrary to this, re-feeding of IF subjects resulted in no apparent differences in glucose levels (Halberg et al., 2005; Becskei et al., 2008, 2009). This suggests that CR establishes a new homeostatic state that persists following *ad libitum* feeding most likely by reducing thermogenesis, where IF does not (Lane et al., 1996; Overton and Williams, 2004; Mahoney et al., 2006). Reduced thermogenesis and subsequent increased activity-related energy expenditure allows less energy to be lost as heat by modulating uncoupling proteins (UCP1) (Klaus et al., 2005; Neschen et al., 2008). UCP1 is a major player in basal and regulatory thermogenesis, overall

energy balance, and body weight regulation.

Moreover, IF generates cycles of lipolysis and fat storage that are not representative of the usual 30-45% reduction in caloric intake (Weindruch et al., 1986; Feige et al., 2008a; Froy et al., 2009). Interestingly, re-feeding of a high fat diet following periods of food deprivation results in a significant decrease in glyceraldehyde phosphate dehydrogenase (GAPDH) activity (Saggerson and Greenbaum, 1969). This enzyme catalyzes the conversion of glyceraldehyde phosphate to D-glycerate 1,3-bisphosphate during glycolysis, compared to other dietary forms of refeeding. A decrease in GAPDH activity results in the diversion of carbohydrate metabolism away from fatty acid synthesis into the formation of glycerol phosphate, the precursor for triglyceride and phospholipids synthesis in liver and adipose tissue (Freedland, 1967; Jansen et al., 1968; Krizova and Simek, 1996; Hillgartner and Charron, 1998; Reshef et al., 2003). Finally, it has been shown that IF can differentially effect circadian rhythms depending on food availability, suggesting that this regimen induces a metabolic state that affects the suprachiasmatic nuclei clock in mice (Froy et al., 2009). Therefore, in contrast to the cyclic IF regimen where body fat deposition increases while decreasing energy expenditure (Lim et al., 1996), CR decreases energy intake, and expenditure along with metabolic rate without increasing fat deposition (McCarter et al., 1985; DeLany et al., 1999; Greenberg and Boozer, 2000; Ramsey et al., 2000; Faulks et al., 2006).

B. Importance of Body Weight as an Independent Measure During CR

Most rodent diets are formulated to promote rapid post-weaning growth with the intent of maximizing body weight gain with age (Ward, 1981). When considering the specific nutrient requirements of mice, it is important to be cognizant of not only the animal age and genotype (male vs. female; wild-type vs. transgenic) (Goodrick et al., 1990), but also the tight association of energy status to physiology (e.g. metabolic energy status, daily energy expenditure, and genetic background) (Felber and Golay, 1995; Ferguson et al., 2008). The observation that CR does not enhance survival in DBA mice despite an apparent reduction in body weight reinforces the importance of the genetic background and diet regimen in determining the neuroprotective effects of CR (Turturro et al., 1999; Forster et al., 2003). Many studies, for instance, evaluating the daily protein requirements for mice have found that a minimal 13.6% casein (~12% protein) is needed for adequate growth, reproduction, and lactation (Goettsch, 1960). This is an important issue regarding the effects of the KD and CR diets in regards to protein and vitamin intake (Likhodii, 2001; Cunnane and Likhodii, 2004; Samala et al., 2008). For example in rodents, interchanging casein and soy peptone as the source of dietary protein has resulted in different survival rates (Iwasaki et al., 1988). Therefore, similar amounts of protein and vitamins are necessary for not only sufficient animal growth and prevention of malnutrition, but also correct data interpretation (Reeves et al., 1993).

The maintenance of metabolic homeostasis relies on the balanced intake of nutrients from food. Although various forms of CR have been implemented each with different levels of feeding restriction, CR typically is administered as a 25-60% decrease in total calorie intake (Martin et al., 2007; Feige et al., 2008a; Masoro, 2009). The most widely used method for implementing CR is pair-feeding. During pair-feeding, CR animals are restricted 70-30% of the daily food intake of the *ad libitum* control group (Feige et al., 2008a). However, measuring food intake for group-housed animals instead of individual housed, raises concerns regarding the efficacy of these diets and data interpretation (Feige et al., 2008a). Usually, group-housing results in varying body weight between animal cohorts, which may result in body weight changes that are independent of any dietary manipulation (Giralt and Armario, 1989; Perez et al., 1997; Ruis et al., 1999; Nyska et al., 2002; Haseman et al., 2003; Ikeno et al., 2005). Thus, housing animals singly reduces data discrepancies. It is important to mention though that the initial stress of housing animals singly sometimes may result in body weight reduction independently of the *ad libitum* fed diet, which is largely due to the animals acclimating to the new environment (Figure 22) (Mantis et al., 2004).

Although it is commonly held that “a calorie is a calorie” and that diets of equal caloric content will result in identical weight change independent of macronutrient composition, the laws of thermodynamics do not support this notion (Feinman and Fine, 2004; Fine and Feinman, 2004). Comparing iso-

calorically restricted diets of different macronutrient composition has shown to induce different changes in body mass (Young et al., 1971; Rabast et al., 1978; Piatti et al., 1994; Golay et al., 1996; Baba et al., 1999; Layman et al., 2003). In particular low carbohydrate diets, such as CR, result in a more significant weight loss than isocaloric diets of high carbohydrate or fat content (such an example is shown in Figure 23). Thus implementing CR regimens as a percent of the daily caloric intake of the control animal may not be informative for data interpretation.

Also when studying the efficacy of diet therapies on a disease phenotype or animal behavior, it is important to mention that exogenous factors as well as the environment modulate/regulate metabolism. In particular, stress, glucocorticoids, G protein-coupled receptors, NPY along with its receptors, and hormones (e.g. leptin, ghrelin, cholecystokinin, peptide YY_{3-36}, a-melanocyte stimulating hormone) all play a pivotal role in body weight and food intake regulation (Marti et al., 1994; Schwartz et al., 2000; Tamashiro et al., 2005; Morton et al., 2006; Beckers et al., 2009; Dietrich and Horvath, 2009; Ferrini et al., 2009). In various animals it has been shown that exposure to stressors (e.g. surgery, various types of drugs or endotoxins, inflammation, etc.) can cause a varying range of biochemical, physiological, and behavioral changes (Armario et al., 1988; Marti et al., 1994; Valles et al., 2000). Specifically, we have shown that tumor implantation reduces body weight (surgery is a body weight modulator) in mice (Zhou et al., 2007; Marsh et al., 2008b). Thus, when CR needs to be co administered along with a stressor, body weight must be allowed to normalize

back to its pre-stressed (e.g. pre-surgery) levels before starting any dietary manipulation. If body weight isn't normalized then active body weight controls need to be used. Similarly to the effect of surgery, the administration of insulin in a dietary animal group can too play a significant role in body weight and food intake regulation (MacKay et al., 1940; Lotter and Woods, 1977; Grossman, 1986; Rushing et al., 2000; Schwartz et al., 2000; Woods et al., 2000). Additionally, drugs such as AEDs, TZDs, corticosteroids, THC, metformin, conglutin-gamma, and fish oil, all have been shown to affect various metabolic parameters, including body weight (Table 12) (Chan et al., 1996; Magni et al., 2004; Yasuda et al., 2004; Gounarides et al., 2008; Hausman et al., 2008; Gonzalez-Periz et al., 2009; Mishra and Mohanty, 2009; Mannaerts et al., 2010). Hormone levels and intact adrenal glands have also been show to be essential in the regulation of food intake and subsequently body weight (Yaktine et al., 1998; Liu et al., 2002). All aforementioned findings point on the modulating effect of a stressor, drug, or hormone levels on body weight regulation reaffirm the importance for the usage of active body weight controls in diet studies.

Consequently, when dividing mice into the various dietary groups, it is important to separate them based on their body weight such that the average body weight is the same between cohorts (similarly to Figures 22 and 23). It was mentioned earlier that CR has indeed a differential effect based on the genetic background of the animal (Forster et al., 2003). It is also know that inbred strains of mice, kept under the same laboratory conditions, differ in body weight at the

same age due to genetic variation (Poiley, 1972). Specifically, it has been shown that the food intake and metabolism of each mouse varies significantly due to gene copy variation (Roberts, 1981; Casellas et al., 2009; Hager et al., 2009; Orozco et al., 2009). It has also been reported that the behavioral assessment of genetically identical mice varies in different environments or test sites and that the genetic background plays a pivotal role in gene expression (Crabbe et al., 1999; Opsahl et al., 2002; Wahlsten et al., 2003; Lathe, 2004). This genetic variation becomes even more important when employing knockout or transgenic mice in dietary studies, since different mouse genotypes or genetic backgrounds can have different behavioral, metabolic, or gene/protein response to a particular diet (Lathe, 1996; Wolfer and Lipp, 2000; Opsahl et al., 2002). Because transgenic or gene knockout mice usually have reduced body weights compared to wild-type mice, further validates body weight as the independent variable when one is implementing a diet study (Joven et al., 2007; Reed et al., 2008).

All aforementioned findings raise the importance of a pre-dietary treatment fasting period as well as active body weight controls when one is about to initiate CR or the KD in animal models. Fasting in adult animals for about 14-16 hr prior to any dietary manipulation establishes a similar metabolic and potentially behavioral baseline at which all animals can start when separated in the various dietary groups (similarly to week 3 in Figure 22). For younger animals (still developing or before sexual maturity) a shorter fasting period (5-7 hr) may be used to avoid any growth retardation effects. Interestingly, in the clinic, the

ketogenic diet is traditionally initiated on an inpatient basis, beginning with a fast, to 'jump-start' ketosis, and is followed by a gradual transition over several days to a diet with a ketogenic ratio of 3:1 or 4:1 (grams of fat to grams of carbohydrate plus protein) (Seyfried et al., 2008a; Seyfried et al., 2009a).

Hence, we have established a new experimental design for the implementation of CR or other forms of DR in mice. In contrast to implementing CR based on the average food consumption of the unrestricted control mice, each restricted mouse serves as its own body weight control. This restriction paradigm reduces variability in body weights and food intake among rodents fed diets widely different in nutritional composition and caloric content (Figure 22) (Mantis et al., 2004). As mentioned earlier, isocaloric restriction of the high fat, low carbohydrate KD was unable to reduce body weight to the same degree as that observed when restricting the high-carbohydrate, high fat standard chow rodent diet (SD) (Figure 23). Using body weight as the independent measure for determining the percentage of CR rather than caloric intake, we can accurately measure blood metabolites, the disease phenotype, as well as study the effect of CR biochemical or behavioral processes (Mantis et al., 2004; Mantis et al., 2009). In addition, establishing metabolic (e.g. body weight, food intake, glucose, ketones, etc.), and disease phenotype (e.g. seizure frequency, behavior, etc.) pre-dietary baseline parameters for each animal further enables us to minimize any data discrepancies (Mantis et al., 2004).

Thus knowing “where and how your animal has been treated” is very

important. When obtaining already treated animals from various vendors for diet studies, one should use caution as to how those animals were housed (grouped vs. singly housed), what age or genotype are they, and of course whether each animal was used as its own body weight control. Nevertheless, we ought to mention that due to raising costs in animal husbandry and per diem charges, if an investigator was to choose group housing for his/her animal diet studies, he/she should be aware of the limitations of this housing paradigm. Employing statistical tools one could omit animals from a group-housed cohort that did not respond similarly to a specific diet treatment regimen (e.g. similar % body weight reduction), thus maximizing the dietary efficacy and normalizing data interpretation.

C. Influence of Diet on Rodent Metabolism

Implementation of the high fat KD is often difficult, as the diet tends to produce an unanticipated loss of body weight. In many studies where the KD is fed to animals in unrestricted amounts the animals lose body weight (Tables 9 and 10) (Su et al., 2000; Thavendiranathan et al., 2000; Likhodii et al., 2002; Ziegler et al., 2002; Noh et al., 2004; Silva et al., 2005; Yamada et al., 2005; Bough et al., 2006; Thio et al., 2006; Kennedy et al., 2007; Samala et al., 2008; Hansen et al., 2009). This observation contradicts what our laboratory and others have observed regarding the effect of UR-fed KD on body weight. Our

data show that when the KD is provided to adult animals in unrestricted amounts, it resulted in no significant reduction of body weight compared to the body weight of the control animals (Figure 22, and Tables 9 and 10) (Muller-Schwarze et al., 1999; Rho et al., 1999; Likhodii et al., 2000; Mantis et al., 2004). This body weight reduction in unrestricted KD fed mice may be due to "self-restriction" associated with the unpalatable diet. It is possible that the various metabolic or behavior differences observed in the self-restricted KD group resulted from CR rather than from the KD alone, which can result in incorrect data interpretation. Thus, in order one to test the hypothesis of whether the underlying therapeutic mechanism of CR and the KD is possibly different, the animals that are fed the unrestricted KD should have similar body weights to normal control animals fed a standard diet (Figure 22) (Todorova et al., 2000; Mantis et al., 2004). As previously shown, whenever the KD is fed unrestricted, the mice should not lose body weight compared to normal control (Figure 22, and Tables 9 and 10) (Muller-Schwarze et al., 1999; Rho et al., 1999; Likhodii et al., 2000; Mantis et al., 2004).

Interestingly, we have previously shown that although an unrestricted KD could delay the onset of seizures in young EL mice with a genetic predisposition to epileptic seizures (Todorova et al., 2000), greater seizure control was achieved when these mice were fed a calorically restricted KD (KD-R) (Mantis et al., 2004). This suggests that KD-R has a greater neuroprotective effect than unrestricted KD, at least in rodent models of epilepsy (Mantis et al., 2004). A

similar observation, on the beneficial effects of a KD-R, was shown involving brain tumor bearing mice (Seyfried et al., 2003; Zhou et al., 2007). Thus, the therapeutic benefits of the ketogenic diet in a variety of diseases may be enhanced further when the diet is carefully administered in restricted amounts to avoid malnutrition or growth retardation.

However, this later notion of malnutrition becomes very important when performing calorically restricted dietary studies in young suckling animal, since the transition from the suckling (fed) state to a calorically restricted state could be associated with the development of malnutrition which in turn can produce a severe loss of body weight (Table 9) (Bough et al., 1999b; Harney et al., 2002; Thavendiranathan et al., 2003). It is imperative that no developmental delays are evident to CR-fed young animals that may affect overall mouse behavior, phenotype (e.g. seizure susceptibility), or metabolism. Specifically, although both hypoglycemia and malnutrition increase seizure incidence in humans by most likely reducing the population of GABAergic neurons (Bennish et al., 1990; Andrade et al., 1995; Andrade and Paula-Barbosa, 1996; Crepin et al., 2009), malnutrition has varying effects on GABA metabolism, the major brain inhibitory neurotransmitter (Smith et al., 1974), as well as overall metabolism (James and Coore, 1970).

Therefore, cautionary measures need to be implemented when dealing with growing/developing animals. Specifically, the percent growth of the young mice in the R-fed mouse groups must be similar to the mice fed normal chow

unrestricted as previously described (Figure 24) (Todorova et al., 2000; Greene et al., 2001). It is vital that the percent body weight reduction for young mice fed a restricted diet is not greater than 8-10%, in order to avoid malnutrition effects (Figure 24). Based on experimental evidence, we propose that for older animals a 30-65% reduction in dietary calories should produce about a 15-25% decrease in body weight and that these parameters are acceptable for dietary studies. The findings of the aforementioned studies further support our contention that it is imperative for each mouse to be used as its own control during the implementation of any DR or KD feeding regimen. In contrast to young animals, older cohorts can withstand a greater degree of CR or body weight reduction without develop malnutrition, as we have previously shown (Mantis et al., 2004; Denny et al., 2005; Mantis et al., 2009). Therefore, during dietary studies the stage of disease progression (early vs. late disease onset) and the suggestive percent of CR or body weight reduction need to be carefully examined.

Taken together, the abovementioned observations suggest that active body weight control groups should be used when one is performing dietary animal studies. Active body weight controls are produced by restricting the total calories consumed by a subgroup of control rodents so that the rodents achieve a similar degree of body weight relative to rodents that have been divided into the experimental or non-standard dietary treatment groups. For example, if a new CR mimetic agent increases life span or improves behavior while also reducing body weight in experimental test cohorts, it is necessary for the investigators to

demonstrate the extent to which the increased survival effect is due specifically to the CR mimetic and not to an involuntary CR regimen.

D. Measurement of Metabolite Biomarkers

It is important to mention that serum metabolites (e.g. insulin, glucose), food intake, and the gastrointestinal/pancreatic response in the whole animal is a multi-dependent process and not just a simple stimulus-secretory response interaction (Gagliardino and Hernandez, 1971; Grossman, 1986; Efendic and Portwood, 2004; Sharma et al., 2008; Dietrich and Horvath, 2009; Ferrini et al., 2009). In light of the observation that circulating biomarkers/metabolites of energy status (e.g. glucose, ketones, insulin, glucagon, cortisol, free fatty acids, triglycerides, etc.) are influenced by the influx of dietary calories and that the levels of these markers fluctuate during the transition from the fed to the fasted state (Fabry and Tepperman, 1970; Romsos et al., 1978; Tunbridge et al., 1991), a subject must fast for at least 3-4 hrs prior to having the metabolites measured in the peripheral circulation.

This is particularly relevant when measuring glucose, insulin, and ketone body levels because it takes at least 2-3 hours for the level of these metabolites to return baseline in the postprandial state. This consideration is also important when one is attempting to connect a significant change in the level of a particular biomarker(s) with the development of a specific disease phenotype, a change in

gene/protein expression levels, or with the expression of a certain animal behavior (Hermus et al., 1994; van 't Veer, 1994; Lathe, 1996; Blanck et al., 2003). Although, biomarkers provide alternative measures of dietary intake, they can vary with absorption, metabolism, genetic background, and disease status. Thus they should be chosen in relationship to both the dietary intake and the chronology of exposure, since proper sample collection, storage, and analytical laboratory technique can all affect the specific level of each corresponding biomarker (Blanck et al., 2003). Interestingly, malnutrition has been shown to result in a slower reduction of blood glucose levels in both humans and mice (James and Coore, 1970; Okitolonda et al., 1987). Thus, because glucose and ketone levels can vary based on prior food intake or malnutrition, blood metabolites should to be carefully measured using validated enzyme assays (Mantis et al., 2004; Zhou et al., 2007). For example, both glucose and ketone body levels should only be measured in the blood (Turan et al., 2008), rather than in the urine (Penders et al., 2005). That's because both metabolites must exceed a specific concentration in the blood before they get excreted into the urine (Taboulet et al., 2007; Turan et al., 2008). Thus, urine may not accurately represent the levels of the markers in the peripheral circulation (Gilbert et al., 2000; Taboulet et al., 2007; Turan et al., 2008). Moreover, the pH of the blood will also affect the extent to which ketone bodies, which are weakly acidic, get produced (ketogenesis) (Wu et al., 1991), or excreted into the urine (Behre, 1931; Lemieux and Plante, 1968; Hood, 1985).

GUIDELINES FOR IMPLEMENTING CALORICALLY RESTRICTED DIETS FOR THE MANAGEMENT OF NEUROLOGICAL AND NEURODEGERATIVE DISEASES

The first issue with therapeutic diet implementation is the non-conventional and non-pharmacological nature of the diet therapy (Seyfried et al., 2008a; Seyfried et al., 2009a). Despite the availability of well-established procedures for acceptable clinical practice, modern medicine has not looked favorably on diet therapies for complex diseases (Seyfried et al., 2008a; Seyfried et al., 2009a), maybe with the exception of type II diabetes. This latter fact is probably due to the lack of a standardized use protocol for implementing calorically restricted diets, which hinders the applicability of dietary therapies to a broad range of patients (Seyfried et al., 2008a; Seyfried et al., 2009a). Therefore, additional animal studies with proper standardized guidelines are needed to provide the clinical basis by which therapeutic diets can have a greater clinical relevance in disease management. Although, similar concerns are often raised for implementing the ketogenic diet as a therapy for epilepsy, several medical groups have successfully established various protocols for implementing the ketogenic diet or low glycemic diets in children (Freeman et al., 2000; Pfeifer and Thiele, 2005; Seo et al., 2007; Kossoff et al., 2008).

We suggest a sequential series of therapeutic phases for the dietary management of neurodegenerative diseases in mice, similar to what we have

recently proposed for the management of brain tumors using a restricted KD (Seyfried et al., 2008a; Seyfried et al., 2009a). During **Phase one** we need to establish the baseline metabolic and behavior parameters of animals housed individually for 4-7 days (pre-trial period). During this pre-trial period animals should be maintained on normal control SD, while body weight, food intake [food intake should be calculated using the formula: FI (g/mouse/day) = $(W_i - W_{fx})/n_{dx}$, where W_i is the weight (g) of food initially given; W_{fx} is the amount of food remaining in the cage for x number of days; and n_{dx} is the number of day for which FI is calculated] as well as plasma glucose, ketone, insulin, and glucagon levels will be measured for each cohort. After, the initial pre-trial period, animals will be separated in their corresponding dietary groups where the average body weight of each group will be similar. For adult mice, a 14-hr fast will then be followed (5-7 hr fast for younger mice). This established pre-dietary baseline should help with data interpretation and reinforce any significant comparisons between groups. It should be again noted that appropriate active body weight controls are needed especially if drugs, various stressors, or different forms of diet application (paste vs. pellet vs. powder) are used within a dietary group regimen. Finally, during phase one, power analysis and other statistical tools are needed for determining cohort size and obtaining statistical significance between the various parameters. Similarly to the hippocampal transciptosome changes observed in male and female rats under DR, statistics are important, because the level of dietary restriction influences differentially metabolism, reproductive

function, the development of age-related diseases, and even cognitive behavior, and gender-specific molecular responses (Martin et al., 2008).

During **Phase Two**, we need to gradually lower circulating glucose levels and elevate circulating β-OHB levels over several days or weeks using restricted diets or therapeutic fasting (Figure 25) (Mantis et al., 2004; Mahoney et al., 2006; Zhou et al., 2007; Mantis et al., 2009). The specific duration of dietary manipulation will depend on what type of genotype, or age of animal cohort we are studying. We suggest that a 20-25% body weight reduction for adult animals (Figure 22), and a 8-10% body weight reduction for younger animals while maintaining a constant rate of developmental growth (Figure 24), is sufficient to lower blood glucose levels between 6-7 mM (108-126 mg/dL) and β-OHB levels between 3-4 mM (31-42 mg/dL). These blood metabolite levels are well within normal physiological ranges and should be effective for disease management (Figure 25). This state in mice is referred as the zone of metabolic disease management (Figure 25). The feeding paradigms for restricting the KD or the SD are similar to those previously described by our laboratory (Mantis et al., 2004; Denny et al., 2005; Mahoney et al., 2006; Marsh et al., 2008b, a; Mantis et al., 2009). Interestingly, with medical supervision, a similar paradigm can be employed for the management of neurodegenerative diseases in patients.

Phase three commences when the animals have reached and maintained their corresponding body weight reduction for a couple days or weeks and are well within their zone of metabolic disease management. During this final phase,

additional observations can be made regarding the influence of the diet on the disease phenotype, animal behavior, and even changes in specific biochemical, molecular (ie. genomic, proteomic, lipidomic analysis), and physiological parameters. In addition to glucose and ketones, other blood biomarkers such as insulin, cholesterol, triglycerides, FFA, glucocorticoids, glucagon, leptin, adiponectin, glucagons, IGF-1, IGF binding protein -1, -2, -3, -5, and -6 may also be relevant to measure in order to confirm DR status. Specifically, previous findings have shown that during CR, insulin, glucose, leptin, and IGF-1 are reduced, while ketone, and glucocorticoids are increased (discussed in the references of Tables 9 and 10) (Duan et al., 2003; Seyfried et al., 2003; Mantis et al., 2004; Bonorden et al., 2009; Fenton et al., 2009).

Maintaining low blood glucose levels is very critical in disease management. Brain does not usually metabolize ketone bodies for energy unless circulating glucose levels are reduced, which is correlated with reduced body weight (Owen et al., 1967; Clarke and Sokoloff, 1999; Greene et al., 2003; Mantis et al., 2004). Specifically, elevated blood glucose levels have been shown to have detrimental effects on disease phenotype and symptomatology (Fabry et al., 1968; McIlwain, 1969; Fabry and Tepperman, 1970; Cornford et al., 2002). Similarly, with respect to the ketogenic diet, “more is not better, as consumption of excessive amounts of the ketogenic diet will maintain high blood glucose levels and thus result in no disease management (Seyfried et al., 2003; Mantis et al., 2004). Supplementation of vitamins and minerals should also not be a

problem as long as their consumption does not change any biochemical or physiological biomarkers (e.g. elevate circulating blood glucose levels).

DISCUSSION

Even though various types of DR are beneficial in most animal models in extending survival and reducing the symptoms associated with neurodegenerative diseases, further research is needed to establish a standardized way of performing these studies in animals, before bringing the therapeutic efficacy of DR into the clinic. Animal data need to be interpreted with caution, as restricted regimens in these animal cases may simply represent a transition from overeating to a healthier diet. Furthermore, in contrast to therapeutic fasting, DR to humans may not work as effectively due to a number of health concerns, which may not be applicable to or impact the life of experimental animals, but may do so in humans (Dirks and Leeuwenburgh, 2006). Potentially, new dietary formulations can be designed with nutritional and caloric compositions more appropriate for managing neurodegenerative and other types of diseases in humans. This could also involve the use of low glycemic diets, which are effective in maintaining low circulating glucose levels and are easier to implement than some ketogenic diets (Kossoff et al., 2007; Pfeifer et al., 2008).

Figure 19. Influence of Intermittent Fasting (IF) on Food Intake in VM Mice. IF resulted in an approximately two-fold increase of the food intake during the 24hr feeding period of the IF. Values are expressed as means of 5 mice per group. The black arrow indicates the initiation of the IF. Data were kindly provided by L. Shelton from the Seyfried laboratory at Boston College.


9,0
6,0
3,0
0,0
Food Intake (g)
0
5
10
15
20
Days of Dietary Treatment

Figure 20. Influence of CR and Re-feeding on Food Intake (A) and Body Weight (B) in C57BL/6J Mice. Upon re-feeding CR mice consumed almost twice as much food compared to the unrestricted mice. Values are expressed as means ± SEM; n = 4–8 mice per group. The black arrow indicates the initiation of CR on day 8. The white arrow indicates the initiation of *ad libitum* re-feeding on day 30. Asterisks indicate that the food intake average of the days 38 to 50 of re-feeding for the R-RF mice was significantly less than their food intake prior to initiation of CR, as determined by the paired *t*-test ($P < 0.05$). Figure reprinted with permission from Mahoney et al., *Lipids Health Dis*., 5:13, 2006.

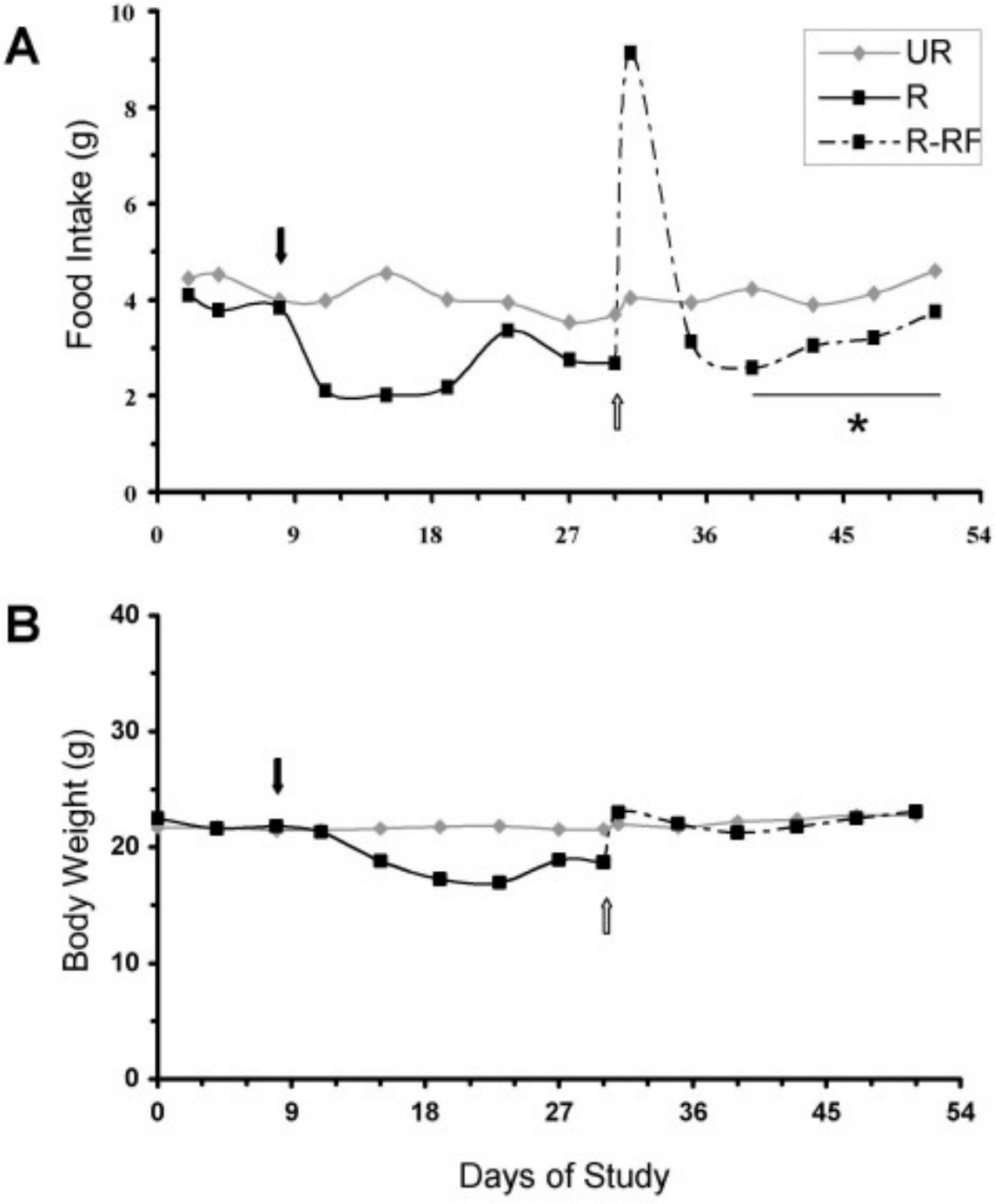
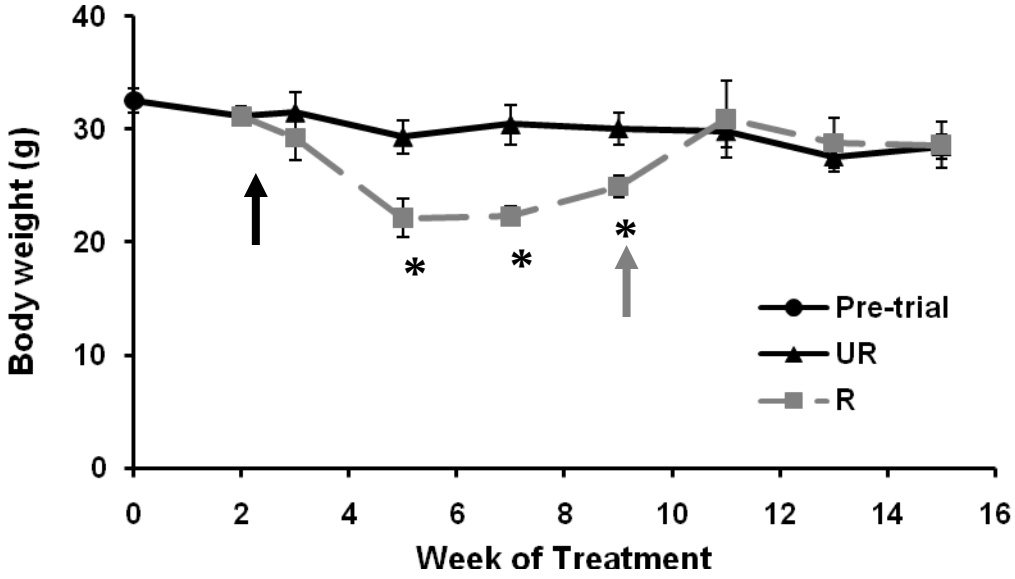

A
Food Intake (g)
UR
R
R-RF
*
B
Body Weight (g)
Days of Study

Figure 21. Influence of Re-feeding on Body Weight of Calorie Restricted Adult EL Mice fed SD (A) or KD (B). Similarly to Mantis et al., adult EL mice (~ 240 days old) fed either a calorie restricted SD, or KD to achieve a 20-24% body weight reduction during a 9-week diet treatment period (Mantis et al., 2004), were switched back to *ad libitum* feeding for weeks 9-15. Upon re-feeding, R-fed mice consumed almost twice as much food compared to their restricted food intake (data not shown), and gradually their body weights matched those of the unrestricted fed mice. Values are expressed as means ± SEM; n = 8-10 mice per group for either UR fed group and n = 3-4 mice per group for either R-fed group. The black arrow indicates the initiation of CR on week 2. The gray arrow indicates the initiation of *ad libitum* re-feeding on week 9. Asterisks indicate that the mean body weight for weeks 5 to 9 of the R-fed mice was significantly less than the body weight of the UR-fed mice, as determined by both the paired *t*-test and ANOVA ($P < 0.01$). No difference in body weight was observed after re-feeding (weeks 10-15) between the UR and R-refed groups.

A.


Body weight (g)
Week of Treatment
Pre-trial
UR
R
*
*
*

B.

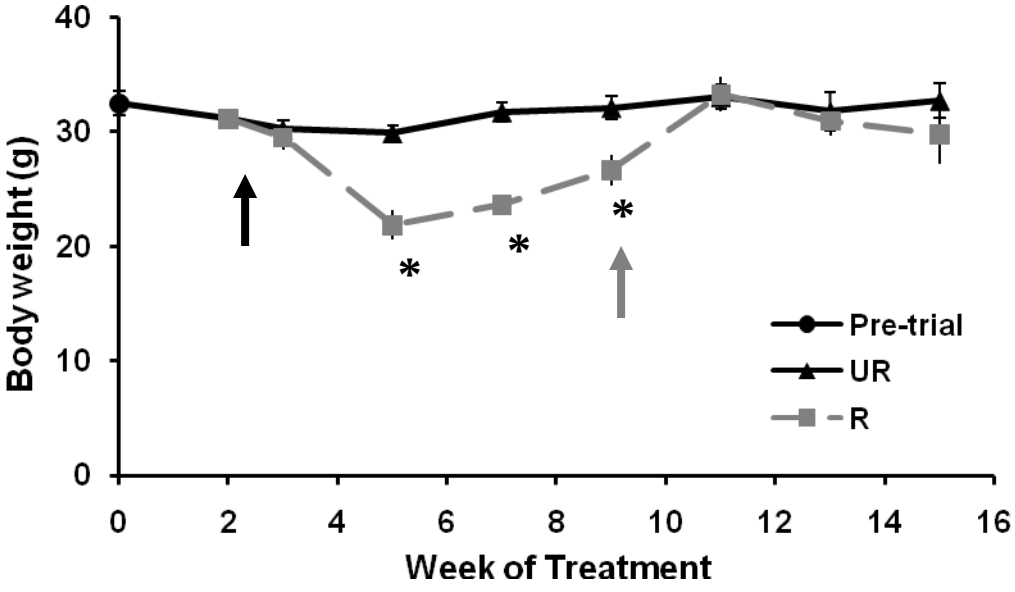

Body weight (g)
Week of Treatment
Pre-trial
UR
R
*
*
*

Figure 22. Influence of Diet on Body Weight in Adult EL Mice Fed a SD (A) or the KD (B). Both R-fed mice lost approximately 20-23% of their pre-dietary body weight. Body weight for both unrestricted groups was similar. Squares represent the pre-trial period when all mice were fed the SD-UR. Circles and triangles represent the UR-fed and R-fed groups, respectively. Values are expressed as the mean ± SEM (n = 6 mice per group). Arrow indicates initiation of CR.

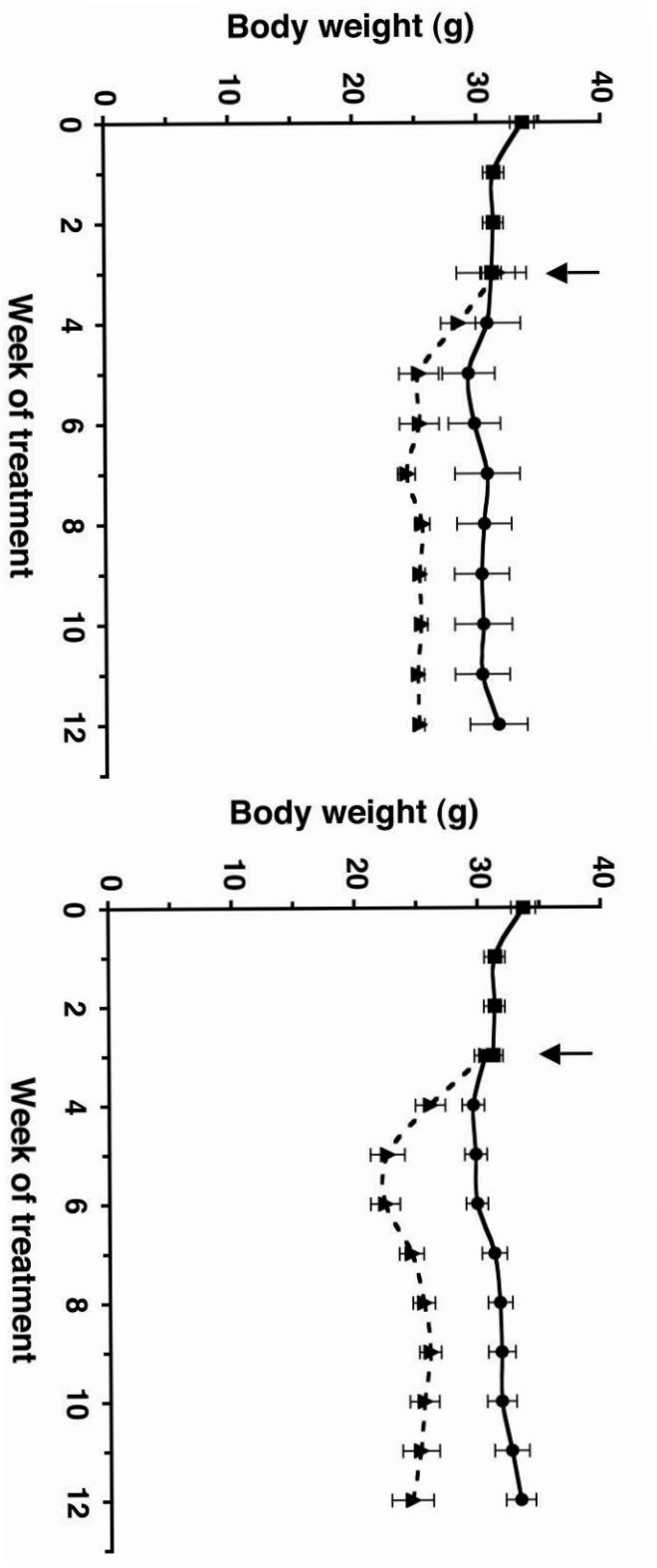

Body weight (g)
0
10
20
30
40
0
2
4
6
8
10
12
Week of treatment
Body weight (g)
0
10
20
30
40
0
2
4
6
8
10
12
Week of treatment

Figure 23. Influence of Isocalorically Restricted Ketogenic and Standard Diets on Body Weight of Adult EL Mice. A four-week 40% isocaloric restriction of the KD and SD diet resulted in varying body weight loss in adult EL mice. Body weight values are expressed as the mean ± SEM (n = 6-7 mice per group) for the 4-week diet treatment period. Asterisk indicates that the body weight of the SD-R mice was significantly reduced compared to their respective SD-UR group, as determined by ANOVA analysis ($P < 0.01$).

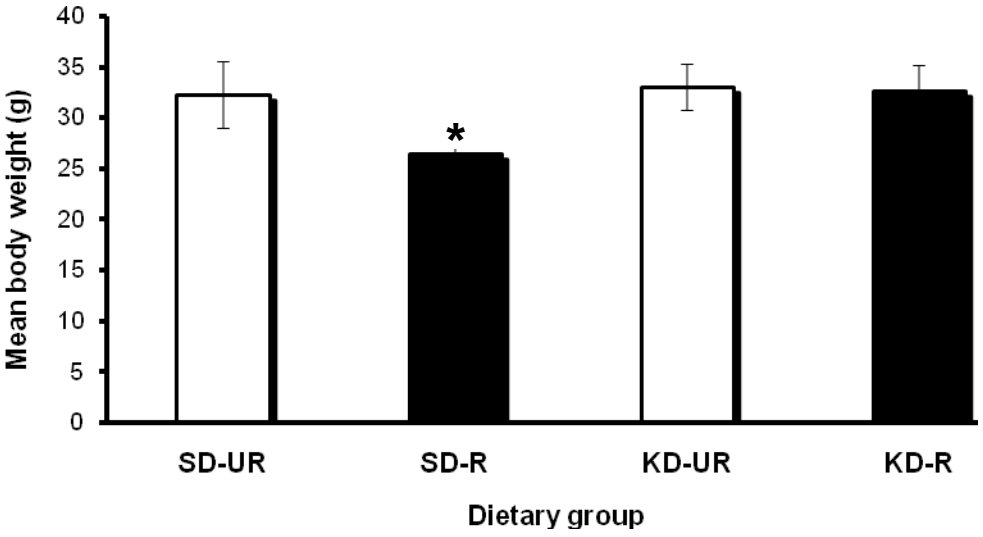

40
35
30
25
20
15
10
5
0
Mean body weight (g)
*
SD-UR
SD-R
KD-UR
KD-R
Dietary group

Figure 24. Influence of CR on Body Weight in Juvenile EL Mice. Although the mean body weight was significantly lower in the juvenile 15% CR mice compared to the *ad libitum* (AL) mice over weeks 1–10 (*$P < 0.01$), the relative growth of the CR mice was similar to that of the AL group. Values are expressed as the mean ± SEM. Figure reprinted with permission from Greene et al., *Epilepsia*., 42(11):1371-78, 2001.

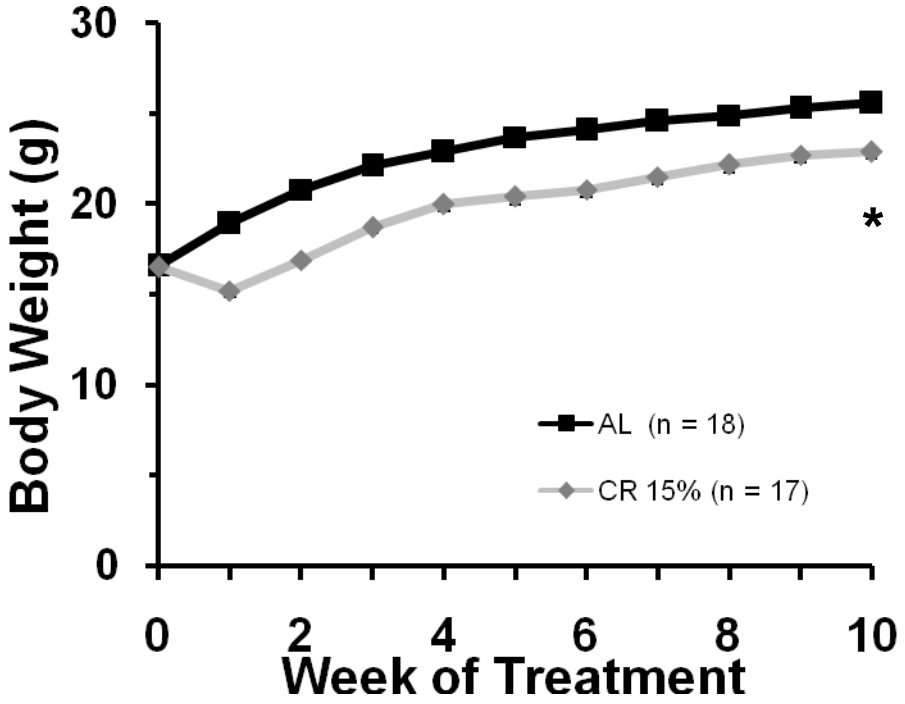

30
20
10
0
Body Weight (g)
0
2
4
6
8
10
Week of Treatment
AL (n = 18)
CR 15% (n = 17)
*

Figure 25. Relationship of Circulating Plasma Metabolites in the Management of Neurodegenerative and Neurological Diseases. These values are within normal physiological ranges of glucose and ketones under fasting conditions in mice. We refer to this state as the zone of metabolic disease management.

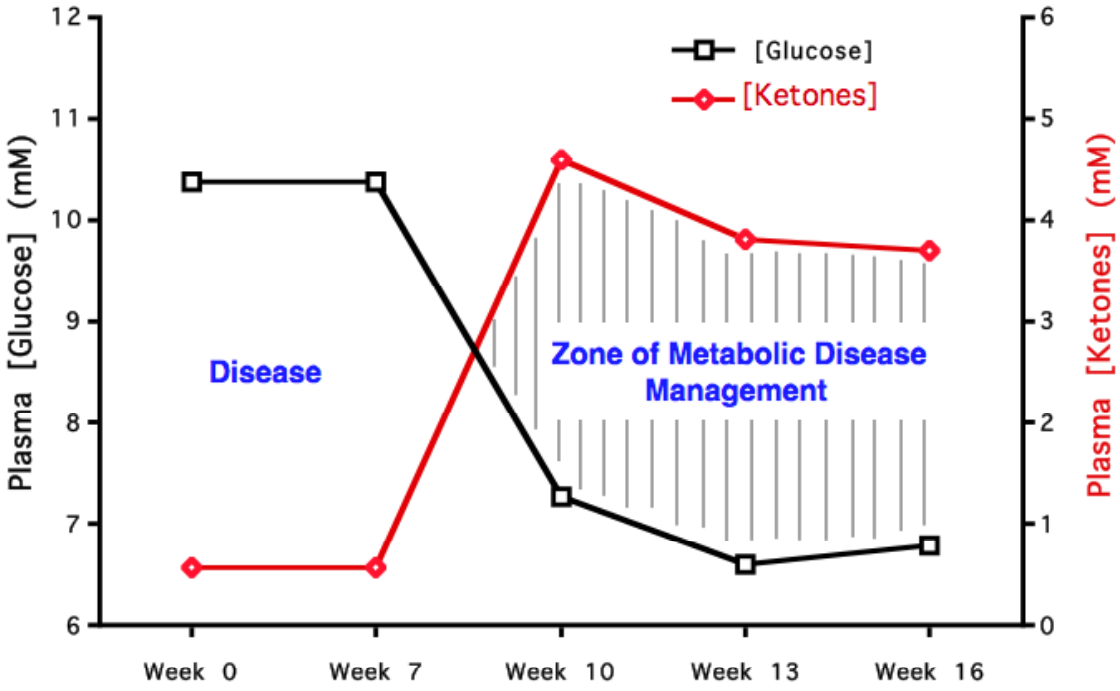

[Glucose]
[Ketones]
Plasma [Glucose] (mM)
Plasma [Ketones] (mM)
12
11
10
9
8
7
6
6
5
4
3
2
1
0
Disease
Zone of Metabolic Disease Management
Week 0
Week 7
Week 10
Week 13
Week 16

Table 9. Influence of Diet Therapies on Animal Models of Epilepsy[1]

Seizure/ Epileptic Model	Animal Age (Diet Initiation)	Diet Paradigm[2]		Biomarker meaurements[3]		Body Weight Measurement	Diet efffect on biomarkers and body weight[4]									References	
							Glucose			Ketone			Body Weight				
							KD		SD	KD		SD	KD		SD		
		UR	R	Glucose	Ketone		UR	R	R	UR	R	R	UR	R	R		
PTZ	Adult	SD	KD	Yes	Yes	Yes		Reduced			Increased			Reduced		Samala et al., 2008	
	Young (P22, 37), Young adult (P50, 63), Adult (P75, 126)	SD	KD, SD	No	Yes	Yes					Increased	Similar		Reduced, Retarded[5]	Reduced, Retarded[5]	Bough et al., 1999	
	Young (P22)	SD	KD, SD	No	Yes	Yes					Increased	Similar		Reduced, Retarded[5]	Reduced, Retarded[5]	Harney et al., 2002	
	Young (P21)	KD, SD		No	Yes	Yes				Increased			Reduced			Hansen et al., 2009	
	Young (P20)	KD, SD		Yes	Yes	Yes	Reduced			Increased			Reduced			Likhodii et al., 2002	
	Young (P20)	KD, SD		Yes	Yes	Yes	Similar			Increased			Similar			Likhodii et al., 2000	
PTZ, MES, Threshold ECS	Young (P22)		KD, SD	No	No	Yes					Increased[6]			Reduced, Retarded[5]	Reduced, Retarded[5]	Thavendiranthan et al., 2003	
PTZ, MES	Adult	KD, SD		Yes	Yes	Yes	Similar			Increased			Similar			Likhodii et al., 2000	
Kainic acid	Young (P32)	KD, SD		No	Yes	Yes				Increased			Reduced			Su et al., 2000	
	Young (P37)		KD, SD	No	No	No					Increased[6]					Bough et al., 2002	
	Young adult (P56)	KD, SD		No	Yes	Yes				Increased			Similar			Muller-Schwarze et al., 1999	
Kainic acid, Fluorothyl	Young (P21)	KD, SD		No	Yes	Yes				Increased			Reduced			Samala et al., 2008	
Fluorothyl	Young ; Adult	KD, SD		No	No; Yes	Yes				Not shown; Increased			Increased but reduced compared to SD-UR; Similar			Rho et al., 1999	
EL mouse (IGE)	Young (P32)	KD, SD		Yes	Yes	Yes	Similar			Increased			Similar			Todorova et al., 2000	*
	Young (P30)	SD	SD	Yes	Yes	Yes	Reduced			Increased					Increased but reduced relative to SD-UR	Greene et al., 2001	*
	Adult	SD	SD	Yes	Yes	Yes	Reduced			Increased					Reduced	Greene et al., 2001	*
	Adult (P210)	KD, SD	KD, SD	Yes	Yes	Yes	Similar	Reduced	Reduced	Increased	Increased	Increased	Similar	Reduced	Reduced	Mantis et al., 2004	*
Kcna1-null mice	Young (P18-20)	KD, SD		No	No	Yes									Similar	Fenoglio-Simeone et al., 2009	
Threshold ECS	Adult	KD, SD		Yes	Yes	No	Similar			Increased						Appleton and DeVivo, 1973	
Kindling	Adult	KD, SD		Yes	Yes	Yes	Similar, Reduced[7]			Similar			Similar			Nylen et al., 2006	
	Adult	SD	SD	No	No	No										Azarbar et al., 2010	
6-Hz	Young (P21)	KD, SD		Yes	Yes	Yes	Varied			Increased			Reduced			Samala et al., 2008	

[1]Table illustrates a representative sample of the neuroprotective effects of dietary therapies in various types of seizure/epileptic animal models
[2]Type of diet (either the KD or the corresponding control SD) that animals were fed (Unrestricted (UR) or Restricted (R) amounts)
[3]Biomarkers measured in the blood (plasma or serum)
[4]Influence of diet on body weight and the biomarkers measured compared to the control group (either SD-UR or KD-UR). Only differences that were statistical significant are reported
[5]Influence of diet on body weight compared to pre-dietary body weight. Body weight of animals is retarded (minimal growth) compared to control-fed animals
[6]Ketone levels were increased relative to the SD-R group
[7]This difference was observed only for the last day of blood sampling
* Asterisks indicate the proper paradigm for diet implementation in young or adult animals
Abbreviations are: ECS, Electroconvulsive shock; IGE, Idiopathic generalized epilepsy; KD, Ketogenic Diet; MES, Maximal electroshock; PTZ, Pentylenetetrazol; SD, Standard Diet

Table 10. Influence of Diet Therapies on Neurodegenerative and Neurological Animal Models[1]

Disease	Animal Model	Animal Age (Diet Initiation)	Diet Paradigm[2]		Biomarker meaurements[3]		Body Weight Measurement	Diet efffect on biomarkers and body weight[4]									References
								Glucose			Ketone			Body Weight			
								KD		SD	KD		SD	KD		SD	
			UR	R	Glucose	Ketone		UR	R	R	UR	R	R	UR	R	R	
Alzheimer's Disease	APP/V717I mice	Adult (P90)	SD	KD	No	Yes	Yes					Increased			Reduced		Van der Auwera et al., 2005
	3xTgAD mice	Adult (P90)	SD	SD, IF	No	No	Yes									Reduced	Halagappa et al., 2007
	Tg2576 mice	Adult (P120)	SD	SD	No	No	No; Yes									Not shown; Reduced	Qin et al., 2008; Qin et al., 2006
Parkinson's Disease	MPTP - Rh. monkeys	Adult	SD	SD	No	No	Yes									Reduced	Maswood et al., 2004
	6-OHDA rats	Adult	SD	IF	No	No	Yes									Reduced, Retarded[5]	Armentero et al., 2008
Huntington's Disease	HD-N171-82Q mice	Young adult (P60)	SD	IF	Yes	No	Yes	Reduced								Reduced but similar to SD	Duan et al., 2003
Sandoff's Disease	*Hexb* -/- mice	Adult	SD	SD	Yes	Yes	Yes			Reduced			Increased			Reduced	Denny et al., 2006
ALS	SOD1-G93A mice	Young (P50)	KD, SD		No	Yes	Yes				Increased			Similar			Zhao et al., 2006
	SOD1-G93A mice	Young (P40)	SD	SD	No	No	Yes									Reduced	Hamadeh et al., 2005
Brain Cancer	Astrocytoma (CT-2A)	Adult	KD, SD	KD, SD	Yes	Yes	Yes	Similar	Reduced	Reduced	Increased	Increased	Increased	Similar	Reduced	Reduced	Seyfried et al., 2003
		Adult	KD, SD	KD	No	No	Yes	Similar	Reduced		Increased	Increased		Similar	Reduced		Zhou et al., 2007
		Adult	SD	KD	No	No	No									Reduced	Marsh et al., 2008a
		Adult	SD	IF-R[6]	Yes	No	Yes			Reduced						Reduced	Marsh et al., 2008b
Rett Syndrome	Mecp2$^{308/y}$ mice	Adult	SD	KD, SD	No	No	Yes								Reduced	Reduced	Mantis et al., 2009
Neuronal damage-injury	Excitotoxin-induced mice	Young (P42)	SD	IF; SD	Yes	Yes	Yes			Reduced; Reduced			Increased; Reduced			Similar; Reduced, Retarded[5]	Anson et al., 2003
	Insulin-induced hypoglycemia	Young (P21)	KD, SD		Yes	Yes	Yes	Reduced			Increased			Reduced, Retarded			Yamada et al., 2005
Ischemia	MCAO	Young (P28)	KD, SD		No	No	No										Puchowicz et al., 2008

[1]Table illustrates a representative sample of the neuroprotective effects of dietary therapies in various neurological and neurodegenerative animal models
[2]Type of diet (either the KD or the corresponding control SD) that animals were fed (Unrestricted (UR), Restricted (R), Alternate 24hr feeding/fasting (IF), or amounts)
[3]Biomarkers measured in the blood (plasma or serum)
[4]Influence of diet on body weight and the biomarkers measured compared to the control group (either SD-UR or KD-UR). Only differences that were statistical significant are reported
[5]Influence of diet on body weight compared to pre-dietary body weight. Body weight of animals is retarded (minimal growth) compared to control-fed animals
[6]IF-R, Alternating days of fasting and calorically restricted feeding. On the feeding days, mice were provided with 75% of the average amount of energy consumed by the UR group on the previous day
Abbreviations are: ALS, Amyotrophic lateral sclerosis; KD, Ketogenic Diet; MCAO, Middle cerebral artery occlusion; SD, Standard Diet

Table 11: Summary of Definitions of Various Dietary Regimens[1]

Dietary energy restriction	Restriction of available energy by limiting caloric intake in absence of an effect on energy
Dietary or food restriction	Reduction of caloric intake by restricted all nutrients and dietary factors
Calorie or energy restriction	Daily reduction of only total caloric intake
Intermittent fasting	Reduction of total caloric intake every other
Fasting	Complete restriction of all available energy resulting in non-pathological conditions
Starvation	Complete restriction of all available energy resulting in pathological conditions

[1]This table is modified from Thompson et al., 2003.

Table 12. Influence of known drugs and herbs on rodent metabolism and body weight

Drug	Metabolism	Body Weight	References
Corticosteroids	Although reduce ability to absorb blood glucose leading to increased fat deposits, the effect may be differential. Existence of a dosage effect	Increased / Reduced	De Vos et al., 1995; Michel & Cabanac, 1999; Bhagavan, 2002; Gounaridis et al., 2008
Antipsychotics	Increase blood sugars, fat deposition, and ketoacidosis. Hyperphagia, and metabolic dysregulation	Increased	Fell et al., 2007; Coccurello et al, 2008; Mishra and Mohanty, 2009
TZDs	Increase insulin sensitivity; increase glucose and fatty acid uptake, increase lipogenesis, reduce gluconeogenesis	Increased	Zhang et al., 2005; Reynolds and Goldberg, 2006; Hausman et al., 2008;
THCs	Decrease oxidative metabolism, metabolic rate, and body temperature, while increase heart rate. Existence of a dosage effect	Reduced	Chiu et al., 1975; Adams et al., 1976; Chan et al., 1996
Metformin	Attenuates hyperglycemia (lowers blood glucose)	Reduced	Bailey & Puah, 1986; Lin et al., 2000; Yasuda et al., 2004; Cleasby et al., 2004; Hull et al.,2005
Conglutin-gamma	Reduces plasma glucose in hyperglycemic animals	Unchanged	Magni et al., 2004; Sirtori et al., 2004
AEDs	Influence lipid and glucose metabolism	Unchanged	Yamashita et al., 1989; Tharappel et al., 2008; Sheth & Montouris, 2008; Mannaerts et al., 2009
Fish oil	Increases glucose uptake; Lowers triglycerides, while increase fatty acid oxidation; Increases lipid metabolism	Reduced	LeBoeuf & Veldee, 1993; Mori et al., 2007; Gonzalez-Periz et al., 2009; Arai et al., 2009

Abbreviations are: AEDs, antiepileptic drugs; THCs, Tetrahydrocannabinol; TZDs, Thiazolidiedione

CHAPTER SIX

Biochemical and Molecular Correlatives in the Management of Seizure Susceptbility in EL mice using the KD and CR

INTRODUCTION

Considering all presented findings, while it becomes evident that both CR and the KD successfully reduce seizure susceptibility in young and adult EL mice, the underlying neuroprotective mechanism of these diets still remains elusive. The one clear emerging mechanism that we can draw from this work is that a reduction in circulating glucose and a subsequent increase in β-hydroxybutyrate levels play an important role in the anticonvulsant efficacy of CR and the KD in EL mice (Mantis et al., 2004; Mantis et al., 2009; Seyfried et al., 2009b). Despite these intriguing observations, it is not yet clear what is the exact mechanism(s) for the neuroprotective effect of these diets.

As described in Chapter 2, under normal physiological conditions the brain derives almost all of its energy from the aerobic oxidation of glucose, via the facilitation of its glucose transporters (GLUT-1) (McIlwain, 1969; Clarke and Sokoloff, 1999; Vannucci and Vannucci, 2000). However, in altered metabolic states, such as fasting or CR, the energetic demands of the brain transition from glycolysis to beta-oxidation. This leads to a production of ketone bodies, which are used as energy substrates. Monocarboxylate transporter 1 (MCT1) and to a

lesser extent MCT2, play a pivotal role in the transfer of ketones bodies (e.g. β-hydroxybutyrate, acetoacetate, and acetone) from the blood circulation into the brain (Nehlig and Pereira de Vasconcelos, 1993; Vannucci and Simpson, 2003; Morris, 2005; Pierre and Pellerin, 2005). This transition in energy substrate metabolism results in part to an increase in ketone body levels, which in turn has been shown to alter the glutamate-glutamine cycle and thus the availability of the major inhibitory neurotransmitter, gamma-aminobutyric acid (GABA) in the brain (Yudkoff et al., 2004; Yudkoff et al., 2005). GABA is synthesized from glutamate in a single rate-limiting enzymatic step by one of the two glutamic acid decarboxylase isoforms (GAD67 and GAD65) (Martin and Rimvall, 1993). The *gad1* and *gad2* genes are responsible for coding these two isoforms, respectively. This alteration in the GABA neurotransmitter pool is important since previous findings have shown that GABA and glutamate mediate fast synaptic inhibitory and excitatory neurotransmission in the CNS, respectively, and plays a major role in epileptogenesis (Meldrum and Garthwaite, 1990; Soghomonian, 1994; Bradford, 1995; Nishimura et al., 2005) (Yudkoff et al., 2001; Kaneez and Saeed, 2009).

Although this single alteration in the glutamate-glutamine cycle may be important in the neuroprotective effects of CR and the KD, emerging evidence suggests that following periods of CR or the KD, a wide range of metabolic and neurochemical changes instead take place (Bough and Rho, 2007; Maalouf et al., 2009; Seyfried et al., 2009b). More specifically, a notable improvement in

mitochondrial function, a decrease in the expression of apoptotic and inflammatory markers, an increase in the activity of neurotrophic factors, alterations in brain energy metabolism, and changes in neuronal activity and neurotransmission pool are all to some extent thought to play an important role in the antiepileptic and anticonvulsant effect of these diets (McIlwain, 1969; Veech et al., 2001; Mazarati and Wasterlain, 2002; Veech, 2004; Yudkoff et al., 2004; Yudkoff et al., 2005; Bough and Rho, 2007; Maalouf et al., 2009; Seyfried et al., 2009b).

In order to better elucidate the mechanism of action of DER, we sought to examine the influence of the KD and CR on gene and protein expression in the EL mouse. Our results show that both CR and the KD have a multifaceted effect in the expression and regulation of various genes and proteins. More specifically, we found that the enzyme glutamic acid decarboxylase (GAD67) and the monocarboxylate transporter 1 (MCT1) may be involved in the anticonvulsant and antiepileptic mechanism of the KD in EL mice.

METHODS AND MATERIALS

Mice

The EL mouse model used in these studies has been described in the Materials and Methods section of Chapter 2. DDY, an inbred non-epileptic EL

background mouse strain, was obtained originally from Clea Japan, Inc.. All mice were maintained and housed as described in the Materials and Methods section of Chapter 2.

Mouse Tissue

At the end of each diet treatment period studying the influence of the diet in seizure susceptibility in adult and young EL mice, the brain, liver, spleen, kindney, heart, and lung, of all various dietary mouse groups were carefully dissected and stored at -80^{o}C. It should be noted that R-fed mice were not fed prior to tissue extraction. Brain tissues were further dissected and separated in left and right cortex, left and right hippocampus, left and right cerebellum, and left and right brain stem. Additional control brain samples were isolated from non-diet treated EL and DDY mice at approximately 40 days, and 360 days of age. DDY mice served as a strain control.

Antibodies, Primers, and Reagents

Anti-GLUT1 (1:3000 dilution), MCT1 (1:1000), and MCT2 (1:1000) antiserum was kindly provided as a gift from I.A. Simpson (Department of Neural and Behavioral Sciences, Hershey Medical Center, Hershey, PA). GAD67 (1:3000) was purchased from Sigma Scientific. COX2 (1:3000) was purchased

from BD Biosciences. Anti–β-actin (1:5000), -VEGF (1:1000), -BDNF (1:1000), goat anti-rabbit IgG-HRP (1:5000), and goat anti-mouse IgG-HRP (1:5000) antibodies were purchased from Santa Cruz Biotechnology, Inc.. The working dilutions for each antibody (shown in parenthesis for each protein) were made in either 5% milk or 5% bovine serum albumin in Tris-buffered Saline (TBS) containing Tween 20 (pH 7.6). Oligo (dT) primers were purchased from MWG-Biotech AG (High Point, NC).

Semi-Quantitative RT-PCR

Total RNA was isolated from either homogenized one half cerebral cortex or hippocampus tissues for each of the four dietary group described in Chapter 2 (SD-UR, SD-R, KD-UR, and KD-R) using TRIzol Reagent (Invitrogen) following the manufacturer's protocol. RNA concentration and purity was determined by spectrophotometric measurements at 260 nm and 280 nm. Single strand cDNA was synthesized from total RNA (3 µg) using oligo (dT) primers (Promega, Madison, WI, USA) in a 20 µL reaction with Moloney murine leukemia virus reverse transcriptase (M-MLV RT; Promega) according to the manufacturer's protocol. Complementary DNA (cDNA) (3 µL) was used for PCR amplification of various genes (Table 10). Gradient PCR was performed to obtain optimal primer annealing temperatures. In order to determine the optimal linear range for the amplification reaction, PCR was performed at increasing cycle numbers (see

Appendix D). PCR amplification was performed with either Taq® DNA polymerase or GoTaq® DNA polymerase (Promega) using the following protocol: initial denaturation at 94°C for 2 min, followed by the previously determined optimal number of cycles (Table 10 and Appendix D) of denaturation at 94°C for 1 min; annealing at the optimal primer annealing temperature for 30-45 sec (Table 10); extension at 72°C for 1 min; and a final extension at 72°C for 6 min following the last cycle. PCR products (5–15 µL) were separated on 0.8–1.5% agarose gels containing ethidium bromide, visualized with UV light, and analyzed using either the 1d Kodak Software (Eastman Kodak Co, Rochester, NY, USA) or the FluorChem 8900 software. RT-PCR was performed on the total RNA of each sample in the absence of reverse transcriptase to control for possible DNA contamination. The relative expression of each gene analyzed was normalized to the expression of β-actin. The difference in PCR number of cycles was also used in the normalization of each gene expression.

Western Blot Analysis

One half of the cerebral cortex or the hippocampus tissue from each dietary group were homogenized in either 500 ml or 200 ml of ice-cold 1X Lysis buffer (Cell Signaling), respectively. Lysis buffer contained 20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L Na_2EDTA, 1 mmol/L EGTA, 1% Triton, 2.5 mmol/L $NaPP_i$, 1 mmol/L -glycerophosphate, 1 mmol/L Na_3PO_4, 1 mg/mL

leupeptin, and 1 mmol/L phenylmethylsufonyl fluoride. Lysates were transferred to Eppendorf tubes, mixed for 1 hr at 4^0C, and then centrifuged at 8,100 x g for 20 min. Supernatants were then collected and protein concentrations were estimated (Bio-Rad DC assay). Either 5, 20, or 40 μg of total protein from each sample were denatured with either SDS-PAGE sample buffer (63 mmol/L Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, 0.0025% bromphenol blue, and 5% 2-mercaptoethanol) or NuPAGE® 4X LDS Sample buffer (Invitrogen) and were then loaded and resolved by SDS-PAGE on NuPAGE 4% to 12% Bis-Tris gels (Invitrogen) at 120 Volts. 40 μg of protein were loaded only for the detection of BDNF and VEGF. Proteins were transferred to a PVDF membrane overnight at 4 ^{0}C and blocked in either 5% nonfat powdered milk or 5% bovine serum albumin in Tris-buffered Saline (TBS) with Tween 20 (pH 7.6) for 1 to 3 hr at room temperature. Blots were then probed with corresponding primary antibodies overnight at 4 ^{0}C. The blots were then incubated with the appropriate animal specific whole HRP-conjugated secondary antibody for 1hr at room temperature. Protein bands were visualized using ECL-Plus chemiluminescence. Blots were thoroughly washed in TBS with Tween 20 (pH 7.6) and then reprobed with additional primary antibodies. The specific ratio of the indicated protein to β-actin was analyzed by scanning densitometry (FluorChem 8900 Software). A similar protocol was used for the analysis of the control brain samples isolated from non-diet treated EL and DDY mice at approximately 40 days, and 360 days of age.

Statistical Analysis

Both ANOVA and a two-tailed *t*-test were used to evaluate the significance of differences in gene and protein expression between unrestricted and restricted groups (Statview). Differences were considered significant at $P \leq 0.05$. All values are expressed as mean ± SEM.

RESULTS

Neuroprotective Effects of CR and the KD by Modulating Brain Glutamic Acid Decarboxylase Levels

Western blot analysis showed that the protein expression of glutamic acid decarboxylase (GAD67) was significantly higher in the cortex and the hippocampus of adult EL mice fed the KD either in restricted or unrestricted amounts compared to the SD-UR fed mice (Figure 26). KC fed either in unrestricted or restricted amounts also resulted in a similar significant increase in GAD67 expression in the cortex of young adult EL mice (Figure 27). In order to confirm that the increased GAD67 protein expression levels was related to the diet and not due to some other factors, such as age- or mouse strain-related effects, GAD67 protein expression was compared between young seizure free

EL mice, and old EL mice highly seizure susceptibility. Brain samples from DDY mice, an inbred non-epileptic EL background mouse strain, were used as additional tissue control. Our results showed no differences on GAD67 protein expression in the cortex of young adult EL mice when compared to old EL and DDY mice (Figure 28). This latter finding suggests no influence of age or mouse strain on GAD67 protein expression.

To further validate any influence of the diet on GAD expression, *gad1* mRNA expression was analyzed in diet-treated EL mice. The results showed that although *gad1* mRNA expression was slightly increased in the KD groups compared to the normal control SD-UR mice, this change did not reach a significance level (Figure 29). Contrary to the effect of the KD on GAD67 protein expression, restriction of the SD had no effect on GAD67 or *gad1* expression (Figures 26 and 29). Furthermore, we sought to determine whether our system is able to detect accurately even the smallest changes on protein expression levels. For this purpose, a standard curve of increasing amounts of protein were loaded on a SDS-PAGE gel (similarly to the protocol described in the methods above) and the levels of GAD67 and β-actin were compared. With the linear range of detection of our system being between 5-20 μg of protein (Figure 30), we are confident whatever changes observed are real.

Overall, these data suggest that the KD resulted in a significant increase in the protein expression of GAD67 in EL mice, and thus may play a role in the anticonvulsant properties of the KD.

Influence of CR and the KD in the Expression of MCT1 and GLUT1

Western blot analysis was also used to examine the influence of the diet on the protein expression of MCT1 in the cortex and hippocampus of adult EL mice. Although both the KD fed unrestricted and restriction of either the KD or the SD resulted in a slight increase on the expression of MCT1 in both the cortex and the hippocampus of adult EL mice compared to the MCT1 levels in the SD-UR mice, this effect was more evident in the hippocampus (Figure 31). In particularly, the hippocampus of both the SD-R and KD-UR groups had a significantly higher expression of MCT1 levels compared to the the SD-UR mice (Figure 31). No significant differences in the expression of MCT1 were observed in the cortex of adult EL mice.

Similarly, in order to confirm that the changes in MCT1 protein expression was related to the diet and not due to some other factors, such as age- or mouse strain-related effects, MCT1 protein expression was compared between young seizure free and old highly seizure susceptibility EL mice, and DDY mice. Our results, although not significant, indicate that young EL mice have a higher expression of MCT1 compared to both old EL and DDY mice (Figure 32). This finding is consistent with the developmental profile of MCT1 expression in mice having just recently been weaned from their mother. Furthermore, the diet had

no effect in the gene expression of glut1 mRNA (Figure 33). Viewing these results together, we can suggest that both CR and the KD may up-regulate MCT1 in the brain of EL mice.

Influence of CR and the KD in Neuroinflammation

Using RT-PCR, we sought to examine any potential effect that the diets may have on neuroinflammation. Our results show that both the KD and CR had no effect in modulating inflammation in the cortex of adult EL mice (Figure 34). The only interesting trend we observed is a slight reduction of CD68 in the cortex of the KD-R group. These findings suggest that both the KD and CR do not directly influence neuroinflammatory markers in EL mice.

DISCUSSION

In this study, we sought to further investigate the underlying anticonvulsant and neuroprotective mechanism of CR and the KD in EL mice. Our results are somewhat consistent with previous reports showing that both GAD67 and MCT1 may play a role in the neuronal and metabolic modulation that occurs in the diseased brain during various dietary regimes (Nehlig and Pereira de Vasconcelos, 1993; Cheng et al., 2003; Yudkoff et al., 2004; Yudkoff et al.,

2005; Bough and Rho, 2007; Nehlig et al., 2009). Specifically, we show for the first time that EL mice fed the KD in either restricted or unrestricted amounts resulted in an increase in cortical and hippocampal expression of GAD67 compared to the mice fed the SD unrestricted. Similarly GAD67 was increased in young EL mice fed the commercially available KC compared to the SD-UR mouse group.

It has been previously shown that GABA, a major inhibitory neurotransmitter of the brain, is involved in both the regulation of neuroendocrine function, as well as mediates glutamate fast synaptic inhibitory and excitatory neurotransmission in the CNS, and thus plays a major role in epileptogenesis (Soghomonian, 1994; Bradford, 1995; Meldrum, 1996; Nishimura et al., 2005) (Yudkoff et al., 2001; Kaneez and Saeed, 2009). Specifically, it was shown that GAD67 mRNA levels are increased after lesions of dopaminergic afferent neurons in the striatum, or in the hippocampus of kainic-acid induced rats, and even in humans with temporal lobe epilepsy (Feldblum et al., 1990; Soghomonian and Chesselet, 1992; Baran et al., 2004; Malfatti et al., 2007; Yamamoto and Soghomonian, 2009). In contrast to these findings, our gene expression analysis showed that *gad1* was not significantly higher in the seizure susceptible SD-UR group compared to the other three dietary treated mice. This latter finding is somewhat also confirmed from our GAD67 protein analysis, where no significant differences were observed in GAD67 expression between old, highly seizure susceptible EL mice, and young, seizure free EL mice. These

findings suggest that in this natural animal model of epilepsy, the increase in cerebral GAD67 protein levels may be a result of the diet and not an effect of the epileptic EL brain. Thus, we can suggest that the neuroprotective effect of the KD may be by acting on brain excitability via an alteration in glutamate and GABA levels. In particular, the KD may accelerate the flux through glutamate decarboxylase; hence, increasing the concentration and rate of formation of GABA and reducing epileptic hyperexcitability (Erecinska et al., 1996; Yudkoff et al., 2001).

Furthermore, it has been suggested by others that convulsions may arise from either an impairment of GABAergic, or excessive glutamatergic function (Scheyer, 1998; Treiman, 2001; Mody and Pearce, 2004). Since the brain expression of GAD67 (*gad1)* was similar between young and old EL mice, we can suggest that seizures in the EL mice aren't a result of abnormalities in GABAergic function. This finding is further supported by our previous work, where no aberrant GABA levels were measured in the brains of EL mice (Flavin et al., 1991; Flavin and Seyfried, 1994). Interestingly, reports of altered gene expression in epilepsy are inconsistent, probably due to discrepancies from the experimental models used. Seizure-induced nonspecific pathological changes, such as surgical lesion, hypoxia, stress, and cell degeneration may also induce alterations in gene expression that may be difficult to separate from effects of hyperactivity (Fengyi Liang and Jones E.G, J Neurosci. 1997). Immunoreactivity, mRNA levels, and/or receptor binding for glutamic acid decarboxylase (GAD),

NMDA receptor and/or AMPA receptor subunits have been reported to be increased (Feldblum et al., 1990; Najlerahim et al., 1992; Pollard et al., 1993; Kamphuis et al., 1994; Kraus et al., 1994; Marianowski et al., 1995), unchanged (Lerner-Natoli et al., 1985; Akiyama et al., 1992; Friedman et al., 1994; Gerfen-Moser et al., 1995), or decreased (Ribak et al., 1979; Gall et al., 1990; Akiyama et al., 1992; Mitsuyoshi et al., 1993; Obenaus et al., 1993; DeFelipe et al., 1994; Friedman et al., 1994; Lee et al., 1994; Bayer et al., 1995; Prince et al., 1995).

In previous studies, it was reported that MCT1 protein and mRNA levels to be significantly increased during ketosis (Leino et al. 2001;(Noh et al., 2004). Although our changes in MCT1 expression were not as pronounced as those previously described, the KD was able to increase MCT1 expression in EL mice fed either the KD in restricted or unrestricted amounts. In particular, MCT1 expression was significantly different in the hippocampus of the KD-UR and SD-R mouse groups compared to the control SD-UR mouse group. This finding may support the notion that a metabolic transition in brain utilization of different energy substrates (away from glycolysis to beta-oxidation) has take place, and the up-regulation of MCT1 expression would facilitate the transport of ketone bodies in the brain as a source of energy (Owen et al., 1967; Clarke and Sokoloff, 1999). MCT1 has long been considered a predominant factor in determining the rate at which the brain can use ketone bodies and the capacity of transport at the level of blood brain barrier endothelial cells (Halestrap and Meredith, 2004).

Based on the findings presented in Chapter 2, it was observed that the R-fed mice as well as the KD-UR mice had significantly high plasma ketone body levels (Figures 6). Hence the increased ketone levels observed in these mice along with the slight increase in MCT1 levels in the brain of the same EL mice may further support a metabolic transition in brain energy substrate utilization. Although, in this current analysis the KD and CR had no effect on *glut*1 expression, these findings are consistent with previous reports. Interestingly, such a report recently showed that the KD did not affect the level of expression of the GLUT1 or MCT1 and MCT2, in SWD rats (Nehlig et al., 2009). Furthermore, a restricted KD was able to increase only parenchymal and not endothelial *glut*1 expression in young rats (Cheng et al 2004 Eagles).

Finally, previous findings have shown inflammation to be linked with seizure activity and vice versa (Jankowsky and Patterson, 2001; Peltola et al., 2001; Ravizza et al., 2005; Vezzani, 2005b, a), and DR to differentially influence pro-and anti-inflammatory markers (Cullingford, 2004; Branch-Mays et al., 2008; Crujeiras et al., 2008; Pan et al., 2008; Jung et al., 2009; Reynolds et al., 2009). On Chapter 1, it was mentioned that the EL mouse is associated with gross neurochemical and neuropathological symptoms associated with human idiopathic epilepsy (Flavin et al., 1991; Murashima et al., 1992; Flavin and Seyfried, 1994; Lambert et al., 1996; Fueta et al., 1998; Drage et al., 2002; Todorova et al., 2006). In this current mRNA analysis expression of the glial fibrillary acidic protein (GFAP) although present in the EL brain, it was not

differentially affected by either the KD or CR. Also both the KD and CR had no effect in modulating the expression of the pro-inflammatory cytokine CD68. Taken these findings together, although, the anticonvulsant effect of the KD and that of CR does not seem to involve a reduction in GFAP or CD68 associated neuroinflammation, we can not exclude the influence of an inflammatory component in the development and progression of seizures in the EL mouse.

Overall these data suggest that the anticonvulsant and antiepileptogenic effect of both the KD and that of CR in managing seizures in both young and old EL mice, is dependent on the reduction of glucose levels and the subsequent increase in ketone body levels. This metabolic transition from glycolysis to beta-oxidation seems to result in the alteration of the glutamate-glutamine cycle facilitated by an increase in GAD67 levels, thus increasing the production of GABA in the synapses of neurons. This increase in GABA results in the hyperpolarization of synaptic membranes and consequently a decrease in neuronal excitability (seizures). Despite these intriguing observations, the exact mechanism(s) for the neuroprotective effect of the KD and that of CR are not yet clear and remain(s) to be elucidated in future studies.

Table 13. RT-PCR: Sequence of Primers and PCR Conditions

Gene	GenBank Accession	Primer sequence	Nucleotide position[a]	Annealing Temp. °C	Annealing Time (sec)	Product size (bp)	Cycle Number
b-actin[b]	NM007393	For 5'-TGT GAT GGT GGG AAT GGG TCA G-3' Rev 5'-TTT GAT GTC ACG CAC GAT TTC C-3'	126-639	58	35	514	22
NPY	NM_023456	For 5'-GAT GCT AGG TAA CAA GCG AATG G-3' Rev 5'-CAC ATG GAA GGG TCT TCA AGC C-3'	73-363	64	64	291	28
NPYr1	NM_010934	For 5'-GTC ACT TGC GGC GTT CAA GGA C-3' Rev 5'-GAA GAT GGT AAG GGG CAG CCA G-3'	558-843	65	30	286	28
NPYr5	AF049329	For 5'-CTG TCT GGA CAC TGG GCT TTG-3' Rev 5' -GGT AAC ACG CAT GCC GTC TTC-3'	548-956	65	30	409	30
GFAP	NM_010277	For 5'-TGG TCG AGC TGG ACG GCG ACG-3' Rev 5' -GTC ACG AAC TCC AGC AGG ACC ATG-3'	137-844	70	30	708	28
GAD2	NM_008078	For 5'-GGA TTG GAT ATG GTT GGA TTA GC-3' Rev 5'-CAG ATG TCA GCT ACA GCC AAG-3'	553-1061	67	30	509	28
GAD1	AF326547	For 5'-CCG GCG CAC AGA GAC CGA CTT C-3' Rev 5'-GTT TGG GCA CAG CCG CCA TGC C -3'	261-841	70	30	581	29
CD68	BC021637	For 5'-CAT CCT TCA CGA TGA CAC CTA CAG-3' Rev 5'-CTC TGA TGT AGG TCC TGT TTG AAT C-3'	95-638	65	35	544	27
Glut1	M23384	For 5'-CAC CAG CTG GGA ATC GTC GTT G-3' Rev 5'-CCA AAG ATG GCC ACG ATG CTC AG-3'	667-1310	70	30	644	28
SCOT	AB085609	For 5'-CTT CGA AAT TAT ACT CAA AAT ATA G-3' Rev 5'-CAC GAT GGA GAG AAC AAT GTC TAT G-3'	393-1346	67	35	954	27

[a]Primer nucleotide positions are based on the CDS of each given gene

[b]Primer sequence for b-actin is based upon Kitakata et al., 2002

Figure 26. Influence of CR and the KD on GAD67 Protein Expression in the Cortex (*A*), and Hippocampus (*B*), of Adult EL Mice. Degree of restriction was aimed to produce a 20-23% body weight reduction. Values are expressed as the ratio of the normalized protein intensity to 1, relative to the normalized intensity of β-actin, and are represented as mean ± SEM. Mean protein expression of GAD67 in the KD-fed groups was significantly higher than that in the SD-UR mice (*$P < 0.05$, Student's *t*-test). 5-20 μg of protein were loaded for each sample. For positive controls, fasted brain (F), or Heart (H) samples were used, whereas brain CT-2A tumor (T) samples served as our negative control. Conditions of dietary treatment are shown in Figure 3; (n = 3 mice per group for both cortex and hippocampus analysis).

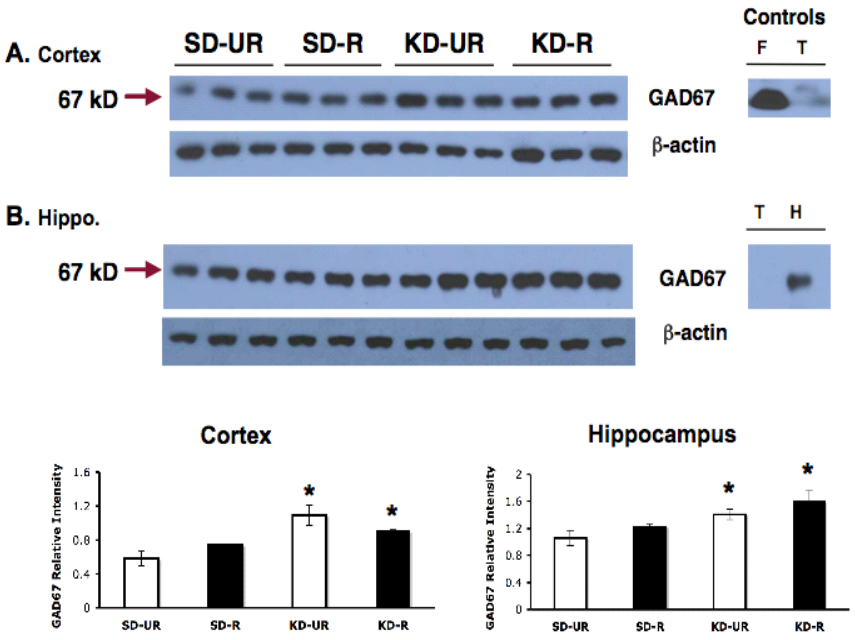

A. Cortex
SD-UR
SD-R
KD-UR
KD-R
Controls
F
T
67 kD
GAD67
β-actin
B. Hippo.
T
H
67 kD
GAD67
β-actin
Cortex
Hippocampus
GAD67 Relative Intensity
SD-UR
SD-R
KD-UR
KD-R

Figure 27. Influence of CR and the KC Diet on GAD67 Protein Expression in the Cortex of Young Adult EL Mice. Degree of restriction was such to produce a 15-18% body weight reduction. Mean protein expression of GAD67 in the KC-fed groups was significantly higher than that in the SD-UR mice (*$P < 0.05$, Student's *t*-test). 5-20 μg of protein were loaded for each sample. For positive and negative controls, fasted brain (F), or Tumor (T) were used respectively. Conditions of dietary treatment are shown in Figure 9; (n = 4 mice per group for both cortex and hippocampus analysis). Similar protein normalization was performed as described in Figure 26.

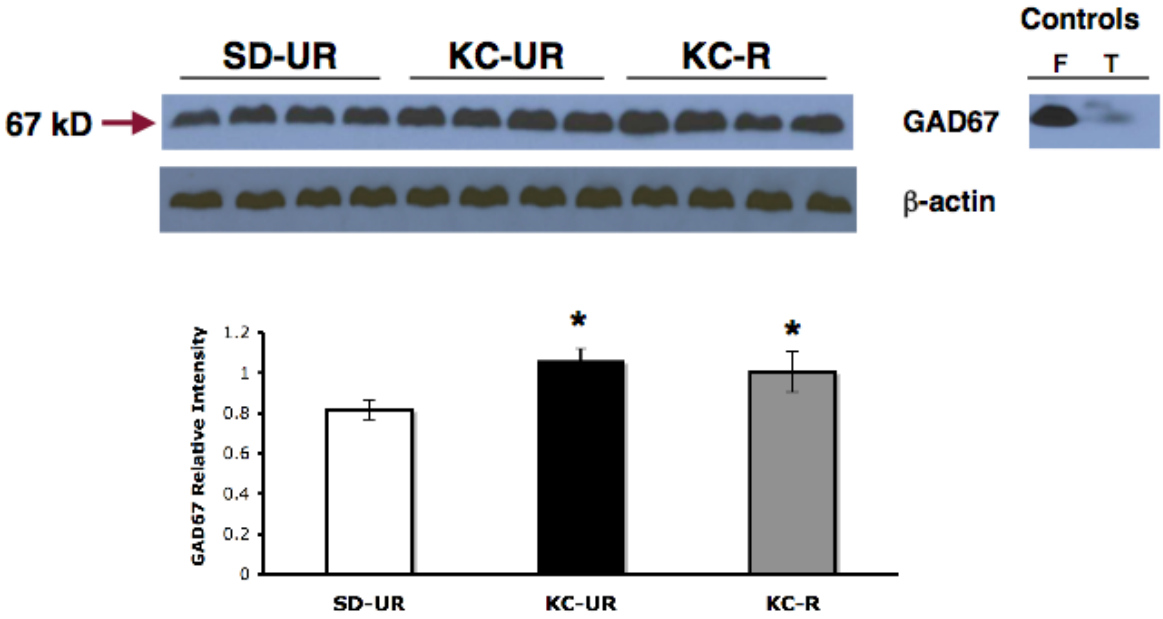

SD-UR
KC-UR
KC-R
Controls
F
T
67 kD
GAD67
β-actin
GAD67 Relative Intensity
1.2
1
0.8
0.6
0.4
0.2
0
*
*
SD-UR
KC-UR
KC-R

Figure 28. Comparative Analysis of GAD67 Protein Expression in the Cortex of Young and Old EL Mice. Although, the mean protein expression of GAD67 was slightly higher in the older mice, this difference was not significant. 5-20 μg of protein were loaded for each sample; (n = 3 - 4 mice per group). Similar protein normalization was performed as described in Figure 26.

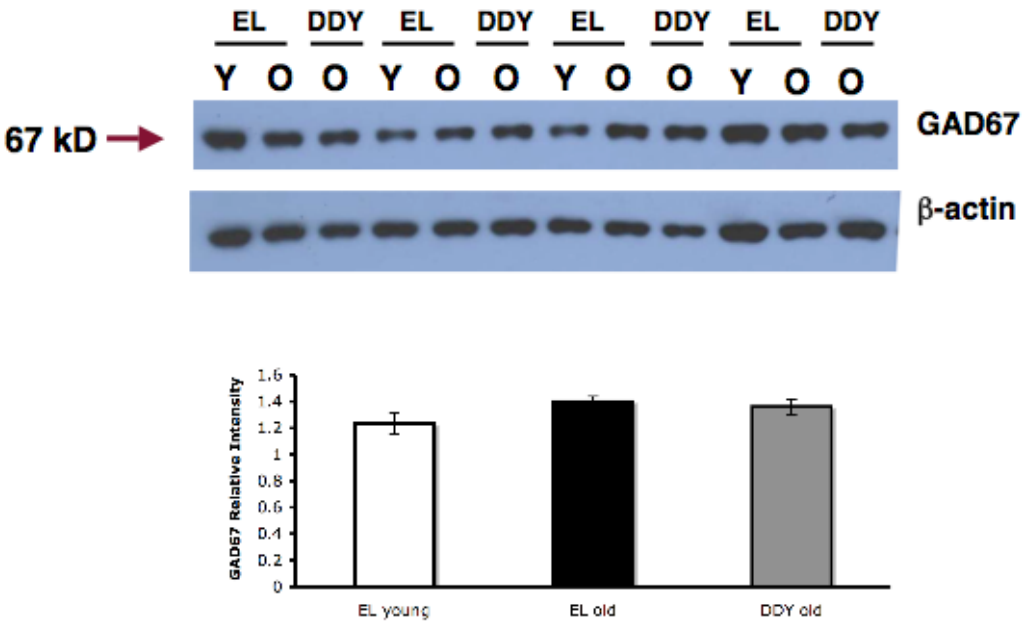
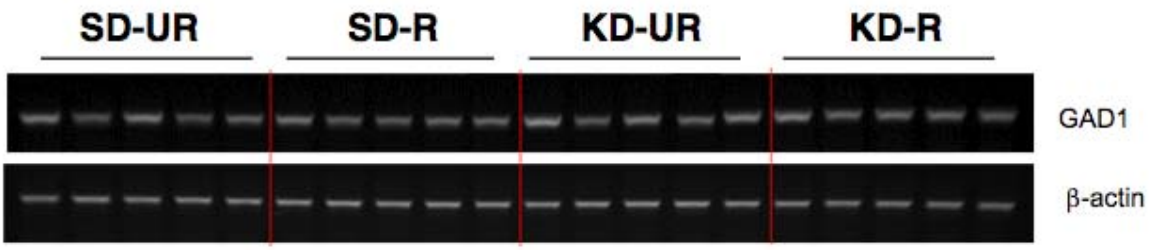

EL DDY EL DDY EL DDY EL DDY
Y O O Y O O Y O O Y O O
67 kD
GAD67
β-actin
GAD67 Relative Intensity
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
EL young
EL old
DDY old

Figure 29. Influence of CR and the KD on *gad1* Gene Expression in the Cortex of Adult EL Mice. Although the mean gene expression of *gad1* in the KD-fed groups was higher compared to the *gad*1 expression in the SD-UR mice, this difference was not significant. Values are expressed as the ratio of each sample's normalized gene expression intensity to 1, relative to the normalized intensity of β-actin after corrected for the difference in PCR cycle number, and are represented as mean ± SEM. Conditions of dietary treatment are shown in Figure 3; (n = 5 mice per group for both cortex and hippocampus analysis).


SD-UR
SD-R
KD-UR
KD-R
GAD1
β-actin

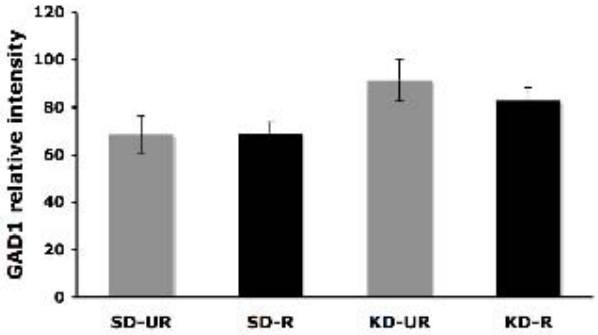

GAD1 relative intensity
120
100
80
60
40
20
0
SD-UR
SD-R
KD-UR
KD-R

Figure 30. Testing the Linear Range of Protein Detection in our Western Blot System. Increasing amounts of protein were loaded on an SDS-PAGE gel and the linearity of our chemiluminescence detection was measured for GAD67 and β-actin. Individual protein intensities (levels) are shown in Appendix E. Our data indicate that the detection of GAD67 is linear up to 40 μg of protein. Similar protein normalization was performed as described in Figure 26.

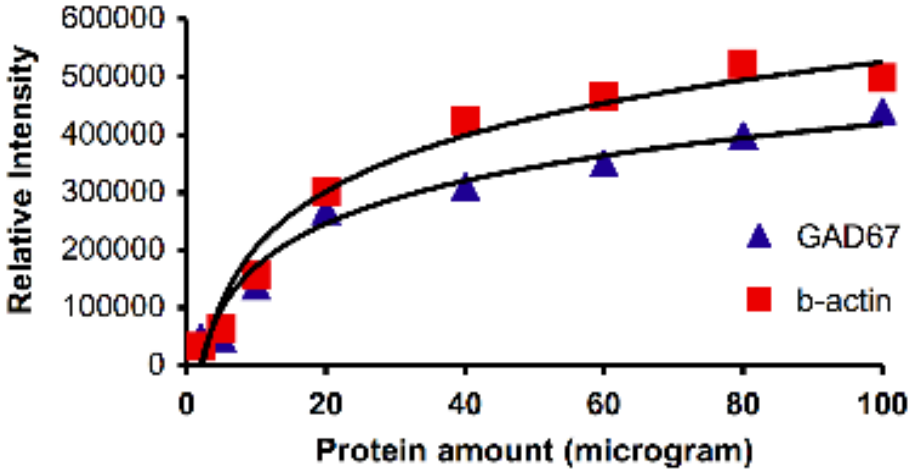

600000
500000
400000
300000
200000
100000
0
Relative Intensity
0
20
40
60
80
100
Protein amount (microgram)
GAD67
b-actin

Figure 31. Influence of CR and the KD on MCT1 Protein Expression in the Cortex (*A*), and Hippocampus (*B*), of Adult EL Mice. Degree of restriction was aimed to produce a 20-23% body weight reduction. The mean protein expression of MCT1 in the hippocampus was significantly higher in the KD-UR and SD-R mouse groups compared to the SD-UR mice MCT1 expression (*$P < 0.05$, Student's *t*-test). 5-20 μg of protein were loaded for each sample. For positive controls, fasted brain (F), or Spleen (S) samples were used, whereas Lung (L) samples served as a partial negative control, since MCT1 is partially (slightly) expressed in lung. Conditions of dietary treatment are shown in Figure 3; (n = 3 mice per group for both cortex and hippocampus analysis). Similar protein normalization was performed as described in Figure 26.

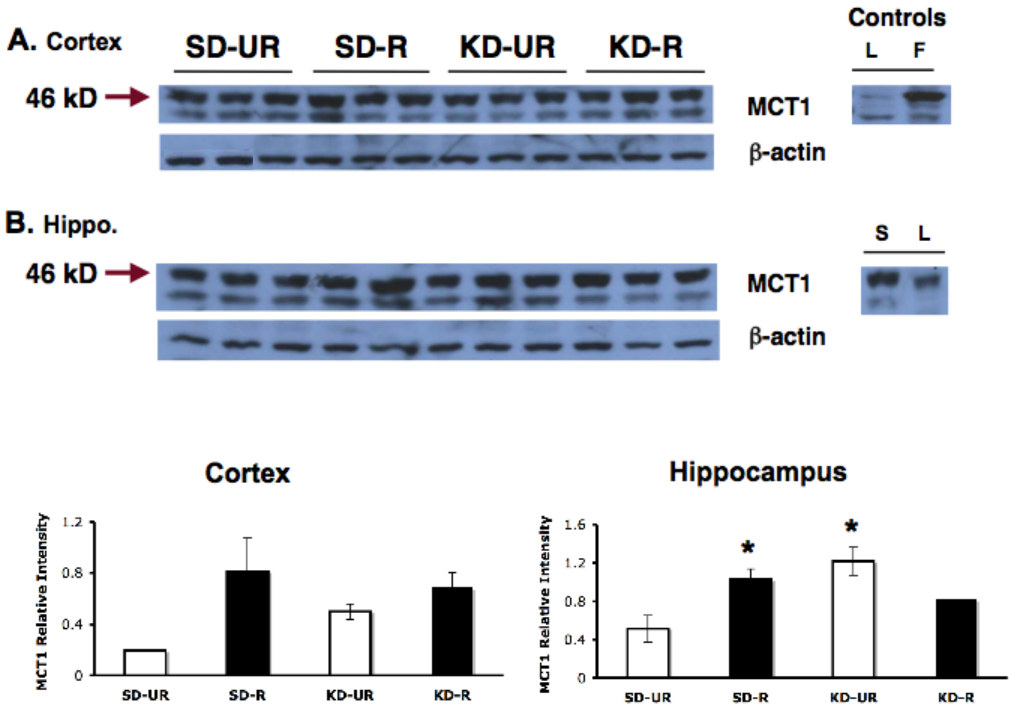
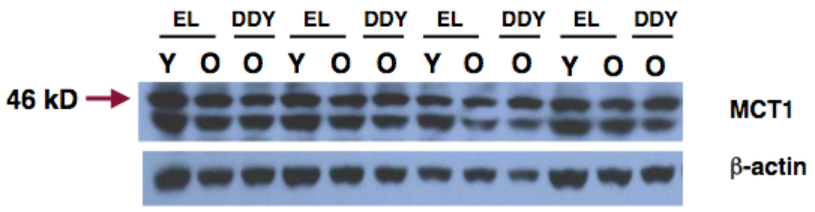

A. Cortex
SD-UR
SD-R
KD-UR
KD-R
Controls
L
F
46 kD
MCT1
β-actin
B. Hippo.
S
L
46 kD
MCT1
β-actin
Cortex
MCT1 Relative Intensity
1.2
0.8
0.4
0
SD-UR
SD-R
KD-UR
KD-R
Hippocampus
MCT1 Relative Intensity
1.6
1.2
0.8
0.4
0
*
*
SD-UR
SD-R
KD-UR
KD-R

Figure 32. Comparative Analysis of MCT1 Protein Expression in the Cortex of Young and Old EL Mice. Although, the mean protein expression of MCT1 was slightly lower in the older mice these values did not reach significance. 5-20 μg of protein were loaded for each sample; (n = 3 - 4 mice/group). Similar protein normalization was performed as described in Figure 26.


EL DDY EL DDY EL DDY EL DDY
Y O O Y O O Y O O Y O O
46 kD
MCT1
β-actin

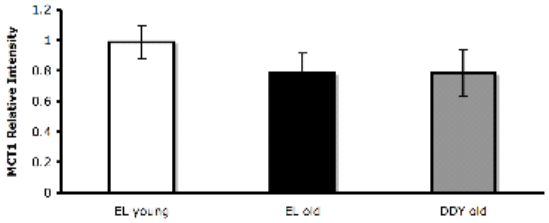

MCT1 Relative Intensity
1.2
1
0.8
0.6
0.4
0.2
0
EL young
EL old
DDY old

Figure 33. Influence of CR and the KD on *glut1* Gene Expression in the Cortex of Adult EL Mice. Although the mean gene expression of *glut1* was higher in all 3 dietary groups compared to the *glut1* in the SD-UR mice, this difference was not significant. Conditions of dietary treatment are shown in Figure 3; (n = 5 mice per group for both cortex and hippocampus analysis). Similar gene normalization was performed as described in Figure 29.

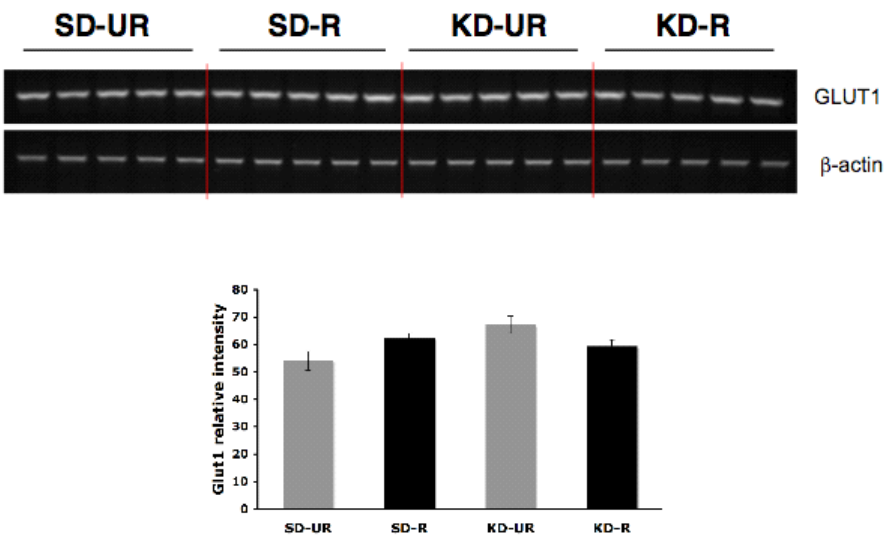
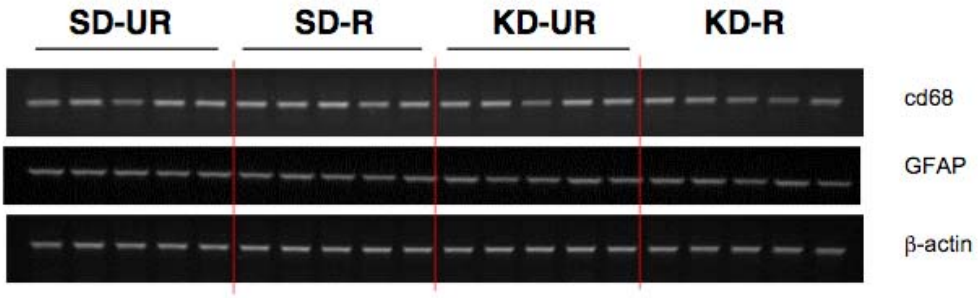
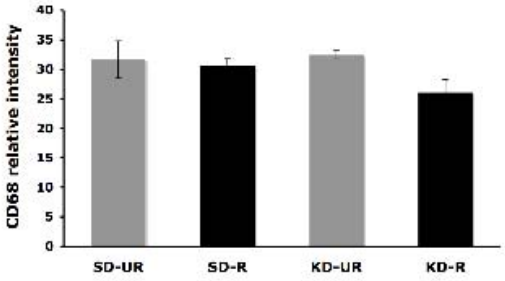
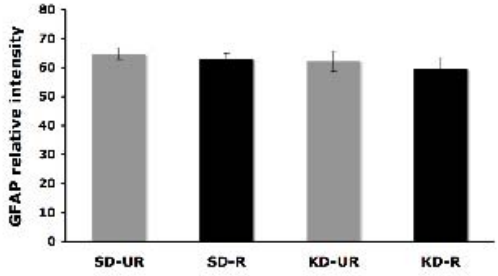

SD-UR
SD-R
KD-UR
KD-R
GLUT1
β-actin
Glut1 relative intensity
80
70
60
50
40
30
20
10
0
SD-UR
SD-R
KD-UR
KD-R

Figure 34. Influence of CR and the KD on Neuroinflammation. Cortex expression of *CD68* (*A*), and *GFAP* (*B*), of adult EL mice fed the KD or SD diet in unrestricted or restricted amounts. The mean gene expression of both *CD68* and *GFAP* was the same for all groups. Conditions of dietary treatment are shown in Figure 3; (n = 5 mice per group for both cortex and hippocampus analysis). Similar gene normalization was performed as described in Figure 29.


SD-UR
SD-R
KD-UR
KD-R
cd68
GFAP
β-actin


CD68 relative intensity
40
35
30
25
20
15
10
5
0
SD-UR
SD-R
KD-UR
KD-R


GFAP relative intensity
80
70
60
50
40
30
20
10
0
SD-UR
SD-R
KD-UR
KD-R

CHAPTER SEVEN

CONCLUSION

Epilepsy is a disabling chronic and neurological disorder involving recurrent abnormal discharges of neurons that produce epileptic seizures (Engel and Pedley, 1997; Johnston and Smith, 2008), and afflicts about 1% of the US population. Despite intensive antiepileptic drug (AED) research, seizures remain unmanageable in many persons with epilepsy (Jallon, 1997; Freeman et al., 2000; Browne and Holmes, 2001). As an alternative to AEDs diet therapies have been shown to be effective in the management or control of epilepsy.

My dissertation research tested the therapeutic efficacy of different dietary regimes, such as calorie restriction (CR) and the ketogenic diet (KD), in the management of both neurological and neurodegenerative diseases, including epilepsy and a mouse model of Rett Syndrome. We successfully investigated the relationship among ketones, glucose, and seizure susceptibility under long-term antiepileptic diet therapies, and provided new evidence in the neuroprotective mechanism(s) of CR and the KD.

Implementation of CR and the KD in adult seizure susceptible EL mice resulted in the seizure control of EL seizures. This neuroprotective effect of the KD was mainly controlled through CR (Mantis et al., 2003; Mantis et al., 2004). Also seizure susceptibility in EL mice was dependent on circulating plasma glucose levels and that seizure control in EL mice depended more on the amount

than on the origin of dietary calories (Mantis et al., 2003; Mantis et al., 2004). A reduction of glucose and a subsequent increase in ketone bodies resulted in the zone of seizure management in the EL mice. A transition from glucose to ketone bodies for energy was predicted to manage EL epileptic seizures through multiple integrated changes of inhibitory and excitatory neural systems.

KetoCal®, a commercially available KD, was later evaluated for its antiepileptic and antiepileptogenic efficacy in young adult EL mice. The results supported our previous findings that seizure control in the EL mice is associated more with the amount rather than the origin of dietary calories. Also KC has both an anticonvulsant and antiepileptogenic effect in EL mice. Furthermore, my thesis for the first time showed that glucose supplementation in the drinking water of restricted mice prior to seizure testing resulted in the amelioration of the anticonvulsant efficacy of CR in a natural model of epilepsy, the EL mouse. This thesis also indicated that an unrestricted KC diet was able to reduce the seizure severity in young adult EL mice. Interestingly, this anticonvulsant effect of the KC was not depended on reduced glucose levels.

Based on the above finding with the EL mouse, next we examined the therapeutic efficacy of the KD and CR in a mouse model of Rett Syndrome. The results in this thesis showed that colorie restricted diets had a positive influence on the anxiety behavior and motor characteristics in $Mecp2^{308/y}$ mice. In particular, CR and the KD reduced the anxiety associated with the exploration of an unfamiliar environment (Mantis et al., 2009). These findings indicate that

calorically restricted diets may be of clinical importance in improving various aspects of the behavior in individuals with Rett.

Although comparisons of a variety of age-related changes have been made between energy-restricted and unrestricted animals in an effort to identify the specific physiological and biochemical processes that may mediate the improvement of the disease phenotype, many studies fail to provide a standardized regimen for diet implementation. This thesis sought to address some of the issues of diet implementation in disease management and to provide guidelines for data interpretation. The points raised in this thesis will help facilitate data analysis across various disease animal models and studies, as well as provide insight on the mechanism(s) by which restricted diet therapies might manage neurological and neurodegenerative diseases. Moreover, these diet implementation guidelines, on a “standard” calorically restricted paradigm for disease animal models, should maximize the therapeutic efficacy of these diets while facilitating cross-study comparisons and data interpretation.

Finally, this thesis discussed the potential biochemical mechanism(s) by which CR and the KD might reduce seizure susceptibility in EL mice. We suggest that the transition from glucose to ketone bodies as a major energy fuel for the brain produces multiple changes in gene-linked metabolic networks. It is these changes that gradually adjust neurotransmitter pools and membrane excitability to restore the physiological balance of excitation and inhibition (Greene et al., 2003). This thesis showed that the metabolic transition from

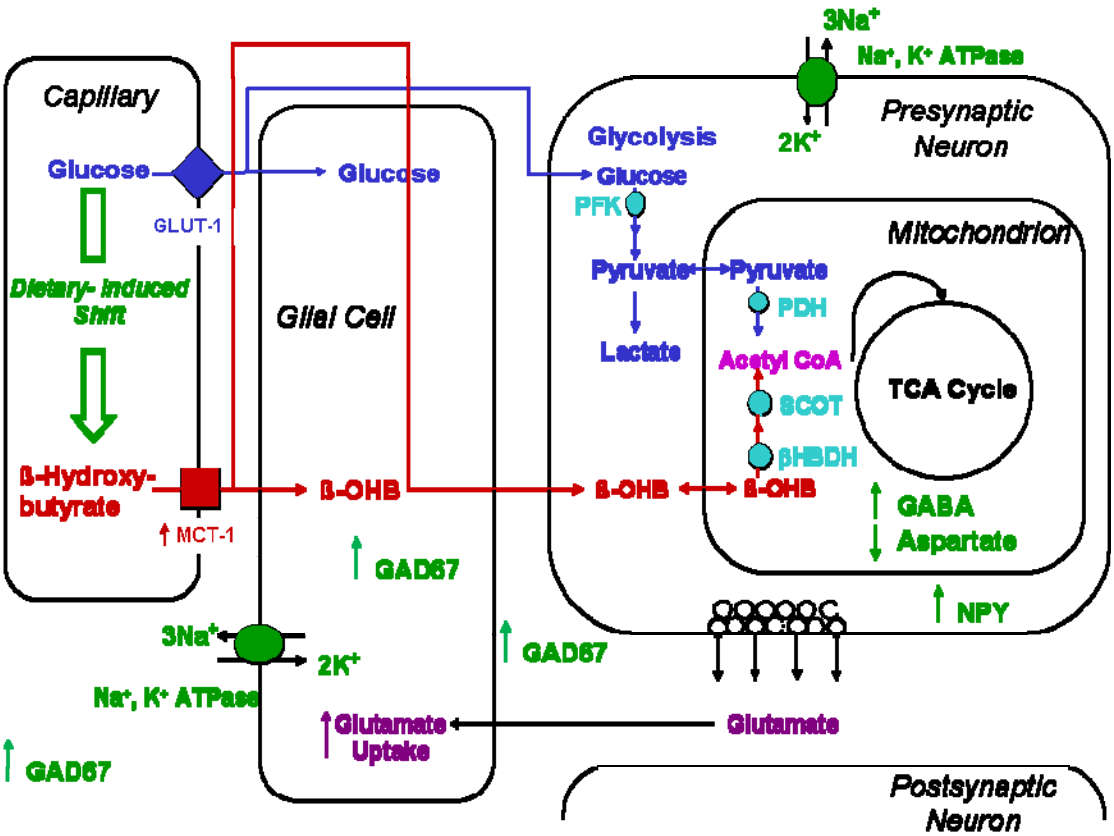
glycolysis to beta-oxidation seems to result in the alteration of the glutamate-glutamine cycle facilitated by an increase in GAD67 levels, thus increasing the production of GABA in the synapses of neurons. This increase in GABA results in the hyperpolarization of synaptic membranes and consequently a decrease in neuronal excitability (seizures). Interestingly, while the levels of γ-aminobutyric acid (GABA) are increased in synaptosomes via the increased action of glutamic acid decarboxylase during the metabolism of ketone bodies for energy, the levels of aspartate decrease due to the formation of glutamate (Yudkoff et al., 2001).

In addition, this thesis showed that MCT1 expression was significantly different in the hippocampus of the KD-UR and SD-R mouse groups compared to the control SD-UR mouse group. This finding may support the notion that a metabolic transition in brain utilization of different energy substrates (away from glycolysis to beta-oxidation) has take place, and the up-regulation of MCT1 expression would facilitate the transport of ketone bodies in the brain as a source of energy. CR could also influence seizure susceptibility through the neuroendocrine system involving leptin signaling and increased levels of neuropeptide-Y, a peptide with antiepileptic and anticonvulsant effects (Mazarati and Wasterlain, 2002; Colmers and El Bahh, 2003; Husum et al., 2004; Richichi et al., 2004). In addition, ketone body metabolism could increase membrane ionic pump activity (Kaur and Kaur, 1990; Veech et al., 2001). Increased pump activity could increase membrane potential in neurons while also increasing neurotransmitter uptake in glia (Greene et al., 2003). We do not exclude the

possibility that CR may reduce seizure susceptibility in EL mice through additional mechanisms (Schwartzkroin, 1999; Stafstrom and Bough, 2003). Some of the cellular systems that are described above and potentially are modulated through CR to influence brain excitability are illustrated in Figure 35 (Mantis et al., 2004).

Despite these intriguing observations, the exact mechanism(s) for the neuroprotective effect of the KD and that of CR are not yet clear and remain(s) to be elucidated in future studies. Nevertheless, it is our contention that CR reduces seizure susceptibility through multiple integrated systems providing a multifactorial therapy to a multifactorial disease.

Figure 35. Perspectives on the Metabolic Management of Epilepsy Through a Dietary Reduction of Glucose and Elevation of Ketone Bodies. A dietary reduction in blood glucose levels will increase ketone utilization for energy. This is expected to shift the neural environment from excitation to inhibition through multiple integrated systems. Abbreviations: GLUT-1 (glucose transporter), MCT (monocarboxylate transporter), PFK (phosphofructokinase), PDH (pyruvate dehydrogenase), SCOT (succinyl-CoA-acetoacetate-CoA transferase), β-OHB (β-hydroxybutyrate), β-HBDH (β-hydroxybutyrate dehydrogenase), NPY (Neuropeptide Y), GABA (gamma-aminobutyric acid). The figure is modified from Mantis et al., *Nutr Metab* (London), 1(1):11, 2004 .



Modified from Mantis et al., 2004

APPENDIX A.

PUBLICATIONS RELATED TO DISSERTATION

Mantis JG, Marsh J, Ta L, and Seyfried TN. Implementation of calorically restricted diets for the management of neurological and neurodegenerative disease in murine models (in preparation).

Mantis JG, Zimick NC, Centeno NA, and Seyfried TN. Glucose reduces the antiepileptic and anticonvulsant effects of the ketogenic diet in EL mice (in preparation).

Mantis JG, Fritz CL, Marsh J, Heinrichs SC, and Seyfried TN. (2009) Improvement in motor and exploratory behavior in Rett syndrome mice with restricted ketogenic and standard diets. *Epilepsy Behav*. **15**:133-141

Mantis JG, Centeno NA, Todorova MT, McGowan R, and Seyfried TN. (2004) Management of multifactorial idiopathic epilepsy in EL mice with caloric restriction and the ketogenic diet: role of glucose and ketone bodies. *Nutr Metab* (Lond). Oct 19; **1**(1):11.

Other Publications

Meidenbauer J, **Mantis JG**, and Seyfried TN. The EL Mouse: A Natural Model of Autism and Epilepsy (in preparation).

Seyfried TN, **Mantis JG**, Todorova MT, Greene AE. Amanda E. (2009) Greene Dietary Management of Epilepsy: Role of Glucose and Ketone Bodies, in Encyclopedia of Basic Epilepsy Research (Schwartzkroin P. ed) Vol. 2, pp 687-693. Academic Press, Oxford.

Seyfried TN, Heinecke KA, **Mantis JG**, Denny CA. (2008) Brain lipid analysis in mice with Rett syndrome. *Neurochem Res*. Jun;**34**(6):1057-65.

Zhou W, Mukherjee P, Kiebish MA, Markis WT, **Mantis JG**, Seyfried TN. (2007) The calorically restricted ketogenic diet, an effective alternative therapy for malignant brain cancer. *Nutr Metab* (Lond). Feb 21;**4**:5.

Todorova MT, **Mantis JG**, Le M, Kim CY, Seyfried TN. (2006) Genetic and environmental interactions determine seizure susceptibility in epileptic EL mice. *Genes Brain Behav*. Oct;**5**(7):518-27.

ABSTRACTS

Mantis JG, Meidenbauer JJ, and Seyfried TN. (2009) The EL Mouse: A Natural Model of Autism and Epilepsy *Epilepsia* **50** (S11), 372.

Mantis JG, Zimick NC, McGowan R, and Seyfried TN. (2006) Efficacy of KetoCal® as a diet therapy for seizure management in mice with idiopathic generalized epilepsy. *Epilepsia* **47** (S4), 225-226.

Todorova MT, **Mantis JG**, Le M, Kim CY, Seyfried TN. (2005) Gene-environmental interactions determine seizure susceptibility in epileptic mice. *Epilepsia* **46** (S8), 300.

Mantis JG, Kim CY, Centeno N, McGowan R, and Seyfried TN. (2005) Influence of diet on gene expression and seizure susceptibility in multifactorial idiopathic epilepsy. *Epilepsia* **46** (S8), 293-294.

Mantis JG, Kim CY, Centeno N, McGowan R, and Seyfried TN. (2004) Gene-environmental interactions in the metabolic control of multifacactorial idiopathic epilepsy. *Epilepsia* **45** (S7), 211-212.

Mantis JG, Kim CY, Centeno N, McGowan R, and Seyfried TN. (2004) Control of multifactorial idiopathic epilepsy with the ketogenic diet and caloric restriction. JAX Neurogenetics Conference

Mantis JG, Centeno N, Todorova MT, McGowan R, and Seyfried TN. (2003) Metabolic control of epilepsy in adult EL mice with the ketogenic diet and caloric restriction. *Epilepsia* **44** (S9), 64-65.

APPENDIX B.

Sequencing Analysis of GluR4 in EL mice

INTRODUCTION

Glutamate and activation of its four different types of receptors (AMPA, NMDA, kainate, and metaboropic) play a critical role in brain development, such as synaptic plasticity, neuronal migration, and synaptogenesis, as well as neurotoxicity (Meldrum and Garthwaite, 1990; Collard et al., 1993; Yen et al., 1993; Behar et al., 1994; Dingledine et al., 1999; Ritter et al., 2002; Cossart et al., 2005). Epileptic seizures, in general, stem from an abnormal balance of excitation and inhibition, and thus considerable effort has been expended on characterizing inhibitory and excitatory neurotransmission abnormalities in the epileptic brain (Engel, 1992; Loscher, 1993; Chapman et al., 1996; Engel and Pedley, 1997). Previous findings have shown both a decrease in GABAergic inhibition and an increase in glutamatergic excitation to be involved in the cellular mechanisms underlying epileptogenesis in human and animal models of epilepsy (Dichter, 1989; Löscher, 1989; Loscher, 1993; Chapman et al., 1996; Chapman, 1998; Dingledine et al., 1999; Meldrum et al., 1999; Chapman, 2000; Silva et al., 2002).

Antagonists of glutamatergic NMDA and AMPA receptors have been shown to be anticonvulsant in many animal models of epilepsy (Meldrum and

Horton, 1980; Chapman et al., 1982; Meldrum et al., 1992). Although, in epileptic mice several genetic alterations have been shown to be epileptogenic, no specific mutation relating to glutamatergic function has yet been linked to human epilepsy (Chapman, 1998). We recently identified a novel QTL, *El-N,* for age-dependent predisposition to seizures on proximal Chromosome 9 of naïve EL mice, that were tested for seizures only once at 150 days of age (Todorova et al., 2006). Although, this region of chromosome 9 had not been previously associated with seizures in other animal epilepsy models and no epilepsy locus has been mapped on human chromosome 11, which is syntenic to the region on mouse chromosome 9 containing *El-N* (Todorova et al., 2006), glutamate receptor subunit 4 gene (*GluR4*) maps to this same region of Chromosome 9. Interestingly, GluR4, one of four genes (GluR1-4) that code for the AMPA receptor, is differentially expressed in the brain (Sato et al., 1993; Myers et al., 1999), and has also been associated with epilepsy (van de Bovenkamp-Janssen et al., 2006).

In this study, we performed a sequencing analysis of GluR4, in epileptic and non-epileptic mice in order to identify potential seizure-associated polymorphisms of GluR4 in EL mice. Our results revealed a few conserved single polymorphisms, suggesting that GluR4 may not be responsible for EL seizures.

METHODS AND MATERIALS

Mice

EL, EL/Frk, DDY, and C57BL/6J were used in this study. EL/Frk is a strain of EL mice that were obtained from The Jackson Laboratory (Bar Harbor, ME). As mentioned in chapter 6, DDY mice, are an inbred non-epileptic EL background mouse strain. The C57BL/6J strain is another control non-epileptic strain. All mice were maintained and housed as described in the Materials and Methods section of Chapter 2.

GluR4 sequencing

Total RNA was isolated from homogenized whole brain of EL/suz, EL/Frk, DDY, and C57BL/6J mice using TRIzol Reagent (Invitrogen) according to the manufacturer's protocol. cDNA libraries for the EL, DDY, and C57BL/6J mice, were made following a similar protocol as described in the RT-PCR protocol in the chapter 6 (n= 3 mice per group). The whole cds sequence of GluR4 was then amplified using the GluR4_214F and GluR_2922R primer sets (Forward 5'-ATG AGG ATT ATT TGC AGG CAG ATT G-3' and Reverse 5'-GGG GAA GCT TGG TGT GAT GAG-3') (Figure 1). PCR amplification was performed with Taq DNA polymerase (Promega) using the following protocol: initial denaturation at 94°C for 2 min, followed by 33 cycles at 94°C for 1 min; annealing at 65°C for 40 s; extension at 72°C for 2 min adding 3 s every cycle; and a final extension at 72°C for 6 min following the last cycle. Amplified PCR products were purified

using a PCR purification kit (MO BIO Laboratories, Solana Beach, CA) and were then visualized by 1% agarose gel electrophoresis. DNA concentration was estimated using a Low DNA Mass Ladder (Invitrogen, Carlsbad, CA) by gel electrophoresis. Approximately 25 fmol of the purified PCR product was used in the sequencing reaction following manufacturer's instructions for the CEQ DTCS kit (Beckman Coulter, Fullerton, CA). The amount of cDNA used varied depending on the size of the PCR fragment. The nested primers illustrated in Figure 1, were for the sequencing reactions to obtain double-stranded sequence of GluR4. Their sequence is shown in Table 3.

Sequencing reactions were performed at 96°C for 20 s, 50°C for 20 s, and 60°C for 240 s based on a 32 cycle reaction. Sequencing products were ethanol precipitated in cold 95% ethanol, washed twice with cold 70% ethanol, dried for 20 minutes, and then re-dissolved in sample loading solution provided by the manufacturer (Beckman Coulter, Fullerton, CA). The individual mouse strain cDNA sequence alignments were then compared to that of GluR4 from *Mus Musculus* [GenBank: AB022913]

RESULTS

The comparative sequencing analysis of GluR4 to the in-house C57BL/6J GluR sequence revealed a single nucleotide base pair change, at position 1029, found in EL, EL/Frk, and DDY mice. This caused a C->A transition, but resulted

in no amino acid change (Table 1). Another single nucleotide base pair change, at position 2338, was found only in EL mice, resulted in a G->A transition and caused an amino acid change from Glycine to Arginine (Table 1). However, when compared to the *Mus Musculus* GenBank sequence [AB022913], this GluR4 change in EL mice was not observed (inferred from Table 2). A third single nucleotide base pair change, at position 2344, was again found only in EL mice, which caused an amino acid change from Alanine to Proline (Table 1). However, once again this change was not observed between the EL and the GenBank GluR4 sequences (inferred from Table 2). Finally, a fourth single nucleotide base pair change, at position 2358, was found only in EL mice when compared to the in-house C57BL/6J GluR4 sequence. This latter A->C transition was though conserved. It should be noted that the same polymorphic changes were observed when partial GluR4 amplicons were TA cloned in a pCR® 2.1 vector, subsequently transformed in E-coli, and then later purified and sequenced (data not shown).

DISCUSSION

The few GluR4 variations that we observed between the EL and in-house C57BL/6J inbred mouse strain were either conserved or not observed in the *Mus Musculus* GluR4 sequence previously published at GenBank [AB022913]. It is

not yet clear if this later discrepancy represents an error in the already published GenBank GluR4 sequence or a population genetic variation among B6 mouse strains. However, since the two single polymorphisms that resulted in an amino acid change are only present in the EL mice and not in the epileptic EL/Frk mice, it is unlikely that GluR4 may be associated with the seizure phenotype in EL mice. Taken together, we can conclude that GluR4 is not associated with the seizure phenotype in the EL mice. However, we cannot exclude the possibility that GluR4 expression might be influenced by different seizure testing environments (Naka et al., 2005).

Figure 1: GluR4 PCR Amplification and Sequencing Primer Construct. Forward primer 167F and reverse primer 2922R were used for the amplification of the GluR4 cDNA libraries. All primers were used as nesting primers for GluR4 sequencing.

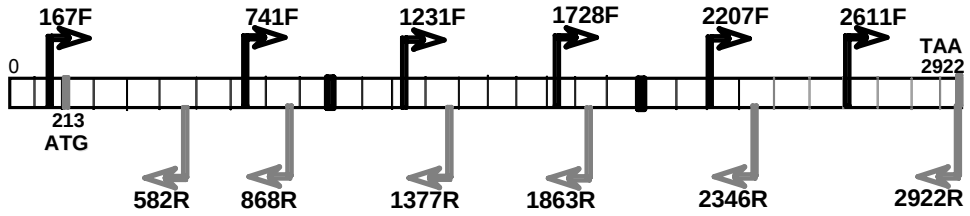


Table 1. Summary of GluR4 Nucleotide Variations in EL and DDY mice[a]

Mouse strain	Nucleotide position	cDNA change	Amino Acid change
EL	1029	C -> A	Ala -> Ala
EL/Frk	1029	C -> A	Ala -> Ala
DDY	1029	C -> A	Ala -> Ala
EL	2338	G -> A	Gly -> Arg
EL	2344	G -> C	Ala -> Pro
EL	2358	A -> C	Ala -> Ala

[a]Nucleotide variation compared to the C57BL/6J GluR4 sequence from the Boston College animal facility at Higgins

Table 2. Summary of GluR4 Nucleotide Variations in EL and DDY mice[a]

Mouse strain	Nucleotide position	cDNA change	Amino Acid change
EL	1029	C -> A	Ala -> Ala
EL/Frk	1029	C -> A	Ala -> Ala
DDY	1029	C -> A	Ala -> Ala
EL/Frk	2338	A -> G	Arg -> Gly
DDY	2338	A -> G	Arg -> Gly
EL/Frk	2344	C -> G	Pro -> Ala
DDY	2344	C -> G	Pro -> Ala
EL/Frk	2358	C -> A	Ala -> Ala
DDY	2358	C -> A	Ala -> Ala

[a]Nucleotide variation compared to the GenBank [AB022913] mouse GluR4 sequence from Sakimura & Ikeno, 1999

Table 3. GluR4 nested primer sequences[a]

GluR4 primer s	Primer sequence
167F	For 5'-GTG AGA GAA AGA GAG GAG AGC G-3'
582R	Rev 5'-GGG GAA GCT TGG TGT GAT GAG-3'
741F	For 5'-GAA TGG ATG GCA TGT CAG TGC G-3'
868R	Rev 5' -GAA GCC TTT CTA TCT CAC AAT C-3'
1231F	For 5'-GGC CAG GGA ATT GAC ATG GAG-3'
1377R	Rev 5'-GCC AAC CTT TCG AGG TCC TGT G3'
1728F	For 5'-GGT GCG AGA GGA GGT CAT CGA C-3'
1863R	Rev 5'-GCA CAT CCA GAT CTC ATA GGC C-3'
2207F	For 5'-CAG AAA TTG CCT ATG GAA CAC-3'
2346R	Rev 5'-GCC CTC AGC TGT AGT TCT AGT G-3'
2611F	For 5'-GGT GAC TCC AAG GAC AAG ACG-3'
2922R	Rev 5'-TTA TGG TAG GTC CGA TGC AAT GAC-3'

[a]Primer nucleotide positions are based on the CDS of each given gene

APPENDIX C.

Assay for the Sectrophotometric Measurement of β-Hydroxybutyrate (D-3-hydroxybutyrate)

METHODOLOGY

A. Assay Principle (ref. Williamson, D.H., and J. Mellanby, pp. 1836-1839, Bergmeyer)

$$\beta\text{-Hydroxybutyric acid} + NAD^+ \xrightarrow[\text{}]{\beta\text{-HBDH}} \text{Acetoacetate} + NADH + H^+$$

$$K_{eq} = [\text{acetoacatetate}][NADH][H^+] / [\text{b-hydroxybutyrate}][NAD^+]$$

Notes: At pH 7, the K_{eq} is 1.42×10^{-2} M and the formation of β-hydroxybutyrate is favored. At pH 7, the formation of acetoacetate can also be determined from the above reaction without modifications. Increasing the pH above 7 (lowering $[H^+]$) the formation of acetoacetate can still be determined by shifting the equilibrium to the right. For i.e., at pH 9.5 the K_{eq} becomes 4.5, but the formation of acetoacaetate is favored slightly. Finally, for β–OHB the molar absorptivity (extinction coefficient) is 6.22 1/M* cm, thus for 1M β-OHB the change in Abs should equal to 6.22.

B. General Instrument/supplies Requirements

1. Molecular Devices SpectraMax M2 or M5 microplate reader with a 340 nm excitation filter (wavelength). The program name is labeled as b-OHB new 2.
2. Half-area clear microplates (Corning cat. No. 3695) (100 µl working volume, with flat bottom wells).
3. Eppendorff repeater Plus pipette (2-20 µl volume), plus regular p20, p200, and p1000 pipettors.

C. Reagent/Buffers

1. *1 M pH 9.9 2-Amino-2-methylpropanol (AMP) (pK_a 9.7)**

Notes: To prepare 10 ml of 1 M, add 959 µl of stock of 2-Amino-2-methylpropanol at a concentration of 10.43 M (Sigma cat. No. A-9199) to 6 ml of ddH_2O. pH solution to 9.9, then bring total solution volume to 10 ml using ddH_2O. Store AMP at room temperature.

The purpose for AMP or a buffer with a free amine group is to remove the acetoacetate and the proton (H^+) generated in the above reaction. If acetoacetate remains in the rxn it can drive reaction backwards thus interfering

with the accurate estimation of β-hydroxybutyrate. The acetoacetate is removed in a shift base reaction (see below) and the proton is captured by the high pH.

Shift base rxn: -C=O + -NH2 ⇔ C=N + H_2O

2. *0.050 M NAD^+ dissolved in CO_3/HCO_3 buffer* *0.0995 g/3ml*

Notes: The NAD^+ can be purchased from Roche Applied Science (cat. No. 775762). Store NAD^+ at dessicator at 4°C. If you make a 0.050M stock NAD^+ solution you can store it at –20°C but it is recommended to make it fresh each time.

3. *0.1 M EDTA* *.372 g/10 ml*

Notes: Store 0.1M EDTA solution at room temperature

4. *1.33% (w/v) β-Hydroxybutyrate dehydrogenase (enzyme) dissolved in 3.2 M* $(NH_4)_2SO_4$

Notes: β-Hydroxybutyrate dehydrogenase stock solution is made as follows: Weigh enzyme and dissolve it ddH_2O (ie. 1.97 mg of enzyme in 1.5 ml ddH_2O). Then add solid $(NH_4)_2SO_4$ to get a 3-3.2 M solution (~0.62 grams for 1.5 ml). Upon adding ammonium sulfate the solution will turn turbid. As soon as enzyme is dissolved, you can store it at 4°C indefinitely. To run assay for a whole half size plate take 150 μl from enzyme stock and add 240 μl of ddH_2O (a 2.6 fold

dilution), otherwise prepare as much enzyme as needed. β-Hydroxybutyrate dehydrogenase can be bought from Biocatalytics Inc. (cat. No. HBDH-101).

5. *1.2% BSA (Bovine serum albumin)* *1.2 g/100 ml*

6. (R)-(-)-3-hydroxybutyric acid, sodium salt

Notes: β-hydroxybutyrate salt can be purchased from SigmaAldrich (cat. No. 298360-1G). Powder is stored at room temperature.

D. Collection and Perchloric acid Treatment of blood (*This step is optional since assay can be done on plasma or serum alone too*)

1. Collect blood in heparinized tube and spin at 6000 rpm for 10 minutes.

2. Immediately separate plasma from pellet. (Plasma may then be stored at -80° C for later use in the assay).

3. Plasma is then treated with 0.055 M Perchloric acid (denatures all proteins/enzyme that you may have in your blood sample that may interfere or degrade β-hydroxybutyrate) (3.6%) by a dilution factor of 5 (1 part plasma to 4 parts $HClO_4$). Vortex sample and put it on ice for 15 minutes.

4. Centrifuge sample for 2 minutes at ~12,000 rpm on table centrifuge (or maximum speed).

5. Take a known aliquot volume of acidified plasma and neutralize it with 3.0 M potassium bicarbonate to a pH of 5.0-6.5 (test with pH strips) (ie. 900 μl of acidified plasma add 90 μl of 3.0 M $KHCO_3$).

6. Let partially neutralized solution sit on ice for 15 minutes (longer incubation will result in complete neutralization—up to 1-2 hrs) with top open.

Notes: If you do not neutralize your sample with a base, the perchloric acid will degrade your β-hydroxybutyrate, thus making the above reaction go slower.

7. Centrifuge solution at about 12,000 rpm for 4 minutes and then aspirate supernatant to new tube for usage in the assay. Store treated plasma in a few aliquots in –80°C. As an option you can test again with pH strip to be sure of pH of solution. Final plasma is diluted approximately 5.2 times.

E. Cocktail Reagent Buffer: (sufficient for a full half size microplate at 50 μl of cocktail/well)

Amount (ml)	**Concentration in cocktail (mM)**	**Reagent**	**Concentration in well (mM)**
0.5	100.0	1M AMP pH 9.9	50.0
3.0	30.0	50 mM NAD^+	15.0
0.2	4.0	100 mM EDTA	2.0
1.3		ddH_2O	
5.0 ml total volume			

Notes: If not running a whole plate, make as much cocktail reagent buffer as needed. Cocktail should be stored at room temperature after it is prepared and while waiting to run assay. Cocktail should always be fresh for each assay run.

F. Preparation of β-hydroxybutyrate standards

1. Prepare a standard stock solution of β-hydroxybutyrate at a concentration of 165 mM in ddH_2O. Stock standard should be stored at 4^oC.

Notes: To prepare a 165 mM stock standard solution of β-hydroxybutyrate, dissolve 0.0208 gram of β-hydroxybutyrate in 1 ml of ddH_2O (0.165 M * 0.001 L* 126.09 g/mole).

2. Using the stock 165 mM solution, prepare the following β-hydroxybutyrate standards: 16.5 mM, 1.65mM, 0.25 mM, 0.165 mM, 0.0825 mM, 0.04125 mM, 0.033 mM, 0.0165 mM. All standards should be stored at - 20^oC.

G. Enzyme protocol

1. Prepare cocktail reagents as indicated above (sections C and E).

2. Prepare Standards as indicated above (section F).

3. Pipette 50 μl of cocktail in each well (this depends on how many samples you have).

4. Pipette first 50 μl of ddH_2O in triplicate and then 50 μl of the standards. Set up a standard curve from your plate by pipetting the following standard solutions in rows 1-3 (in triplicate), wells A through H.

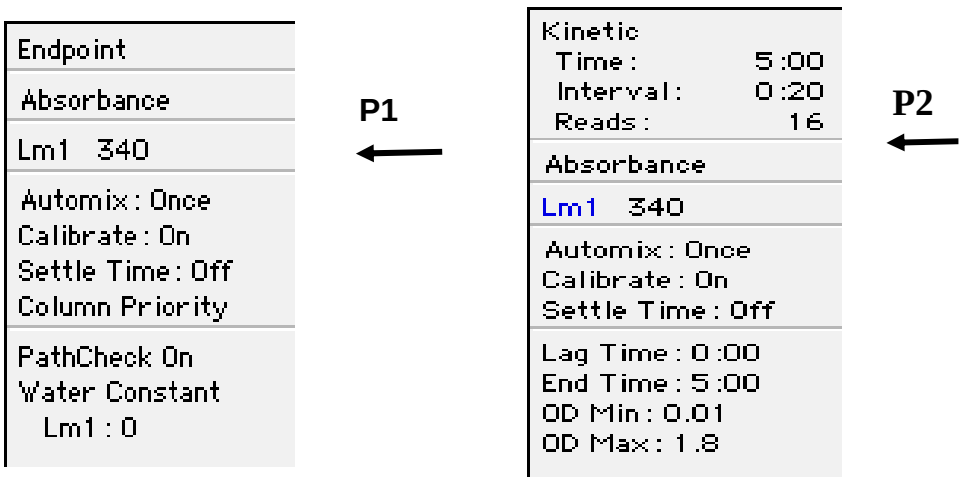
Well (row 1-3)	Standard solution (μl)	ddH_2O (μl)	Concentration in well (μg)	Concentration in well (mM)	Dilution factor (DF) of standards
A	0	50	-	-	-
B	50 of H	0	0.02080485	0.0165	10000
C	50 of G	0	0.0832194	0.0330	5000
D	50 of F	0	0.13003031	0.04125	4000
E	50 of E	0	0.52012125	0.0825	2000
F	50 of D	0	1.0402425	0.165	1000
G	50 of C	0	1.576125	0.250	660
H	0	50	-	-	-

Notes: If your standard curve does not give you a good r^2 value, you may want to make each of the standards individually, instead of making serial dilutions of other standards. Also make sure pipette is calibrated, since that may affect your r^2 value. Using an automatic repeat pipette may reduce variability.

5. Pipet in triplicates your plasma samples to the rest of the plate (treated with perchloric acid or not) to make a final volume of sample or diluted sample of 100µl per well.

6. Open the bOHB template program at the microplate reader and read pathcheck. This is plate one (P1) from the bOHB new 2 assay protocol from the lab assay menu (see left box below).

Notes: Pathcheck measures the depth (optical pathlength) of your samples in the microplate reader and in coordination with the machine's SoftMax®Pro software, the program automatically normalizes the well absorbance to a cuvette of an equivalent pathlength of 1cm. This function allows you to eliminate standard curves, for compounds with known absorptivity and the concentration of a sample can be calculated directly from its absorbance.



7. After reading the pathcheck (P1), read plate two (P2) (right box above) for baseline from the bOHB new 2 assay protocol. This is your initial absorbance ($Abs_{initial}$).

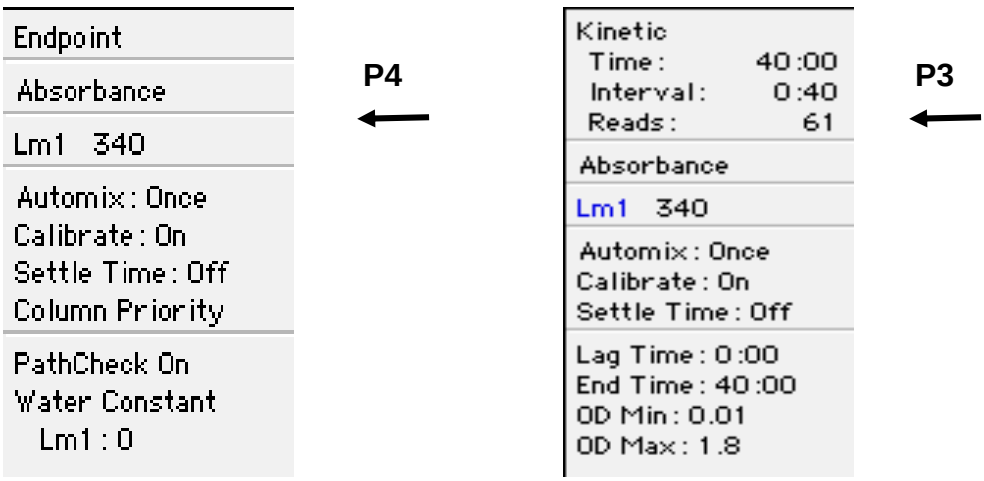
8. After reading baseline, initiate reaction by adding 5 μl of β-hydroxybutyrate dehydrogenase enzyme to each well. This enzyme solution is made by diluting 150 μl of the stock enzyme (see section C step 4) in 240 μl of ddH_2O. Mix stock enzyme before making this enzyme solution, since the enzyme will precipitate over time due to the ammonium sulfate suspension.

Notes: If you are not planning to use whole 96-well plate, make as much enzyme as needed.

9. After adding the enzyme to each well read pathcheck again. This is plate four (P4) (see left box below) from the bOHB new 2 assay protocol.

Notes: The pathcheck principle function, is explained Step 6 (see above).

10. Follow reaction to completion by reading the plate for 40-50 times at 50-60 sec intervals (assay usually takes about 50 minutes to got to completion), by reading plate three (P3) (see right box below) from the bOHB new 2 assay protocol. This is your final absorbance (Abs_{final}).



11. Prepare similar excel template in order to calculate standard curve and assay sample results (ie. my folder (John) > Research Protocols > Ketone assay > Veach's > Spectr. bOHB spreadsheet).

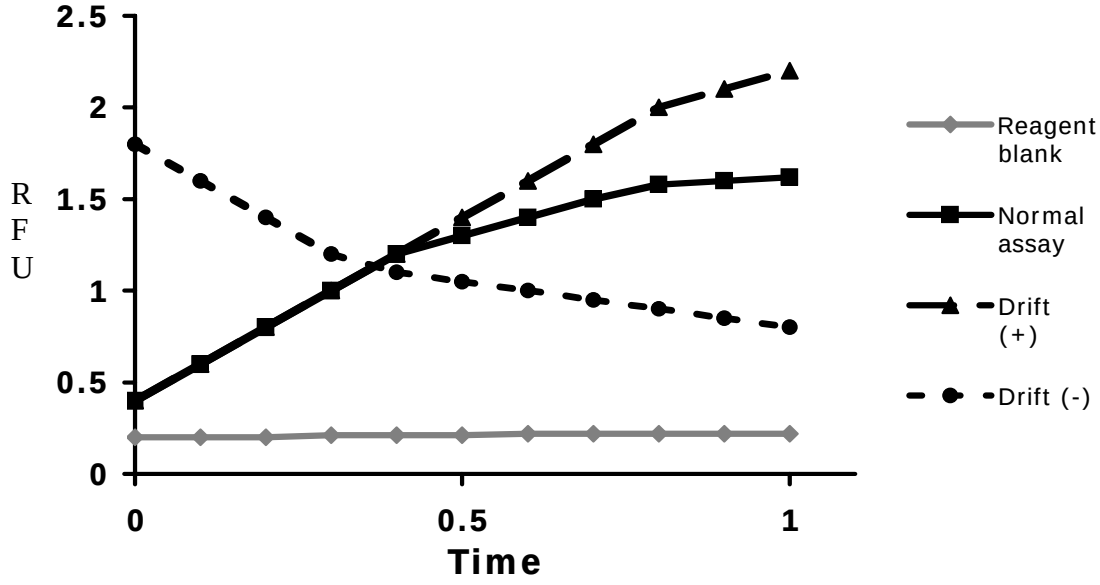
Notes: If you use a M2 or M5 microplate reader, export the data in excel format (from file menu > export data as .txt. The open .txt file using excel).

12. Based on the standard curve (plotting well b-OHB concentration and net absorbance change), calculate the b-OHB concentrations of your unknown samples.

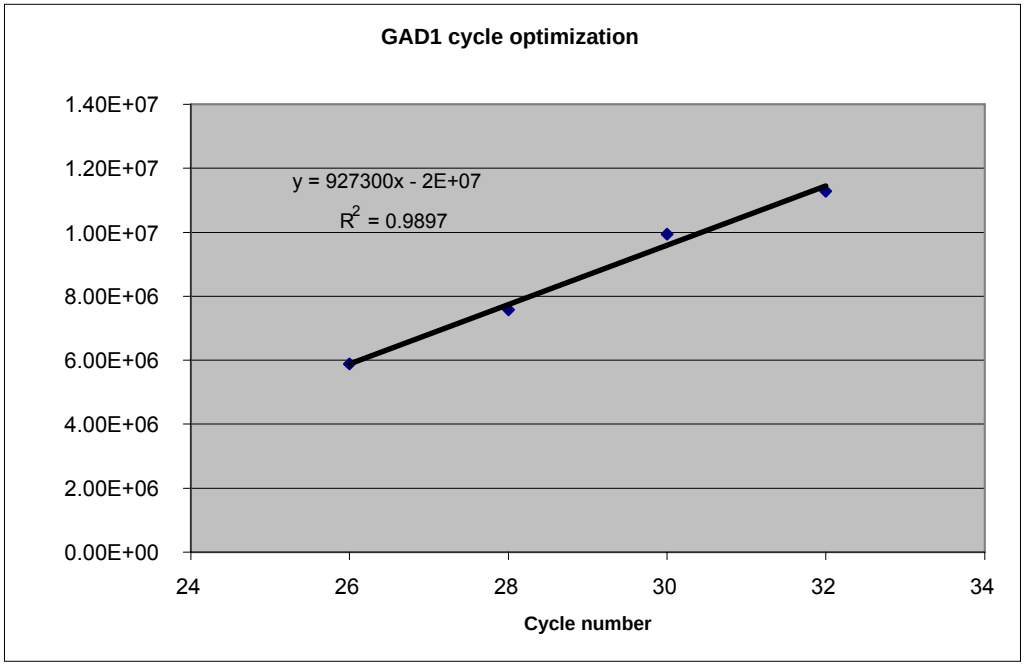
Notes: You may need to calculate the drift for both your standard and your samples, in order to get an exact estimate of your β–OHB concentrations. Drift results from other metabolites (lactate, malate, etc) that may exist in your enzyme suspension that use or produce NADH or NAD^+ in a metabolic reaction may interfere with the kinetics of your ketone assay, thus resulting in incorrect fluorescence reaction signal. If drift is positive (positive slope) you need to

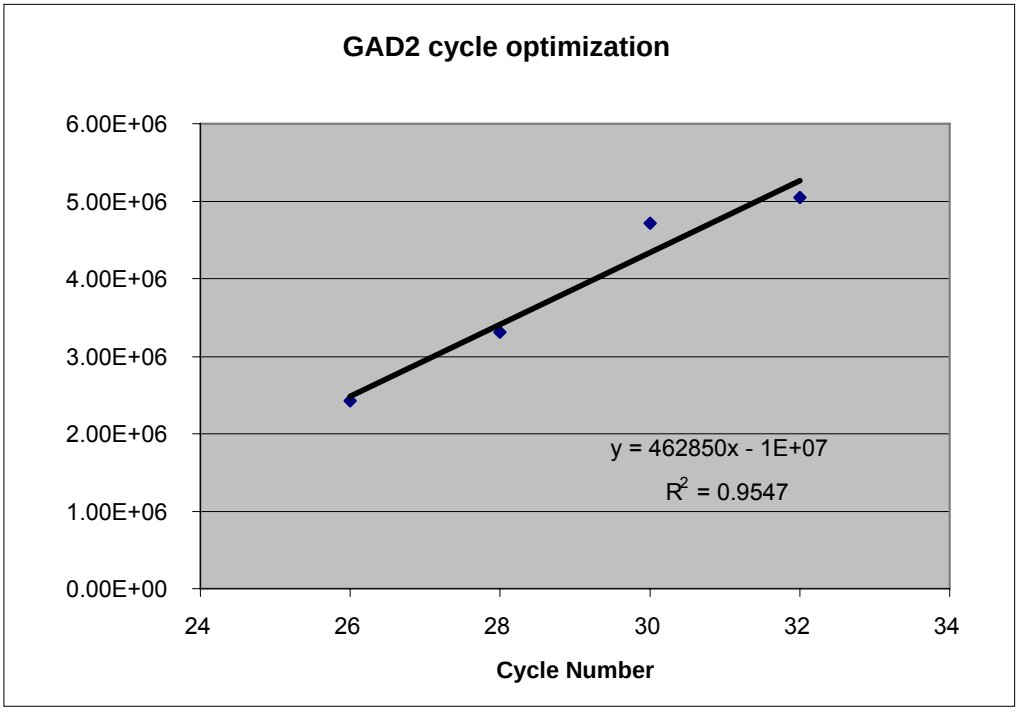
subtract your drift from your DAbs □Abs_{final} - $Abs_{initial}$), whereas if the drift is negative (negative slope) you need to add your drift to your DAbs □Abs_{final} - $Abs_{initial}$). An example is shown below.

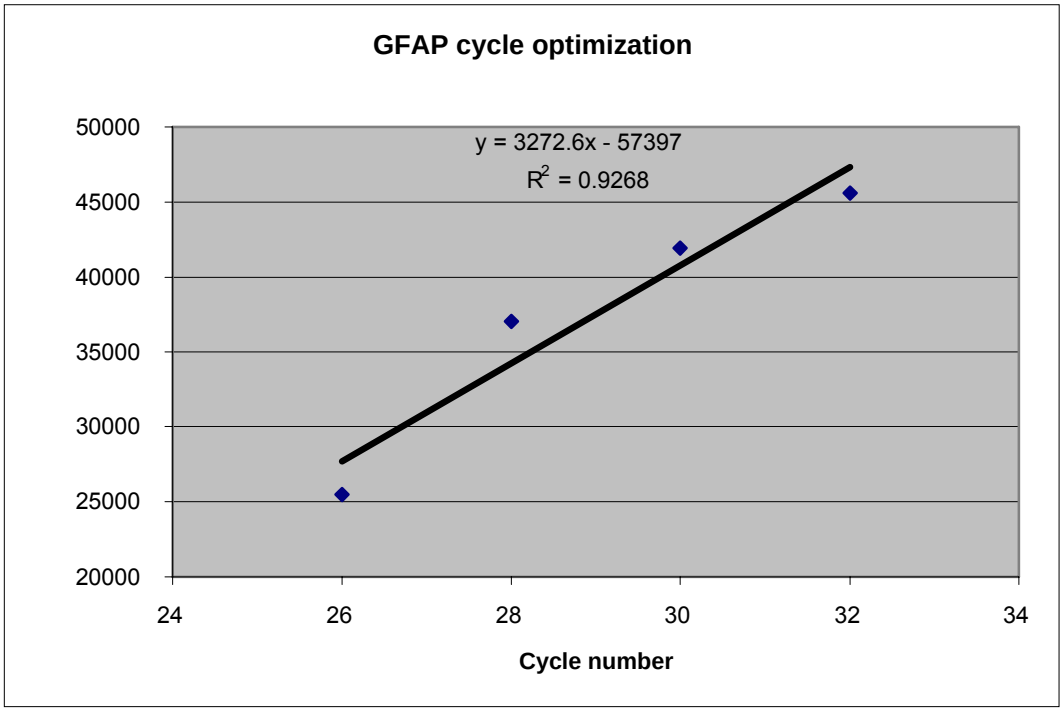
Figure 2: Drifting analysis of ketone assay.

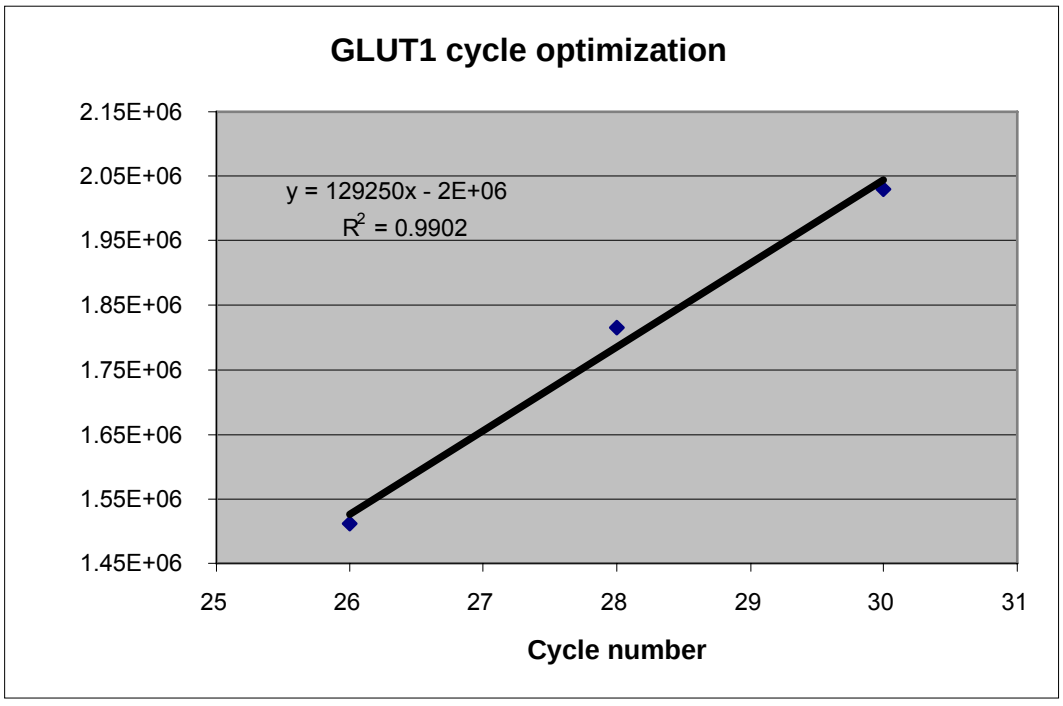


APPENDIX D. Analyses of Primer Cycle Optimization






GFAP cycle optimization
y = 3272.6x - 57397
R2 = 0.9268
50000
45000
40000
35000
30000
25000
20000
24
26
28
30
32
34
Cycle number


GLUT1 cycle optimization
y = 129250x - 2E+06
R2 = 0.9902
2.15E+06
2.05E+06
1.95E+06
1.85E+06
1.75E+06
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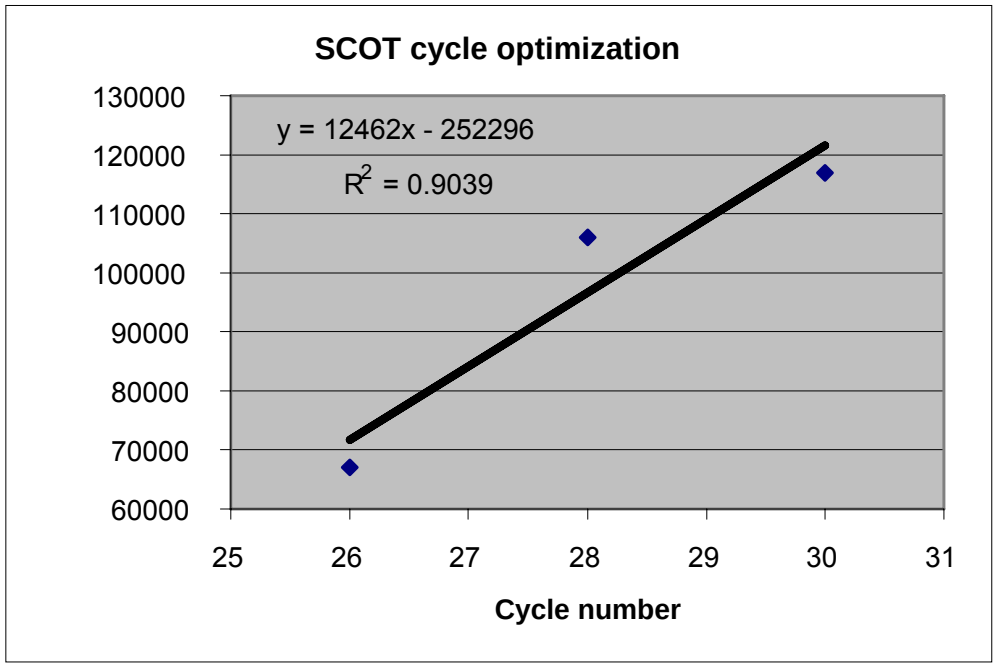
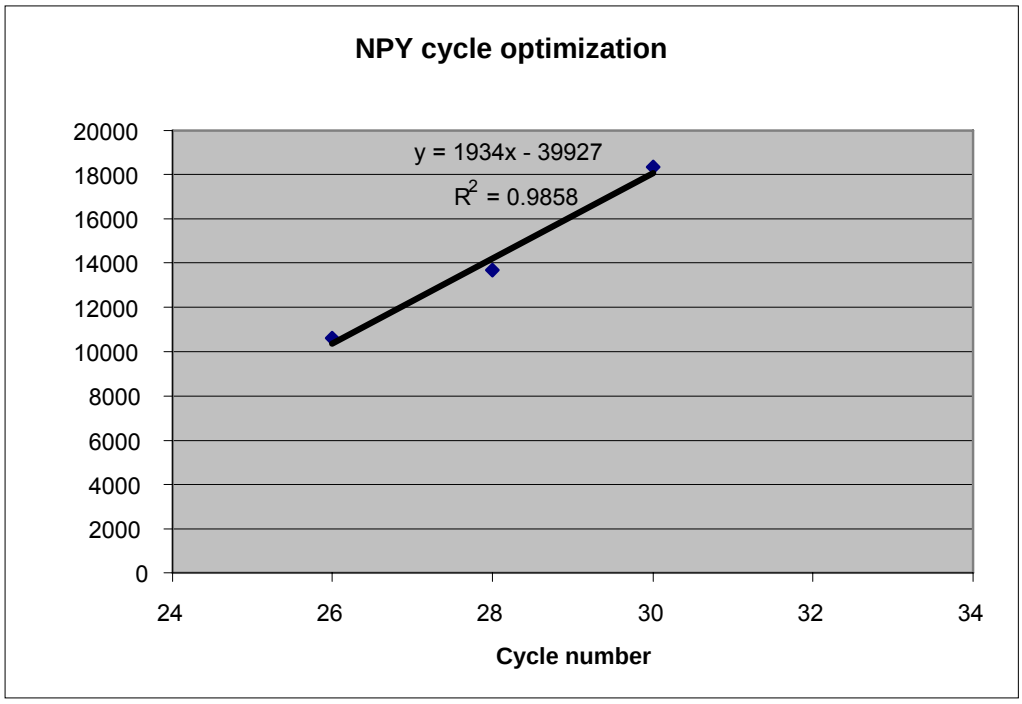
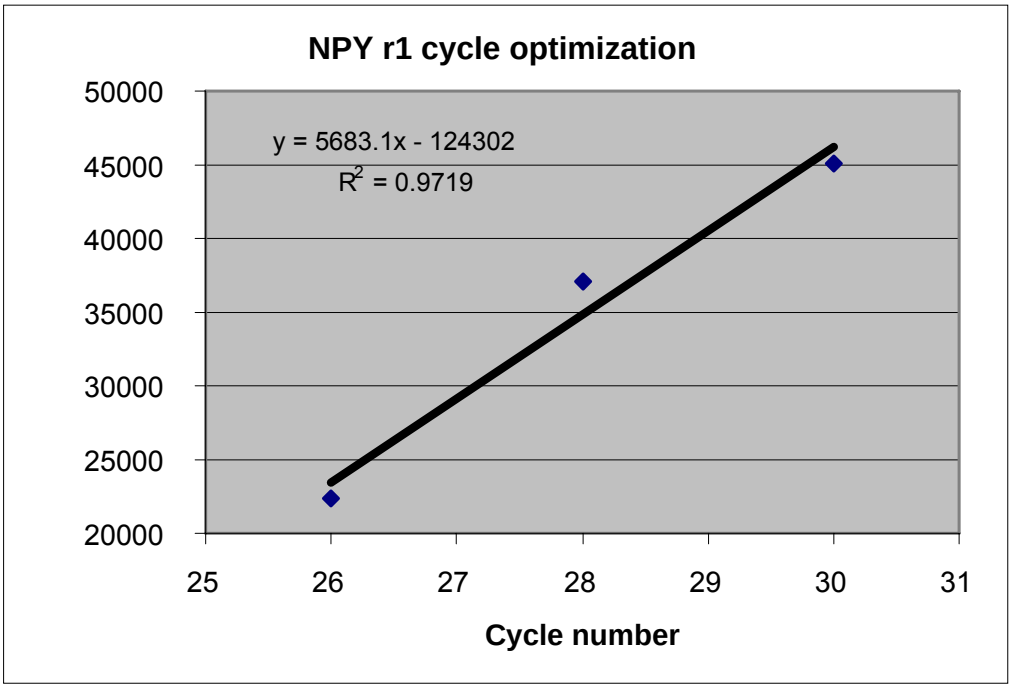
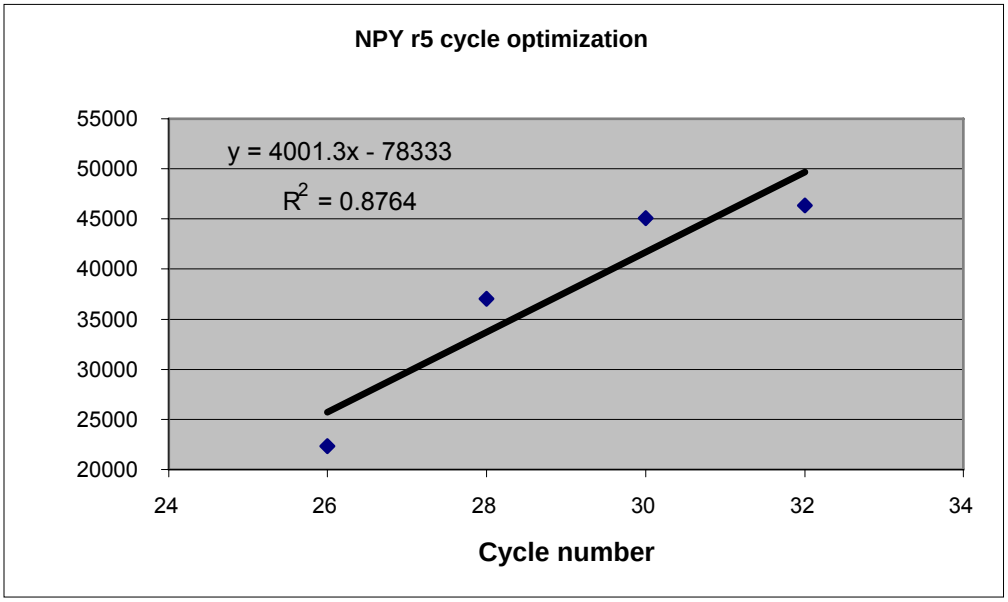
1.55E+06
1.45E+06
25
26
27
28
29
30
31
Cycle number


SCOT cycle optimization
y = 12462x - 252296
R2 = 0.9039
130000
120000
110000
100000
90000
80000
70000
60000
25
26
27
28
29
30
31
Cycle number


NPY cycle optimization
y = 1934x - 39927
R2 = 0.9858
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
24
26
28
30
32
34
Cycle number


NPY r1 cycle optimization
y = 5683.1x - 124302
R2 = 0.9719
50000
45000
40000
35000
30000
25000
20000
25
26
27
28
29
30
31
Cycle number


NPY r5 cycle optimization
y = 4001.3x - 78333
R2 = 0.8764
55000
50000
45000
40000
35000
30000
25000
20000
24
26
28
30
32
34
Cycle number

APPENDIX E.

Detection of Western Blot Linearity

Table 4: Detection of Western Blot System Linearity[a]

Protein (μg)	GAD67	β-actin
2	48990	32660
5	48990	65320
10	138805	155135
20	269445	302105
40	310270	424580
60	351095	465405
80	400085	522560
100	440910	498065

a. Relative Intensity of GAD67 and β-actin using chemiluminesce

REFERENCES

Ackermann R. F. and Lear J. L. (1989) Glycolysis-induced discordance between glucose metabolic rates measured with radiolabeled fluorodeoxyglucose and glucose. J Cereb Blood Flow Metab 9, 774-785.

Ahmet I., Wan R., Mattson M. P., Lakatta E. G. and Talan M. (2005) Cardioprotection by intermittent fasting in rats. Circulation 112, 3115-3121.

Amir R. E., Van den Veyver I. B., Wan M., Tran C. Q., Francke U. and Zoghbi H. Y. (1999) Rett syndrome is caused by mutations in X-linked MECP2, encoding methyl-CpG-binding protein 2. Nature genetics 23, 185-188.

Anderson R. M., Shanmuganayagam D. and Weindruch R. (2009) Caloric restriction and aging: studies in mice and monkeys. Toxicologic pathology 37, 47-51.

Andrade J. P. and Paula-Barbosa M. M. (1996) Protein malnutrition alters the cholinergic and GABAergic systems of the hippocampal formation of the adult rat: an immunocytochemical study. Neuroscience letters 211, 211-215.

Andrade J. P., Madeira M. D. and Paula-Barbosa M. M. (1995) Effects of long-term malnutrition and rehabilitation on the hippocampal formation of the adult rat. A morphometric study. Journal of anatomy 187 (Pt 2), 379-393.

Anson R. M., Guo Z., de Cabo R., Iyun T., Rios M., Hagepanos A., Ingram D. K., Lane M. A. and Mattson M. P. (2003) Intermittent fasting dissociates beneficial effects of dietary restriction on glucose metabolism and neuronal resistance to injury from calorie intake. Proceedings of the National Academy of Sciences of the United States of America 100, 6216-6220.

Appleton D. B. and De Vivo D. C. (1973) An experimental animal model for the effect of ketogenic diet on epilepsy. Proceedings of the Australian Association of Neurologists 10, 75-80.

Appleton D. B. and DeVivo D. C. (1974) An animal model for the ketogenic diet. Epilepsia 15, 211-227.

Armario A., Hidalgo J. and Giralt M. (1988) Evidence that the pituitary-adrenal axis does not cross-adapt to stressors: comparison to other physiological variables. Neuroendocrinology 47, 263-267.

Armentero M. T., Levandis G., Bramanti P., Nappi G. and Blandini F. (2008) Dietary restriction does not prevent nigrostriatal degeneration in the 6-hydroxydopamine model of Parkinson's disease. Experimental neurology 212, 548-551.

Azarbar A., McIntyre D. C. and Gilby K. L. Caloric restriction alters seizure disposition and behavioral profiles in seizure-prone (fast) versus seizure-resistant (slow) rats. Behavioral neuroscience 124, 106-114.

Baba N. H., Sawaya S., Torbay N., Habbal Z., Azar S. and Hashim S. A. (1999) High protein vs high carbohydrate hypoenergetic diet for the treatment of obese hyperinsulinemic subjects. Int J Obes Relat Metab Disord 23, 1202-1206.

Ballaban-Gil K. (2004) Complications of the Ketogenic Diet, in Epilepsy and the Ketogenic Diet (Stafstrom C. and Rho J. M., eds), pp 123-128. Humana Press, Inc., Totowa, NJ.

Baran H., Kepplinger B., Draxler M. and Skofitsch G. (2004) Choline acetyltransferase, glutamic acid decarboxylase and somatostatin in the kainic acid model for chronic temporal lobe epilepsy. Neuro-Signals 13, 290-297.

Baranano K. W. and Hartman A. L. (2008) The ketogenic diet: uses in epilepsy and other neurologic illnesses. Curr Treat Options Neurol 10, 410-419.

Barzilai N. and Bartke A. (2009) Biological approaches to mechanistically understand the healthy life span extension achieved by calorie restriction and modulation of hormones. The journals of gerontology 64, 187-191.

Bates H. E., Kiraly M. A., Yue J. T., Goche Montes D., Elliott M. E., Riddell M. C., Matthews S. G. and Vranic M. (2007) Recurrent intermittent restraint delays fed and fasting hyperglycemia and improves glucose return to baseline levels during glucose tolerance tests in the Zucker diabetic fatty rat--role of food intake and corticosterone. Metabolism: clinical and experimental 56, 1065-1075.

Baumann R. J. (1982) Classification and population studies of epilepsy, in Genetic basis of the epilepsies (Anderson V. E., Hauser W. A., Penry J. K. and Sing C. F., eds), pp 11-20. Raven Press, New York.

Bebbington A., Anderson A., Ravine D., Fyfe S., Pineda M., de Klerk N., Ben-Zeev B., Yatawara N., Percy
A., Kaufmann W. E. and Leonard H. (2008) Investigating genotype-phenotype relationships in Rett syndrome using an international data set. Neurology 70, 868-875.

Beckers S., Zegers D., Van Gaal L. F. and Van Hul W. (2009) The role of the leptin-melanocortin signalling pathway in the control of food intake. Critical reviews in eukaryotic gene expression 19, 267-287.

Becskei C., Lutz T. A. and Riediger T. (2008) Glucose reverses fasting-induced activation in the arcuate nucleus of mice. Neuroreport 19, 105-109.

Becskei C., Lutz T. A. and Riediger T. (2009) Diet-derived nutrients mediate the inhibition of hypothalamic NPY neurons in the arcuate nucleus of mice during refeeding. American journal of physiology 297, R100-110.

Behar T., Ma W., Hudson L. and Barker J. L. (1994) Analysis of the anatomical distribution of GAD67 mRNA encoding truncated glutamic acid decarboxylase proteins in the embryonic rat brain. Brain Res Dev Brain Res 77, 77-87.

Behre J. A. (1931) Studies in Ketone Body Excretion: I. Daily Variations in the Ketone Bodies of Normal and Ketonuria of Short Fasts, with a Note on Diabetic Ketonuria during Insulin Treatment. The Journal of biological chemistry 92, 679-697.

Bennish M. L., Azad A. K., Rahman O. and Phillips R. E. (1990) Hypoglycemia during diarrhea in childhood. Prevalence, pathophysiology, and outcome. The New England journal of medicine 322, 1357-1363.

Berkovic S. F. (1998) Genetics of epilepsy syndromes, in Epilepsy: A comprehensive textbook (Engel J. and Pedley T. A., eds), pp 217-224. Lippincott-Raven, Philadelphia.

Bhagavan N. V. (2002) Medical Biochemistry, Fourth Edition, pp 80, 1016. Harcourt, New York.

Bishop N. A. and Guarente L. (2007) Genetic links between diet and lifespan: shared mechanisms from yeast to humans. Nat Rev Genet 8, 835-844.

Blanck H. M., Bowman B. A., Cooper G. R., Myers G. L. and Miller D. T. (2003) Laboratory issues: use of nutritional biomarkers. The Journal of nutrition 133 Suppl 3, 888S-894S.

Blanco M. M., Costa C. A., Freire A. O., Santos J. G., Jr. and Costa M. (2009) Neurobehavioral effect of essential oil of Cymbopogon citratus in mice. Phytomedicine 16, 265-270.

Blander G. and Guarente L. (2004) The Sir2 family of protein deacetylases. Annual review of biochemistry 73, 417-435.

Bodenant M., Moreau C., Sejourne C., Auvin S., Delval A., Cuisset J. M., Derambure P., Destee A. and Defebvre L. (2008) [Interest of the ketogenic diet in a refractory status epilepticus in adults]. Revue neurologique 164, 194-199.

Bonorden M. J., Rogozina O. P., Kluczny C. M., Grossmann M. E., Grambsch P. L., Grande J. P., Perkins S., Lokshin A. and Cleary M. P. (2009) Intermittent calorie restriction delays prostate tumor detection and increases survival time in TRAMP mice. Nutrition and cancer 61, 265-275.

Bough K. (2008) Energy metabolism as part of the anticonvulsant mechanism of the ketogenic diet. Epilepsia 49 Suppl 8, 91-93.

Bough K. J. and Eagles D. A. (1999) A ketogenic diet increases the resistance to pentylenetetrazole-induced seizures in the rat. Epilepsia 40, 138-143.

Bough K. J. and Rho J. M. (2007) Anticonvulsant mechanisms of the ketogenic diet. Epilepsia 48, 43-58.

Bough K. J., Chen R. S. and Eagles D. A. (1999a) Path analysis shows that increasing ketogenic ratio, but not beta-hydroxybutyrate, elevates seizure threshold in the Rat. Developmental neuroscience 21, 400-406.

Bough K. J., Matthews P. J. and Eagles D. A. (2000) A ketogenic diet has different effects upon seizures induced by maximal electroshock and by pentylenetetrazole infusion. Epilepsy research 38, 105-114.

Bough K. J., Valiyil R., Han F. T. and Eagles D. A. (1999b) Seizure resistance is dependent upon age and calorie restriction in rats fed a ketogenic diet. Epilepsy research 35, 21-28.

Bough K. J., Wetherington J., Hassel B., Pare J. F., Gawryluk J. W., Greene J. G., Shaw R., Smith Y., Geiger J. D. and Dingledine R. J. (2006) Mitochondrial biogenesis in the anticonvulsant mechanism of the ketogenic diet. Annals of neurology 60, 223-235.

Bourin M. and Hascoet M. (2003) The mouse light/dark box test. European journal of pharmacology 463, 55-65.

Bouzier-Sore A. K., Merle M., Magistretti P. J. and Pellerin L. (2002) Feeding active neurons: (re)emergence of a nursing role for astrocytes. J Physiol Paris 96, 273-282.

Bradford H. F. (1995) Glutamate, GABA and epilepsy. Progress in neurobiology 47, 477-511.

Branch-Mays G. L., Dawson D. R., Gunsolley J. C., Reynolds M. A., Ebersole J. L., Novak K. F., Mattison J. A., Ingram D. K. and Novak M. J. (2008) The effects of a calorie-reduced diet on periodontal inflammation and disease in a non-human primate model. Journal of periodontology 79, 1184-1191.

Brigande J. V., Wieraszko A., Albert M. D., Balkema G. W. and Seyfried T. N. (1992) Biochemical correlates of epilepsy in the El mouse: analysis of glial fibrillary acidic protein and gangliosides. J. Neurochem. 58, 752-760.

Bross T. G., Rogina B. and Helfand S. L. (2005) Behavioral, physical, and demographic changes in Drosophila populations through dietary restriction. Aging cell 4, 309-317.

Browne T. R. and Holmes G. L. (2001) Epilepsy. The New England journal of medicine 344, 1145-1151.

Cahill G. F., Jr. (1970) Starvation in man. The New England journal of medicine 282, 668-675.

Casellas J., Farber C. R., Gularte R. J., Haus K. A., Warden C. H. and Medrano J. F. (2009) Evidence of maternal QTL affecting growth and obesity in adult mice. Mamm Genome 20, 269-280.

Chan P. C., Sills R. C., Braun A. G., Haseman J. K. and Bucher J. R. (1996) Toxicity and carcinogenicity of delta 9-tetrahydrocannabinol in Fischer rats and B6C3F1 mice. Fundam Appl Toxicol 30, 109-117.

Chapman A., Keane P. E., Meldrum B. S., Simiand J. and Vernieres J. C. (1982) Mechanism of anticonvulsant action of valproate. Progress in neurobiology 19, 315-359.

Chapman A. G. (1998) Glutamate receptors in epilepsy. Progress in brain research 116, 371-383.

Chapman A. G. (2000) Glutamate and epilepsy. The Journal of nutrition 130, 1043S-1045S.

Chapman A. G., Elwes R. D., Millan M. H., Polkey C. E. and Meldrum B. S. (1996) Role of glutamate and aspartate in epileptogenesis; contribution of microdialysis studies in animal and man. Epilepsy Res Suppl 12, 239-246.

Chen R. Z., Akbarian S., Tudor M. and Jaenisch R. (2001) Deficiency of methyl-CpG binding protein-2 in CNS neurons results in a Rett-like phenotype in mice. Nature genetics 27, 327-331.

Cheng C. M., Hicks K., Wang J., Eagles D. A. and Bondy C. A. (2004) Caloric restriction augments brain glutamic acid decarboxylase-65 and -67 expression. Journal of neuroscience research 77, 270-276.

Cheng C. M., Kelley B., Wang J., Strauss D., Eagles D. A. and Bondy C. A. (2003) A ketogenic diet increases brain insulin-like growth factor receptor and glucose transporter gene expression. Endocrinology 144, 2676-2682.

Clarke D. D. and Sokoloff L. (1999) Circulation and energy metabolism of the brain, in Basic Neurochemistry: Molecular, Cellular and Medical Aspects, 6 Edition (Siegel G. J., Agranoff B. W., Albers R. W., Fisher S. K. and Uhler M. D., eds), pp 637-669. Lippincott-Raven, New York.

Collard K. J., Edwards R. and Liu Y. (1993) Changes in synaptosomal glutamate release during postnatal development in the rat hippocampus and cortex. Brain Res Dev Brain Res 71, 37-43.

Colman R. J., Anderson R. M., Johnson S. C., Kastman E. K., Kosmatka K. J., Beasley T. M., Allison D. B., Cruzen C., Simmons H. A., Kemnitz J. W. and Weindruch R. (2009) Caloric restriction delays disease onset and mortality in rhesus monkeys. Science (New York, N.Y 325, 201-204.

Colmers W. F. and El Bahh B. (2003) Neuropeptide Y and Epilepsy. Epilepsy currents / American Epilepsy Society 3, 53-58.

Coppola G., Verrotti A., Ammendola E., Operto F. F., Corte R. D., Signoriello G. and Pascotto A. (2009) Ketogenic diet for the treatment of catastrophic epileptic encephalopathies in childhood. Eur J Paediatr Neurol.

Coppola G., Veggiotti P., Cusmai R., Bertoli S., Cardinali S., Dionisi-Vici C., Elia M., Lispi M. L., Sarnelli C., Tagliabue A., Toraldo C. and Pascotto A. (2002) The ketogenic diet in children, adolescents and young adults with refractory epilepsy: an Italian multicentric experience. Epilepsy research 48, 221-227.

Cornford E. M., Nguyen E. V. and Landaw E. M. (2000) Acute upregulation of blood-brain barrier glucose transporter activity in seizures. Am J Physiol Heart Circ Physiol 279, H1346-1354.

Cornford E. M., Shamsa K., Zeitzer J. M., Enriquez C. M., Wilson C. L., Behnke E. J., Fried I. and Engel J. (2002) Regional analyses of CNS microdialysate glucose and lactate in seizure patients. Epilepsia 43, 1360-1371.

Cossart R., Bernard C. and Ben-Ari Y. (2005) Multiple facets of GABAergic neurons and synapses: multiple fates of GABA signalling in epilepsies. Trends in neurosciences 28, 108-115.

Crabbe J. C., Wahlsten D. and Dudek B. C. (1999) Genetics of mouse behavior: interactions with laboratory environment. Science (New York, N.Y 284, 1670-1672.

Crawley J. N. (1999) Behavioral phenotyping of transgenic and knockout mice: experimental design and evaluation of general health, sensory functions, motor abilities, and specific behavioral tests. Brain research 835, 18-26.

Crawley J. N. (2007) Mouse behavioral assays relevant to the symptoms of autism. Brain Pathol 17, 448-459.

Crawley J. N. and Paylor R. (1997) A proposed test battery and constellations of specific behavioral paradigms to investigate the behavioral phenotypes of transgenic and knockout mice. Hormones and behavior 31, 197-211.

Crawley J. N., Belknap J. K., Collins A., Crabbe J. C., Frankel W., Henderson N., Hitzemann R. J., Maxson S. C., Miner L. L., Silva A. J., Wehner J. M., Wynshaw-Boris A. and Paylor R. (1997) Behavioral phenotypes of inbred mouse strains: implications and recommendations for molecular studies. Psychopharmacology 132, 107-124.

Crepin S., Godet B., Chassain B., Preux P. M. and Desport J. C. (2009) Malnutrition and epilepsy: a two-way relationship. Clinical nutrition (Edinburgh, Scotland) 28, 219-225.

Crowe T. C. (2005) Safety of low-carbohydrate diets. Obes Rev 6, 235-245.

Crujeiras A. B., Parra D., Milagro F. I., Goyenechea E., Larrarte E., Margareto J. and Martinez J. A. (2008) Differential expression of oxidative stress and inflammation related genes in peripheral blood mononuclear cells in response to a low-calorie diet: a nutrigenomics study. Omics 12, 251-261.

Cullingford T. E. (2004) The ketogenic diet; fatty acids, fatty acid-activated receptors and neurological disorders. Prostaglandins, leukotrienes, and essential fatty acids 70, 253-264.

Cunnane S. C. and Likhodii S. S. (2004) Claims to identify detrimental effects of the ketogenic diet (KD) on cognitive function in rats. Pediatric research 56, 663-664; author reply 664.

Dahlin M., Elfving A., Ungerstedt U. and Amark P. (2005) The ketogenic diet influences the levels of excitatory and inhibitory amino acids in the CSF in children with refractory epilepsy. Epilepsy research 64, 115-125.

Dayer A. G., Bottani A., Bouchardy I., Fluss J., Antonarakis S. E., Haenggeli C. A. and Morris M. A. (2007) MECP2 mutant allele in a boy with Rett syndrome and his unaffected heterozygous mother. Brain & development 29, 47-50.

DeLany J. P., Hansen B. C., Bodkin N. L., Hannah J. and Bray G. A. (1999) Long-term calorie restriction reduces energy expenditure in aging monkeys. The journals of gerontology 54, B5-11; discussion B12-13.

Denny C. A., Kasperzyk J. L., Gorham K. N., Bronson R. T. and Seyfried T. N. (2006) Influence of caloric restriction on motor behavior, longevity, and brain lipid composition in Sandhoff disease mice. Journal of neuroscience research 83, 1028-1038.

Descamps O., Riondel J., Ducros V. and Roussel A. M. (2005) Mitochondrial production of reactive oxygen species and incidence of age-associated lymphoma in OF1 mice: effect of alternate-day fasting. Mechanisms of ageing and development 126, 1185-1191.

DeVivo D. C., Leckie M. P., Ferrendelli J. S. and McDougal D. B., Jr. (1978) Chronic ketosis and cerebral metabolism. Annals of neurology 3, 331-337.

Di Pasquale E., Keegan K. D. and Noebels J. L. (1997) Increased excitability and inward rectification in layer V cortical pyramidal neurons in the epileptic mutant mouse Stargazer. J-Neurophysiol 77, 621-631.

Dichter M. A. (1989) Cellular mechanisms of epilepsy and potential new treatment strategies. Epilepsia 30 Suppl 1, S3-12; discussion S64-18.

Dietrich M. O. and Horvath T. L. (2009) Feeding signals and brain circuitry. The European journal of neuroscience 30, 1688-1696.

Dingledine R., Borges K., Bowie D. and Traynelis S. F. (1999) The glutamate receptor ion channels. Pharmacological reviews 51, 7-61.

Dirks A. J. and Leeuwenburgh C. (2006) Caloric restriction in humans: potential pitfalls and health concerns. Mechanisms of ageing and development 127, 1-7.

Drage M. G., Holmes G. L. and Seyfried T. N. (2002) Hippocampal neurons and glia in epileptic EL mice. J Neurocytol 31, 681-692.

Duan W., Guo Z., Jiang H., Ware M., Li X. J. and Mattson M. P. (2003) Dietary restriction normalizes glucose metabolism and BDNF levels, slows disease progression, and increases survival in huntingtin mutant mice. Proceedings of the National Academy of Sciences of the United States of America 100, 2911-2916.

Eadie M. J. and Bladin P. F. (2001) A Disease Once Sacred: A History of the Medical Understanding of Epilepsy, p 248. John Libby, Eastleigh.

Eagles D. A., Boyd S. J., Kotak A. and Allan F. (2003) Calorie restriction of a high-carbohydrate diet elevates the threshold of PTZ-induced seizures to values equal to those seen with a ketogenic diet. Epilepsy research 54, 41-52.

Efendic S. and Portwood N. (2004) Overview of incretin hormones. Hormone and metabolic research = Hormon- und Stoffwechselforschung = Hormones et metabolisme 36, 742-746.

Engel J., Jr. (1992) Experimental animal models of epilepsy: classification and relevance to human epileptic phenomena. Epilepsy Res Suppl 8, 9-20.

Engel J. J. and Pedley T. A. (1997) Introduction: What is Epilepsy?, in Epilepsy: A Comprehensive Textbook, Vol. 1 (Englel J. J. and Pedley T. A., eds), pp 1-10. Lipponcott-Raven, New York.

Erecinska M., Nelson D., Daikhin Y. and Yudkoff M. (1996) Regulation of GABA level in rat brain synaptosomes: fluxes through enzymes of the GABA shunt and effects of glutamate, calcium, and ketone bodies. J-Neurochem 67, 2325-2334.

Evangeliou A., Vlachonikolis I., Mihailidou H., Spilioti M., Skarpalezou A., Makaronas N., Prokopiou A., Christodoulou P., Liapi-Adamidou G., Helidonis E., Sbyrakis S. and Smeitink J. (2003) Application of a ketogenic diet in children with autistic behavior: pilot study. Journal of child neurology 18, 113-118.

Everitt A. V. and Le Couteur D. G. (2007) Life extension by calorie restriction in humans. Annals of the New York Academy of Sciences 1114, 428-433.

Fabry P. and Tepperman J. (1970) Meal frequency--a possible factor in human pathology. The American journal of clinical nutrition 23, 1059-1068.

Fabry P., Fodor J., Hejl Z., Geizerova H. and Balcarova O. (1968) Meal frequency and ischaemic heart-disease. Lancet 2, 190-191.

Faulks S. C., Turner N., Else P. L. and Hulbert A. J. (2006) Calorie restriction in mice: effects on body composition, daily activity, metabolic rate, mitochondrial reactive oxygen species production, and membrane fatty acid composition. The journals of gerontology 61, 781-794.

Feige J. N., Lagouge M. and Auwerx J. (2008a) Dietary manipulation of mouse metabolism. Current protocols in molecular biology / edited by Frederick M. Ausubel ... [et al Chapter 29, Unit 29B 25.

Feige J. N., Lagouge M., Canto C., Strehle A., Houten S. M., Milne J. C., Lambert P. D., Mataki C., Elliott P. J. and Auwerx J. (2008b) Specific SIRT1 activation mimics low energy levels and protects against diet-induced metabolic disorders by enhancing fat oxidation. Cell metabolism 8, 347-358.

Feinman R. D. and Fine E. J. (2004) "A calorie is a calorie" violates the second law of thermodynamics. Nutrition journal 3, 9.

Felber J. P. and Golay A. (1995) Regulation of nutrient metabolism and energy expenditure. Metabolism: clinical and experimental 44, 4-9.

Feldblum S., Ackermann R. F. and Tobin A. J. (1990) Long-term increase of glutamate decarboxylase mRNA in a rat model of temporal lobe epilepsy. Neuron 5, 361-371.

Fenoglio-Simeone K. A., Wilke J. C., Milligan H. L., Allen C. N., Rho J. M. and Maganti R. K. (2009) Ketogenic diet treatment abolishes seizure periodicity and improves diurnal rhythmicity in epileptic Kcna1-null mice. Epilepsia 50, 2027-2034.

Fenton J. I., Nunez N. P., Yakar S., Perkins S. N., Hord N. G. and Hursting S. D. (2009) Diet-induced adiposity alters the serum profile of inflammation in C57BL/6N mice as measured by antibody array. Diabetes, obesity & metabolism 11, 343-354.

Ferguson M., Rebrin I., Forster M. J. and Sohal R. S. (2008) Comparison of metabolic rate and oxidative stress between two different strains of mice with varying response to caloric restriction. Experimental gerontology 43, 757-763.

Ferrini F., Salio C., Lossi L. and Merighi A. (2009) Ghrelin in central neurons. Current neuropharmacology 7, 37-49.

Fine E. J. and Feinman R. D. (2004) Thermodynamics of weight loss diets. Nutrition & metabolism 1, 15.

Flavin H. J. and Seyfried T. N. (1994) Enhanced aspartate release related to epilepsy in (EL) mice. Journal of neurochemistry 63, 592-595.

Flavin H. J., Wieraszko A. and Seyfried T. N. (1991) Enhanced aspartate release from hippocampal slices of epileptic (El) mice. Journal of neurochemistry 56, 1007-1011.

Fontana L. (2008) Calorie restriction and cardiometabolic health. Eur J Cardiovasc Prev Rehabil 15, 3-9.

Fontana L. (2009) The scientific basis of caloric restriction leading to longer life. Current opinion in gastroenterology 25, 144-150.

Forster M. J., Morris P. and Sohal R. S. (2003) Genotype and age influence the effect of caloric intake on mortality in mice. Faseb J 17, 690-692.

Fox W. M. (1965) Reflex-ontogeny and behavioural development of the mouse. Anim Behav 13, 234-241.

Francois L. L., Manel V., Rousselle C. and David M. (2003) [Ketogenic regime as anti-epileptic treatment: its use in 29 epileptic children]. Arch Pediatr 10, 300-306.

Frankel W. N., Johnson E. W. and Lutz C. M. (1995a) Congenic strains reveal effects of the epilepsy quantitative locus, El2, separate from other El loci. Mamm. Genome 6, 839-843.

Frankel W. N., Valenzuela A., Lutz C. M., Johnson E. W., Dietrich W. F. and Coffin J. M. (1995b) New seizure frequency QTL and the complex genetics of epilepsy in EL mice. Mamm. Genome 6, 830-838.

Freedland R. A. (1967) Effect of progressive starvation on rat liver enzyme activities. The Journal of nutrition 91, 489-495.

Freeman J. M. (2001) The ketogenic diet and epilepsy. Nestle Nutrition workshop series 5, 307-318; discussion 318-321.

Freeman J. M. and Vining E. P. (1999) Seizures decrease rapidly after fasting: preliminary studies of the ketogenic diet. Archives of pediatrics & adolescent medicine 153, 946-949.

Freeman J. M., Freeman J. B. and Kelly M. T. (2000) The Ketogenic Diet: A Treatment for Epilepsy, third Edition, p 236. Demos, New York.

Freeman J. M., Kossoff E. H. and Hartman A. L. (2007) The ketogenic diet: one decade later. Pediatrics 119, 535-543.

French J. A. (2007) Refractory epilepsy: clinical overview. Epilepsia 48 Suppl 1, 3-7.

Froy O., Chapnik N. and Miskin R. (2009) Effect of intermittent fasting on circadian rhythms in mice depends on feeding time. Mechanisms of ageing and development 130, 154-160.

Fueta Y., Kawano H., Ono T., Mita T., Fukata K. and Ohno K. (1998) Regional differences in hippocampal excitability manifested by paired-pulse stimulation of genetically epileptic El mice. Brain research 779, 324-328.

Gagliardino J. J. and Hernandez R. E. (1971) Circadian variation of the serum glucose and immunoreactive insulin levels. Endocrinology 88, 1532-1534.

Gasior M., Rogawski M. A. and Hartman A. L. (2006) Neuroprotective and disease-modifying effects of the ketogenic diet. Behav Pharmacol 17, 431-439.

Gasior M., French A., Joy M. T., Tang R. S., Hartman A. L. and Rogawski M. A. (2007) The anticonvulsant activity of acetone, the major ketone body in the ketogenic diet, is not dependent on its metabolites acetol, 1,2-propanediol, methylglyoxal, or pyruvic acid. Epilepsia 48, 793-800.

Gates J. R. (2000) Side Effect Profiles and Behavioral Consequences of Antiepileptic Medications. Epilepsy Behav 1, 153-159.

Geng Y. Q., Guan J. T., Xu M. Y., Xu X. H. and Fu Y. C. (2007) Behavioral study of calorie-restricted rats from early old age. Conf Proc IEEE Eng Med Biol Soc 2007, 2393-2395.

Gilbert D. L., Pyzik P. L. and Freeman J. M. (2000) The ketogenic diet: seizure control correlates better with serum beta-hydroxybutyrate than with urine ketones. Journal of child neurology 15, 787-790.

Giralt M. and Armario A. (1989) Individual housing does not influence the adaptation of the pituitary-adrenal axis and other physiological variables to chronic stress in adult male rats. Physiology & behavior 45, 477-481.

Goettsch M. (1960) Comparative protein requirements of the rat and mouse for growth, reproduction and lactation using casein diets. J. Nutr. 70, 307-312.

Golay A., Allaz A. F., Morel Y., de Tonnac N., Tankova S. and Reaven G. (1996) Similar weight loss with low- or high-carbohydrate diets. The American journal of clinical nutrition 63, 174-178.

Goldberg A. A., Bourque S. D., Kyryakov P., Gregg C., Boukh-Viner T., Beach A., Burstein M. T., Machkalyan G., Richard V., Rampersad S., Cyr D., Milijevic S. and Titorenko V. I. (2009) Effect of calorie restriction on the metabolic history of chronologically aging yeast. Experimental gerontology.

Gonzalez-Periz A., Horrillo R., Ferre N., Gronert K., Dong B., Moran-Salvador E., Titos E., Martinez-Clemente M., Lopez-Parra M., Arroyo V. and Claria J. (2009) Obesity-induced insulin resistance and hepatic steatosis are alleviated by omega-3 fatty acids: a role for resolvins and protectins. Faseb J 23, 1946-1957.

Goodrick C. L., Ingram D. K., Reynolds M. A., Freeman J. R. and Cider N. (1990) Effects of intermittent feeding upon body weight and lifespan in inbred mice: interaction of genotype and age. Mechanisms of ageing and development 55, 69-87.

Gounarides J. S., Korach-Andre M., Killary K., Argentieri G., Turner O. and Laurent D. (2008) Effect of dexamethasone on glucose tolerance and fat metabolism in a diet-induced obesity mouse model. Endocrinology 149, 758-766.

Gowers W. R. (1901) Epilepsy and other chronic convulsive diseases: their causes, symptoms and treatment., 2nd edition Edition, p 320. Old Hickory Bookshop, Brinklow, MD, London.

Grand T. C. and Millar J. S. (1990) The effects of intermittent dietary restriction on weight gain and body fat in white-footed mice, Peromyscus leucopus. Physiology & behavior 48, 221-224.

Greenberg J. A. and Boozer C. N. (2000) Metabolic mass, metabolic rate, caloric restriction, and aging in male Fischer 344 rats. Mechanisms of ageing and development 113, 37-48.

Greene A. E., Todorova M. M. and Seyfried T. N. (2003) Perspectives on the metabolic management of epilepsy through dietary reduction of glucose and elevation of ketone bodies. J. Neurochem. 86, 529-537.

Greene A. E., Todorova M. T., McGowan R. and Seyfried T. N. (2001) Caloric restriction inhibits seizure susceptibility in epileptic EL mice by reducing blood glucose. Epilepsia 42, 1371-1378.

Grossman S. P. (1986) The role of glucose, insulin and glucagon in the regulation of food intake and body weight. Neuroscience and biobehavioral reviews 10, 295-315.

Guarente L. and Picard F. (2005) Calorie restriction--the SIR2 connection. Cell 120, 473-482.

Guy J., Hendrich B., Holmes M., Martin J. E. and Bird A. (2001) A mouse Mecp2-null mutation causes neurological symptoms that mimic Rett syndrome. Nature genetics 27, 322-326.

Haas R. H., Rice M. A., Trauner D. A. and Merritt T. A. (1986) Therapeutic effects of a ketogenic diet in Rett syndrome. Am J Med Genet Suppl 1, 225-246.

Haas R. H., Light M., Rice M. and Barshop B. A. (1995a) Oxidative metabolism in Rett syndrome: 1. Clinical studies. Neuropediatrics 26, 90-94.

Haas R. H., Nasirian F., Hua X., Nakano K. and Hennessy M. (1995b) Oxidative metabolism in Rett syndrome: 2. Biochemical and molecular studies. Neuropediatrics 26, 95-99.

Hagan M. M. and Moss D. E. (1997) Persistence of binge-eating patterns after a history of restriction with intermittent bouts of refeeding on palatable food in rats: implications for bulimia nervosa. The International journal of eating disorders 22, 411-420.

Hagan M. M., Wauford P. K., Chandler P. C., Jarrett L. A., Rybak R. J. and Blackburn K. (2002) A new animal model of binge eating: key synergistic role of past caloric restriction and stress. Physiology & behavior 77, 45-54.

Hager R., Cheverud J. M. and Wolf J. B. (2009) Relative contribution of additive, dominance, and imprinting effects to phenotypic variation in body size and growth between divergent selection lines of mice. Evolution; international journal of organic evolution 63, 1118-1128.

Halagappa V. K., Guo Z., Pearson M., Matsuoka Y., Cutler R. G., Laferla F. M. and Mattson M. P. (2007) Intermittent fasting and caloric restriction ameliorate age-related behavioral deficits in the triple-transgenic mouse model of Alzheimer's disease. Neurobiology of disease 26, 212-220.

Halberg N., Henriksen M., Soderhamn N., Stallknecht B., Ploug T., Schjerling P. and Dela F. (2005) Effect of intermittent fasting and refeeding on insulin action in healthy men. J Appl Physiol 99, 2128-2136.

Halestrap A. P. and Meredith D. (2004) The SLC16 gene family-from monocarboxylate transporters (MCTs) to aromatic amino acid transporters and beyond. Pflugers Arch 447, 619-628.

Hamadeh M. J., Rodriguez M. C., Kaczor J. J. and Tarnopolsky M. A. (2005) Caloric restriction transiently improves motor performance but hastens clinical onset of disease in the Cu/Zn-superoxide dismutase mutant G93A mouse. Muscle & nerve 31, 214-220.

Hammer S., Snel M., Lamb H. J., Jazet I. M., van der Meer R. W., Pijl H., Meinders E. A., Romijn J. A., de Roos A. and Smit J. W. (2008) Prolonged caloric restriction in obese patients with type 2 diabetes mellitus decreases myocardial triglyceride content and improves myocardial function. Journal of the American College of Cardiology 52, 1006-1012.

Hansen S. L., Nielsen A. H., Knudsen K. E., Artmann A., Petersen G., Kristiansen U., Hansen S. H. and Hansen H. S. (2009) Ketogenic diet is antiepileptogenic in pentylenetetrazole kindled mice and decrease levels of N-acylethanolamines in hippocampus. Neurochemistry international 54, 199-204.

Harney J. P., Madara J. and I'Anson H. (2002) Effects of acute inhibition of fatty acid oxidation on latency to seizure and concentrations of beta hydroxybutyrate in plasma of rats maintained on calorie restriction and/or the ketogenic diet. Epilepsy research 49, 239-246.

Hartman A. L. and Vining E. P. (2007) Clinical aspects of the ketogenic diet. Epilepsia 48, 31-42.
Hartman A. L., Gasior M., Vining E. P. and Rogawski M. A. (2007) The neuropharmacology of the ketogenic diet. Pediatric neurology 36, 281-292.

Haseman J. K., Ney E., Nyska A. and Rao G. N. (2003) Effect of diet and animal care/housing protocols on body weight, survival, tumor incidences, and nephropathy severity of F344 rats in chronic studies. Toxicologic pathology 31, 674-681.

Hassan A. M., Keene D. L., Whiting S. E., Jacob P. J., Champagne J. R. and Humphreys P. (1999) Ketogenic diet in the treatment of refractory epilepsy in childhood. Pediatric neurology 21, 548-552.

Hauser W. A. (1982) Genetics and clinical characteristics of seizures, in Genetic basis of the epilepsies (Anderson V. E., Hauser W. A., Penry J. K. and Sing C. F., eds), pp 3-9. Raven Press, New York.

Hauser W. A. (1992) Seizure disorders: the changes with age. Epilepsia 33, S6-14.

Hauser W. A. (1997) Incidence and prevalence, in Epilepsy: A Comprehensive Textbook, Vol. 1 (Engel J. J. and Pedley T. A., eds), pp 47-57. Lippincott-Raven, New York.

Hausman G. J., Poulos S. P., Pringle T. D. and Azain M. J. (2008) The influence of thiazolidinediones on adipogenesis in vitro and in vivo: potential modifiers of intramuscular adipose tissue deposition in meat animals. Journal of animal science 86, E236-243.

Helmholz H. F. and Keith H. M. (1930) Eight years' experience with the ketogenic diet in the treatment of epilepsy. Journal of American Medical Association 95, 707-709.

Hemingway C., Freeman J. M., Pillas D. J. and Pyzik P. L. (2001) The ketogenic diet: a 3- to 6-year follow-up of 150 children enrolled prospectively. Pediatrics 108, 898-905.

Hermus R. J., Verhagen H. and van Poppel G. (1994) Biomarkers in nutritional assessment. Bibliotheca nutritio et dieta, 116-125.

Hillgartner F. B. and Charron T. (1998) Glucose stimulates transcription of fatty acid synthase and malic enzyme in avian hepatocytes. The American journal of physiology 274, E493-501.

Hipkiss A. R. (2008) Energy metabolism, altered proteins, sirtuins and ageing: converging mechanisms? Biogerontology 9, 49-55.

Hoag W. G. and Dickie M. M. (1968) Nutrition, in Biology of the Laboratory Mouse, second Edition (Green E. L., ed.). Dover, New York.

Hood V. L. (1985) pH regulation of endogenous acid production in subjects with chronic ketoacidosis. The American journal of physiology 249, F220-226.

Huppke P., Maier E. M., Warnke A., Brendel C., Laccone F. and Gartner J. (2006) Very mild cases of Rett syndrome with skewed X inactivation. J Med Genet 43, 814-816.

Husum H., Bolwig T. G., Sanchez C., Mathe A. A. and Hansen S. L. (2004) Levetiracetam prevents changes in levels of brain-derived neurotrophic factor and neuropeptide Y mRNA and of Y1- and Y5-like receptors in the hippocampus of rats undergoing amygdala kindling: implications for antiepileptogenic and mood-stabilizing properties. Epilepsy Behav 5, 204-215.

Huttenlocher P. R. (1976) Ketonemia and seizures: metabolic and anticonvulsant effects of two ketogenic diets in childhood epilepsy. Pediatr-Res 10, 536-540.

Huttenlocher P. R., Wilbourn A. J. and Signore J. M. (1971) Medium-chain triglycerides as a therapy for intractable childhood epilepsy. Neurology 21, 1097-1103.

Ikemoto A., Bole D. G. and Ueda T. (2003) Glycolysis and Glutamate Accumulation into Synaptic Vesicles. Role of glyceraldehyde phosphate dehydrogenase and 3-phosphoglycerate kinase. The Journal of biological chemistry 278, 5929-5940.

Ikeno Y., Hubbard G. B., Lee S., Richardson A., Strong R., Diaz V. and Nelson J. F. (2005) Housing density does not influence the longevity effect of calorie restriction. The journals of gerontology 60, 1510-1517.

Ishida N., Kasamo K., Nakamoto Y. and Suzuki J. (1993) Epileptic seizure of El mouse initiates at the parietal cortex: depth EEG observation in freely moving condition using buffer amplifier. Brain Res. 608, 52-57.

Iwasaki K., Gleiser C. A., Masoro E. J., McMahan C. A., Seo E. J. and Yu B. P. (1988) The influence of dietary protein source on longevity and age-related disease processes of Fischer rats. Journal of gerontology 43, B5-12.

Jallon P. (1997) The problem of intractability: the continuing need for new medical therapies in epilepsy. Epilepsia 38 Suppl 9, S37-42.

James W. P. and Coore H. G. (1970) Persistent impairment of insulin secretion and glucose tolerance after malnutrition. The American journal of clinical nutrition 23, 386-389.

Jankowsky J. L. and Patterson P. H. (2001) The role of cytokines and growth factors in seizures and their sequelae. Progress in neurobiology 63, 125-149.

Jansen G. R., Zanetti M. E. and Hutchison C. F. (1968) Studies on lipogenesis in vivo. Lipogenesis during extended periods of re-feeding after starvation. The Biochemical journal 106, 345-353.

Jazet I. M., Schaart G., Gastaldelli A., Ferrannini E., Hesselink M. K., Schrauwen P., Romijn J. A., Maassen J. A., Pijl H., Ouwens D. M. and Meinders A. E. (2008) Loss of 50% of excess weight using a very low energy diet improves insulin-stimulated glucose disposal and skeletal muscle insulin signalling in obese insulin-treated type 2 diabetic patients. Diabetologia 51, 309-319.

Jedele K. B. (2007) The overlapping spectrum of rett and angelman syndromes: a clinical review. Seminars in pediatric neurology 14, 108-117.

Jeukendrup A. E., Wagenmakers A. J., Stegen J. H., Gijsen A. P., Brouns F. and Saris W. H. (1999a) Carbohydrate ingestion can completely suppress endogenous glucose production during exercise. The American journal of physiology 276, E672-683.

Jeukendrup A. E., Raben A., Gijsen A., Stegen J. H., Brouns F., Saris W. H. and Wagenmakers A. J. (1999b) Glucose kinetics during prolonged exercise in highly trained human subjects: effect of glucose ingestion. The Journal of physiology 515 (Pt 2), 579-589.

Jian L., Nagarajan L., de Klerk N., Ravine D., Christodoulou J. and Leonard H. (2007) Seizures in Rett syndrome: an overview from a one-year calendar study. Eur J Paediatr Neurol 11, 310-317.

Jiang J. C., Jaruga E., Repnevskaya M. V. and Jazwinski S. M. (2000) An intervention resembling caloric restriction prolongs life span and retards aging in yeast. Faseb J 14, 2135-2137.

Jiang W., Zhu Z. and Thompson H. J. (2008) Dietary energy restriction modulates the activity of AMP-activated protein kinase, Akt, and mammalian target of rapamycin in mammary carcinomas, mammary gland, and liver. Cancer research 68, 5492-5499.

Johnston A. and Smith P. (2008) Epilepsy: A General Overview, in Epilepsy and Intellectual Disabilities (Prasher V. P. and Kerr M. P., eds), pp 7-29. Springer-Verlag, New York, LLC.

Joven J., Rull A., Ferre N., Escola-Gil J. C., Marsillach J., Coll B., Alonso-Villaverde C., Aragones G., Claria J. and Camps J. (2007) The results in rodent models of atherosclerosis are not interchangeable: the influence of diet and strain. Atherosclerosis 195, e85-92.

Jung K. H., Chu K., Lee S. T., Kim J. H., Kang K. M., Song E. C., Kim S. J., Park H. K., Kim M., Lee S. K. and Roh J. K. (2009) Region-specific plasticity in the epileptic rat brain: a hippocampal and extrahippocampal analysis. Epilepsia 50, 537-549.

Kaneez F. S. and Saeed S. A. (2009) Investigating GABA and its function in platelets as compared to neurons. Platelets 20, 328-333.

Kang H. C., Chung da E., Kim D. W. and Kim H. D. (2004) Early- and late-onset complications of the ketogenic diet for intractable epilepsy. Epilepsia 45, 1116-1123.

Kankirawatana P., Jirapinyo P., Kankirawatana S., Wongarn R. and Thamanasiri N. (2001) Ketogenic diet: an alternative treatment for refractory epilepsy in children. Journal of the Medical Association of Thailand = Chotmaihet thangphaet 84, 1027-1032.

Kasamo K., Ishida N., Murashima Y. L., Ozawa N., Nakamoto Y. and Suzuki J. (1992) The depth EEG and the multiunit activity in the hippocampal CA1 region during the epileptic seizure of an El mouse: involvement of the hippocampal neurons in seizure manifestations. Neurosciences 18 (Suppl. 2), 129-136.

Kasischke K. A., Vishwasrao H. D., Fisher P. J., Zipfel W. R. and Webb W. W. (2004) Neural activity triggers neuronal oxidative metabolism followed by astrocytic glycolysis. Science (New York, N.Y 305, 99-103.

Katare R. G., Kakinuma Y., Arikawa M., Yamasaki F. and Sato T. (2009) Chronic intermittent fasting improves the survival following large myocardial ischemia by activation of BDNF/VEGF/PI3K signaling pathway. Journal of molecular and cellular cardiology 46, 405-412.
Kaur G. and Kaur K. (1990) Effect of acute starvation on monoamine oxidase and Na+,K(+)-ATPase activity in rat brain. Mol Chem Neuropathol 13, 175-183.

Keenan K. P., Ballam G. C., Soper K. A., Laroque P., Coleman J. B. and Dixit R. (1999) Diet, caloric restriction, and the rodent bioassay. Toxicol Sci 52, 24-34.

Kelley D. E., Wing R., Buonocore C., Sturis J., Polonsky K. and Fitzsimmons M. (1993) Relative effects of calorie restriction and weight loss in noninsulin-dependent diabetes mellitus. The Journal of clinical endocrinology and metabolism 77, 1287-1293.

Kennedy A. R., Pissios P., Otu H., Xue B., Asakura K., Furukawa N., Marino F. E., Liu F. F., Kahn B. B., Libermann T. A., Maratos-Flier E. and Roberson R. (2007) A high-fat, ketogenic diet induces a unique metabolic state in mice. American journal of physiology 292, E1724-1739.

Kim D. W., Kang H. C., Park J. C. and Kim H. D. (2004) Benefits of the nonfasting ketogenic diet compared with the initial fasting ketogenic diet. Pediatrics 114, 1627-1630.

Klass M. R. (1977) Aging in the nematode Caenorhabditis elegans: major biological and environmental factors influencing life span. Mechanisms of ageing and development 6, 413-429.

Klaus S., Rudolph B., Dohrmann C. and Wehr R. (2005) Expression of uncoupling protein 1 in skeletal muscle decreases muscle energy efficiency and affects thermoregulation and substrate oxidation. Physiological genomics 21, 193-200.

Klepper J., Leiendecker B., Bredahl R., Athanassopoulos S., Heinen F., Gertsen E., Florcken A., Metz A. and Voit T. (2002) Introduction of a ketogenic diet in young infants. Journal of inherited metabolic disease 25, 449-460.

Knowlton R. C., Abou-Khalil B., Sawrie S. M., Martin R. C., Faught R. E. and Kuzniecky R. I. (2002) In vivo hippocampal metabolic dysfunction in human temporal lobe epilepsy. Archives of neurology 59, 1882-1886.

Kossoff E. H. and McGrogan J. R. (2005) Worldwide use of the ketogenic diet. Epilepsia 46, 280-289.

Kossoff E. H. and Rho J. M. (2009) Ketogenic diets: evidence for short- and long-term efficacy. Neurotherapeutics 6, 406-414.

Kossoff E. H., Pyzik P. L., McGrogan J. R., Vining E. P. and Freeman J. M. (2002) Efficacy of the ketogenic diet for infantile spasms. Pediatrics 109, 780-783.

Kossoff E. H., Turner Z., Bluml R. M., Pyzik P. L. and Vining E. P. (2007) A randomized, crossover comparison of daily carbohydrate limits using the modified Atkins diet. Epilepsy Behav 10, 432-436.

Kossoff E. H., Laux L. C., Blackford R., Morrison P. F., Pyzik P. L., Hamdy R. M., Turner Z. and Nordli D. R., Jr. (2008) When do seizures usually improve with the ketogenic diet? Epilepsia 49, 329-333.

Krizova E. and Simek V. (1996) Influence of intermittent fasting and high-fat diet on morphological changes of the digestive system and on changes of lipid metabolism in the laboratory mouse. Physiological research / Academia Scientiarum Bohemoslovaca 45, 145-151.

Kwiterovich P. O., Jr., Vining E. P., Pyzik P., Skolasky R., Jr. and Freeman J. M. (2003) Effect of a high-fat ketogenic diet on plasma levels of lipids, lipoproteins, and apolipoproteins in children. Jama 290, 912-920.

Lambert J. D., Fueta Y., Roepstorff A. and Andreasen M. (1996) Analysis of the kinetics of synaptic inhibition points to a reduction in GABA release in area CA1 of the genetically epileptic mouse, El. Epilepsy-Res 26, 15-23.

Lane M. A., Baer D. J., Rumpler W. V., Weindruch R., Ingram D. K., Tilmont E. M., Cutler R. G. and Roth G. S. (1996) Calorie restriction lowers body temperature in rhesus monkeys,

consistent with a postulated anti-aging mechanism in rodents. Proceedings of the National Academy of Sciences of the United States of America 93, 4159-4164.

Lang T. A. and Secic M. (1997) How to Report Statistics in Medicine. Amer. College Physicians, Philadelphia.

Lathe R. (1996) Mice, gene targeting and behaviour: more than just genetic background. Trends in neurosciences 19, 183-186; discussion 188-189.

Lathe R. (2004) The individuality of mice. Genes, brain, and behavior 3, 317-327.

Layman D. K., Boileau R. A., Erickson D. J., Painter J. E., Shiue H., Sather C. and Christou D. D. (2003) A reduced ratio of dietary carbohydrate to protein improves body composition and blood lipid profiles during weight loss in adult women. The Journal of nutrition 133, 411-417.

Lemieux G. and Plante G. E. (1968) The effect of starvation in the normal dog including the Dalmatian coach hound. Metabolism: clinical and experimental 17, 620-630.

Lennox W. G. (1960) Epilepsy and Related Disorders, Vol. 1, p 1168. Little, Brown and Company, Boston.

Lennox W. G. and Cobb S. (1928) Studies in epilepsy VIII. The clinical effect of fasting. Arch. Neurol. Psychiat. 20, 771-779.

Levay E. A., Govic A., Penman J., Paolini A. G. and Kent S. (2007) Effects of adult-onset calorie restriction on anxiety-like behavior in rats. Physiology & behavior 92, 889-896.

Li S. and Quock R. M. (2001) Comparison of N2O- and chlordiazepoxide-induced behaviors in the light/dark exploration test. Pharmacology, biochemistry, and behavior 68, 789-796.

Li S. and Quock R. M. (2002) Effects of a nitric oxide donor on behavior and interaction with nitrous oxide in the mouse light/dark exploration test. European journal of pharmacology 447, 75-78.

Li X., Yokono K. and Okada Y. (2000) Phosphofructokinase, a glycolytic regulatory enzyme has a crucial role for maintenance of synaptic activity in guinea pig hippocampal slices. Neuroscience letters 294, 81-84.

Liebhaber G. M., Riemann E. and Baumeister F. A. (2003) Ketogenic diet in Rett syndrome. Journal of child neurology 18, 74-75.

Likhodii S. (2001) Experiments in the rat pentylenetetrazole infusion threshold model of the ketogenic diet. Epilepsy research 44, 83-86.

Likhodii S. S., Musa K. and Cunnane S. C. (2002) Breath acetone as a measure of systemic ketosis assessed in a rat model of the ketogenic diet. Clinical chemistry 48, 115-120.

Likhodii S. S., Musa K., Mendonca A., Dell C., Burnham W. M. and Cunnane S. C. (2000) Dietary fat, ketosis, and seizure resistance in rats on the ketogenic diet. Epilepsia 41, 1400-1410.

Likhodii S. S., Serbanescu I., Cortez M. A., Murphy P., Snead O. C., 3rd and Burnham W. M. (2003) Anticonvulsant properties of acetone, a brain ketone elevated by the ketogenic diet. Annals of neurology 54, 219-226.

Lim K., Murakami E., Lee S., Shimomura Y. and Suzuki M. (1996) Effects of intermittent food restriction and refeeding on energy efficiency and body fat deposition in sedentary and exercised rats. Journal of nutritional science and vitaminology 42, 449-468.

Lin C., Blank W., Ceriani R. L. and Baker N. (1992) Effect of human mammary MX-1 tumor on plasma free fatty acids in fasted and fasted-refed nude mice. Lipids 27, 33-37.

Liu Y., Wang W., Hawley J. and Birt D. F. (2002) Adrenalectomy abrogates reduction of 12-O-tetradecanoylphorbol-13-acetate-induced extracellular signal-regulated protein kinase activity in the epidermis of dietary energy-restricted SENCAR mice: implications of glucocorticoid hormone. Cancer Epidemiol Biomarkers Prev 11, 299-304.

Livingston S. (1972) Dietary treatment of epilepsy, in Comprehensive management of epilepsy in infancy, childhood and adolescence (Livingston S., ed.), pp 378-405. Charles C. Thomas, Springfield, IL.

Loscher W. (1993) Basic aspects of epilepsy. Current opinion in neurology and neurosurgery 6, 223-232.

Löscher W. (1989) GABA and the epilepsies. Experimental and clinical considerations, pp 260-300. Pythagora Press, Rome.

Lotter E. C. and Woods S. C. (1977) Injections of insulin and changes of body weight. Physiology & behavior 18, 293-297.

Lueker C. E., Meyer J. H. and Smith J. D. (1956) Influence of food and energy restriction and subsequent recovery on body composition and food utilization of rats. The Journal of nutrition 60, 121-128.

Maalouf M. and Rho J. M. (2008) Oxidative impairment of hippocampal long-term potentiation involves activation of protein phosphatase 2A and is prevented by ketone bodies. Journal of neuroscience research 86, 3322-3330.

Maalouf M., Rho J. M. and Mattson M. P. (2009) The neuroprotective properties of calorie restriction, the ketogenic diet, and ketone bodies. Brain research reviews 59, 293-315.

MacKay E. M., Calloway J. W. and Barnes R. H. (1940) Hyperalimentation in normal animals produced by protamine insulin. J. Nutr. 20, 59-66.

Madorsky I., Opalach K., Waber A., Verrier J. D., Solmo C., Foster T., Dunn W. A., Jr. and Notterpek L. (2009) Intermittent fasting alleviates the neuropathic phenotype in a mouse model of Charcot-Marie-Tooth disease. Neurobiology of disease 34, 146-154.

Mady M. A., Kossoff E. H., McGregor A. L., Wheless J. W., Pyzik P. L. and Freeman J. M. (2003) The ketogenic diet: adolescents can do it, too. Epilepsia 44, 847-851.

Mager D. E., Wan R., Brown M., Cheng A., Wareski P., Abernethy D. R. and Mattson M. P. (2006) Caloric restriction and intermittent fasting alter spectral measures of heart rate and blood pressure variability in rats. Faseb J 20, 631-637.

Magni C., Sessa F., Accardo E., Vanoni M., Morazzoni P., Scarafoni A. and Duranti M. (2004) Conglutin gamma, a lupin seed protein, binds insulin in vitro and reduces plasma glucose levels of hyperglycemic rats. The Journal of nutritional biochemistry 15, 646-650.

Mahoney L. B., Denny C. A. and Seyfried T. N. (2006) Caloric restriction in C57BL/6J mice mimics therapeutic fasting in humans. Lipids Health Dis 5, 13.

Mair W., Piper M. D. and Partridge L. (2005) Calories do not explain extension of life span by dietary restriction in Drosophila. PLoS biology 3, e223.

Malfatti C. R., Perry M. L., Schweigert I. D., Muller A. P., Paquetti L., Rigo F. K., Fighera M. R., Garrido-Sanabria E. R. and Mello C. F. (2007) Convulsions induced by methylmalonic acid are associated with glutamic acid decarboxylase inhibition in rats: a role for GABA in the seizures presented by methylmalonic acidemic patients? Neuroscience 146, 1879-1887.

Mannaerts I., Nuytten N. R., Rogiers V., Vanderkerken K., van Grunsven L. A. and Geerts A. (2010) Chronic administration of valproic acid inhibits activation of mouse hepatic stellate cells in vitro and in vivo. Hepatology (Baltimore, Md 51, 603-614.

Mantis J. G., Centeno N., Todorova M. T., McGowan R. and Seyfried T. N. (2003) Metabolic control of epilepsy in adult EL mice with the ketogenic diet and caloric restriction. Epilepsia 44, 64-65.

Mantis J. G., Centeno N. A., Todorova M. T., McGowan R. and Seyfried T. N. (2004) Management of multifactorial idiopathic epilepsy in EL mice with caloric restriction and the ketogenic diet: role of glucose and ketone bodies. Nutrition & metabolism 1, 11.

Mantis J. G., Fritz C. L., Marsh J., Heinrichs S. C. and Seyfried T. N. (2009) Improvement in motor and exploratory behavior in Rett syndrome mice with restricted ketogenic and standard diets. Epilepsy Behav 15, 133-141.

Marini C., Scheffer I. E., Crossland K. M., Grinton B. E., Phillips F. L., McMahon J. M., Turner S. J., Dean J. T., Kivity S., Mazarib A., Neufeld M. Y., Korczyn A. D., Harkin L. A., Dibbens L. M., Wallace R. H., Mulley J. C. and Berkovic S. F. (2004) Genetic architecture of idiopathic generalized epilepsy: clinical genetic analysis of 55 multiplex families. Epilepsia 45, 467-478.

Marsh E. B., Freeman J. M., Kossoff E. H., Vining E. P., Rubenstein J. E., Pyzik P. L. and Hemingway C. (2006) The outcome of children with intractable seizures: a 3- to 6-year follow-up of 67 children who remained on the ketogenic diet less than one year. Epilepsia 47, 425-430.

Marsh J., Mukherjee P. and Seyfried T. N. (2008a) Drug/diet synergy for managing malignant astrocytoma in mice: 2-deoxy-D-glucose and the restricted ketogenic diet. Nutrition & metabolism 5, 33.

Marsh J., Mukherjee P. and Seyfried T. N. (2008b) Akt-dependent proapoptotic effects of dietary restriction on late-stage management of a phosphatase and tensin homologue/tuberous sclerosis complex 2-deficient mouse astrocytoma. Clin Cancer Res 14, 7751-7762.

Marti O., Marti J. and Armario A. (1994) Effects of chronic stress on food intake in rats: influence of stressor intensity and duration of daily exposure. Physiology & behavior 55, 747-753.

Marti O., Gavalda A., Jolin T. and Armario A. (1993) Effect of regularity of exposure to chronic immobilization stress on the circadian pattern of pituitary adrenal hormones, growth hormone, and thyroid stimulating hormone in the adult male rat. Psychoneuroendocrinology 18, 67-77.

Martin B., Mattson M. P. and Maudsley S. (2006) Caloric restriction and intermittent fasting: two potential diets for successful brain aging. Ageing research reviews 5, 332-353.

Martin B., Golden E., Egan J. M., Mattson M. P. and Maudsley S. (2007) Reduced energy intake: the secret to a long and healthy life? IBS journal of science 2, 35-39.

Martin B., Pearson M., Brenneman R., Golden E., Keselman A., Iyun T., Carlson O. D., Egan J. M., Becker K. G., Wood W., 3rd, Prabhu V., de Cabo R., Maudsley S. and Mattson M. P. (2008) Conserved and differential effects of dietary energy intake on the hippocampal transcriptomes of females and males. PloS one 3, e2398.

Martin D. L. and Rimvall K. (1993) Regulation of gamma-aminobutyric acid synthesis in the brain. Journal of neurochemistry 60, 395-407.

Masoro E. J. (2009) Caloric restriction-induced life extension of rats and mice: a critique of proposed mechanisms. Biochimica et biophysica acta 1790, 1040-1048.

Maswood N., Young J., Tilmont E., Zhang Z., Gash D. M., Gerhardt G. A., Grondin R., Roth G. S., Mattison J., Lane M. A., Carson R. E., Cohen R. M., Mouton P. R., Quigley C., Mattson M. P. and Ingram D. K. (2004) Caloric restriction increases neurotrophic factor levels and attenuates neurochemical and behavioral deficits in a primate model of Parkinson's disease. Proceedings of the National Academy of Sciences of the United States of America 101, 18171-18176.

Mattson M. P. and Wan R. (2005) Beneficial effects of intermittent fasting and caloric restriction on the cardiovascular and cerebrovascular systems. The Journal of nutritional biochemistry 16, 129-137.

Mattson M. P., Cutler R. G. and Camandola S. (2007) Energy intake and amyotrophic lateral sclerosis. Neuromolecular medicine 9, 17-20.

Mattson R. H. (2001) Monotherapy trials: endpoints. Epilepsy research 45, 109-117; discussion 119, 121-102.

Mavropoulos J. C., Buschemeyer W. C., 3rd, Tewari A. K., Rokhfeld D., Pollak M., Zhao Y., Febbo P. G., Cohen P., Hwang D., Devi G., Demark-Wahnefried W., Westman E. C., Peterson B. L., Pizzo S. V. and Freedland S. J. (2009) The effects of varying dietary carbohydrate and fat content on survival in a murine LNCaP prostate cancer xenograft model. Cancer prevention research (Philadelphia, Pa 2, 557-565.

Mazarati A. and Wasterlain C. G. (2002) Anticonvulsant effects of four neuropeptides in the rat hippocampus during self-sustaining status epilepticus. Neuroscience letters 331, 123-127.

McCarter R., Masoro E. J. and Yu B. P. (1985) Does food restriction retard aging by reducing the metabolic rate? The American journal of physiology 248, E488-490.

McGill B. E., Bundle S. F., Yaylaoglu M. B., Carson J. P., Thaller C. and Zoghbi H. Y. (2006) Enhanced anxiety and stress-induced corticosterone release are associated with increased Crh expression in a mouse model of Rett syndrome. Proceedings of the National Academy of Sciences of the United States of America 103, 18267-18272.

McIlwain H. (1969) Cerebral energy metabolism and membrane phenomena, in Basic Mechanisms of the Epilepsies (Jasper H. H., Ward A. A. J. and Pope A., eds), pp 83-103. Little, Brown, and Co., Boston.

Meldrum B. (1996) Action of established and novel anticonvulsant drugs on the basic mechanisms of epilepsy. Epilepsy Res Suppl 11, 67-77.

Meldrum B. and Horton R. (1980) Effects of the bicyclic GABA agonist, THIP, on myoclonic and seizure responses in mice and baboons with reflex epilepsy. European journal of pharmacology 61, 231-237.

Meldrum B. and Garthwaite J. (1990) Excitatory amino acid neurotoxicity and neurodegenerative disease. Trends in pharmacological sciences 11, 379-387.

Meldrum B. and Chapman A. (1999) Epileptic seizures and epilepsy, in Basic Neurochemistry: Molecular, Cellular and Medical Aspects, 6 Edition (Siegel G. J., Agranoff B. W., Albers R. W., Fisher S. K. and Uhler M. D., eds), pp 755-768. Lippincott-Raven, New York.

Meldrum B. S., Akbar M. T. and Chapman A. G. (1999) Glutamate receptors and transporters in genetic and acquired models of epilepsy. Epilepsy research 36, 189-204.

Meldrum B. S., Craggs M. D., Durmuller N., Smith S. E. and Chapman A. G. (1992) The effects of AMPA receptor antagonists on kindled seizures and on reflex epilepsy in rodents and primates. Epilepsy Res Suppl 9, 307-311.

Meric P., Barrere B., Peres M., Gillet B., Berenger G., Beloeil J. C. and Seylaz J. (1994) Effects of kainate-induced seizures on cerebral metabolism: a combined 1H and 31P NMR study in rat. Brain research 638, 53-60.

Merry B. J. (2002) Molecular mechanisms linking calorie restriction and longevity. The international journal of biochemistry & cell biology 34, 1340-1354.

Metcalf B. M., Mullaney B. C., Johnston M. V. and Blue M. E. (2006) Temporal shift in methyl-CpG binding protein 2 expression in a mouse model of Rett syndrome. Neuroscience 139, 1449-1460.

Meyer O. A., Tilson H. A., Byrd W. C. and Riley M. T. (1979) A method for the routine assessment of fore- and hindlimb grip strength of rats and mice. Neurobehav Toxicol 1, 233-236.

Minamiyama Y., Bito Y., Takemura S., Takahashi Y., Kodai S., Mizuguchi S., Nishikawa Y., Suehiro S. and Okada S. (2007) Calorie restriction improves cardiovascular risk factors via reduction of mitochondrial reactive oxygen species in type II diabetic rats. The Journal of pharmacology and experimental therapeutics 320, 535-543.

Mishra A. and Mohanty B. (2009) Effect of lactational exposure of olanzapine on body weight of mice: a comparative study on neonates of both the sexes during post-natal development. Journal of psychopharmacology (Oxford, England).

Mody I. and Pearce R. A. (2004) Diversity of inhibitory neurotransmission through GABA(A) receptors. Trends in neurosciences 27, 569-575.

Moretti P., Bouwknecht J. A., Teague R., Paylor R. and Zoghbi H. Y. (2005) Abnormalities of social interactions and home-cage behavior in a mouse model of Rett syndrome. Human molecular genetics 14, 205-220.

Moretti P., Levenson J. M., Battaglia F., Atkinson R., Teague R., Antalffy B., Armstrong D., Arancio O., Sweatt J. D. and Zoghbi H. Y. (2006) Learning and memory and synaptic plasticity are impaired in a mouse model of Rett syndrome. J Neurosci 26, 319-327.

Morris A. A. (2005) Cerebral ketone body metabolism. Journal of inherited metabolic disease 28, 109-121.

Morton G. J., Cummings D. E., Baskin D. G., Barsh G. S. and Schwartz M. W. (2006) Central nervous system control of food intake and body weight. Nature 443, 289-295.

Mosek A., Natour H., Neufeld M. Y., Shiff Y. and Vaisman N. (2009) Ketogenic diet treatment in adults with refractory epilepsy: a prospective pilot study. Seizure 18, 30-33.

Motil K. J., Schultz R., Brown B., Glaze D. G. and Percy A. K. (1994) Altered energy balance may account for growth failure in Rett syndrome. Journal of child neurology 9, 315-319.

Mount R. H., Hastings R. P., Reilly S., Cass H. and Charman T. (2001) Behavioural and emotional features in Rett syndrome. Disabil Rehabil 23, 129-138.

Mount R. H., Charman T., Hastings R. P., Reilly S. and Cass H. (2003) Features of autism in Rett syndrome and severe mental retardation. J Autism Dev Disord 33, 435-442.

Mukherjee P., Abate L. E. and Seyfried T. N. (2004) Anti-angiogenic and pro-apoptotic effects of dietary restriction on experimental mouse and human brain tumors. Clinical. Cancer Res. (in press).

Mukherjee P., Sotnikov A. V., Mangian H. J., Zhou J. R., Visek W. J. and Clinton S. K. (1999) Energy intake and prostate tumor growth, angiogenesis, and vascular endothelial growth factor expression. J. Natl. Cancer Inst. 91, 512-523.

Muller-Schwarze A. B., Tandon P., Liu Z., Yang Y., Holmes G. L. and Stafstrom C. E. (1999) Ketogenic diet reduces spontaneous seizures and mossy fiber sprouting in the kainic acid model. Neuroreport 10, 1517-1522.

Murashima Y. L., Kasamo K. and Suzuki J. (1992) Developmental abnormalities of GABAergic system are involved in the formation of epileptogenesis in the EL. Neurosciences 18 (Suppl. 2), 63-73.

Murashima Y. L., Suzuki J. and Yoshii M. (2005) Developmental program of epileptogenesis in the brain of el mice. Epilepsia 46 Suppl 5, 10-16.

Myers S. J., Dingledine R. and Borges K. (1999) Genetic regulation of glutamate receptor ion channels. Annual review of pharmacology and toxicology 39, 221-241.

Nag N. and Berger-Sweeney J. E. (2007) Postnatal dietary choline supplementation alters behavior in a mouse model of Rett syndrome. Neurobiology of disease 26, 473-480.

Nagatomo I., Akasaki Y., Nagase F., Nomaguchi M. and Takigawa M. (1996) Relationships between convulsive seizures and serum and brain concentrations of phenobarbital and zonisamide in mutant inbred strain EL mouse. Brain Res. 731, 190-198.

Naka F., Narita N., Okado N. and Narita M. (2005) Modification of AMPA receptor properties following environmental enrichment. Brain & development 27, 275-278.

Nakano H., Saito K. and Suzuki K. (1994) Chronic implantation technique for monopolar EEG monitoring of epileptic seizures in mice. Brain research bulletin 35, 261-268.

Naruse H. and Kurokawa M. (1992) The beginnings of studies on EL mice. Neurosciences (Suppl. 2) 18, 1-3.

Nehlig A. and Pereira de Vasconcelos A. (1993) Glucose and ketone body utilization by the brain of neonatal rats. Progress in neurobiology 40, 163-221.

Nehlig A., Dufour F., Klinger M., Willing L. B., Simpson I. A. and Vannucci S. J. (2009) The ketogenic diet has no effect on the expression of spike-and-wave discharges and nutrient transporters in genetic absence epilepsy rats from Strasbourg. Journal of neurochemistry 109 Suppl 1, 207-213.

Neschen S., Katterle Y., Richter J., Augustin R., Scherneck S., Mirhashemi F., Schurmann A., Joost H. G. and Klaus S. (2008) Uncoupling protein 1 expression in murine skeletal muscle increases AMPK activation, glucose turnover, and insulin sensitivity in vivo. Physiological genomics 33, 333-340.

Nishimura T., Schwarzer C., Gasser E., Kato N., Vezzani A. and Sperk G. (2005) Altered expression of GABA(A) and GABA(B) receptor subunit mRNAs in the hippocampus after kindling and electrically induced status epilepticus. Neuroscience 134, 691-704.

Noebels J. L. (1999) Single-gene models of epilepsy. Advances in neurology 79, 227-238.

Noebels J. L. (2001) Modeling human epilepsies in mice. Epilepsia 42 Suppl 5, 11-15.

Noh H. S., Lee H. P., Kim D. W., Kang S. S., Cho G. J., Rho J. M. and Choi W. S. (2004) A cDNA microarray analysis of gene expression profiles in rat hippocampus following a ketogenic diet. Brain Res Mol Brain Res 129, 80-87.

Nylen K., Likhodii S., Abdelmalik P. A., Clarke J. and Burnham W. M. (2005) A comparison of the ability of a 4:1 ketogenic diet and a 6.3:1 ketogenic diet to elevate seizure thresholds in adult and young rats. Epilepsia 46, 1198-1204.

Nyska A., Hester S. D., Cooper R. L., Goldman J. M., Stoker T. E., House D. and Wolf D. C. (2002) Single or group housing altered hormonal physiology and affected pituitary and interstitial cell kinetics. The Journal of toxicological sciences 27, 449-457.

Oddy W. H., Webb K. G., Baikie G., Thompson S. M., Reilly S., Fyfe S. D., Young D., Anderson A. M. and Leonard H. (2007) Feeding experiences and growth status in a Rett syndrome population. J Pediatr Gastroenterol Nutr 45, 582-590.

Okitolonda W., Brichard S. M. and Henquin J. C. (1987) Repercussions of chronic protein-calorie malnutrition on glucose homeostasis in the rat. Diabetologia 30, 946-951.

Opsahl M. L., McClenaghan M., Springbett A., Reid S., Lathe R., Colman A. and Whitelaw C. B. (2002) Multiple effects of genetic background on variegated transgene expression in mice. Genetics 160, 1107-1112.

Orozco L. D., Cokus S. J., Ghazalpour A., Ingram-Drake L., Wang S., van Nas A., Che N., Araujo J. A., Pellegrini M. and Lusis A. J. (2009) Copy number variation influences gene expression and metabolic traits in mice. Human molecular genetics.

Ortinski P. and Meador K. J. (2004) Cognitive side effects of antiepileptic drugs. Epilepsy Behav 5 Suppl 1, S60-65.

Overton J. M. and Williams T. D. (2004) Behavioral and physiologic responses to caloric restriction in mice. Physiology & behavior 81, 749-754.

Owen O. E., Morgan A. P., Kemp H. G., Sullivan J. M., Herrera M. G. and Cahill G. F., Jr. (1967) Brain metabolism during fasting. The Journal of clinical investigation 46, 1589-1595.

Owen O. E., Caprio S., Reichard G. A., Jr., Mozzoli M. A., Boden G. and Owen R. S. (1983) Ketosis of starvation: a revisit and new perspectives. Clinics in endocrinology and metabolism 12, 359-379.

Pan J. W., Williamson A., Cavus I., Hetherington H. P., Zaveri H., Petroff O. A. and Spencer D. D. (2008) Neurometabolism in human epilepsy. Epilepsia 49 Suppl 3, 31-41.

Paylor R., Nguyen M., Crawley J. N., Patrick J., Beaudet A. and Orr-Urtreger A. (1998) Alpha7 nicotinic receptor subunits are not necessary for hippocampal-dependent learning or sensorimotor gating: a behavioral characterization of Acra7-deficient mice. Learn Mem 5, 302-316.

Pedersen C. R., Hagemann I., Bock T. and Buschard K. (1999) Intermittent feeding and fasting reduces diabetes incidence in BB rats. Autoimmunity 30, 243-250.

Peltola J., Eriksson K. and Keranen T. (2001) Cytokines and seizures. Archives of neurology 58, 1168-1169.

Penders J., Fiers T., Giri M., Wuyts B., Ysewyn L. and Delanghe J. R. (2005) Quantitative measurement of ketone bodies in urine using reflectometry. Clin Chem Lab Med 43, 724-729.

Percy A. K. (2002) Rett syndrome. Current status and new vistas. Neurologic clinics 20, 1125-1141.

Percy A. K. and Lane J. B. (2005) Rett syndrome: model of neurodevelopmental disorders. Journal of child neurology 20, 718-721.

Perez C., Canal J. R., Dominguez E., Campillo J. E., Guillen M. and Torres M. D. (1997) Individual housing influences certain biochemical parameters in the rat. Laboratory animals 31, 357-361.

Peterman M. G. (1928) The ketogenic diet. J. Amer. Med. Assoc. 90, 1427-1429.

Pfeifer H. H. and Thiele E. A. (2005) Low-glycemic-index treatment: a liberalized ketogenic diet for treatment of intractable epilepsy. Neurology 65, 1810-1812.

Pfeifer H. H., Lyczkowski D. A. and Thiele E. A. (2008) Low glycemic index treatment: implementation and new insights into efficacy. Epilepsia 49 Suppl 8, 42-45.

Piatti P. M., Monti F., Fermo I., Baruffaldi L., Nasser R., Santambrogio G., Librenti M. C., Galli-Kienle M., Pontiroli A. E. and Pozza G. (1994) Hypocaloric high-protein diet improves glucose

oxidation and spares lean body mass: comparison to hypocaloric high-carbohydrate diet. Metabolism: clinical and experimental 43, 1481-1487.

Pierre K. and Pellerin L. (2005) Monocarboxylate transporters in the central nervous system: distribution, regulation and function. Journal of neurochemistry 94, 1-14.

Piper M. D. and Bartke A. (2008) Diet and aging. Cell metabolism 8, 99-104.

Pitkanen A. and Sutula T. P. (2002) Is epilepsy a progressive disorder? Prospects for new therapeutic approaches in temporal-lobe epilepsy. Lancet neurology 1, 173-181.

Poderycki M. J., Simoes J. M., Todorova M., Neumann P. E. and Seyfried T. N. (1998) Environmental influences on epilepsy gene mapping in EL mice. J. Neurogenetics 12, 67-86.

Poiley S. M. (1972) Growth tables for 66 strains and stocks of laboratory animals. Laboratory animal science 22, 758-779.

Porter R. J., Meldrum B. S., Macdonald R. L., Dicter M. A., Dam M., Treiman D. M., Chadwick D., Gram L. and Pedley T. A. (1997) Antiepileptic drugs, in Epilepsy: A Comprehensive Textbook, Vol. 2 (Engel J. J. and Pedley T. A., eds), pp 1379-1670. Lippincott-Raven, New York. Prins M. L. (2008) Cerebral metabolic adaptation and ketone metabolism after brain injury. J Cereb Blood Flow Metab 28, 1-16.

Pryor G. T., Uyeno E. T., Tilson H. A. and Mitchell C. L. (1983) Assessment of chemicals using a battery of neurobehavioral tests: a comparative study. Neurobehav Toxicol Teratol 5, 91-117.

Puchowicz M. A., Zechel J. L., Valerio J., Emancipator D. S., Xu K., Pundik S., LaManna J. C. and Lust W. D. (2008) Neuroprotection in diet-induced ketotic rat brain after focal ischemia. J Cereb Blood Flow Metab 28, 1907-1916.

Putnam T. J. and Merritt H. H. (1937) Experimental Determination of the Anticonvulsant Properties of Some Phenyl Derivatives. Science (New York, N.Y 85, 525-526.

Qin W., Zhao W., Ho L., Wang J., Walsh K., Gandy S. and Pasinetti G. M. (2008) Regulation of forkhead transcription factor FoxO3a contributes to calorie restriction-induced prevention of Alzheimer's disease-type amyloid neuropathology and spatial memory deterioration. Annals of the New York Academy of Sciences 1147, 335-347.

Qin W., Yang T., Ho L., Zhao Z., Wang J., Chen L., Zhao W., Thiyagarajan M., MacGrogan D., Rodgers J. T., Puigserver P., Sadoshima J., Deng H., Pedrini S., Gandy S., Sauve A. A. and Pasinetti G. M. (2006) Neuronal SIRT1 activation as a novel mechanism underlying the prevention of Alzheimer disease amyloid neuropathology by calorie restriction. The Journal of biological chemistry 281, 21745-21754.

Rabast U., Kasper H. and Schonborn J. (1978) Comparative studies in obese subjects fed carbohydrate-restricted and high carbohydrate 1,000-calorie formula diets. Nutrition and metabolism 22, 269-277.

Raffo E., Francois J., Ferrandon A., Koning E. and Nehlig A. (2008) Calorie-restricted ketogenic diet increases thresholds to all patterns of pentylenetetrazol-induced seizures: critical importance of electroclinical assessment. Epilepsia 49, 320-328.

Ramsey J. J., Harper M. E. and Weindruch R. (2000) Restriction of energy intake, energy expenditure, and aging. Free radical biology & medicine 29, 946-968.

Ravizza T., Rizzi M., Perego C., Richichi C., Veliskova J., Moshe S. L., De Simoni M. G. and Vezzani A. (2005) Inflammatory response and glia activation in developing rat hippocampus after status epilepticus. Epilepsia 46 Suppl 5, 113-117.

Reed D. R., Lawler M. P. and Tordoff M. G. (2008) Reduced body weight is a common effect of gene knockout in mice. BMC genetics 9, 4.

Reeves P. G., Rossow K. L. and Lindlauf J. (1993) Development and testing of the AIN-93 purified diets for rodents: results on growth, kidney calcification and bone mineralization in rats and mice. The Journal of nutrition 123, 1923-1931.

Reilly S. and Cass H. (2001) Growth and nutrition in Rett syndrome. Disabil Rehabil 23, 118-128.

Renieri A., Meloni I., Longo I., Ariani F., Mari F., Pescucci C. and Cambi F. (2003) Rett syndrome: the complex nature of a monogenic disease. J Mol Med 81, 346-354.

Reshef L., Olswang Y., Cassuto H., Blum B., Croniger C. M., Kalhan S. C., Tilghman S. M. and Hanson R. W. (2003) Glyceroneogenesis and the triglyceride/fatty acid cycle. The Journal of biological chemistry 278, 30413-30416.

Reynolds M. A., Dawson D. R., Novak K. F., Ebersole J. L., Gunsolley J. C., Branch-Mays G. L., Holt S. C., Mattison J. A., Ingram D. K. and Novak M. J. (2009) Effects of caloric restriction on inflammatory periodontal disease. Nutrition (Burbank, Los Angeles County, Calif 25, 88-97.

Rho J. M., Anderson G. D., Donevan S. D. and White H. S. (2002) Acetoacetate, acetone, and dibenzylamine (a contaminant in l-(+)-beta-hydroxybutyrate) exhibit direct anticonvulsant actions in vivo. Epilepsia 43, 358-361.

Rho J. M., Kim D. W., Robbins C. A., Anderson G. D. and Schwartzkroin P. A. (1999) Age-dependent differences in flurothyl seizure sensitivity in mice treated with a ketogenic diet. Epilepsy research 37, 233-240.

Rice M. A. and Haas R. H. (1988) The nutritional aspects of Rett syndrome. Journal of child neurology 3 Suppl, S35-42.

Richichi C., Lin E. J., Stefanin D., Colella D., Ravizza T., Grignaschi G., Veglianese P., Sperk G., During M. J. and Vezzani A. (2004) Anticonvulsant and antiepileptogenic effects mediated by

adeno-associated virus vector neuropeptide Y expression in the rat hippocampus. J Neurosci 24, 3051-3059.

Ritter L. M., Vazquez D. M. and Meador-Woodruff J. H. (2002) Ontogeny of ionotropic glutamate receptor subunit expression in the rat hippocampus. Brain Res Dev Brain Res 139, 227-236.

Roberts R. C. (1981) Genetical Influences on growth and fertility, Vol. 47, pp 231-524. Academic press, London.

Romsos D. R., Belo P. S., Bergen W. G. and Leveille G. A. (1978) Influence of meal frequency on body weight, plasma metabolites, and glucose and cholesterol metabolism in the dog. The Journal of nutrition 108, 2,8-47.

Ruis M. A., te Brake J. H., Buwalda B., De Boer S. F., Meerlo P., Korte S. M., Blokhuis H. J. and Koolhaas J. M. (1999) Housing familiar male wildtype rats together reduces the long-term adverse behavioural and physiological effects of social defeat. Psychoneuroendocrinology 24, 285-300.

Rushing P. A., Lutz T. A., Seeley R. J. and Woods S. C. (2000) Amylin and insulin interact to reduce food intake in rats. Hormone and metabolic research = Hormon- und Stoffwechselforschung = Hormones et metabolisme 32, 62-65.

Saggerson E. D. and Greenbaum A. L. (1969) The effect of dietary and hormonal conditions on the activities of glycolytic enzymes in rat epididymal adipose tissue. The Biochemical journal 115, 405-417.

Samala R., Willis S. and Borges K. (2008) Anticonvulsant profile of a balanced ketogenic diet in acute mouse seizure models. Epilepsy research 81, 119-127.

Sampath A., Kossoff E. H., Furth S. L., Pyzik P. L. and Vining E. P. (2007) Kidney stones and the ketogenic diet: risk factors and prevention. Journal of child neurology 22, 375-378.

Sarkisian M. R. (2001) Overview of the Current Animal Models for Human Seizure and Epileptic Disorders. Epilepsy Behav 2, 201-216.

Sato H. (1985) The development of EEG background activities in El mouse. Folia Psychiatr Neurol Jpn 39, 581-587.

Sato K., Kiyama H. and Tohyama M. (1993) The differential expression patterns of messenger RNAs encoding non-N-methyl-D-aspartate glutamate receptor subunits (GluR1-4) in the rat brain. Neuroscience 52, 515-539.

Saywell V., Viola A., Confort-Gouny S., Le Fur Y., Villard L. and Cozzone P. J. (2006) Brain magnetic resonance study of Mecp2 deletion effects on anatomy and metabolism. Biochemical and biophysical research communications 340, 776-783.

Scheyer R. D. (1998) Involvement of glutamate in human epileptic activities. Progress in brain research 116, 359-369.

Schwartz M. W., Woods S. C., Porte D., Jr., Seeley R. J. and Baskin D. G. (2000) Central nervous system control of food intake. Nature 404, 661-671.

Schwartz R. H., Eaton J., Bower B. D. and Aynsley Green A. (1989) Ketogenic diets in the treatment of epilepsy: short-term clinical effects. Dev-Med-Child-Neurol 31, 145-151.

Schwartzkroin P. A. (1999) Mechanisms underlying the anti-epileptic efficacy of the ketogenic diet. Epilepsy research 37, 171-180.

Schwechter E. M., Veliskova J. and Velisek L. (2003) Correlation between extracellular glucose and seizure susceptibility in adult rats. Annals of neurology 53, 91-101.

Seo J. H., Lee Y. M., Lee J. S., Kang H. C. and Kim H. D. (2007) Efficacy and tolerability of the ketogenic diet according to lipid:nonlipid ratios--comparison of 3:1 with 4:1 diet. Epilepsia 48, 801-805.

Seyfried T. N. and Todorova M. (1999) Experimental models of epilepsy, in The Epilepsies: Etiologies and Prevention (Kotagal P. and Luders H. O., eds), pp 527-542. Academic Press, New York.

Seyfried T. N., Poderycki M. J. and Todorova M. T. (1999) Genetics of the EL mouse: a multifactorial epilepsy model, in Genetics of Focal Epilepsies (Genton P., Berkovic S. F., Hirsch E. and Marescaux C., eds), pp 229-238. J. Libby, London.

Seyfried T. N., Greene A. E. and Todorova M. M. (2004) Caloric restriction and epilepsy: Historical perspectives, relationship to the ketogenic diet, and analysis in epileptic EL mice, in Epilepsy and the Ketogenic Diet (Stafstrom C. E. and Rho J. M., eds), pp 247-264. Humana Press Inc., Totowa, NJ.

Seyfried T. N., Kiebish M. A. and Mukherjee P. (2009a) Targeting Energy Metabolism in Brain Cancer with Restricted Diets, in Glioblastoma: Molecular Mechanisms of Pathogenesis and Current Therapeutic Strategies (Ray S. K., ed.), pp 341-363. Springer, New York, NY.

Seyfried T. N., Kiebish M., Mukherjee P. and Marsh J. (2008a) Targeting energy metabolism in brain cancer with calorically restricted ketogenic diets. Epilepsia 49 Suppl 8, 114-116.

Seyfried T. N., Heinecke K. A., Mantis J. G. and Denny C. A. (2008b) Brain Lipid Analysis in Mice with Rett Syndrome. Neurochemical research.

Seyfried T. N., Mantis J. G., Todorova M. T. and Greene A. E. (2009b) Dietary Management of Epilepsy: Role of Glucose and Ketone Bodies, Vol. 2, pp 687-693. Academic Press, Oxford.

Seyfried T. N., Sanderson T. M., El-Abbadi M. M., McGowan R. and Mukherjee P. (2003) Role of glucose and ketone bodies in the metabolic control of experimental brain cancer. Br J Cancer 89, 1375-1382.

Shahbazian M., Young J., Yuva-Paylor L., Spencer C., Antalffy B., Noebels J., Armstrong D., Paylor R. and Zoghbi H. (2002a) Mice with truncated MeCP2 recapitulate many Rett syndrome features and display hyperacetylation of histone H3. Neuron 35, 243-254.

Shahbazian M. D., Antalffy B., Armstrong D. L. and Zoghbi H. Y. (2002b) Insight into Rett syndrome: MeCP2 levels display tissue- and cell-specific differences and correlate with neuronal maturation. Human molecular genetics 11, 115-124.

Sharma M. D., Garber A. J. and Farmer J. A. (2008) Role of insulin signaling in maintaining energy homeostasis. Endocr Pract 14, 373-380.

Sheth R. D. (2004) Metabolic concerns associated with antiepileptic medications. Neurology 63, S24-29.

Silva A. V., Sanabria E. R., Cavalheiro E. A. and Spreafico R. (2002) Alterations of the neocortical GABAergic system in the pilocarpine model of temporal lobe epilepsy: neuronal damage and immunocytochemical changes in chronic epileptic rats. Brain research bulletin 58, 417-421.

Silva M. C., Rocha J., Pires C. S., Ribeiro L. C., Brolese G., Leite M. C., Almeida L. M., Tramontina F., Ziegler D. R. and Goncalves C. A. (2005) Transitory gliosis in the CA3 hippocampal region in rats fed on a ketogenic diet. Nutritional neuroscience 8, 259-264.

Smith J. V., Heilbronn L. K. and Ravussin E. (2004) Energy restriction and aging. Current opinion in clinical nutrition and metabolic care 7, 615-622.

Smith S. R., Pozefsky T. and Chhetri M. K. (1974) Nitrogen and amino acid metabolism in adults with protein-calorie malnutrition. Metabolism: clinical and experimental 23, 603-618.

Soghomonian J. J. (1994) Differential regulation of glutamate decarboxylase and preproenkephalin mRNA levels in the rat striatum. Brain research 640, 146-154.

Soghomonian J. J. and Chesselet M. F. (1992) Effects of nigrostriatal lesions on the levels of messenger RNAs encoding two isoforms of glutamate decarboxylase in the globus pallidus and entopeduncular nucleus of the rat. Synapse (New York, N.Y 11, 124-133.

Sohal R. S., Ferguson M., Sohal B. H. and Forster M. J. (2009) Life span extension in mice by food restriction depends on an energy imbalance. The Journal of nutrition 139, 533-539.

Stafstrom C. E. (1999) Animal models of the ketogenic diet: what have we learned, what can we learn? Epilepsy research 37, 241-259.

Stafstrom C. E. (2004) Dietary approaches to epilepsy treatment: old and new options on the menu. Epilepsy currents / American Epilepsy Society 4, 215-222.

Stafstrom C. E. and Bough K. J. (2003) The ketogenic diet for the treatment of epilepsy: a challenge for nutritional neuroscientists. Nutritional neuroscience 6, 67-79.

Stafstrom C. E. and Sutula T. P. (2005) Models of epilepsy in the developing and adult brain: implications for neuroprotection. Epilepsy Behav 7 Suppl 3, S18-24.

Su S. W., Cilio M. R., Sogawa Y., Silveira D. C., Holmes G. L. and Stafstrom C. E. (2000) Timing of ketogenic diet initiation in an experimental epilepsy model. Brain Res Dev Brain Res 125, 131-138.

Sullivan P. G., Rippy N. A., Dorenbos K., Concepcion R. C., Agarwal A. K. and Rho J. M. (2004) The ketogenic diet increases mitochondrial uncoupling protein levels and activity. Annals of neurology 55, 576-580.

Suzuki J. (1976) Paroxysmal discharges in the electroencephalogram of the El mouse. Experientia 32, 336-338.

Suzuki J. (2004) Investigations of epilepsy with a mutant animal (EL mouse) model. Epilepsia 45 Suppl 8, 2-5.

Suzuki J. and Nakamoto Y. (1977) Seizure patterns and electroencephalograms of El mouse. Electroencephalogr. Clin. Neurophysiol. 43, 299-311.

Suzuki J., Kasamo K., Ishida N. and Murashima Y. L. (1991) Initiation, propagation and generalization of paroxysmal discharges in an epileptic mutant animal. Jpn. J. Psychiatry Neurol. 45, 271-274.

Taboulet P., Deconinck N., Thurel A., Haas L., Manamani J., Porcher R., Schmit C., Fontaine J. P. and Gautier J. F. (2007) Correlation between urine ketones (acetoacetate) and capillary blood ketones (3-beta-hydroxybutyrate) in hyperglycaemic patients. Diabetes & metabolism 33, 135-139.

Takahashi S., Ohinata J., Makita Y., Suzuki N., Araki A., Sasaki A., Murono K., Tanaka H. and Fujieda K. (2008) Skewed X chromosome inactivation failed to explain the normal phenotype of a carrier female with MECP2 mutation resulting in Rett syndrome. Clin Genet 73, 257-261.

Tamashiro K. L., Nguyen M. M. and Sakai R. R. (2005) Social stress: from rodents to primates. Frontiers in neuroendocrinology 26, 27-40.

Tan N. C., Mulley J. C. and Berkovic S. F. (2004) Genetic association studies in epilepsy: "the truth is out there". Epilepsia 45, 1429-1442.

Tannenbaum A. (1959) Nutrition and cancer, in Physiopathology of Cancer (Homburge F., ed.), pp pp. 517-562. Paul B. Hober, NY.

Tannis A. J., Barban J. and Conquer J. A. (2004) Effect of glucosamine supplementation on fasting and non-fasting plasma glucose and serum insulin concentrations in healthy individuals. Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society 12, 506-511.

Temkin O. (1971) The Falling Sickness: A History of Epilepsy from the Greeks to the Beginnings of Modern Neurology, 2nd Edition, pp 67-68. Johns Hopkins University Press, Baltimore.

Thavendiranathan P., Chow C., Cunnane S. and McIntyre Burnham W. (2003) The effect of the 'classic' ketogenic diet on animal seizure models. Brain research 959, 206-213.

Thavendiranathan P., Mendonca A., Dell C., Likhodii S. S., Musa K., Iracleous C., Cunnane S. C. and Burnham W. M. (2000) The MCT ketogenic diet: effects on animal seizure models. Experimental neurology 161, 696-703.

Thio L. L., Wong M. and Yamada K. A. (2000) Ketone bodies do not directly alter excitatory or inhibitory hippocampal synaptic transmission. Neurology 54, 325-331.

Thio L. L., Erbayat-Altay E., Rensing N. and Yamada K. A. (2006) Leptin contributes to slower weight gain in juvenile rodents on a ketogenic diet. Pediatric research 60, 413-417.

Thommessen M., Kase B. F. and Heiberg A. (1992) Growth and nutrition in 10 girls with Rett syndrome. Acta Paediatr 81, 686-690.

Thompson H. J., Zhu Z. and Jiang W. (2003) Dietary energy restriction in breast cancer prevention. J Mammary Gland Biol Neoplasia 8, 133-142.

Ting Y. L. and Degani H. (1993) Energetics and glucose metabolism in hippocampal slices during depolarization: 31P and 13C NMR studies. Brain research 610, 16-23.

Todorova M. T., Burwell T. J. and Seyfried T. N. (1999a) Environmental risk factors for multifactorial epilepsy in EL mice. Epilepsia 40, 1697-1707.

Todorova M. T., Dangler C. A., Drage M. G., Fox J. G. and Seyfried T. N. (1999b) Ketogenic diet prevents urinary retention and sudden death in epileptic male EL mice. Epilepsia 40, 41.

Todorova M. T., Tandon P., Madore R. A., Stafstrom C. E. and Seyfried T. N. (2000) The ketogenic diet inhibits epileptogenesis in EL mice: a genetic model for idiopathic epilepsy. Epilepsia 41, 933-940.

Todorova M. T., Mantis J. G., Le M., Kim C. Y. and Seyfried T. N. (2006) Genetic and environmental interactions determine seizure susceptibility in epileptic EL mice. Genes, brain, and behavior 5, 518-527.

Todorova M. T., Dangler C. A., Drage M. G., Sheppard B. J., Fox J. G. and Seyfried T. N. (2003) Sexual dysfunction and sudden death in epileptic male EL mice: inheritance and prevention with the ketogenic diet. Epilepsia 44, 25-31.

Treiman D. M. (2001) GABAergic mechanisms in epilepsy. Epilepsia 42 Suppl 3, 8-12.

Tunbridge F. K., Home P. D., Murphy M. and Alberti K. G. (1991) Does flexibility at mealtimes disturb blood glucose control on a multiple insulin injection regimen? Diabet Med 8, 833-838.

Turan S., Omar A. and Bereket A. (2008) Comparison of capillary blood ketone measurement by electrochemical method and urinary ketone in treatment of diabetic ketosis and ketoacidosis in children. Acta diabetologica 45, 83-85.

Turturro A., Witt W. W., Lewis S., Hass B. S., Lipman R. D. and Hart R. W. (1999) Growth curves and survival characteristics of the animals used in the Biomarkers of Aging Program. The journals of gerontology 54, B492-501.

Uchibori M., Saito K., Yokoyama S., Sakamoto Y., Suzuki H., Tsuji T. and Suzuki K. (2002) Foci identification of spike discharges in the EEGs of sleeping El mice based on the electric field model and wavelet decomposition of multi monopolar derivations. J Neurosci Methods 117, 51-63.

Ugochukwu N. H. and Figgers C. L. (2007) Attenuation of plasma dyslipidemia and oxidative damage by dietary caloric restriction in streptozotocin-induced diabetic rats. Chemico-biological interactions 169, 32-41.

Vaisleib, II, Buchhalter J. R. and Zupanc M. L. (2004) Ketogenic diet: outpatient initiation, without fluid, or caloric restrictions. Pediatric neurology 31, 198-202.

Valles A., Marti O., Garcia A. and Armario A. (2000) Single exposure to stressors causes long-lasting, stress-dependent reduction of food intake in rats. American journal of physiology 279, R1138-1144.

van 't Veer P. (1994) Measuring nutritional exposures including biomarkers. The Proceedings of the Nutrition Society 53, 27-35.

van de Bovenkamp-Janssen M. C., van der Kloet J. C., van Luijtelaar G. and Roubos E. W. (2006) NMDA-NR1 and AMPA-GluR4 receptor subunit immunoreactivities in the absence epileptic WAG/Rij rat. Epilepsy research 69, 119-128.

Van der Auwera I., Wera S., Van Leuven F. and Henderson S. T. (2005) A ketogenic diet reduces amyloid beta 40 and 42 in a mouse model of Alzheimer's disease. Nutrition & metabolism 2, 28.

Vannucci R. C. and Vannucci S. J. (2000) Glucose metabolism in the developing brain. Seminars in perinatology 24, 107-115.

Vannucci S. J. and Simpson I. A. (2003) Developmental switch in brain nutrient transporter expression in the rat. American journal of physiology 285, E1127-1134.

Vaquero A. and Reinberg D. (2009) Calorie restriction and the exercise of chromatin. Genes & development 23, 1849-1869.

Veech R. L. (2004) The therapeutic implications of ketone bodies: the effects of ketone bodies in pathological conditions: ketosis, ketogenic diet, redox states, insulin resistance, and mitochondrial metabolism. Prostaglandins, leukotrienes, and essential fatty acids 70, 309-319.

Veech R. L., Chance B., Kashiwaya Y., Lardy H. A. and Cahill G. F., Jr. (2001) Ketone bodies, potential therapeutic uses. IUBMB life 51, 241-247.

Vermeulen J. and Aldenkamp A. P. (1995) Cognitive side-effects of chronic antiepileptic drug treatment: a review of 25 years of research. Epilepsy research 22, 65-95.

Vezzani A. (2005a) VEGF and seizures: cross-talk between endothelial and neuronal environments. Epilepsy currents / American Epilepsy Society 5, 72-74.

Vezzani A. (2005b) Inflammation and epilepsy. Epilepsy currents / American Epilepsy Society 5, 1-6.

Vielhaber S., Von Oertzen J. H., Kudin A. F., Schoenfeld A., Menzel C., Biersack H. J., Kral T., Elger C. E. and Kunz W. S. (2003) Correlation of Hippocampal Glucose Oxidation Capacity and Interictal FDG-PET in Temporal Lobe Epilepsy. Epilepsia 44, 193-199.

Villard L. (2007) MECP2 mutations in males. J Med Genet 44, 417-423.

Villard L., Kpebe A., Cardoso C., Chelly P. J., Tardieu P. M. and Fontes M. (2000) Two affected boys in a Rett syndrome family: clinical and molecular findings. Neurology 55, 1188-1193.

Wahlsten D., Metten P., Phillips T. J., Boehm S. L., 2nd, Burkhart-Kasch S., Dorow J., Doerksen S., Downing C., Fogarty J., Rodd-Henricks K., Hen R., McKinnon C. S., Merrill C. M., Nolte C., Schalomon M., Schlumbohm J. P., Sibert J. R., Wenger C. D., Dudek B. C. and Crabbe J. C. (2003) Different data from different labs: lessons from studies of gene-environment interaction. Journal of neurobiology 54, 283-311.

Wan M., Lee S. S., Zhang X., Houwink-Manville I., Song H. R., Amir R. E., Budden S., Naidu S., Pereira J. L., Lo I. F., Zoghbi H. Y., Schanen N. C. and Francke U. (1999) Rett syndrome and beyond: recurrent spontaneous and familial MECP2 mutations at CpG hotspots. Am J Hum Genet 65, 1520-1529.

Ward B. C., Kolodny N. H., Nag N. and Berger-Sweeney J. E. (2009) Neurochemical changes in a mouse model of Rett syndrome: changes over time and in response to perinatal choline nutritional supplementation. Journal of neurochemistry 108, 361-371.

Ward R. J. (1981) Diet and Nutrition, 255-266 Edition, Vol. 47. Academic Press, London.

Weindruch R. and Walford R. L. (1988) The retardation of aging and disease by dietary restriction, p 436. Thomas, Springfield, IL.

Weindruch R., Walford R. L., Fligiel S. and Guthrie D. (1986) The retardation of aging in mice by dietary restriction: longevity, cancer, immunity and lifetime energy intake. The Journal of nutrition 116, 641-654.

Westphal C. H., Dipp M. A. and Guarente L. (2007) A therapeutic role for sirtuins in diseases of aging? Trends in biochemical sciences 32, 555-560.

Wheless J. W., Baumgartner J. and Ghanbari C. (2001) Vagus nerve stimulation and the ketogenic diet. Neurologic clinics 19, 371-407.

Wilder R. (1921) The effects of ketonemia on the course of epilepsy. Mayo Clinic Proc 2, 307-308.

Williamson D. H., Mellanby J. and Krebs H. A. (1962) Enzymatic determination of D(--)-b-hydroxybutyric acid and acetoacetic acid in blood. Biochem. J. 82, 90-96.

Williamson S. L. and Christodoulou J. (2006) Rett syndrome: new clinical and molecular insights. Eur J Hum Genet 14, 896-903.

Witt Engerstrom I. (1992) Age-related occurrence of signs and symptoms in the Rett syndrome. Brain & development 14 Suppl, S11-20.

Wolf P. (1994) Historical aspects: the concept of idiopathy, in Idiopathic generalized epilepsies: clinical, experimental, and genetic aspects (Malafosse A., Genton P., Hirsch E., Marescaux C., Broglin D. and Bernasconi R., eds), pp 3-6. John Libbey & Co. Ltd, London.

Wolf P. (2005) Historical aspects of idiopathic generalized epilepsies. Epilepsia 46 Suppl 9, 7-9.

Wolfer D. P. and Lipp H. P. (2000) Dissecting the behaviour of transgenic mice: is it the mutation, the genetic background, or the environment? Experimental physiology 85, 627-634.

Woods S. C., Schwartz M. W., Baskin D. G. and Seeley R. J. (2000) Food intake and the regulation of body weight. Annual review of psychology 51, 255-277.

Wu G. Y., Gunasekara A., Brunengraber H. and Marliss E. B. (1991) Effects of extracellular pH, CO2, and HCO3- on ketogenesis in perfused rat liver. The American journal of physiology 261, E221-226.

Wylie-Rosett J. and Davis N. J. (2009) Low-carbohydrate diets: an update on current research. Current diabetes reports 9, 396-404.

Yaktine A. L., Vaughn R., Blackwood D., Duysen E. and Birt D. F. (1998) Dietary energy restriction in the SENCAR mouse: elevation of glucocorticoid hormone levels but no change in distribution of glucocorticoid receptor in epidermal cells. Molecular carcinogenesis 21, 62-69.

Yamada K. A., Rensing N. and Thio L. L. (2005) Ketogenic diet reduces hypoglycemia-induced neuronal death in young rats. Neuroscience letters 385, 210-214.

Yamamoto N. and Soghomonian J. J. (2009) Metabotropic glutamate mGluR5 receptor blockade opposes abnormal involuntary movements and the increases in glutamic acid decarboxylase mRNA levels induced by l-DOPA in striatal neurons of 6-hydroxydopamine-lesioned rats. Neuroscience.

Yamashita M., Matsuki A. and Oyama T. (1976) General anaesthesia for a patient with progressive muscular dystrophy. Der Anaesthesist 25, 76-79.

Yasuda N., Inoue T., Nagakura T., Yamazaki K., Kira K., Saeki T. and Tanaka I. (2004) Metformin causes reduction of food intake and body weight gain and improvement of glucose intolerance in combination with dipeptidyl peptidase IV inhibitor in Zucker fa/fa rats. The Journal of pharmacology and experimental therapeutics 310, 614-619.

Yen L. H., Sibley J. T. and Constantine-Paton M. (1993) Fine-structural alterations and clustering of developing synapses after chronic treatments with low levels of NMDA. J Neurosci 13, 4949-4960.

Young C. M., Scanlan S. S., Im H. S. and Lutwak L. (1971) Effect of body composition and other parameters in obese young men of carbohydrate level of reduction diet. The American journal of clinical nutrition 24, 290-296.

Young J. I. and Zoghbi H. Y. (2004) X-chromosome inactivation patterns are unbalanced and affect the phenotypic outcome in a mouse model of rett syndrome. Am J Hum Genet 74, 511-520.

Yudkoff M., Daikhin Y., Nissim I. and Lazarow A. (2004) Ketogenic diet, brain glutamate metabolism and seizure control. Prostaglandins, leukotrienes, and essential fatty acids 70, 277-285.

Yudkoff M., Daikhin Y., Nissim I., Lazarow A. and Nissim I. (2001) Ketogenic diet, amino acid metabolism, and seizure control. Journal of neuroscience research 66, 931-940.

Yudkoff M., Daikhin Y., Nissim I., Horyn O., Lazarow A., Luhovyy B. and Wehrli S. (2005) Response of brain amino acid metabolism to ketosis. Neurochemistry international 47, 119-128.

Zhao Z., Lange D. J., Voustianiouk A., MacGrogan D., Ho L., Suh J., Humala N., Thiyagarajan M., Wang J. and Pasinetti G. M. (2006) A ketogenic diet as a potential novel therapeutic intervention in amyotrophic lateral sclerosis. BMC neuroscience 7, 29.

Zhou W., Mukherjee P., Kiebish M. A., Markis W. T., Mantis J. G. and Seyfried T. N. (2007) The calorically restricted ketogenic diet, an effective alternative therapy for malignant brain cancer. Nutrition & metabolism 4, 5.

Ziegler D. R., Araujo E., Rotta L. N., Perry M. L. and Goncalves C. A. (2002) A ketogenic diet increases protein phosphorylation in brain slices of rats. The Journal of nutrition 132, 483-487.

Ziegler D. R., Gamaro G. D., Araujo E., Bassani M. G., Perry M. L., Dalmaz C. and Goncalves C. A. (2005) Nociception and locomotor activity are increased in ketogenic diet fed rats. Physiology & behavior 84, 421-427.

Ziegler D. R., Ribeiro L. C., Hagenn M., Siqueira I. R., Araujo E., Torres I. L., Gottfried C., Netto C. A. and Goncalves C. A. (2003) Ketogenic diet increases glutathione peroxidase activity in rat hippocampus. Neurochemical research 28, 1793-1797.

Zimmerman E. and Wylie-Rosett J. (2003) Nutrition therapy for hypertension. Current diabetes reports 3, 404-411.

Zoghbi H. Y. (2002) Introduction: Rett syndrome. Ment Retard Dev Disabil Res Rev 8, 59-60.

Zoghbi H. Y. (2005) MeCP2 dysfunction in humans and mice. Journal of child neurology 20, 736-740.