5-2009

Alterations of High-Energy Brain Metabolites Across Multiple Neurodegenerative Disorders

Jeremy W. Gawryluk

Follow this and additional works at: https://commons.und.edu/theses

Part of the Psychology Commons

Recommended Citation

https://commons.und.edu/theses/902
ALTERATIONS OF HIGH-ENERGY BRAIN METABOLITES ACROSS MULTIPLE NEURODEGENERATIVE DISORDERS

by

Jeremy W. Gawryluk
Bachelor of Science, University of Manitoba, 2003

A Dissertation
Submitted to the Graduate Faculty
of the
University of North Dakota
in partial fulfillment of the requirements

for the degree of
Doctor of Philosophy

Grand Forks, North Dakota
May
2009
This dissertation, submitted by Jeremy W. Gawryluk in partial fulfillment of the requirements for the Degree of Doctor of Philosophy from the University of North Dakota, has been read by the Faculty Advisory Committee under whom the work has been done and is hereby approved.

This dissertation meets the standards for appearance, conforms to the style and format requirements of the Graduate School of the University of North Dakota, and is hereby approved.

Dean of the Graduate School

May 5, 2009
PERMISSION

Title Alterations of High-Energy Brain Metabolites across Multiple Neurodegenerative Disorders

Department Pharmacology, Physiology & Therapeutics

Degree Doctor of Philosophy

In presenting this dissertation in partial fulfillment of the requirements for a graduate degree from the University of North Dakota, I agree that the library of this University shall make it freely available for inspection. I further agree that permission for extensive copying for scholarly purposes may be granted by the professor who supervised my dissertation work or, in his absence, by the chairperson of the department or the dean of the Graduate School. It is understood that any copying or publication or other use of this dissertation or part thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of North Dakota in any scholarly use which may be made of any material in my dissertation.

Signature

Date April 20, 2009
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF FIGURES</td>
<td>ix</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xiv</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>xv</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>xvii</td>
</tr>
<tr>
<td>CHAPTER</td>
<td></td>
</tr>
<tr>
<td>I.  INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>Energetics</td>
<td>1</td>
</tr>
<tr>
<td>Alzheimer's Disease</td>
<td>11</td>
</tr>
<tr>
<td>Autism</td>
<td>17</td>
</tr>
<tr>
<td>Down Syndrome</td>
<td>24</td>
</tr>
<tr>
<td>HIV-1 Dementia</td>
<td>33</td>
</tr>
<tr>
<td>Ketogenic Diet &amp; Epilepsy</td>
<td>35</td>
</tr>
<tr>
<td>II. METHOD</td>
<td>43</td>
</tr>
<tr>
<td>General Method</td>
<td>43</td>
</tr>
<tr>
<td>Analysis of Cerebral Energetics</td>
<td>43</td>
</tr>
<tr>
<td>Cholesterol fed Rabbits as a Model for Alzheimer's Disease</td>
<td>45</td>
</tr>
<tr>
<td>Animals and Dietary Treatment</td>
<td>45</td>
</tr>
<tr>
<td>Analysis of Cerebral Energetics</td>
<td>46</td>
</tr>
<tr>
<td>ADSL Transgenic Mice as a Model for Autism</td>
<td>46</td>
</tr>
</tbody>
</table>
III. RESULTS

Cholesterol Fed Rabbits as a Model for Alzheimer's Disease 65
ADSL Transgenic Mice as a Model for Autism 68
Ts65Dn and GART Transgenic Mice as Models for Down Syndrome 81
Treatment of Rat Cerebral Cortical Neurons with HIV-1 Protein Tat as a Model for HIV-Dementia 99
Creatine Protection Against Tat172-Induced Neuronal Cell Death 100
Effects of Tat and Creatine on Levels of Adenine Nucleotides and Phosphocreatine 101
Creatine Protects Against Tat-Induced Decreases in Mitochondrial Membrane Potential 105
Creatine Protects Against Tat-Induced Increases in the Opening of Mitochondrial Permeability Transition Pores 106
Ketogenic Diet as a Treatment for Epilepsy 107
KD Treatment Induces Rapid Ketonemia but Delayed Seizure Protection 107
Metabolic Genes are Coordinately Upregulated after KD 110
Increased Number of Mitochondrial Profiles in the Hippocampus after KD 116
Energy Reserves are Increased after KD 118
Synaptic Transmission is More Resistant to Low Glucose after KD 128
Exogenously Applied Adenosine Decreases fEPSP Amplitude after KD 131
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Glycolysis</td>
<td>3</td>
</tr>
<tr>
<td>2.</td>
<td>The Citric Acid Cycle</td>
<td>4</td>
</tr>
<tr>
<td>3.</td>
<td>The Electron Transport Chain</td>
<td>5</td>
</tr>
<tr>
<td>4.</td>
<td>Creatine Biosynthesis</td>
<td>8</td>
</tr>
<tr>
<td>5.</td>
<td>Creatine-Phosphocreatine Energy Shuttle</td>
<td>10</td>
</tr>
<tr>
<td>6.</td>
<td>Pathways of Purine Metabolism</td>
<td>20</td>
</tr>
<tr>
<td>7.</td>
<td>Effects of Administration of a High Cholesterol Diet on Levels of Creatine and Phosphocreatine in Rabbit Cerebral Cortex</td>
<td>66</td>
</tr>
<tr>
<td>8.</td>
<td>Effects of Administration of a High Cholesterol Diet on Levels of Adenine Nucleotides in Rabbit Cerebral Cortex</td>
<td>67</td>
</tr>
<tr>
<td>9.</td>
<td>Effects of Administration of a High Cholesterol Diet on Energetic Ratios in Rabbit Cerebral Cortex</td>
<td>68</td>
</tr>
<tr>
<td>10.</td>
<td>Effects of ADSL Genetic Modification on Levels of Creatine and Phosphocreatine in Mouse Cerebral Cortex</td>
<td>69</td>
</tr>
<tr>
<td>11.</td>
<td>Effects of ADSL Genetic Modification on Levels of Creatine and Phosphocreatine in Mouse Cerebral Cortex</td>
<td>71</td>
</tr>
<tr>
<td>12.</td>
<td>Effects of ADSL Genetic Modification on Levels of Creatine and Phosphocreatine in Mouse Cerebellum</td>
<td>72</td>
</tr>
<tr>
<td>13.</td>
<td>Effects of ADSL Genetic Modification on Levels of Adenine Nucleotides in Mouse Cerebral Cortex</td>
<td>73</td>
</tr>
<tr>
<td>14.</td>
<td>Effects of ADSL Genetic Modification on Levels of Adenine Nucleotides in Mouse Cerebral Cortex</td>
<td>74</td>
</tr>
<tr>
<td>Chapter</td>
<td>Title</td>
<td>Page</td>
</tr>
<tr>
<td>---------</td>
<td>----------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>15</td>
<td>Effects of ADSL Genetic Modification on Levels of Adenine Nucleotides in Mouse Cerebellum</td>
<td>75</td>
</tr>
<tr>
<td>16</td>
<td>Effects of ADSL Genetic Modification on Energetic Ratios in Mouse Cerebral Cortex</td>
<td>76</td>
</tr>
<tr>
<td>17</td>
<td>Effects of ADSL Genetic Modification on Energetic Ratios in Mouse Cerebral Cortex</td>
<td>77</td>
</tr>
<tr>
<td>18</td>
<td>Effects of ADSL Genetic Modification on Energetic Ratios in Mouse Cerebellum</td>
<td>78</td>
</tr>
<tr>
<td>19</td>
<td>Effects of ADSL Genetic Modification on SAICAR Levels in Mouse Cerebral Cortex</td>
<td>79</td>
</tr>
<tr>
<td>20</td>
<td>Effects of ADSL Genetic Modification on SAICAR and Adenylosuccinate Levels in Mouse Cerebral Cortex</td>
<td>80</td>
</tr>
<tr>
<td>21</td>
<td>Effects of ADSL Genetic Modification on SAICAR and Adenylosuccinate Levels in Mouse Cerebellum</td>
<td>80</td>
</tr>
<tr>
<td>22</td>
<td>Effects of Trisomy 21 on Levels of Creatine and Phosphocreatine in Mouse Cerebral Cortex</td>
<td>82</td>
</tr>
<tr>
<td>23</td>
<td>Effects of Trisomy 21 on Levels of Adenine Nucleotides in Mouse Cerebral Cortex</td>
<td>83</td>
</tr>
<tr>
<td>24</td>
<td>Effects of Trisomy 21 on Energetic Ratios in Mouse Cerebral Cortex</td>
<td>84</td>
</tr>
<tr>
<td>25</td>
<td>Effects of Trisomy 21 on Levels of Adenosine in Mouse Cerebral Cortex</td>
<td>85</td>
</tr>
<tr>
<td>26</td>
<td>Levels of Creatine and Phosphocreatine in Wildtype Mouse Cerebral Cortex as a Function of Age</td>
<td>86</td>
</tr>
<tr>
<td>27</td>
<td>Levels of Adenine Nucleotides in Wildtype Mouse Cerebral Cortex as a Function of Age</td>
<td>87</td>
</tr>
<tr>
<td>28</td>
<td>Energetic Ratios in Wildtype Mouse Cerebral Cortex as a Function of Age</td>
<td>87</td>
</tr>
<tr>
<td>29</td>
<td>Levels of Adenosine in Wildtype Mouse Cerebral Cortex as a Function of Age</td>
<td>88</td>
</tr>
<tr>
<td>30</td>
<td>Levels of Creatine and Phosphocreatine in Ts65Dn Mouse Cerebral Cortex as a Function of Age</td>
<td>89</td>
</tr>
</tbody>
</table>

Produced with permission of the copyright owner. Further reproduction prohibited without permission.
46. Effects of Tat, Mutant-Tat and Creatine on Levels of Creatine and Phosphocreatine in Primary Rat Cortical Neurons ........................................ 104

47. Effects of Tat, Mutant-Tat and Creatine on Mitochondrial Membrane Potential as Determined with the Fluorescent Dye JC-1 ........................................ 105

48. Effects of Tat, Mutant-Tat and Creatine on Opening of Mitochondrial Permeability Transition Pores as Determined with Calcein-AM Fluorescence and Cobalt Chloride Quenching ........................................ 106

49. Effects of Tat, Mutant-Tat and Creatine on Formation of Reactive Oxygen Species as Determined with the Fluorescent Dye H$_2$DCFDA.. 107

50. The Anticonvulsant Effect of the KD Develops Slowly .................................. 109

51. Changes in Gene Expression after KD .......................................................... 114

52. Metabolic Genes Changed after KD ............................................................ 116

53. Mitochondrial Biogenesis in Hippocampus of Rats fed a Ketogenic Diet .............................................................. 118

54. Effects of KD Administration on Levels of Creatine and Phosphocreatine in Discrete Rat Brain Regions .......................................................... 121

55. Effects of KD Administration on Levels of Adenine Nucleotides in Discrete Rat Brain Regions .......................................................... 122

56. Effects of KD Administration on Energetic Ratios in Discrete Rat Brain Regions .......................................................... 123

57. Effects of KD Administration on Levels of Adenosine in Discrete Rat Brain Regions .......................................................... 123

58. Effects of KD Administration on Levels of Creatine and Phosphocreatine in Discrete Rat Brain Regions .......................................................... 125

59. Effects of KD Administration on Levels of Adenine Nucleotides in Discrete Rat Brain Regions .......................................................... 126

60. Effects of KD Administration on Energetic Ratios in Discrete Rat Brain Regions .......................................................... 127

61. Effects of KD Administration on Levels of Adenosine in Discrete Rat Brain Regions .......................................................... 127
62. Synaptic Transmission is More Resistant to Metabolic Challenge in Slices taken from KD-Fed Animals than those from Control-Fed Animals......................................................................................................130
63. Effect of KD on fEPSP Amplitude in Hippocampus..............................132
64. Hypothesis for Anticonvulsant Effect of the KD .....................................157
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Levels of Energy Metabolites following Ketogenic Diet</td>
<td>119</td>
</tr>
<tr>
<td>2.</td>
<td>Dietary Constituents for Standard Rodent Chow (2018 Harlan-Teklad)</td>
<td>163</td>
</tr>
<tr>
<td>3.</td>
<td>Dietary Constituents for Bio-Serv Ketogenic Diet (F3666 Bio-Serv)</td>
<td>164</td>
</tr>
<tr>
<td>4.</td>
<td>Ketogenic Diet affects Expression of Metabolism Genes</td>
<td>165</td>
</tr>
<tr>
<td>5.</td>
<td>List of ADSL Mutations</td>
<td>167</td>
</tr>
<tr>
<td>6.</td>
<td>Genes on Chromosome 21 Important for Mitochondrial Metabolism</td>
<td>168</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

The Alzheimer’s disease project was funded by COBRE for Dr. Othman Ghribi.

The ADSL studies were funded by NIH Grant MH65431 and grants from the BonWils-Stanton Foundation and the Ludlow-GriYth Foundation to Dr. David Patterson. Thanks to Dr. Erin Spiegel for her help with SAICAR analyses.

Ts65Dn mouse studies were funded by the National Institute of Neurological Disease and Stroke to Dr. David Patterson and AG17628 and RR017699 grants to Dr. Jonathan D. Geiger. Thanks Dr. Guido Vacano for his constant assistance on the Down syndrome project.

The HIV-1 dementia studies were supported by grants from the NCRR (P20 RR17699-01), the National Institute of Aging (AG17628), and the Canadian Institutes of Health Research (HOP-8901, MOP-53329). Thanks to Patrick Stevens for his insight on the HIV1-dementia project as well as for his careful and dedicated experimentation that is included in this dissertation. Thank you to Dr. Avindra Nath for supplying us with Tat.

The ketogenic diet and epilepsy studies were supported by the Charlie Foundation (Dr. Kristopher Bough), the NIH - National Institute of Neurological Disorders and Stroke (Dr. Raymond Dimgledine [NS 177701], Dr. Yoland Smith, Dr. James Greene), the Norwegian Defense Research Establishment (Dr. Bjornar Hassel), and by P20 RR17699 from the National Center for Research...
Resources, a component of the NIH, AG17628 (Dr. Jonathan Geiger). Thank you to David Knorr for performing the glycogen measurements and to John Wagener for assisting with animal sacrifice and ketogenic diet analyses.
To my parents, Ray & Linda Gawryluk, my son, Kailas Gawryluk, & wife, Sangeeta Kauldher for providing encouragement, support, and patience throughout my doctoral studies.
ABSTRACT

Brain energy metabolism is vital for many cellular processes including homeostasis and thus disturbances to metabolism can be the cause or consequence of neurodegeneration. Metabolic discrepancies have been hypothesized to be involved but less frequently demonstrated to be manipulated by acute and chronic neurodegenerative disorders such as stroke, traumatic brain injury, and epilepsy. I hypothesized that cerebral energy levels are decreased in my model systems for Alzheimer’s disease, Autism, Down syndrome, and HIV-1 dementia and that dietary treatments could enhance energy reserves and protect against neurodegenerative disease. For rodent studies, I utilized a high-energy head focused microwave irradiation system to kill animals but most importantly to snap-inactivate all cerebral enzymes, including those that contribute to the rapid degradation of high-energy phosphate compounds. I found that energy levels are diminished in a high-cholesterol diet model for Alzheimer’s disease in rabbit, a trisomic mouse model for Down syndrome (Ts65Dn), and following administration of Tat to primary mouse cortical cultures as a model for HIV-1 dementia. My experiments also examined the extent to which protection is provided by creatine supplementation and the ketogenic diet in models of HIV-1 dementia and epilepsy, respectively. Creatine bioenergetically protected against Tat-induced decreases in cellular levels of ATP, Tat-induced mitochondrial hypopolarization, and Tat-induced
mitochondrial permeability transition pore opening. My calorie restricted ketogenic diet studies demonstrated this diet's ability to protect against chemically induced seizures. As well, I observed a coordinated upregulation of all differentially regulated transcripts encoding energy metabolism enzymes, increased numbers of mitochondrial profiles, and ultimately augmented high-energy phosphate levels in seizure naïve rats. My studies demonstrate compromised brain energy levels in the aforementioned neurodegenerative disorders and that dietary treatments such as creatine supplementation for HIV-1 dementia and the ketogenic diet for epilepsy, may protect cerebral function by enhancing neuroenergetics.
CHAPTER I
INTRODUCTION

Energetics

The brain uses a disproportionately large amount of energy for its size and it is of utmost importance that this organ is able to maintain its proper energy. The brain uses approximately 20% of the body’s energy even though it only comprises approximately 2% of the body’s mass (Clarke & Sokoloff, 1999). The brain has a high priority for glucose. Energy is responsible for all the active processes that occur. For example, adenosine triphosphate (ATP) is used for transcription, translation, lipid production, and for channels and transport proteins (Clarke & Sokoloff, 1999). Large amounts of brain energy are needed to drive the Na⁺/K⁺ ATPase; transport proteins that exchange 2 extracellular K⁺ ions for 3 intracellular Na⁺ ions. The Na⁺/K⁺ ATPases are directly responsible for maintaining ion gradients across the plasma membrane in neurons and thus readying neurons for action potential generation.

Mitochondrial oxidative phosphorylation is regulated by three metabolites; adenosine diphosphate (ADP), O₂, and nitric oxide (Wallace, 2001). The rate of ATP synthesis and that of respiration are set by cellular energy needs, which are represented by the concentration of cytosolic ADP or as phosphorylation potential ([ATP]/[ADP][Pi]), where the ratio of [ATP]/[ADP] is the fundamental component (Navarro & Boveris, 2007). ATP metabolizes to ADP and inorganic...
phosphate (Pi), and these products increase when cellular energy demand is elevated, thus lowering the phosphorylation potential. Increased availability of ADP raises the rate of respiration and this regenerates ATP. The phosphorylation potential is finely regulated and only minimally fluctuates between tissues (Navarro & Boveris, 2007).

Mitochondria contribute approximately 90% of the required energy for cellular functions (Wallace, 1997) by two metabolic processes; the citric acid cycle (TCA; anaerobic) and the electron transport chain (ETC; aerobic). Bioenergetic homeostasis requires the production and deliverance of phosphoryls and nicotinamide adenine dinucleotide (NAD⁺) oxidizing power (Dzeja et al., 2000; Saks et al., 1994). During aerobic conditions, the majority of ATP generated by the oxidation of the glycolytic product pyruvate is used to maintain cellular homeostasis (Figure 1). Under anaerobic conditions, glycolysis results in lactate as the product. ATP generating systems, including glucose uptake, glycolysis, NADH, and fatty acid oxidation (Vannucci & Hawkins, 1983), are stimulated by an elevated ratio of AMP to ATP and thus activation of AMP-activated protein kinase (Carling, 2004; Hardie et al., 2003). However, this process is inadequate to satisfy all cellular energetic demands (Dzeja et al., 2000) and therefore, other processes including adenylate kinase, creatine kinase, and glycolytic enzymes contribute to maintenance of ATP-generating and ATP-consuming processes (Dzeja & Terzic, 1998; Saks et al., 1994; Wallace, 1999).
Figure 1: Glycolysis. Glycolysis links the metabolism of sugars to that of organic acids in the Citric acid cycle. Glycolysis has three key functions: (1) to generate high-energy molecules such as ATP and NADH as energy sources for either aerobic or anaerobic respiration; (2) to produce pyruvate for the citric acid cycle; and (3) to produce various three and six carbon intermediate products that can be removed and utilized for other cellular purposes.
Figure 2: The Citric Acid Cycle. Two carbons are oxidized to CO$_2$ and energy from these reactions is transferred to other metabolic processes by either GTP or ATP, and as electrons in ubiquinol (QH$_2$) and nicotinamide adenine dinucleotide (NADH). The NADH generated in the citric acid cycle will donate its electrons in oxidative phosphorylation to drive ATP synthesis. Flavin adenine dinucleotide (FADH$_2$) is covalently attached to succinate dehydrogenase, an enzyme functioning both in the citric acid cycle and the ETC in oxidative phosphorylation. FADH$_2$ facilitates transfer of electrons to coenzyme Q, the final electron acceptor of the reaction.

Cerebral cells create most of their energy as ATP, through the mitochondrial ETC coupled to oxidative phosphorylation from the oxidation of NADH, a substrate for complex I, and FADH$_2$, a substrate for complex II. NADH and FADH$_2$ function as electron carriers by transferring glycolytic-derived electrons (Figure 1) and/or those resulting from the citric acid (TCA) or Kreb's cycle (Figure 2) into the ETC (Figure 3) through NADH; ubiquinone.
oxidoreductase (complex I) and succinate:ubiquinone oxidoreductase (complex II). Ubiquinone:cytochrome c-oxidoreductase (complex III) couples the oxidation of ubiquinol to cytochrome c reduction (Rieske, 1976), whereas cytochrome c:oxygen oxidoreductase (complex IV) catalyzes the oxidation of cytochrome c, ultimately reducing molecular oxygen to water (Capaldi, 1990; Higgins & Greenamyre, 1996). The course of redox cycling, releases potential energy that drives extrusion of protons across the inner mitochondrial membrane at complexes I, III, and IV. The generated electrochemical gradient from proton translocation powers ATP synthesis by the activity of F<sub>1</sub>F<sub>0</sub> ATP synthase (Complex V; Love et al., 1999).

**Mitochondrial Electron Transport Chain**

Figure 3: The Electron Transport Chain. Enzymatic complexes couple a chemical reaction between an electron donor (such as NADH) and an electron acceptor (such as O<sub>2</sub>) to the transfer of H<sup>+</sup> ions across a membrane, through a set of mediating biochemical reactions. These H<sup>+</sup> ions generate an electrochemical gradient that drives ATP synthase and thus produces ATP.
Glycogen represents the principal store of glucose equivalents in brain, with its equivalent concentration far exceeding physiological glucose concentration. Glycogen is essential for normal brain function, however its acceptance as a reserve has been discounted based on its low concentration compared to liver and muscle. This limited amount of glycogen, taken together with a metabolic rate of 1.4 μmol/g/min glucose in rodents, has led to an argument that 3 μmol/g of glycogen can merely sustain glycolysis for 10 min and therefore glycogen must not serve as a significant energy store (Siegel & Agranoff, 1999). However, there are indeed substantial brain glycogen levels, that are further elevated following hypoglycemia (Choi et al., 2003), sleep deprivation (Kong et al., 2002), and ischemia (Brucklacher et al., 2002). Brain glycogen is a significant store of glucose equivalents and can protect brain during episodes of diminished glucose or insult (Gruetter, 2003).

The basal rate of glucose utilization of astrocytes is higher than that observed for neurons (Magistretti & Pellerin, 1996). A metabolic compartmentation exists whereby astrocytic glucose is glycolytically metabolized to lactate, which is then released to the extracellular space to be utilized by neurons. Lactate can act as an energy source, i.e. sustain synaptic activity, through pyruvate by being enzymatically metabolized by lactate dehydrogenase (Bittar et al., 1996). Pyruvate is therefore an important energetic substrate that is distributed abundantly in brain synapses (Nicklas et al., 1971). Both lactate and pyruvate have limited permeability to the blood-brain barrier and thus cannot
replace plasma glucose to maintain then normal function of brain (Pardridge & Oldendorf, 1977) however they remain useful metabolic substrates for neurons if formed inside brain parenchyma (Ide et al., 1969; Teller et al., 1977). Glucose is the brain’s essential energy substrate (Edvinsson et al, 1993), but fatty acids are also used (Ebert et al., 2003). However, fatty acid oxidation is limited by the low activity of mitochondrial 3-ketoacyl-CoA thiolase (Yang et al., 1987). As an alternative substrate, brain cells can metabolize ketones during fasting-induced reductions to blood glucose (Cahill, 1970; Greene et al., 2003). Ketone bodies are derived from hepatic fat metabolism and are transported into brain across the blood-brain barrier via monocarboxylic transporters (Koehler-Stec et al., 1998; Pellerin et al., 1998). The ketones consist of β-hydroxybutyrate, acetoacetate, and acetone and are important metabolites in our ketogenic diet studies described later.

Creatine (N-[[aminoiminomethyl]-N-methyl glycine) is an amino acid that is produced endogenously (Figure 4) as well as being ingested in meat-based diets. Though creatine is not a direct brain energetic substrate, it does serve an intimate role for maintaining energy levels. Creatine is stored mainly in the muscle with the remainder found in brain, liver, kidneys and testes (Mujika & Padilla, 1997). In a typical western diet, approximately 1g of creatine is consumed per day from protein containing foods (Jacobs, 1999). Endogenous creatine is primarily produced from the amino acids arginine, glycine and methionine in liver, with a minor contribution from the kidneys, pancreas and testes. The average human body will synthesize approximately 1g of creatine per
day. The kidneys excrete creatine in the form of creatinine at the rate of approximately 2 grams per day (Thorell et al., 1999).

Figure 4: Creatine Biosynthesis. Endogenous formation of creatine occurs primarily in liver from arginine, glycine and methionine. Removal of creatine from the body results from nonenzymatic degradation to creatinine through glomerular filtration within kidneys.

Greater than 95% of creatine is stored in muscle where it appears to enhance muscle performance; however creatine can be taken up into brain where evidence suggests that it is neuroprotective (Sullivan et al., 2000). Transport of creatine across the blood brain barrier is limited as there is an absence of creatine transporter expression on astrocytes in contact with capillary
endothelial cells. The brain relies instead on \textit{de novo} synthesis of creatine (Figure 4; Braissant et al., 2001).

Creatine enters target tissue cells via sodium dependent transport through creatine transporters (CreaT; Snow & Murphy, 2001) that are down-regulated in some myopathies (Persky & Brazeau, 2001). CreaT regulates tissue levels in response to low dietary intake or high endogenous creatine levels (Guerrero-Ontiveros & Wallimann, 1998). Reverse enzymatic phosphorylation of creatine by mitochondrial creatine kinase, in the presence of ATP, thereby forms phosphocreatine (creatine phosphate) and ADP in the mitochondrial intermembrane space. Phosphocreatine is then transported into the cytosol where creatine kinase can enzymatically catalyze high-energy phosphate donation from phosphocreatine to ADP and thus yield the products creatine and ATP (Greenhaff et al., 1994). This process in which creatine accepts and donates a high-energy phosphate to ATP is termed the creatine-phosphocreatine energy shuttle (Figure 5). This mechanism permits phosphocreatine to act as a cytosolic energy store.
Figure 5: Creatine-Phosphocreatine Energy Shuttle. Creatine enters the cell through its plasma membrane transporter and passes into mitochondria via the outer mitochondrial membrane creatine transporter. Mitochondrial creatine kinase reversibly phosphorylates creatine in the presence of adenosine triphosphate (ATP) thus forming phosphocreatine. Phosphocreatine, after leaving a mitochondrion, donates a high-energy phosphate group to adenosine diphosphate (ADP) by cytosolic creatine kinase to form ATP and creatine.

The creatine-phosphocreatine energy shuttle and adenylate kinase systems facilitate processes in the brain that would otherwise depend entirely on the diffusion of ATP and ADP. Creatine kinase is a major phosphotransfer system in cells specifically located at places of energy metabolism (Wallimann et al., 1992) and acts in concert with other enzymatic systems to facilitate
intracellular energetic communication (Dzeja & Terzic, 2003; Joubert et al., 2002; Neumann et al., 2003) and enhancement of cytoplasmic high-energy phosphates (Brustovetsky et al., 2001). Due to its location at the adenine nucleotide transporter on the mitochondrial membrane, intramitochondrial ATP is released from mitochondria by interconversion to phosphocreatine (Schlattner et al., 2006). Phosphocreatine has an important role in protection from heart attacks (Saks et al., 2006) and as a result of creatine treatment for brain insult (e.g. traumatic brain injury (Sullivan et al., 2000), and other acute and chronic neurological disorders (Klein & Ferrante, 2007). Both mitochondrial and cytosolic creatine kinase isozymes are active in brain cells with high and variable ATP metabolic rates (Holtzman et al., 1997). The importance of phosphocreatine to energetic homeostasis is indicated by promotion of hypoxic seizures (Holtzman et al., 1999).

Alzheimer's Disease

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized primarily by deteriorating cognitive function with memory loss, reduced self-care, behavioral and psychological symptoms, and emotional troubles (Mimura, 2008). The most vulnerable regions of brain in AD include the basal forebrain, amygdale, hippocampus, cerebral cortex, and discrete brain stem nuclei (Auld et al., 2002; Selkoe, 2001). For the most part, AD cases are sporadic without known genetic linkage, however approximately 5% of cases are caused by known genetic abnormalities. There have been many documented pathogenic pathways that lead to neurodegeneration observed in AD patients;
these include accumulation of misfolded proteins, excitotoxic reactions, oxidative stress, mitochondrial injury, ubiquitin-proteasome system dysfunction, synaptic failure, altered metal homeostasis, dysfunction of axonal and dendritic transport, and misoperation of chaperones (Cacabelos et al., 2005; Kayed et al., 2003). The hallmark pathologies of disease for both sporadic and genetic (familial) AD are the presence of extracellular neuritic plaques containing amyloid-β (Aβ) peptide, intracellular neurofibrillary tangles consisting of hyperphosphorylated microtubule associated protein (Tau), and loss of specific cerebral synapses. Aβ plaque deposition is composed of a 39- to 42-amino acid peptide, the proteolytic product of the amyloid precursor protein (APP; Miller et al., 1993). The underlying root for selective neuronal loss is unknown, however evidence suggests that Aβ accumulation may initiate and/or advance neurodegeneration in brain (Clippingdale et al., 2001; Yankner, 1996). Fernandez-Vizarra and colleagues (2004) hypothesized that Aβ may be inhibiting mitochondrial function and thus contributing to bioenergetic failure. A loss of mitochondrial metabolism regulation, combined with plausible oxidative abnormalities, may be an underlying link to the known pathological events (Zhu et al., 2004c).

Brain hypoperfusion and heart disease are two important risk factors that have been linked to AD (de la Torre, 2006). Evidence from an assortment of clinical and basic studies (Alves & Busatto, 2006; Breteler, 2000; Korf et al., 2004) have suggested that AD is a vasocognopathy (a vascular-related, cognitive disorder; de la Torre, 2004). Hypoperfusion-induced oxidative stress contributes to the pathogenesis of AD (de la Torre, 2002; de la Torre, 1997). AD patients...
also collect a larger fraction of oxygen from the vasculature (Fukuyama et al., 1994; Hoyer, 1993; Galle et al., 1995). It is possible that low cerebrovascular blood flow could be one of the chief initiating factors of mitochondrial abnormalities during AD progression (Risberg, 1980). De la Torre (2000) predicted that aging in the presence of a comorbid condition, such as a vascular risk factor, promotes a critically attained threshold of chronic cerebral hypoperfusion and over time induces degeneration of brain capillaries and hindered delivery of energy substrates to neurons (de la Torre, 2000).

Diminished metabolism occurs with age in frontal areas, temporal and parietal cortices, as measured by high-resolution positron emission tomography (PET) Loessner et al., 1995). Impaired cerebral energy metabolism can facilitate neuronal damage or the deleterious effects of some excitotoxic agents such as glutamate (Schinder et al., 1996). The most direct evidence of abnormal metabolism was demonstrated from in vivo PET (Benson et al., 1983; Frackowiak et al., 1981; Rhee, 1999) and diagnosis of AD using PET are roughly equivalent to those using diagnostic criteria. Decreases in the metabolic rate for glucose in cortex have been demonstrated (Vander Borght et al., 1997) appear early in the disease, perhaps even earlier than the onset of clinical symptoms in some patients (Kennedy et al., 1995; Small et al., 2000), and increase in magnitude as AD becomes more severe (Smith et al., 1992).

Since brain cells thrive on glucose as a principal fuel, its impaired delivery and poor delivery of oxygen compromises neuronal stability as aerobic glycolysis' supply does not adequately satisfy the brain's requirements. Most importantly,
cerebral metabolic rate abnormalities precede evidence for functional impairment by neuropsychological testing or of brain atrophy by neuroimaging (Baloyannis et al., 2004). Metabolic derangements comparable to those observed in AD, such as hypoxia, hypoglycemia, vitamin deficiency, are sufficient to provoke mental and neurological deficits as is evident in neuropsychiatric disorders associated with oxidative metabolism abnormalities (Blass & Gibson, 1999). Overall, chronic cerebral hypoperfusion is a metabolic cascade involving mitochondrial dysfunction, elevated oxidative stress, and diminished production of ATP and phosphocreatine (Pettegrew et al., 1994) that likely contributes to the progressive cognitive decline characteristic of AD. Chronic cerebral hypoperfusion also results in anatomic pathology common to AD, such as synaptic loss, senile plaques, and neurofibrillary tangles.

Since impaired energy metabolism greatly precedes clinical symptoms and oxidative stress is one of the initial features of the disease (Nunomura et al., 2000; Nunomura et al., 2001), it is likely that mitochondria play a critical role in the pathogenesis of the disease. Mitochondria, the powerhouses of the cell, are located within dendrites, axons and synaptic terminals and generate ATP, oxidizing power, and calcium buffering (Mattson & Liu, 2003). These subcellular regions have greater metabolic demands from mitochondria, compared to other subcellular neuronal regions, and therefore are endowed with superior oxidative and calcium burdens. Indirect evidence for this enhanced burden can be extrapolated from the location of early neurodegeneration in AD, the synapse (Coleman et al., 2004). For example, synapses are missing within dense Aβ
cores of classic senile plaques (Lassmann et al., 1993) and synapse degeneration correlates strongly with cognitive decline (DeKosky et al., 1996). Synaptic compartments have high concentrations of glutamate receptors and calcium channels. Following depolarization with calcium influx there is increased oxidative stress and ATP demand, and possible apoptosis (Bergles & Jahr, 1998; Guo, 1999). Considerable evidence suggesting that synapses are primary sites of calcium deregulation in AD (LaFerla, 2002). As well, apoptosis has been demonstrated to occur in vulnerable AD neuronal populations and may be activated locally in synaptic compartments following exposure to Aβ (Mattson et al., 1998). Synaptic loss associated with downstream processes may elicit neurodegeneration through reduction in metabolic activity, regional cerebral blood flow (Liu et al., 1999), and activation of microglia (Ji et al., 2001).

The most dependable defect in mitochondrial components in AD has been a deficit in key enzymes of oxidative metabolism, including alpha-ketoglutarate dehydrogenase complex and pyruvate dehydrogenase complex, two enzymes in the rate-limiting step of the tricarboxylic acid cycle, and cytochrome oxidase, complex IV of the mitochondrial respiratory chain that reduces molecular oxygen to water (Cottrell et al., 2001; Gibson et al., 1998). Reductions in alpha-ketoglutarate dehydrogenase complex activity correlates much better to the extent of dementia than can senile plaques and neurofibrillary tangles in the brains of ApoE4-positive AD patients (Gibson et al., 2000). Upon reductions to enzymatic activity there is a substantial increase in aberrant production of reactive oxygen species and this has been demonstrated in mitochondrial DNA
isolated from AD (Coskun et al., 2004; Hirai et al., 2001; Mecocci et al., 1993; Mecocci et al., 1994).

Changes to mitochondrial function are well documented in AD and can have a significant impact on neurons. Recently, Liang and colleagues (2008) performed analyses of nuclear genes influencing mitochondrial energy metabolism and demonstrated that they have a significantly lowered expression in AD brain. The majority of these nuclear genes encode electron transport chain subunits while the remaining examined genes encode mitochondrial translocases that are responsible for the entry of electron transport chain subunits into mitochondria. The largest percentage of underexpressed genes was found in the posterior cingulate cortex, where up to 70% of genes were underexpressed, this region has been shown to be metabolically affected in the earliest stages of AD (Liang et al., 2008). The regional underexpression of genes encoding ETC subunits correspond to the regional pattern of cerebral metabolic rate of glucose reductions observed in PET studies of AD patients (Mielke et al., 1994; Minoshima et al., 1994; Minoshima et al., 1997; Pietrini et al., 1993; Smith et al., 1992).

Findings of abnormal brain energy metabolism in AD patients have come from imaging studies largely of patients with the sporadic form of the disease. However, there exist alternative strategies to help elucidate these metabolic changes. For example, direct biochemical measurements from animal models may better define disease etiology. Cholesterol has been suggested to be a risk factor for AD (Pappolla et al., 2003; Wolozin 2004) and has been demonstrated...
in both animal (Refolo et al., 2000; Sparks et al., 1994) and cellular (Racchi et al., 1997) models to promote production of Aβ, a major pathological hallmark of AD. Examination of brain energy levels in this AD model system may provide better insight into the universality of altered energetics in AD and our work was aimed to determine whether changes in brain bioenergetics was associated with this disease.

Autism

Autism is a syndrome of early childhood characterized by abnormal social relationships, language, impaired understanding, echolalia, and pronominal reversal. Individuals with autism may perform ritual and compulsive behaviors and typically display an uneven intellectual development (State et al., 2000). Affecting approximately 1 in 2000 individuals (Wassink & Piven, 2000), the syndrome emerges prior to three years of age and affects approximately three times more boys than girls. However, girls with the disorder are often more severely affected (Wassink & Piven, 2000). Extreme environmental deprivation is one means to cause an autism-like syndrome however the relatively constant rate of occurrence across cultures suggests a strong biological component. A biological factor has been demonstrated by family studies (Wassink & Piven, 2000). The recurrence rate among siblings of an affected proband is approximately 5% and therefore the risk for siblings is 100-200 times higher than that for the general population. The concordance of between monozygotic and dizygotic twins is 60% versus 0% in the general population. These findings taken together argue for a strong genetic component to autism. Unfortunately, of the
three large genome-wide scans of autism groups none were successful in identifying a significant linkage.

It is generally agreed that there is a large genetic component to autism however no single gene has been found to be responsible; although chromosomal regions possibly containing susceptibility genes have been identified and account for approximately 10-20% of cases. However, no single identified cause accounts for greater than 1-2% of cases. Regardless of this heterogeneity, hypotheses have been generated to link genetic changes with altered biological function, including faulty synaptic function, irregular brain connectivity (Geschwind & Levitt, 2007), changes to mitochondrial reactive oxygen species and the purine synthetic pathway (Spiegel et al., 2006). Several parallel approaches are essential to progress our understanding of the genetic factors causal of the ASDs. These approaches comprise whole-genome and pathway-based association experiments, dense resequencing to discover mutations, and sustained gathering of large well characterized patient cohorts and their relatives for genotype-phenotype studies (Abrahams & Geschwind, 2008). One genetic disease, adenylsuccinate lyase (ADSL) deficiency, causes a syndrome of profound developmental delay wherein approximately 30% of those affected develop autistic features (Jaeken & Van den Berghe, 1984). Over 20 different genetic mutations causing ADSL deficiency are recognized while little is known about how this deficiency leads to the syndrome. In fact, this is not an isolated example of disruption to the purine synthetic pathway as defects are
known to cause other neurodevelopmental abnormalities, including Lesch-Nyhan syndrome (Baumeister & Frye, 1985).

If there is a biological component to autism then one would expect there to be a biochemical correlate and indeed there is; approximately 20 to 30% of individuals have significantly elevated levels of the end product of purine metabolism in humans, uric acid, as well as significant increases in de novo purine synthesis (Page, 2000; Page & Coleman, 2000). These or other purine synthesis abnormalities may be important for the development of autistic features. Specifically, fault in the purine biosynthetic pathway, the enzyme ADSL, has been associated with a high incidence of autistic features (Jaeken et al., 1984).

An infantile autistic syndrome has been described that is characterized by the presence of succinylpurines in bodily fluids (Jaeken & Van den Berghe, 1984). These findings are likely attributable to significantly reduced activity of ADSL in liver and its absence in kidney from one patient. This enzyme is responsible for both the eighth step of de novo purine biosynthesis as well as the second step of conversion from IMP to AMP (Figure 6). Both reactions involve the release of a fumarate molecule from the succinylpurine substrate, in the first case from phosphoribosylsuccinylaminomidazole carboxamide (SAICAR) and in the second case from adenylosuccinate. As a result of the deficiency in ADSL, the nucleoside derivatives of the precursor compounds SAICA and succinyladenosine accumulate in the bodily fluids, including cerebrospinal fluid, of affected individuals.
Figure 6: Pathways of Purine Metabolism. The first 7 steps of the *de novo* synthetic pathway are represented by a broken line. The ADSL defect is indicated by solid bars. PRPP, phosphoribosyl pyrophosphate; SAICAR, succinylaminomidazolecarboxamide ribotide; AICAR, aminomidazolecarboxamide ribotide; IMP, inosine monophosphate; S-AMP, adenylosuccinate; S-Ado, succinyladenosine; XMP, xanthosine monophosphate. 1, adenylosuccinate lyase; 2, adenylosuccinate synthetase; 3, AMP deaminase; 4, cytosolic 5'-nucleotidase; 5, adenosine deaminase; 6, purine nucleoside phosphorylase; 7, xanthine oxidase; 8, hypoxanthine-guanine phosphoribosyltransferase; 9, adenine phosphoribosyltransferase.

Mutations in ADSL are single base pair substitutions that translate into changed ADSL protein (Baron-Cohen et al., 2000; Kmoch et al., 2000; Marie et al., 1999; Valik et al., 1997; van den Berghe et al., 1998). In 1999, Kohler et al., reported a patient with a single base substitution that had especially severe autism together with severe early infantile epileptic encephalopathy amid reduced myelination (Kohler et al., 1999). There have not been any reported...
cases where enzymatic activity is completely missing; all affected individuals have been compound heterozygotes (Van den Berghe et al., 1993a).

Our collaborator, Dr. David Patterson, and his group mapped the gene encoding ADSL to human chromosome 22, a location that was later confirmed by the DNA sequence of this chromosome (Dunham et al., 1999; Van Keuren et al., 1987). The ADSL gene and cDNA have been cloned as well as their structures determined (Kmoch et al., 2000). The mouse gene has also been cloned and characterized and is located on mouse chromosome 15 (Wong & O'Brien, 1995). The clinical presentation of ADSL deficiencies is quite varied, but commonly includes severe psychomotor delay, epileptic seizures, and autism features (Jaeken et al., 1984; Kohler et al., 1999; Maaswinkel-Mooij et al., 1997; Race et al., 2000; Valik et al., 1997; Van den Berghe et al., 1998). Other characteristics noted include profound hypotonia, atypical brain glucose utilization, and muscle energy metabolism malfunction (Salerno et al., 1997; Salerno et al., 1999; Valik et al., 1997). Some individuals with mutations to ADSL are only mildly developmentally delayed.

Residual ADSL activity is observed to some extent in tissues of ADSL-deficient individuals (Kmoch et al., 2000; Marie et al., 1999; Van Den Bergh et al., 1993b). However, the severity of clinical features manifest by these mutations does not correlate well with decreased purine levels and altered patterns of purine metabolism and stability. Because of this, researchers have divided the disorder into two groups; ADSL type I & II. For ADSL type I, individuals are severely affected and contain a ratio of S-ado/SAICA of roughly 1:1 in bodily...
fluids. Whereas for ADSL type II, the ratio spans from four to five to one (Van Den Berge et al., 1993) and individuals are not severely affected. It is possible that it is not the lack of purines that leads to this pathology, but rather an accumulation of SAICA, the precursor to SAICAR. Some investigators have proposed that accumulation of S-ado may even be protective (Van Den Bergh, 1993b) against the damaging effects of SAICA (Stone et al., 1998).

Approximately 30% of individuals with autism appear to have elevated serum levels of urate. This increase is associated with greater rates of de novo purine biosynthesis in fibroblasts originating from these individuals (Page & Coleman, 2000). This condition, hyperuricemia, has an incidence of approximately 5 to 8% among adult American males and appears to have no clinical significance for these individuals (Becker & Roessler, 1995). Thus, 30% of individuals with autism having elevated de novo purine synthesis are strongly suggestive of a relationship between the purine biosynthetic pathway and autism. A large percentage of individuals with autism have elevated serum urate and many have an elevated auditory startle response (Page & Coleman, 2000). For these individuals with hyperuricosuric autism, a low purine diet has been reported to improve symptoms (Page, 2000).

ADSL also contributes to the purine nucleotide cycle with AMP deaminase and adenylosuccinate synthetase by controlling the levels of fumarate and free AMP, which influences the concentration of ATP (Van den Berghe et al., 1992). Furthermore in muscle, the purine nucleotide cycle is essential to preserve the ratio of ATP/AMP (Van den Berghe et al., 1992). An ADSL deficient patient has...
been reported with impaired energy metabolism and a low energy reserve in muscle. This patient's ATP levels were rapidly depleted from mild exercise (Salerno et al., 1997). Thus, ADSL deficiency may contribute to muscle wasting and growth retardation observed in some patients.

In a 1999 study by Kent and colleagues, the comorbidity of autism spectrum disorders and Down syndrome (DS or trisomy 21) was calculated (Kent et al., 1999). A comorbidity rate of at least 7% was determined, meaning that individuals with DS are at least 100-times more likely to have autism than persons without DS. Remarkably, people with DS have increased serum urate levels (Pant et al., 1968). Dr. Patterson's group has produced transgenic mice that express the human GART gene (steps 2, 3, 5 of Figure 6) that resides on human chromosome 21 (Daubner et al., 1985), and these mice do have elevated auditory startle responses (Page & Coleman, 2000). Koch & Schnitzler (1997) predicted on the basis of increased purine production. Thus, abnormalities in the purine pathway may influence auditory startle response systems.

Animal models of chemically-induced autism have been generated, for example, by treating rats with ibotenic acid or with valproic acid during gestation in an attempt to mimic the prenatal teratogenic effects generated by compounds such as thalidomide (Ingram et al., 2000; Rodier et al., 1997; Wolterink et al., 2001). These animals have neuronal abnormalities in the amygdale and/or cerebellum and in cranial nerve motor nuclei associated with autism (Ingram et al., 2000; Rodier et al., 1997; Wolterink et al., 2001). A major focus among
researchers continues to be generating transgenic mouse models of autism that compare favorably with rodent models generated through chemical treatment.

Down Syndrome

Down syndrome (DS), or trisomy 21, is the most common genetic cause of cognitive disability, affecting approximately 1 in every 1000 births (Collins et al., 2002). DS can be detected prenatally, through a tri- or quad-screening or a maternal blood screening test, and the incidence of the syndrome is increasing (Alexander, 2000). The most pervasive and considerable characteristic of DS is cognitive impairment as it not only encompasses lifelong cognitive impairment, but also a cognitive decline with age. The life expectancy of persons with DS continues to increase and this increases the significance of cognitive decline for these individuals.

Improving the cognitive abilities of persons with DS would provide a major benefit to persons with DS, their families, and society as a whole. The IQ for people with DS ranges from 30 to 70, with an average of 50 (Chapman & Hesketh, 2000). The American Association of Mental Retardation (AAMR) regards individuals with an IQ less than 70 as having mental retardation. Therefore an improvement of 20 IQ points would imply that the average IQ of people with DS would be close to normal. An improvement of 20 points was obtained for persons home-reared rather than institutionalized (Wishart, 1995).

The predominance of experimental and clinical support suggests that DS results from altered expression of several genes. Chromosome analyses occasionally reveal that only a segment of human chromosome 21 (HSA21) is
trisomic for some individuals, and these findings led to attempts to identify a small region of the chromosome responsible for DS (Delabar et al., 1993; Korenberg et al., 1994). This strategy has some success as some aspects of DS have been regionally mapped (Barlow et al., 2001); however regional mapping of the cognitive phenotype of the syndrome has not been successful (Shapiro, 1999). To date, about 430 genes have been identified on HSA21 with about 45% of these genes having an unknown function (Kahlem, 2006). The sequence of HSA21 has exposed the complexity of genome structure and in gene regulation that will have profound implications for understanding genotype/phenotype relationships. The trisomic region reaches from Mrp139 to Znf295 and contains approximately 136 genes that are orthologous to genes on HSA21. Partial trisomy 21 often manifests a mild to moderate DS phenotype (Habedank & Rodewald, 1982; Miyazaki et al., 1987). These observations indicate that there are genes in particular regions of chromosome 21 that are not trisomic in the mild or moderate cases of DS. Partial trisomy also suggests that there are genes in the trisomic region that lead to a clinical diagnosis of DS for the individual. Mice have been developed with segmental trisomies that offer a similar situation to the human condition. Three trisomic mouse lines exists, including Ts65Dn, Ts1Cje, and Ms1Ts65 and all three lines have trisomy to different regions of chromosome 16 (equivalent to human chromosome 21).

All three trisomic mouse lines have cognitive and behavioral impairment (Sago et al., 2000), although the phenotypes vary between lines. The Ts65Dn mice have a trisomic region that is the summation of the Ts1Cje and Ms1Ts65.
Notably, the Ts65Dn mouse does not have all of the cognitive features of DS and this signifies that there are genes essential for cognition on chromosome 21 that are not trisomic in the Ts65Dn mouse. The Ts65Dn mouse is the most robust and commonly used model for DS. These mice contain an additional chromosome, spanning most of the region of MMU16 that is homologous to HSA21, translocated to a MMU17 centromere. This model system has several physical, behavioral, and neurological features observed in persons with DS. These features include craniofacial dysmorphogenesis, loss of age-related cholinergic markers in the basal forebrain, and prominent learning and memory deficits (Cataldo et al., 2003; Holtzman et al., 1996; Hyde et al., 2001; Olson et al., 2004; Reeves et al., 1995).

Mice have also been developed to be transgenic for a single gene on chromosome. For example, mice transgenic for Dyrk1A (Altafaj et al., 2001; Smith & Rubin, 1997) Sim2 (Ema et al., 1999; Chrast et al., 2000), SOD1 (Avraham et al., 1988; Rando et al., 1998), APP (Cairns, 2001; Koistinaho et al., 2001), and S100beta (Wincour et al., 2001) all demonstrate neurocognitive abnormalities. These genes map to different locations on chromosome 21, however none of these alone cause all of the features linked with DS. Thus, results argue that a different approach may be more advantageous. We hypothesized that examining consequences of the trisomy, involving numerous genes (not all on chromosome 21), may explain the neurocognitive impairments observed in DS 21 and this argues against any individual gene causing DS. The effects of modifying gene expression, protein levels, or even enzyme activities
should have quantifiable metabolic, anatomic, and/or physiological consequences if the change affects cognitive ability.

Depending on tissue or cell type, the expression of single HSA21 genes appeared to be different (Li et al., 2006). This situation is present in the Ts65Dn mouse model of DS where expression of GABPA protein is prominent only in brain and skeletal muscle (O'Leary et al., 2004). It is important to determine HSA21 gene expression levels, the tissues that specific genes are expressed in and at what developmental stage expression occurs as functions of homologous genes are not identical in mice and humans and even their regulation can differ between the two species. An example is folate/one-carbon/trans-sulfuration; the tissue specificity of expression of critical genes in this biological system is different in mice and in humans (Whetstine et al., 2002; Liu et al., 2005; Butler et al., 2006) and may mean that these pathways provide somewhat different functions in humans and mice. The purine metabolic pathway is another example of gene differences between the species. The end product of purine catabolism in humans is urate, whereas mice metabolize urate to allantoin. Pant and colleagues (1968) observed dramatically increased urate levels in AD patients (Pant et al., 1968). In particular the GART (phosphoribosylglycineamide transformylase) gene is on HSA21, and trisomy of this gene may be guilty of causing excessive urate accumulation in people with DS (Brodsky et al., 1997). Therefore, data arising from mouse studies have to ultimately be confirmed for their significance to humans.
According to gene dosage in DS, many genes are expressed at high levels; yet tissue and temporal specificity has been observed (Antonarakis & Epstein, 2006; Butler et al., 2006; Li et al., 2006; O'Leary et al., 2004; Rachidi et al., 2005; Reymond et al., 2002b; Roper & Reeves, 2006). Overexpression of a gene may have varying consequences depending on an individual's developmental stage. For instance, overexpression of a particular gene may cause developmental abnormalities at a particular stage of development while the same gene's overexpression at another developmental stage could compensate for developmental irregularities that are associated with the syndrome (Head et al., 2007). It is possible that a gene's overexpression may only occur at a specific timepoint and to specific cells or tissues yet still manifest a permanent phenotypic effect. Conversely, there may also be genes that require continual overexpression to preserve their phenotypic effect (Patterson, 2007).

Trisomy of particular genes may generate functional consequences through alterations to protein levels in cells. For instance, many proteins interact with other proteins in their immediate environment, such as enzymes. Enzymes are highly regulated proteins through events such as phosphorylation and endproduct inhibition among other mechanisms. Thus, increased levels of an enzyme may not correctly reveal the same increases to enzyme activity and therefore may not predict disturbed metabolism (Cornish-Bowden & Cardenas, 2000).

The majority of genes on chromosome 21 contribute to either metabolic or developmental pathways, and this increases the difficulty in predicting how
altering expression of one gene in a multi-step pathway will ultimately affect the whole pathway. For example mitochondrial reactive oxygen species pathways, many genes are located on chromosome 21 that are vital for the pathways (Hattori et al., 2000; see table 6 in the Appendices). All 18 genes listed in Table 6 are expressed in mouse with the first 14 being trisomic in the Ts65Dn mouse (Akeson et al., 2001; Gardiner et al., 2002; Gitton et al., 2002; Reymond et al., 2002a; Reymond et al., 2002b). Because the enzymes themselves are subject to biochemical or metabolic regulation, this complicates the extraction of definite conclusions based solely on increased gene or protein expression. It is widely hypothesized that mitochondrial reactive oxygen species and one-carbon metabolism play a role in many cognitive disabilities and neurodegenerative diseases including DS. Preliminary findings by our collaborator, Dr. David Patterson as well as by others, demonstrate that similar to persons with DS having trouble performing tasks that involves hippocampal function (Pennington et al., 2003) Ts65Dn mice exhibit deficits in hippocampal tasks (Hyde et al., 2001; Hyde & Cynic, 2001; Granholm et al., 2000). The observed deficits entail both failure of appropriate development of the brain as well as age-related loss of functional cholinergic neurons of the basal forebrain, reminiscent of what occurs in persons with DS (Granholm et al., 2000), and this cognitive loss can be reversed in old Ts65Dn mice (Granholm et al., 2002).

Mitochondrial oxidative phosphorylation is accountable for the majority of ATP generation in cells as well as production of the bulk of reactive oxygen species (Halliwell & Gutteridge, 1985; Wallace, 2001). The collapse of the
mitochondrial transmembrane potential can initiate apoptosis. Mitochondrial energy metabolism is carried out by five multienzyme complexes and mutations in several of the mitochondrial reactive oxygen species genes lead to human disorders, including mental retardation, developmental abnormalities, and neuromuscular and neurodegenerative disorders (Wallace, 2001). Altered mitochondrial function and reactive oxygen species metabolism have been hypothesized to play a significant role in DS, Alzheimer disease, other neurodegenerative diseases, and aging (Busciglio et al., 2002; Butterfield et al., 2002; Capone et al., 2002; Cecchi et al., 2002; Floyd & Hensley, 2002; Iannello et al., 1999; Prince et al., 1994; Rao & Balachandran, 2002; Schon et al., 2000). For example in DS and AD there are decreased levels of Complex I subunits, Complex III core protein 1, and Complex V subunits (Kim et al., 2000; Kim et al., 2001). Abnormal expression of these genes may affect the stoichiometry of these complexes and could result in diminished function or stability. These changes could lead to impaired energy metabolism in DS and could also promote generation of reactive oxygen species and therefore enhance neuronal cell death. Another well-known gene on chromosome 21 is amyloid precursor protein (APP). Trisomy of APP may elicit mitochondrial dysfunction in DS (Busciglio et al., 2002) and thus provides a link between AD and DS. Defective mitochondrial energy metabolism is consistent with the finding that individuals with DS have a low resting metabolic rate (Roizen & Patterson, 2003) with an elevated threat for obesity (Luke et al., 1996; Prasher, 1995).
The GART gene, trisomic in the Ts65Dn mouse, encodes a trifunctional protein carrying out steps 2 (GARS), 3 (GART), and 5 (AIRS) of de novo purine synthesis (see figure 6). These three activities are performed by distinct domains of the protein ordered GARS-AIRS-GART from the N-terminal end. In DS cerebellum, the production of the petite GARS protein declines slower than the entire GARS-AIRS-GART protein (Brodsky et al., 1997). Though the function of the small GARS protein is unknown we hypothesize that it may contribute to the regulation of purine synthesis. Therefore, intron 11 was deleted from the GART P1 clone, P8E5, via the method of Yu et al (2000) as customized by Swaminathan et al (2001) and Lee et al (2001) to produce a GART gene that can solely fabricate the GARS-AIRS-GART protein but not the single GARS protein. Since GART gene overexpression affects the de novo purine synthesis pathway, it is likely to have effects on both DS and autism and could possibly be a link between the high comorbidity between the disorders.

Mitochondria have gained attention in aging biology due to their vital role in generating ATP to satisfy cellular energy demands. Aging leads to a decline in basal metabolic rate and to physical performance in energy-requiring tasks (McCarter, 1995). Mitochondrial function is impaired with age and may encompass slower rates of electron transfer, increased inner membrane H⁺ permeability, and H⁺-driven ATP synthesis impairment (Navarro & Boveris, 2007). An underlying concept for the mitochondrial hypothesis of aging is that oxidative damage accumulates in the organs and tissues of aged animals as mitochondrial ATP production declines. This age-dependent diminished capacity

produced with permission of the copyright owner. Further reproduction prohibited without permission.
to generate ATP is not due to a reduced mitochondrial mass in brain nor to the
phosphorylating capacity of ATP synthase (Navarro et al., 2004; Navarro et al.,
2005; Navarro & Boveris, 2004) but this mitochondrial dysfunction has been
attributed to abnormal electron transfer (Navarro et al., 2002; Navarro et al.,
2004; Navarro et al., 2005; Navarro & Boveris, 2004). Upon further investigation,
Navarro & Boveris demonstrated that the activities of mitochondrial complexes I
and IV decrease by approximately 30% with age and are therefore effective
markers of aging (Navarro & Boveris, 2007). Diminished complex I activity
predisposes to excitotoxicity by changing levels of ATP and by altering calcium
homeostasis (Dauer & Przedborski, 2003). Reductions to the levels of ATP
negatively affect the activity of plasma membrane Na⁺/K⁺-ATPase, resulting in
partial neuronal depolarization. Under these circumstances, even physiological
levels of extracellular glutamate can cause excitotoxicity through the activation of
N-methyl-D-aspartate (NMDA) receptors and elevations to intracellular calcium,
and complex I defects disturb normal calcium signaling in neurons (Giasson &
Lee, 2003). Even under ideal conditions mitochondrial electron transfer is not
perfect; some electrons escape from the ETC and subsequently interact with
oxygen to produce superoxide anion among other reactive oxygen-nitrogen
species. This electron leakage increases significantly upon mitochondrial
dysfunction as a function of age.

Individuals with DS have a lifelong cognitive impairment that worsens with
age. Biochemical studies are difficult, if not impossible, to perform on these
individuals but we do have transgenic animal models in which to perform these
studies, namely Ts65Dn mice and GART overexpressing mice. We hypothesize that the features of DS are partially due to changes to de novo purine synthesis resulting from mitochondrial dysfunction that result in decreased brain energy levels in these animals and likely the human population.

HIV-1 Dementia

Human immunodeficiency virus type 1 (HIV-1) infection can lead to HIV-1 associated dementia characterized clinically by memory loss, and cognitive, motor and behavioral abnormalities, and pathologically by neuronal loss, dendritic pruning, astrogliosis, demyelination, neuroinflammation, and the presence of multinucleated giant cells (Everall et al., 1999; Masliah et al., 1992; Navia et al., 1986; Rappaport et al., 1999; Zhou et al., 2004). With the advent of highly active antiretroviral therapeutics the incidence but not the prevalence of HIV-1 associated dementia has decreased (Bouwman et al., 1998). Neuronal cell dysfunction and death, as a result of HIV-1 infection, appears to be caused by the release of soluble factors secreted from infected non-neuronal cells including macrophages, microglia and astrocytes because HIV-1 only rarely infects neurons (Giulian et al., 1990). One such soluble factor is HIV-1 Tat, a nonstructural viral protein essential for viral replication that is actively released from cells infected with HIV-1 and that can be taken up by non-infected cells (Cheng et al., 1998).

HIV-1 Tat, composed of 72 amino acids in the first exon and up to an additional 32 amino acids in the second exon, is present in brain of HIV-1 infected patients (Hofman et al., 1994; Kruman et al., 1999; Nath et al., 1998).
Neuronal cell death can be induced by Tat (Aksenov et al., 2006; Gavril et al., 2000; Kruman et al., 1998; Langford et al., 2004; Nath & Geiger, 1998; New et al., 1997; Perry et al., 2005; Shi et al., 1998; Singh et al., 2005), as it depolarizes neurons through the activation of glutamate receptors (Cheng et al., 1998; Chandra et al., 2005; Haughey et al., 2001; Longordo et al., 2006; Magnuson et al., 1995; Self et al., 2004; Song et al., 2003), increases levels of intracellular calcium at least in part through the release of calcium from intracellular stores (Haughey et al., 1999; Lipton, 1994; Nath & Geiger, 1998), and leads to mitochondrial dysfunction as indicated by changes to the levels of reactive oxygen species, levels of ATP, and/or mitochondrial membrane polarization (Kruman et al., 1998; Nath et al., 2000; Perry et al., 2005; Price et al., 2005; Rappaport et al., 1999).

Creatine, a normal component of meat-based diets that is produced endogenously and may be ingested as a dietary supplement is present at high levels in brain (Mujika & Padilla, 1997). Creatine is an effective neuroprotectant against a number of acute and chronic neurodegenerative conditions including traumatic brain and spinal cord injuries (Rabchevsky et al., 2003; Scheff & Dhillon, 2004; Sullivan et al., 2000), ischemia and hypoxia (Adcock et al., 2002; Zhu et al., 2004a), Huntington's disease (Bender et al., 2005; Dedeoglu et al., 2003; Matthews et al., 1998; Ryu et al., 2005), Alzheimer's disease (Brewer & Wallimann, 2000), amyotrophic lateral sclerosis (Dupuis et al., 2004; Ferrante et al., 2000; Klivenyi et al., 2004; Pena-Altamira et al., 2005), and Parkinson's disease (Beal, 2003; Matthews et al., 1999).
Mechanistically, the neuroprotective properties of creatine appear to be related mostly to stabilizing mitochondrial bioenergetics and preventing mitochondrial redox catastrophe. In terms of stabilizing mitochondrial bioenergetics, creatine is phosphorylated by mitochondrial creatine kinase to phosphocreatine that is then transported to the cytoplasm where cytoplasmic creatine kinase catalyzes the transfer of high-energy phosphate from phosphocreatine to ADP thus yielding ATP. For the prevention of redox catastrophe, creatine can reduce the production of reactive oxygen species by various actions including acting as a free-radical scavenger (Lawler et al., 2002) and prevention of mitochondrial permeability transition pore opening (Dolder et al., 2003; O’Gorman et al., 1997; Sullivan et al., 2000).

Ketogenic Diet & Epilepsy

Epilepsy is a brain disorder that is characterized by a predisposition to generate epileptic seizures. In the general population, the incidence of epilepsy is around 50 cases per 100,000 people per year, with higher proportions for infants and elderly people (Duncan et al., 2006; Forsgren et al., 2005; Patel, 2004). The disorder is poorly classified as it has many known and unknown causes, manifestations, seizure types, and identifiable syndromes. Epilepsy associated with head trauma, central nervous system infections, and tumors may occur at any age whereas cerebrovascular disease is the most common risk factor in people older than 60 years (Duncan et al., 2006).

Seizures are transient occurrences of signs or symptoms that are due to abnormally excessive or synchronous neuronal activity in brain (Fisher et al.,
During a seizure, groups of neurons undergo a prolonged depolarization that may last for seconds. The prolonged depolarization of a neuron increases the probability that an inadequate stimulus can elicit an action potential and this can result in neurons firing at a very high rate (Walker et al., 1990). Excessive neuronal activity causes a large flux of ions across the plasma membrane and will lead to the intracellular accumulation of sodium and calcium ions. Each of these ions' influx generates a predicament for cellular homeostasis in that the cell expends large amounts of energy in order to maintain low intracellular sodium and calcium concentrations. The fate for sodium is active transport from the cell, via sodium-potassium-ATPase, whereas calcium may accumulate within the mitochondria, endoplasmic reticulum, or be extruded from the cell. These ion movement processes directly impact a neuron's bioenergetics and may lead to redox catastrophe. ATP is necessary to maintain the resting membrane potential. During seizure activity the resting membrane potential depolarizes, as the sodium-potassium-ATPase is unable to exchange sufficient amounts of intracellular sodium for extracellular potassium. A decreased electrical potential across the neuronal membrane increases the probability of a subsequent action potential train occurring and further sodium and calcium influx.

The mitochondrion is commonly termed the powerhouse of the cell as it converts dietary calories, through oxidative phosphorylation by the ETC, into ATP. The rate of ATP production is augmented as ATP is consumed by cellular processes such as via the sodium-potassium-ATPase to ADP (Wallace et al., 2005). Under basal conditions, estimations are that this ATPase consumes 25-
40% of the brain's energy (Albers & Siegel, 1999) furthermore, after seizure activity it is probable that this ATPase consumes an even greater percentage of brain energy to restore the resting membrane potential. The ETC not only creates useable ATP energy for the cell but also produces most of the cell's endogenous reactive oxygen species, as a toxic byproduct of energy production (Wallace, 2005).

Antiepileptic drugs as well as non-pharmacological treatments are available for the treatment of epilepsy (Duncan et al., 2006). The aim of antiepileptic drug treatment is to control seizures without adverse effects; unfortunately antiepileptic drugs are effective for only 60–70% of epileptic individuals. Findings that epileptic seizures were reduced in frequency and intensity lead to the hypothesis that ketosis effectively decreases seizure disorders (Freeman & Vining, 1998; Schwartzkroin, 1999). The ketogenic diet (KD) induces ketosis and is a common non-pharmacological treatment for individuals with epilepsy that do not respond well to traditional AEDs.

R.M. Wilder, at the Mayo Clinic, first proposed the KD in 1921 to mimic the biochemical changes such as acidosis, dehydration, and ketosis that occur during fasting (Schwartzkroin, 1999). The KD is a high fat and low carbohydrate diet that can be expressed as a ratio of fat to protein (plus carbohydrate) of 4:1 (fat: protein + carbohydrates). Approximately 90% of dietary calories are derived from fat whereas only 2-3% comes from carbohydrates (Freeman et al., 2000). Proteins are added to the diet to meet requirements for the individual’s age and size. In order to maintain ketosis in the patient, fluids and calories are restricted.
and total caloric intake is targeted to 75% of recommendations for the individual's age (Sinha & Kossoff, 2005). The diet reduces seizure frequency by more than 90% for a third of children and by greater than 50% for an additional third of children (Thiele, 2003). It appears that the KD is more or less as effective in adults as it is in children however there have been few studies on adults (Barborka, 1930; Sirven et al., 1999). Most patients that respond well to the KD are weaned from the diet after two years as the anticonvulsant effect achieved becomes permanent (Edelstein & Chrisholm, 1996). The KD may even be more efficient in the management of childhood seizures than all other currently available AEDs (Freeman et al., 2006). The KD might also slow the progression of this disorder (Bough et al., 2003; Su et al., 2000). Unfortunately the mechanism(s) by which the KD provides protection remains uncertain. There are many hypothesizes for the protective effects of the KD. Investigators have attributed the diet’s protection to; alterations in neurotransmitter levels, circulating factors that function as neuromodulators, modifications to the extracellular milieu, decreased neuronal excitability, and transformations of cerebral energy metabolism (Schwartzkroin, 1999). We hypothesize that changes to gene expression and ultimately altered energy metabolism are the strongest candidates.

Because the KD may require several days and weeks to become maximally effective (Appleton & DeVivo, 1974), we in collaboration with Drs. Raymond Dingledine and Kristopher Bough at Emory University in Atlanta hypothesized that alterations in gene expression and ultimately altered energy metabolism...
metabolism were responsible for its anticonvulsant actions. Microarrays were used to identify functional groups of genes induced or repressed in rat hippocampus after KD. Although microarray analysis of brain regions is complicated by massive heterogeneity of cell types, global changes in gene expression have provided important insights into underlying mechanisms of neurological disease. For example, transcriptional profiling has yielded insights into epilepsy, schizophrenia, Alzheimer's disease, and multiple sclerosis (Arion et al., 2006; Glanzer et al., 2004; Mintics & Pevsner, 2004; Pierce & Small, 2004).

The hippocampus was chosen for microarray, electron micrographic, biochemical, and electrophysiological studies because of previous findings showing that KD affects several hippocampal processes associated with diminished neuronal excitability and altered epileptogenesis (Bough et al., 2003; Cheng et al., 2003, Muller-Schwarze et al., 1999; Noh et al., 2003; Su et al., 2000; Sullivan et al., 2004; Ziegler et al., 2003). Normal animals were studied rather than 'epileptic' animals because a KD-induced change in seizure frequency would itself change gene expression that would cloud interpretation. We selected adolescent rats (37-41 d; Stansfield et al., 2004) rather than very young rats to circumvent ontogenetic profiles that would be superimposed on diet-induced changes. Age at diet onset is only moderately linked with anticonvulsant efficacy either experimentally (Appleton & DeVivo, 1974; Bough et al., 1999b) or clinically (Freeman et al., 1998; Sirven et al., 1999; Vining et al., 1998). Indeed, in a prospective trial of 150 children and adolescents age less than 2 to greater than 12 years, there was no age-related difference in outcome.
The KD is effective in infants (Nordli et al., 2001), adolescents, (Coppola et al., 2002; Mady et al., 2003) and adults with generalized and partial epilepsy (Sirven et al., 1999). Experimental studies in adult rats support this notion (Appleton & DeVivo, 1974). Although the KD is primarily used for pediatric epilepsy, we chose to study adolescent rats (e.g. Stansfield et al., 2004), an age group in which KD has documented effect (Coppola et al., 2002; Mady et al., 2003). Key variables associated with KD efficacy have been identified in rats (Bough et al., 1999a; Bough et al., 1999b; Bough & Eagles, 1999; Bough et al., 2000a; Bough et al., 2000b). Reports have suggested that KD-fed brain has increased ATP levels (DeVivo et al., 1978) and phosphocreatine to γATP ratio (Pan et al., 1999). During our studies, in collaboration with Drs. Raymond Dingledine & Kristopher Bough, we observed a significantly elevated energetic capacity from KD-fed brain (data presented in results section; Bough et al., 2006). Specifically, we fed young Sprague-Dawley rats a calorie-restricted KD for three weeks prior to sacrifice by high-energy head focused microwave irradiation. In order to accurately determine brain energy levels it is critical to kill the animals by microwave irradiation as it snap-inactivates all brain enzymes and thus prevents the rapid post-mortem degradation of high-energy molecules (Delaney & Geiger, 1996). We found an increased ratio of phosphocreatine to creatine in hippocampus (Bough et al., 2006). This elevation indicates that KD-fed brain has a greater ability to maintain cellular ATP levels. We believe that these animals will also have reduced
mitochondrial free radical production and oxidative damage following seizures however this hypothesis has not been tested.

Adenosine is the brain's endogenous anticonvulsant and an endogenous neuromodulator that is released during seizures, ischemia, and hypoxia (Berman et al., 2000; Fredholm et al., 1984; Olsson et al., 2004). The brain is the tissue by far with the greatest density of the most abundant adenosine A1 receptors (A1R), which play key roles in controlling neuronal excitability and in particular neurotransmitter release (Cunha, 2005). It exerts both anticonvulsant and neuroprotective effects (Fredholm, 1997; Fredholm et al., 2005a; Ribeiro et al., 2003) that are mediated by adenosine receptors, of which the inhibitory A1R is the most abundant in seizure prone regions, such as the hippocampus (Fredholm et al., 2001; Fredholm et al., 2005b). Excitotoxicity and ischemia studies have demonstrated that A1 activation is neuroprotective (Fredholm, 1997; Fredholm et al., 2005a; Lee et al., 2004; de Mendonca et al., 2000; Sweeney, 1997; Vianna et al., 2005). Adenosine is released during seizures and is believed to contribute to the termination of seizures (Dunwiddie et al., 1981; During & Spencer, 1992; Ribeiro et al., 2003) by reducing neuronal excitability via activation of A1Rs. A1R activation inhibits excitatory synaptic transmission, mainly by inhibiting presynaptic glutamate release (Thompson et al., 1992). Postsynaptically, A1R activation inhibits potassium conductances which lead to hyperpolarization of the neuron (Greene & Haas, 1991). This latter effect is of chief importance to manage neuronal firing (Cunha, 2005). The post-synaptic density contains high numbers of A1Rs and could implicate adenosine in the control of signal...
integration at the post-synaptic level. For instance, tonic activation of $A_1$Rs affects synaptic plasticity (de Mendonca & Ribeiro, 1997) and this could be due to $A_1$Rs modulating NMDA receptors (Klishin et al., 1995) as well as post-synaptically located voltage-sensitive calcium channels. Hence, the dominant anticonvulsant role of endogenous and tonic adenosine occurs through the actions of the $A_1$R (Etherington & Frenquelli, 2004) where seizure activity can be suppressed (Anschel et al., 2004; Boison, 2005; Huber et al., 2001).

Furthermore, adenosine agonists have been demonstrated to suppress pharmacoresistant epilepsy through $A_1$Rs (Gouder et al., 2003).
CHAPTER II

METHOD

General Method

Analysis of Cerebral Energetics

Frozen brain tissue samples were weighed in 1.5 ml centrifuge tubes and the total volume of ice-cold H₂O necessary to generate a concentration of 50 mg of tissue per ml was calculated and added. Samples were kept ice-cold while being homogenized with a Polytron (Speed “3”) in short bursts to ensure the samples remained cold. From each sample, 50 μl of homogenate was removed and stored at -20°C until being taken for protein analysis by DC method (Bio-Rad, Hercules, CA). The remaining homogenate volume was calculated and 100% trichloroacetic acid (TCA; Supelco, Bellefonte, PA) was added to yield a 2% final concentration of TCA. Samples were rigorously vortexed for 10s at the highest setting to allow ample opportunity for the acid to interact and precipitate proteins in the tubes prior to centrifugation for 15 min at 4°C at 16,000 x g. The supernatant containing adenine nucleotides, phosphocreatine, creatine and adenosine were analyzed using high pressure liquid chromatography (HPLC)-based method (Delaney & Geiger, 1996). The supernatant was added on ice to a mixture of tri-n-octylamine (TOA) and dichloromethane (DCM) in order to neutralize the TCA (225:775, respectively). The tubes were then vortexed for
15 s and centrifuged at 4°C for 4 min at 16,000 x g to promote separation of the organic and aqueous layers. The organic layer was discarded while the aqueous layer was collected and re-introduced to the same ratio of our TOA/DCM mixture prior to a 15 s vortex and 4 min centrifugation at 16,000 x g. Following the second phase separation, the aqueous layer was collected and placed into polyspring inserts residing in transparent HPLC vials. The contents of the vials were analyzed using a Gemini 3 μm C18 column (4.6 x 150 mm, Phenomenex, Torrance, CA) using a mobile phase consisting of 0.1 M KH$_2$PO$_4$, pH 6.0 run isocratically at 1.0 ml/min. Metabolites were detected by a photodiode array 168 detector (Beckman Coulter) at 210 nm for phosphocreatine and creatine, and at 254 nm for adenine nucleotides. Each sample run was analyzed for 60 min, even though all compounds of interest were eluted in less than 15 min, to ensure no other compounds from a previous run would contaminate a subsequent sample. We then quantified nucleotide levels according to sample spiking and by injecting known amounts of standards to determine peak heights and areas under the peaks. AMP, ADP, ATP, creatine, and phosphocreatine peaks will be identified by retention time of standards. Adenylate energy charge was calculated using the formula $([ATP] + 1/2 [ADP]) / ([ATP] + [ADP]+[AMP])$.

For adenosine, brain samples were homogenized and neutralized in the same fashion as for the adenine nucleotides. Following collection of the second supernatant, we added an equal volume of Ba(OH)$_2$ and samples were mixed rigorously for 10 s - this resulted in a milk-like solution that was mixed with an equal volume of ZnSO$_3$. The resulting solution was vortexed for 10 s and
centrifuged at 4°C for 4 min at 16,000 x g. The supernatant (150 µl) was then added to 25 µl of 5% chloroacetaldehyde and samples were vortex mixed for 10 s prior to being heated for 2h at 85°C to produce adenosine's fluorescent derivative N6-ethenoadenosine (Zhang et al., 1991). After heating, samples and adenosine standards were removed from the block, vortexed for 2 s, and centrifuged at room temperature for 2 min at 16,000 x g. The samples and adenosine standards were then injected onto a Gemini 3 µm C18 column (4.6 x 150 mm) using a mobile phase of 0.01 M NH₄H₂PO₄, 17% methanol, pH 5.4 run isocratically at 1 ml/min. The excitation wavelength for fluorescent detection was set at 275 nm and the emission wavelength at 407 nm. Adenosine levels were quantified according to sample spiking and by injecting a known amount of standard to determine peak heights & areas under the peaks.

The resulting HPLC data were analyzed using 32Karat software (Beckman Coulter) to determine quantitatively levels of adenine nucleotides, creatines, and adenosine. Data were expressed either as moles per mg wet weight or per mg protein and analyzed statistically using GraphPad software (La Jolla, CA). A student's t-test was used to determine statistical significance between experimental groups within discrete brain regions; error bars are standard deviation unless mentioned otherwise, p values were set at 0.05.

Cholesterol Fed Rabbits as a Model for Alzheimer's Disease

Animals and Dietary Treatment

New Zealand white male rabbits (Charles River Laboratories, Wilmington, MA), were obtained between 1.5 to 2 years of age (initial weights 3-5 kg).
Rabbits were assigned randomly to either control diet (n=6) or 2% (w:w) cholesterol-enriched diet (n=6; Harlan-Teklad, Madison, WI) groups and were fed their respective diets for 12 weeks before death. The animals were killed by anesthetic and were perfused with 37°C Dulbecco's phosphate-buffered saline. Brains were rapidly removed, dissected for olfactory bulbs, hippocampus, and cerebral cortex, and were frozen on a liquid nitrogen cooled surface before storage at -80°C. All experiments were performed in accordance with NIH guidelines for the care and use of laboratory animals and approved by the University of North Dakota's Animal Care and Use Committee.

**Analysis of Cerebral Energetics**

Frozen cerebral cortex and hippocampal samples were analyzed for adenine nucleotides, phosphocreatine, and creatine (see General method).

**ADSL Transgenic Mice as a Model for Autism**

**Animals and Dietary Treatment**

As a model for Autism, we received genetically modified mice from Dr. David Patterson's laboratory at the Eleanor Roosevelt Institute at the University of Denver. Mice ranging in age from 3 to 18 months were communally housed and fed a standard diet ad libitum (#2018, Harlan-Teklad, Madison, WI). A detailed description of the constituents of the diet is provided in Table 1 of the Appendix. Control and genetically modified mice were killed with a 6 kW head-focused high-energy microwave irradiation system (Cober Electronics, Norwalk, CT) set to 35% of maximal power for a duration of 0.3 s. Mouse brains were taken and cerebral cortex and cerebellum were dissected and frozen on dry ice.
Brain tissue was stored at -80°C. All experiments were performed in accordance with NIH guidelines for the care and use of laboratory animals and approved by Institutional Animal Care and Use Committees.

Analysis of Cerebral Energetics

In addition to phosphocreatine and creatine detected at 210 nm and adenine nucleotides detected at 254 nm (see General method), SAICAR and adenylosuccinate were detected at 254 nm from cerebral cortex and cerebellum.

Ts65Dn and GART Transgenic Mice as Models for Down Syndrome

Animals and Dietary Treatment

As a model for Down syndrome, we obtained trisomic (Ts65Dn) and standard FVB mice from Dr. David Patterson’s laboratory at the Eleanor Roosevelt Institute at the University of Denver. The Ts65Dn mouse strain originated from Jackson Laboratory (Bar Harbor, ME) and contains triplications of partially overlapping segments in the Down syndrome region and covers the majority of the triplicated region observed in humans (Epstein et al., 1985). Mice ranged in age from 3 to 18 months, were housed communally, and were fed a standard diet ad libitum (#2018, Harlan-Teklad, Madison, WI). A detailed description of the constituents of the diet is provided in Table 1 of the Appendix. Control and genetically modified mice were killed with a 6 kW head-focused high-energy microwave irradiation system (Cober Electronics, Norwalk, CT) set to 35% of maximal power for a duration of 0.3 s. Mouse brains were taken and cerebral cortex and cerebellum were dissected and frozen on dry ice. Brain
tissue was stored at -80°C. All experiments were performed in accordance with NIH guidelines for the care and use of laboratory animals and approved by Institutional Animal Care and Use Committees.

**Analysis of Cerebral Energetics**

Frozen cerebral cortex and cerebellum samples were analyzed for adenine nucleotides, phosphocreatine, creatine, and adenosine (see General method).

**Treatment of Rat Cerebral Cortical Neurons with HIV-1 Protein Tat as a Model for HIV-1 Dementia**

**Preparation of Primary Cortical Neuron Culture**

Primary cultures of mouse cerebral cortical neurons were prepared as follows. E-16 pups were removed aseptically from pregnant C57BL/6 mice (Charles River Laboratories), brains were isolated and placed into ice-cold sterile PBS containing 5.5 mM glucose, 1 μM EDTA and 1 μM EGTA, and meninges were removed. Cerebral cortices were dissected, placed in fresh ice-cold buffer, minced and incubated for 15 minutes at 37°C with 5 ml of trypsin-EDTA (Gibco). Trypsin was de-activated by adding cells to 5 ml of heat-inactivated fetal bovine serum (Atlanta Biologicals) for 1 min at room temperature. Cells were added to 10 ml of Neurobasal media (Gibco) containing B-27 supplement (Gibco), 0.5 mM L-glutamine, and antibiotic antimycotic (Sigma) containing penicillin (100 units), streptomycin (0.10 mg) and amphotericin B (0.25 μg) and were triturated through a 5 ml pipette. Cells were seeded onto uncoated 96-well plates (Nunc, Roskilde, Denmark) or glass bottom 35 mm culture dishes (Matek, Ashland, MA, USA)
coated previously with poly-D-lysine. After 10-14 days in culture, cells were found typically to be >95% neurons and were taken for experimentation as determined by staining and morphology.

**HIV-1 Tat**

Purified (>95%) recombinant Tat_{1-72} protein was produced from the tat gene encoding for the first exon as described (Ma & Nath, 1997). A deletion mutant (Gurwell et al., 2001; Prendergast et al., 2002) was produced by deleting the sequence encoding amino acids 31-61 of Tat (mutant Tat) previously shown to contain the neurotoxic epitope (Nath et al., 1996). Both Tat_{1-72} and mutant Tat_{1-72} were obtained as gifts from Dr. Avindra Nath. Low retention pipette tips and micro-centrifuge tubes were used to reduce the loss of Tat due to its adherent properties. At the time of experimental treatments, Neurobasal media was replaced with Locke’s buffer containing 156 mM NaCl, 5.6 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 3.6 mM NaHCO₃, 5 mM glucose, and 5 mM HEPES in double-distilled sterile water (pH 7.2). Thawed Tat was never re-frozen for later use due to freeze/thaw degradation. Care was taken not to mix vigorously or oxygenate Tat so as to minimize inactivation.

**Analysis of Cerebral Energetics**

Four h after experimental treatments were applied, buffer was removed, cultures were washed three-times with ice-cold PBS, and cells were lysed by three freeze/thaw cycles and proteins were precipitated with 2% trichloroacetic acid (TCA; Supelco, Bellefonte, PA). A small amount of the lysate was removed for protein analysis (Bio-Rad). Lysate was added to an equal amount of
dichloromethane and tri-octylamine (775:225 v:v) and samples were mixed vigorously. Aliquots (30 µl) of the aqueous phase were injected onto a LC-18-T HPLC column (Supelco, Bellefonte, PA) and adenine nucleotides, phosphocreatine, and creatine were analyzed as described in General method.

Cell Viability Assays

Trypan blue exclusion assays were conducted in 96-well plates to determine neuronal cell death (Mattson et al., 1995; Wang et al., 2002). Media was removed from cell cultures 24 h after treatments, 50 µl trypan blue (0.2%) diluted in PBS was added, and after 5 min at room temperature the total number of cells and the number of cells lacking trypan blue were counted; data for dead cells were expressed as a percentage of total cells. Each experiment was conducted in triplicate and experiments were repeated at least four times using different dishes of cultured cells.

Measurement of Mitochondrial Membrane Potential

Mitochondrial membrane potential was determined 4 h after treatments in cells grown on glass bottom 35 mm culture dishes by removing culture media, replacing the media with 2 ml PBS containing 1.0 µg/ml of the cationic dual emission dye JC-1 (Molecular Probes), and incubating cells at 37°C and 5% CO₂ for 25 minutes. After incubation, cells were washed two-times with media at 37°C and fluorescence was measured using a Zeiss Axiovert 200M microscope system at excitation/emission wavelengths of 485/530nm for the monomer and 535/570nm for the J-aggregate. Fluorescence was quantified for each cell using average pixel intensity of both J-aggregate and JC-1 monomers using Image-J.
software (NIH). Data were normalized to the ratio of J-aggregate (average pixel
intensity)/J-monomer (average pixel intensity) in untreated control cultures
(untreated ratios were changed to a value of 1). Each experiment was conducted
in duplicate and experiments were repeated four-times using different batches of
neurons.

Measurements of Mitochondrial Permeability Transition Pore Status

Mitochondrial permeability transition pore opening was determined
fluorometrically (Petronilli et al., 1999). Following treatment of cells for 4 h,
cultures were washed in 2 ml Locke’s buffer and incubated in 2 ml Locke’s buffer
containing calcein-AM (1 µM) and cobalt chloride (1 mM) for 20 minutes at 37°C.
Cells were washed with 2 ml Locke’s buffer and fluorescence was measured with
a Zeiss Axiovert 200M microscope at an excitation wavelength of 488 nm and an
emission wavelength of 525 nm. Following de-esterification, calcein fluorescence
was visible in mitochondria, and cobalt chloride that quenches the fluorescence
only enters intact mitochondria with opening of the mitochondrial permeability
transition pore. Fluorescence was quantified using average pixel intensity using
Image-J software (NIH) and data were expressed as cells with open pores as a
percentage of total cells. Each experiment was conducted in duplicate 35 mm
glass-bottomed plates and repeated four-times using different batches of cultured
cells.
**Analysis of Reactive Oxygen Species**

Levels of reactive oxygen species were measured in 96-well plates using the fluorescent dye dichloro-dihydrofluorescein diacetate (H$_2$DCFDA), a cell membrane permeable dye that fluoresces in the presence of hydrogen peroxide, peroxyl radicals, peroxynitrite anions, and nitric oxide. Following treatment of cells for 4 h, culture media was replaced with 50 µl PBS containing 20 µM H$_2$DCFDA and cells were incubated at 37°C and 5% CO$_2$ for 45 minutes. Cells were washed once with 50 µl PBS and fluorescence was determined using a SpectraMax Plus 384 plate-reader (Molecular Devices). Each experiment was conducted in triplicate and experiments were repeated at least 4-times using different batches of cultured cells.

**Statistical Analyses**

All data were reported as means ± SEM. Statistical comparisons were made using one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison post hoc test, unless indicated otherwise. Significant differences were set at p < 0.05.

**Ketogenic Diet as Treatment for Epilepsy**

**Animals and Dietary Treatment**

For these experiments conducted at Emory University, male Sprague-Dawley rats (Harlan, Indianapolis, IN) were housed individually and fed either a calorie-restricted ketogenic (KD) or an isocaloric normal diet ad libitum (CON) beginning on post-natal day 37-41 (initial body weights 135-175 g). A detailed description of the constituents of the normal (Purina 5001) and ketogenic (Bio-
Serv #F-3666, Frenchtown, NJ) diets is provided (Bough & Eagles, 1999). All experiments were performed in accordance with NIH guidelines for the care and use of laboratory animals and approved by Institutional Animal Care and Use Committees. Seizure threshold was measured every 3 to 4 days via timed venous infusion of pentylenetetrazole (PTZ; Pollack & Shen, 1985). In separate experiments seizure threshold was measured only once, after three weeks on a control or KD, via timed exposure to PTZ (n = 25) or flurothyl (n = 20). A threshold dose of PTZ or flurothyl (mg/kg) was calculated from the time at which the rat first exhibited a bilateral forelimb clonic jerk (Bough et al., 2000a).

Seizures were induced between 13:00 and 17:00 prior to feeding to minimize possible effects of circadian rhythms and postprandial hormonal fluctuations. Plasma levels of β-hydroxybutyrate (BHB) and glucose were measured using a Keto-Site (GDS Technologies, Elkhart, IN) or Precision Xtra (Abbott Labs, Alameda, CA) meter, respectively.

Microarray Analysis

Seizure-naïve male rats (n = 24) were maintained on the diet for 22 days, lightly anesthetized with isoflorane, and decapitated. Their brains were rapidly removed and placed in ice-cold phosphate-buffered saline solution for 10-15 s, and then hippocampi were dissected over ice and frozen. To minimize biologic variability, left hippocampi from either two KD or two control animals were pooled together and stored at -80°C to produce six pooled samples from each treatment group of 12 rats. The right hippocampi (n = 12 per group) were stored individually at -80°C for corroborative analyses. All tissue samples were sent to the NINDS-
NIMH Affymetrix Microarray Consortium (TGEN, Phoenix, AZ) for total RNA isolation, quality control assessment, probe generation, hybridization to rat 230A GeneChips (Affymetrix, Santa Clara, CA), and GeneChip scanning. Total RNA was inadequately isolated from one of the ketogenic samples, thus only 11 total samples were used for array testing (5 KD, 6 CON). Image data from each chip were normalized to a mean target intensity value of 150.

The relative abundance of each probe set and an evaluation of whether a particular transcript was expressed above background were calculated using Microarray suite (MAS 5.0, Affymetrix). The assignment of each probe pair on the rat 230A GeneChip to a gene was originally based by Affymetrix on the sequences available in Unigene build #99, whereas the most recent build is #144. The probe pair assignments were not updated by Affymetrix, and approximately 11% of the original accession numbers assigned to probe sets on the RAE 230A chip either match fewer than half of the probe pairs in the corresponding set or are retired from current databases (Dai et al., 2005). Dai and colleagues (2005) created a custom CDF file (http://brainarray.mbni.med.umich.edu/Brainarray/Database/CustomCDF/genomic_curated_CDF.asp) that was readable by the MAS 5.0 program to assign signal intensities of each probe pair to genes. All probe pairs for a particular transcript were pooled into a single probe set, which eliminated duplicate or triplicate instances of genes on the Chip. Moreover, probes hybridizing to the non-coding strand of a transcript were deleted from analysis, which greatly reduced the number of expressed sequence tags (ESTs) called. Discrimination scores of the signal intensities for each spot
on an individual chip were determined to be significantly different from background (i.e., present, marginally present, or absent calls) using a one-sided Wilcoxon's Sign Ranked test. We selected genes for subsequent statistical analysis if signal intensities were significantly above background (i.e., called "present") in at least 5 of the 11 arrays tested. The Significance Analysis of Microarrays (SAM) program (http://www.stat.stanford.edu/~tibs/SAM; Tusher et al., 2001) was used to determine differences in patterns of gene expression between groups at a 1% false discovery rate (FDR). Functional categories were ascribed to differentially-expressed genes using NetAffx Analysis Center (www.affymetrix.com), and explore Gene Ontology (eGOn; http://nova2.idi.ntnu.no/egon/). Because not all named genes were associated with a GO biological process, functions for the remaining named genes were identified by manual searching through EntrezGene (NIH), the Rat Genome Database, and GeneCards. To determine whether the ratio of induced-to-repressed genes in a particular category was significantly different from the ratio of all induced-to-repressed genes, two-sided Fisher's exact tests for each of the eight categories were performed.

Analysis of Cerebral Metabolites, Enzyme Activities and Nucleotides

Cohorts of KD (n = 29) and CON (n = 28) animals were maintained on diet treatment for 20-28 days. A cohort of KD (n = 10) and CON (n = 10) animals was maintained on diet treatment for 28 days, then lightly anesthetized with isoflurane and decapitated. The head was immediately cooled in ice-cold phosphate-buffered saline for 10 to 15 s to minimize the post-mortem
accumulation of GABA (Hassel et al., 2001) and the brain was rapidly removed. Hippocampi were dissected rapidly over ice, frozen on dry ice, and weighed. For BHB, citrate and amino acid determinations, freshly-frozen hippocampi were homogenized in a 1:20 weight-to-volume ratio of a 7% perchloric acid (v:v) solution that included 100 μM α-amino adipate as an internal standard. BHB was measured fluorometrically according to the principles of Lowry and Passonneau (1972) in a buffer containing Tris-potassium, 0.1 M, hydrazine, 0.1 M, NAD, 0.2 mM, and BHB dehydrogenase, 1 U/ml. Citrate was analyzed as described by Lowry and Passonneau (1972). Amino acid levels were measured by HPLC and fluorescence detection after precolumn derivatization with o-phthaldialdehyde (Hassel et al., 1997).

For enzyme determinations, frozen hippocampi were homogenized in ice-cold 0.32 M sucrose. Hexokinase was measured fluorometrically according to Knull et al (1973) with an ATP concentration of 1 mM; glucose-6-phosphate dehydrogenase was measured with the buffers used for hexokinase, substituting 10 mM glucose-6-phosphate for glucose and ATP. Lactate dehydrogenase was analyzed fluorometrically in Tris-HCl, 0.1 M, hydrazine 0.1 M, pH 8, MgCl₂, 5 mM, lactate, 30 mM, and NAD, 1 mM; BHB dehydrogenase was analyzed in Tris-HCl, 0.1 M without hydrazine, pH 8, MgCl₂, 3 mM, β-mercaptoethanol, 10 mM, BHB, 30 mM, and NAD, 1 mM. Citrate synthase and malate dehydrogenase were analyzed spectrophotometrically according to Srere (1969) and Kitto (1969), respectively. α-Ketoglutarate dehydrogenase was analyzed fluorometrically according to Mastrogiacomo et al. (1993). All enzyme reactions were verified to

produced with permission of the copyright owner. Further reproduction prohibited without permission.
be linear with time and concentration of tissue. Protein was measured according
to Lowry et al (1951).

For cerebral nucleotides and glycogen a separate cohort of KD (n = 9) and
control (n = 8) rats were maintained on their respective diets for 20 days and
killed with a 6 kW head-focused high-energy microwave irradiation system
(Cober Electronics, Norwalk, CT) set to 70% of maximal power for a duration of
1.3 s (Delaney & Geiger, 1996). Hippocampus was dissected and then frozen on
dry ice. The left hippocampus was used to measure AMP, ADP, ATP, creatine,
and phosphocreatine levels as described in General method, whereas
hippocampal tissue from the right hemisphere was used to measure glycogen.
Glycogen levels were determined biochemically using an enzyme-linked assay
as described (Kong et al., 2002). Glycogen was hydrolyzed to glucose in aliquots
(100 µl) of homogenate that were incubated overnight (16 h) at room temperature
with 1 ml of 0.2 M sodium acetate, 20 µl of 1.0 M KHCO₃, and 20 U/ml of
amyloglucosidase. Following incubation, we added 0.5 ml of a perchloric acid
solution containing 6% perchloric acid and 1 mM ethylenediaminetetraacetic acid
(EDTA) that stopped the reaction. The tubes were centrifuged at 25,000 X g for
10 min at 4°C before supernatants were neutralized with a KOH solution
consisting of 3 M KOH, 0.3 M imidazole, and 0.4 M KCl and then centrifuged at
14,000 X g for 10 min at 4°C, and utilized for glucose content assays.
Endogenous glucose levels were determined from nonhydrolyzed samples that
we obtained by centrifuging homogenates as described above and a pH
adjustment to a final pH 6-8 via KOH solution. Neutralized samples were

produced with permission of the copyright owner. Further reproduction prohibited without permission.
vortexed, centrifuged at 25,000 X g for 10 min at 4°C and assayed for background glucose levels. To each well of the 96-well plate 200 µl of a reaction solution containing 50 mM Tris-HCl, pH 8.1, 0.5 mM ATP, 0.5 mM NADP⁺, 5 mM MgSO₄, and 0.1 U/ml glucose-6-phosphate dehydrogenase was added. The plates were then placed in a SpectraMax Gemini fluorescence plate reader (Molecular Devices, Sunnyvale, CA), shaken, and measurements of formed NADPH were executed at 355 nm excitation, 480 nm emission, and 420 nm cutoff wavelengths. Hexokinase (0.3 U) was added to each well, plates were shaken, and after a 30 min incubation period, measurements were collected. Glycogen levels, indicated as glucose units, were calculated by subtracting the final µM concentration of glucose per g of wet weight of the nonhydrolyzed tissue sample from the µM concentration of glucose per g of wet weight of the hydrolyzed tissue sample. Resulting numbers were transposed to GraphPad software (La Jolla, CA) for statistical analyses as well as graphing of data. A student’s t-test was used to determine significance of between experimental groups within discrete brain regions; the p value was set at 0.05.

Electron Microscopy

Nine animals (4 KD, 5 CON) were maintained on diet treatment as above for at least four weeks. After deep anesthesia with halothane, animals were perfused transcardially with 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1M phosphate buffer (pH 7.4). Brains were post-fixed in the same mixture for 8-12 h at 4-8°C. Coronal sections (60 µm) were cut with a vibratome, dehydrated and embedded in Durcupan resin (Electron Microscopy Sciences, Fort...
Regions of the dentate and hilus (~2 mm^2) were microdissected, mounted on blocks, and 60 nm sections were collected onto Pioloform-coated slot grids, then counterstained with uranyl acetate and lead citrate.

Sections were examined using a Zeiss EM10C electron microscope. Electron micrographs were randomly taken at 20,000 x magnification through the dentate-hilus region. Data from 4 KD-fed animals (160 micrographs representing 2680 μm^2) and 5 controls (184 micrographs representing 3080 μm^2) were examined for mitochondrial counts. Images were captured on a Leica DMRBE microscope with a Spot RT color digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI), saved in a jpg-file format, and printed using a Kodak 8660 Thermal printer. Identification of dendrites, terminals, spines, and axons was based on ultrastructural characteristics and size, as described previously (Peters & Palay, 1996). Mitochondria were identified by their localization within identified processes, dual outer membranes, and presence of internal cristae.

**Electrophysiology**

Six pairs of animals were fed either a ketogenic or CON diet for ≥ 20 days as above, anesthetized with halothane, and decapitated. Brains were rapidly removed and chilled in an ice-cold, bubbled with 95% O_2/5% CO_2 cutting ACSF containing the following (in mM): NaCl 130, KCl 3.5, Na_2HPO_4 1.25, NaHCO_3 24, MgSO_4 4, CaCl_2 1, glucose 10, and osmolarity 300 ± 5 mOsm. For each experiment, transverse entorhinal-hippocampal slices (500-550 μM thick) were cut using a vibratome from each pair of animals (1 KD and 1 control).
simultaneously. Slices were transferred to a carbogenated holding chamber in ACSF containing 2 mM each of MgSO₄ and CaCl₂, where they were maintained for approximately 30 min at 25°C until recordings were begun. Slice recordings from pairs of animals were conducted concurrently in a submerged chamber, where slices were continuously perfused with carbogenated bathing medium at a rate of 2-3 ml/min. With our chamber dead volume of 2.2 ml, this allowed for fluid exchange within a few minutes. All experiments were performed at 32-34°C.

Extracellular field excitatory post-synaptic potentials (fEPSPs) were recorded from the dentate molecular layer using glass micropipettes (5-10 MΩ) filled with artificial cerebrospinal fluid (ACSF) and an aliquot (~25 µl) of India ink to visualize recording electrode placement. Slices from the middle hippocampus were used preferentially. Responses were evoked by stimulation of the medial perforant path (MPP, located in the middle third of the molecular layer of the dentate gyrus) using a Teflon-coated, Pt/I monopolar microelectrode (~1 MΩ impedance). Electrode placements in the MPP were corroborated by observing paired-pulse depression (~15%) at a 50 ms interpulse interval (McNaughton, 1980). Responses that did not exhibit consistent paired-pulse depression were not used. Stimulus intensity (0.1 ms duration, ≤70 µA) was adjusted to evoke fEPSPs of approximately 50% maximum amplitude. A solution containing low (2 mM) glucose supplemented with 8 mM mannitol to maintain osmolarity was bath applied for 7 min.

Electrophysiological recordings were performed on cerebral tissue from male Sprague-Dawley rats fed control or KD for 21-35 days at Trinity College.
Animals were decapitated, the hippocampus was dissected from whole brain, after rapid decapitation and cooling, hippocampal slices (400 mm) were prepared. At least 1 hr after surgery, brain slices were maintained at a constant temperature of 31-33°C in a submersion chamber and constantly superfused (2 ml/min) with gassed (95% O2, 5% CO2) artificial cerebrospinal fluid containing 126 mM NaCl, 3 mM KCl, 1.5 mM MgCl2, 2.4 mM CaCl2, 1.2 mM NaH2PO4, 11 mM glucose, and 26 mM NaHCO3 (Sigma-Aldrich, St. Louis, MO). For recordings of field excitatory postsynaptic potential (fEPSP) responses, the recording electrode was placed in stratum radiatum of the CA1 region and the stimulation electrode in stratum radiatum near the border of the CA1 and CA2 regions at 33°C; stimuli were delivered at 15 s intervals (Masino et al., 2002). Adenosine (50 mM) was added at least 15 min prior to recording and all recordings were performed within 3.5 h of slicing. Responses were recorded using an AC amplifier, and a computer was used to digitize and store the responses for further analysis (Dunwiddie & Diao, 1994). A student’s t-test was used to determine significance between experimental groups, p was set at 0.05.

**QRT-PCR of Laser Captured Neurons**

Brains from KD (n=5) and CON (n = 5) fed rats were fresh frozen and sectioned via cryostat into 8-12 μm thick sections for laser-capture microscopy (LCM). Dentate granule and CA1 pyramidal cells were harvested with an Arcturus Pixcell Ile system (Arcturus, CA) using the following parameters: power = 90-100 W, Duration = 800-1500 μs, and spot size = 7.5 μm (Greene et al., 2005). cDNA was transcribed from random hexamer amplified total RNA

*Produced with permission of the copyright owner. Further reproduction prohibited without permission.*
(Invitrogen, Carlsbad, CA) and quantitative PCR (qPCR) was performed using Applied Biosystems SybrGreen Master Mix [Applied Biosystems, Foster City, CA].

Eight metabolic genes showing some degree of regulation on the microarray were examined by qPCR. These genes were Rieske (similar to Ubiquinol cytochrome reductase transmembrane 1 (Uqcrfs1), NADH dehydrogenase (ubiquinone) 1 beta subcomplex 9 (Ndufb9), the delta and O subunits of ATP synthase, H⁺ transporting and mitochondrial F1 complex (Atp5d and ATP5o), succinate dehydrogenase complex (Sdha1), both soluble and mitochondrial forms of malate dehydrogenase, and cytochrome c oxidase subunit VIa polypeptide 1 (Cox6a1). Hypoxanthine guanine phosphoribosyl transferase (HPRT), a gene showing no significant change related to KD, was used to standardize the level of expression of each transcript.

Changes in transcript levels of the genes of interest (GOI) were measured in duplicate and calculated as [(the average cycle threshold (CT) for transcript – average CT for HPRT (standard gene) for a KD-fed animal) – ((average CT for transcript – the average CT for HPRT for a control-fed animal)], resulting in a \( \Delta \Delta CT \) value for each sample (Greene et al., 2005). The mean DG and CA1 \( \Delta \Delta CT \) values were compared with a 2 tailed -test for significance, \( p \) was set at 0.05.

**Analysis of Cerebral Energetics**

For these experiments conducted at University of North Dakota, male Sprague-Dawley rats (Harlan, Indianapolis, IN) were housed individually and fed either a calorie-restricted KD or an ad libitum standard rodent diet beginning on
post-natal day 35 (initial weights 135-175 g). A detailed description of the
cosstituents of the control (Harlan-Teklad #2018) and ketogenic (Bio-Serv #F-
3666, Frenchtown, NJ) diets is provided in Table 2 of the Appendix. Animals
were individually housed at the University of North Dakota in the Center for
Biomedical Research for five days prior to a daily monitoring of each animal's
weight and caloric intake. Rats were weight-matched and evenly split into
experimental (KD) and control groups. Caloric intake data was used to calculate
daily food allowance for all animals with an increase of 1.6% of their previous
day's calories to allow for their normal growth. Ketogenic animals received 90%
of their normal daily caloric intake in the form of a frozen 8% ketogenic paste
smeared on one side of a 35 mm culture dish that was placed atop of the
bedding in their cage. Control animals received an isocaloric amount of their
normal daily caloric intake in the form of chow pellets that were placed on the
hopper of each cage. All rats were fed at approximately 5:00 pm each day. After
21 days of diet treatment, a cohort of KD and control rats were killed with a 6kW
head-focused high-energy microwave irradiation system (Cober Electronics,
Norwalk, CT) set to 70% of maximal power for a duration of 1.3 s. Animals were
killed at 5pm ±2h as to minimize circadian and feeding effects. Discrete rat brain
regions were dissected and then fresh frozen over dry ice. Samples were stored
at -80°C until taken for analyses. All experiments were performed in accordance
with NIH guidelines for the care and use of laboratory animals and approved by
University of North Dakota's Animal Care and Use Committees.
Frozen cerebral cortex, cerebellum, hippocampal, hypothalamus, and striatum samples were analyzed for adenine nucleotides, phosphocreatine, creatine, and adenosine (see General method).
CHAPTER III

RESULTS

Cholesterol Fed Rabbits as a Model for Alzheimer's Disease

Pathological hallmarks of Alzheimer’s disease (AD) include abnormal accumulations of Aβ and Tau. These pathological observations may be associated with changes in brain energy metabolism that could occur as a result of dysregulation of mitochondrial metabolism (Zhu et al., 2004b) and/or altered glucose metabolism that has been observed in patients with AD (Pettegrew et al., 1994). Accordingly, using high-cholesterol diet fed rabbits as a model for the sporadic form of Alzheimer’s disease (Ghribi et al., 2006) we tested the hypothesis that rabbits fed a high cholesterol diet for 12 weeks have decreased levels of high-energy brain metabolites in cerebral cortex and hippocampus.

Compared to a standard diet, 2% cholesterol fed rabbits exhibited decreased phosphocreatine levels (ns, Figure 7A), significantly diminished levels of creatine (Figure 7B, \( p < 0.05 \)), and significantly elevated phosphocreatine/creatine ratio (Figure 7C, \( p < 0.05 \)).
Figure 7: Effects of Administration of a High Cholesterol Diet on Levels of Creatine and Phosphocreatine in Rabbit Cerebral Cortex. (A) Compared with controls, levels of phosphocreatine in cholesterol-fed rabbits were not significantly decreased. (B) Levels of creatine were decreased significantly (p < 0.05) in cerebral cortex compared to control animals. (C) Statistically significant increases (p < 0.01) were observed for the ratio of phosphocreatine/creatine compared to control rabbits. * p < 0.05 versus control, ** p < 0.01 versus control. Values are mean ± SD. Sample sizes are indicated on respective bars.

Adenine nucleotides represent an important energy and demands for energy are particularly high at neuronal synapses; sites where neurodegenerative processes occur early in the disease state (Coleman et al., 2004). We hypothesized that levels of ATP would be diminished in our hypercholesterol model for AD. In cerebral cortex, levels of ATP were reduced significantly (p < 0.05) in cholesterol-fed rabbits (Figure 8A). The levels of ADP (Figure 8B) and AMP (Figure 8C) were also reduced significantly (p < 0.05) as a
result of the diet. These findings suggest that energy stores in the form of adenine nucleotides are decreased in rabbits fed cholesterol-enriched diets.

Figure 8: Effects of Administration of Cholesterol-Enriched Diet on Levels of Adenine Nucleotides in Rabbit Cerebral Cortex. (A) Compared with controls, levels of ATP were decreased significantly (p < 0.05) in cerebral cortex of rabbits fed cholesterol-enriched diet for 12 weeks. ADP (B) and AMP (C) were also significantly (p < 0.05) lower in cerebral cortex compared to control animals (p < 0.05). * p < 0.05 versus control. Values are mean ± SD. Sample sizes are indicated on respective bars.

From the data on adenine nucleotide levels, we calculated the ratio of ATP/ADP and the adenylate energy charge. The ratio of ATP/ADP was increased significantly (p < 0.05) in cholesterol-fed rabbits (Figure 9A) indicating an increased energetic status of the cerebral cortical tissue. However, adenylate energy charge ratios were not significantly different (Figure 9B). However,
adenylate energy charge values were very much lower than the theoretical maximum ratio of 1 because these animals were killed such that post-mortem decreases in bioenergetic environment was unavoidable.

Figure 9: Effects of Administration of Cholesterol-Enriched Diet on Energetic Ratios in Rabbit Cerebral Cortex. (A) Statistically significant (p < 0.05) elevations were observed for the ratio of ATP/ADP in rabbits fed cholesterol-enriched diet for 12 weeks. (B) No statistically significant differences were observed for adenylate energy charge ((ATP + ½ADP) / (ATP + ADP + AMP)). * p < 0.05 versus control. Values are mean ± SD. Sample sizes are indicated on respective bars.

ADSL Transgenic Mice as a Model for Autism

For autism, a group of pervasive neurodevelopmental disorders, genetic mutations and de novo copy number variations have been described (Abrahams & Geschwind, 2008). Here, we examined brain energy levels in mice with genetically modified ADSL, a gene associated with a sub-population of individuals with autism. Using mice genetically modified for D87E, a novel genetic mutation to the ADSL gene that results in severe autism (Spiegel et al., 2006), and ADSL, a deficiency of which causes defects in purine metabolism and severe neurological and physiological symptoms (Spiegel et al., 2006) we
measured levels of phosphocreatine and creatine. Because of limited sample size, we “forced” the statistical program to ignore sample size and generate approximate p values; this applies to data in Figures 10, 13, 16, & 19. For phosphocreatine, we observed significantly (p < 0.05) reduced levels for D87E and ADSL_P9383 transgenic mice (Figure 10A). Levels of creatine were reduced significantly (p < 0.01) for D87E as well (Figure 10B). However, phosphocreatine/creatine ratios were unchanged for all examined groups (Figure 10C).

![Graphs showing effects of ADSL genetic modification on levels of creatine and phosphocreatine in mouse cerebral cortex.](image)

Figure 10: Effects of ADSL Genetic Modification on Levels of Creatine and Phosphocreatine in Mouse Cerebral Cortex. (A) Compared to wildtype founder mice, levels of phosphocreatine were decreased significantly (p < 0.05) in the D87E_M347 and ADSL_P9383 (p < 0.05) transgenic mice. (B) Levels of creatine were decreased significantly (p < 0.01) in cerebral cortex for the D87E_M347 transgenic mice. (C) No statistically significant changes were observed for the ratio of phosphocreatine/creatine ratios. * p < 0.05 versus control, ** p < 0.01

*produced with permission of the copyright owner. Further reproduction prohibited without permission.*
versus control. Values are mean ± SD. Sample sizes are indicated on respective bars.

In a second shipment of mice from our collaborators in Denver we received D87E, ADSL, and R426H animals. The amino acid change of R426H is an ADSL mutation that results in moderate to severe autism (Spiegel et al., 2006). With increased sizes within each group, normal statistical analyses were performed; this applies to Figures 11, 12, 14, 15, 17, 18, 20 & 21. Levels of phosphocreatine were reduced significantly (p < 0.05) in cerebral cortex in the transgenic ADSL_P9383 group (Figure 11A) while levels of creatine were elevated significantly (p < 0.05) in transgenic ADSL_P365 mice (Figure 11B). No statistically significant changes were observed to phosphocreatine/creatine ratios (Figure 11C). No statistically significant changes in metabolite levels were noted in cerebellum (Figure 12).
Figure 11: Effects of ADSL Genetic Modification on Levels of Creatine and Phosphocreatine in Mouse Cerebral Cortex. (A) Compared to wildtype founder mice, levels of phosphocreatine were decreased significantly ($p < 0.05$) for ADSL_P9383 transgenic mice. (B) Levels of creatine were increased significantly ($p < 0.05$) in cerebral cortex for ADSL_P9365 transgenic mice. (C) No statistically significant changes were observed for phosphocreatine/creatine ratios. * $p < 0.05$ versus control. Values are mean ± SD. Sample sizes are indicated on respective bars.
Figure 12: Effects of ADSL Genetic Modification on Levels of Creatine and Phosphocreatine in Mouse Cerebellum. (A) Compared to wildtype founder mice, levels of phosphocreatine were decreased significantly (p < 0.05) in ADSL_P9383 transgenic mice. (B) Levels of creatine were increased significantly (p < 0.05) in cerebellum for ADSL_P9365 transgenic mice. (C) No statistically significant changes were observed for phosphocreatine/creatine ratios. * p < 0.05 versus control. Values are mean ± SD. Sample sizes are indicated on respective bars.

Next, we measured levels of adenine nucleotides. Levels of ATP (Figure 13A, p < 0.01) and ADP (Figure 13B, p < 0.05) were significantly reduced in ADSL_P9383 transgenic mice but remained unchanged for all other transcripts. Levels of AMP were not altered significantly in cerebral cortex for any group (Figure 13C).
ATP ADP
AM

Figure 13: Effects of ADSL Genetic Modification on Levels of Adenine Nucleotides in Mouse Cerebral Cortex. (A) Compared to wildtype founder mice, levels of ATP were decreased significantly (p < 0.01) in the ADSL_P9383 transgenic mice. (B) Levels of ADP were decreased significantly (p < 0.05) in cerebral cortex for ADSL_P9383 transgenic mice. (C) No statistically significant changes were observed for levels of AMP. * p < 0.05 versus control, ** p < 0.01 versus control. Values are mean ± SD. Sample sizes are indicated on respective bars.

In ADSL_P9383 transgenic mice we observed a non-statistically significant decrease in levels of ATP cerebral cortex (Figure 14A) and increase in cerebellum (Figure 15A. The large standard deviations likely accounted for the lack of statistical significance. In R426H_M1036 transgenic mice, levels of ATP in cerebral cortex (Figure 14A, p < 0.05) and ADP in cerebral cortex and cerebellum (Figure 14B & 15B p < 0.05) were elevated significantly whereas levels of AMP were decreased significantly (Figure 14C, p < 0.01) in cerebral cortex and
displayed a non-significant trend toward a decrease in cerebellum (Figure 15C, p > 0.05). These findings of increased ADP and ATP were unexpected in R426H transgenic mice, but may caused by developmental changes (Alexiou & Leese, 1992) or by other as yet unexplained technical reasons.

Figure 14: Effects of ADSL Genetic Modification on Levels of Adenine Nucleotides in Mouse Cerebral Cortex. (A) Compared to wildtype founder mice, levels of ATP were increased significantly (p < 0.05) in R426H_M1036 transgenic mice. (B) Levels of ADP were increased significantly (p < 0.05) in cerebral cortex for R426H_M1036 transgenic mice. (C) Levels of AMP were decreased significantly (p < 0.05) for R426H_M1036 transgenic mice. * p < 0.05 versus control. Values are mean ± SD. Sample sizes are indicated on respective bars.
Figure 15: Effects of ADSL Genetic Modification on Levels of Adenine Nucleotides in Mouse Cerebellum. (A) Compared to wildtype founder mice, levels of ATP were increased significantly (p < 0.05) in R426H_M1036 transgenic mice. (B) Levels of ADP were increased significantly (p < 0.05) in ADSL_P9383 transgenic mice. (C) Levels of AMP were decreased significantly (p < 0.05) in R426H_M1036 transgenic mice. * p < 0.05 versus control. Values are mean ± SD. Sample sizes are indicated on respective bars.

Decreased levels of adenine nucleotides were noted for some transgenic groups born to founder mouse ADSL_P9383 whereas unexpected elevations were found when examining mice with the R426H transcript. To examine further potential differences, we calculated two energetic ratios; ATP/ADP and adenylate energy charge. In cerebral cortex from our first group of ADSL animals, we noted a significantly lower level of ATP from the group born to founder mouse P9383 (Figure 13A), and this effect was confirmed with decreased ATP/ADP ratios (Figure 16A, p < 0.05) and adenylate energy charges (Figure 16B, p < 0.05).
ATP/ADP Ratio

Adenylate Energy Charge

Figure 16: Effects of ADSL Genetic Modification on Energetic Ratios in Mouse Cerebral Cortex. (A) Compared to wildtype founder mouse, the ratio of ATP/ADP was decreased significantly (p < 0.05) in ADSL_P9383 transgenic mice. (B) Adenylate energy charge was decreased significantly (p < 0.05) for ADSL_P9383 transgenic mice. * p < 0.05 versus control. Values are mean ± SD. Sample sizes are indicated on respective bars.

In the second group of genetically modified ADSL mice, animals born to founder ADSL_P9383 had non-statistically significant decreases in levels of ATP in cerebral cortex (Figure 14A), a significantly lower (p < 0.05) amount of ATP compared to ADP (Figure 17A), and no change in adenylate energy charge (Figure 17B). For cerebral cortex of mice from founder R426H_M1036’s litters, ATP and ADP increased significantly (Figure 14A & B) and the ATP/ADP ratios and adenylate energy charges were elevated significantly (Figure 17A & B).
Figure 17: Effects of ADSL Genetic Modification on Energetic Ratios in Mouse Cerebral Cortex. (A) Compared to wildtype founder mice, ATP/ADP ratios were decreased significantly (p < 0.05) for ADSL_P9383 and increased significantly (p < 0.05) in the R426H_M1036 transgenic mice. (B) Adenylate energy charge was increased significantly (p < 0.05) for R426H_M1036 transgenic mice. * p < 0.05 versus control. Values are mean ± SD. Sample sizes are indicated on respective bars.

For cerebellum, we observed a non-significant trend toward elevated ATP levels in mice bred from founder ADSL_P9383 (Figure 15A). The ATP/ADP ratios were not increased significantly (Figure 18A) and no change was observed for adenylate energy charge (Figure 18B). For cerebellum of transgenic mice born to founder R426H_M1036, ATP was non-significantly elevated (Figure 15A), ADP was increased significantly (Figure 15B), AMP was not significantly decreased (Figure 15C). ATP/ADP ratios were reduced significantly (Figure 18A, p < 0.05) whereas no change was demonstrated for adenylate energy charge (Figure 18B).
Figure 18: Effects of ADSL Genetic Modification on Energetic Ratios in Mouse Cerebellum. (A) Compared to wildtype founder mice, ATP/ADP ratios were decreased significantly ($p < 0.05$) in ADSL_P9383 transgenic mice whereas this ratio was increased significantly ($p < 0.05$) in R426H_M1036 transgenic mice. (B) Adenylate energy charge was decreased significantly ($p < 0.05$) for R426H_M1036 transgenic mice. * $p < 0.05$ versus control. Values are mean ± SD. Sample sizes are indicated on respective bars.

Next, we attempted to quantify levels of SAICAR and adenylosuccinate using HPLC-based techniques. Since these animals have genetically altered ADSL genes, it was expected that we would find levels of both our compounds of interest elevated significantly in cortex and cerebellum. We developed an accurate and reliable means to determine these compounds via photo-diode array HPLC and relatively pure SAICAR and adenylosuccinate standards were received and used. Surprisingly, SAICAR was not elevated in brain samples of transgenic mice as was expected and were even decreased significantly ($p < 0.05$) in ADSL_P9383 (Figure 19).
Figure 19: Effects of ADSL Genetic Modification on SAICAR Levels in Mouse Cerebral Cortex. Compared to the wildtype founder mice, levels of SAICAR were significantly (p < 0.05) lower for ADSL_P9383 transgenic mice. * p < 0.05 versus control. Values are mean ± SD. Sample sizes are indicated on respective bars.

We then analyzed cerebral cortex and cerebellum from our second set of ADSL animals for SAICAR and adenylosuccinate. We found that in cerebral cortex SAICAR levels were not changed significantly as a result of modifying the ADSL gene (Figure 20A). For adenylosuccinate levels, only R426H_M1036 mice exhibited statistically significant decreases (Figure 20B, p < 0.05). In cerebellum, we observed significantly (p < 0.05) elevated levels of SAICAR in D87E_M347 mice (Figure 21A) however no significant changes were noted for adenylosuccinate levels (Figure 20B & Figure 21B).
Figure 20: Effects of ADSL Genetic Modification on SAICAR and Adenylosuccinate Levels in Mouse Cerebral Cortex. (A) Compared to wildtype founder mice, levels of SAICAR were not significantly altered for any of the tested groups. (B) Levels of adenylosuccinate were significantly decreased (p < 0.05) in R426H_M1036 transgenic mice. * p < 0.05 versus control. Values are mean ± SD. Sample sizes are indicated on respective bars.

Figure 21: Effects of ADSL Genetic Modification on SAICAR and Adenylosuccinate Levels in Mouse Cerebellum. (A) Compared to the wildtype founder mice, levels of SAICAR were not significantly altered for any of the tested groups. (B) Levels of adenylosuccinate were decreased significantly (p < 0.05) in R426H_M1036 transgenic mice. * p < 0.05 versus control. Values are mean ± SD. Sample sizes are indicated on respective bars.

In an attempt to confirm that the SAICAR and adenylosuccinate peaks observed using HPLC techniques were indeed the compounds of interest we applied additional analytical techniques. Having collected fractions containing the...
apparent SAICAR peak, samples were lyophilized and analyzed by Dr. Erin Spiegel using the Bratton-Marshall assay method (Laikind et al., 1986). SAICAR was undetectable in our brain samples. We then collaborated with Dr. Alena Kubatova of the Mass Spectrometry Core at the University of North Dakota to apply electro-spray ionization mass spectrometer as well as through an HPLC-coupled tandem mass spectrometer. Although we detected SAICAR from spiked-samples, we could not measure definitively SAICAR in our brain samples. Therefore, we remain uncertain as to the validity of the results illustrated in Figures 19-21.

Ts65Dn and GART Transgenic Mice as Models for Down Syndrome

The Ts65Dn mouse model of Down syndrome (Reeves et al., 1995) has trisomic genes important for mitochondrial metabolism (see Table 5). With these mice we tested the hypothesis that trisomy leads to increased energy production. In 3 month old trisomic mice, phosphocreatine levels were decreased significantly (Figure 22A, p < 0.05) and were increased significantly (p < 0.05) in old animals (Figure 22A); levels of creatine and phosphocreatine/creatine ratios were not significantly changed (Figure 22B & C).
Phosphocreatine

Figure 22: Effects of Trisomy 21 on Levels of Creatine and Phosphocreatine in Mouse Cerebral Cortex. (A) Compared to 3 month old wildtype mice, levels of phosphocreatine were decreased significantly ($p < 0.05$) in 3 month old Ts65Dn mice. Levels of phosphocreatine were elevated significantly ($p < 0.05$) in old Ts65Dn animals. (B & C) There were no statistically significant changes in creatine levels or to ratios of phosphocreatine/creatine. * $p < 0.05$ versus control, ** $p < 0.01$ versus control. Values are mean ± SD. Sample sizes are indicated on respective bars.

We next examined levels of adenine nucleotides between trisomic and normal mice for differing ages. Similar to preliminary data obtained at the University of Manitoba, we observed that levels of ATP were reduced significantly ($p < 0.05$) in trisomic mice 6 months of age compared to wildtype controls (Figure 23A). In addition, we found levels of ATP were increased significantly ($p < 0.05$) in old trisomic mice (Figure 23A) and that levels of ADP produced with permission of the copyright owner. Further reproduction prohibited without permission.
were reduced significantly \( (p < 0.05) \) at 6 months in trisomic mice (Figure 23B); ADP levels were not changed in old animals and no changes were observed for AMP.

![Figure 23: Effects of Trisomy 21 on Levels of Adenine Nucleotides in Mouse Cerebral Cortex. (A) Compared to 6 month old wildtype mice, levels of ATP were decreased significantly \( (p < 0.05) \) at 6 months in Ts65Dn mice. Levels of ATP were elevated significantly \( (p < 0.05) \) in old Ts65Dn animals compared to age-matched controls. (B) Levels of ADP were decreased significantly \( (p < 0.05) \) at 6 months in Ts65Dn mice compared to 6 month control mice. (C) Levels of AMP were not statistically different at any age between groups. * \( p < 0.05 \) versus control. Values are mean ± SD. Sample sizes are indicated on respective bars.](image)

Next we calculated the ratios of ATP/ADP and adenylate energy charge as a function of each age from these groups. This ratio was reduced significantly in 3 (Figure 24A, \( p < 0.05 \)) and 6 month old (Figure 24A, \( p < 0.001 \)) animals, but was altered in old mice. Adenylate energy charge ratios were decreased.

---

Produced with permission of the copyright owner. Further reproduction prohibited without permission.
significantly ($p < 0.01$) in 6 month old Ts65Dn mice compared to wildtypes (Figure 24B).

Figure 24: Effects of Trisomy 21 on Energetic Ratios in Mouse Cerebral Cortex. (A) The ratio of ATP/ADP was decreased significantly in 3 month old ($p < 0.05$) and 6 month old ($p < 0.001$) Ts65Dn mice compared to age-matched controls. (B) Adenylate energy charge was decreased significantly ($p < 0.01$) in 6 month old Ts65Dn mice. * $p < 0.05$ versus control. ** $p < 0.01$ versus control. *** $p < 0.001$ versus control. Values are mean ± SD. Sample sizes are indicated on respective bars.

In Ts65Dn mice, adenosine levels were elevated significantly ($p < 0.05$) in 3 and 6 month old mice (Figure 25); in old animals the levels were similar to those in control.
Adenosine

- vs. wildtype 3 month
- vs wildtype 6 month

Figure 25: Effects of Trisomy 21 on Levels of Adenosine in Mouse Cerebral Cortex. Adenosine levels were elevated significantly (p < 0.05) in 3 and 6 month old Ts65Dn mice compared to age-matched controls. * p < 0.05 versus control. Values are mean ± SD. Sample sizes are indicated on respective bars.

Using wildtype control animal data from the Ts65Dn experiments, we analyzed energy measurements as a function of age. Aging decreases basal metabolic rates, decreases efficiency of the ETC, increases production of reactive oxygen species, and diminishes energy levels. Therefore, we hypothesized that aged wildtype animals would have lower energy levels in cerebral cortex compared to young and middle-aged adult mice. Levels of phosphocreatine were elevated significantly in old wildtype mice compared to 3 (Figure 26A, p < 0.05) and 6 month old animals (Figure 26A, p < 0.001) whereas levels of creatine (Figure 26B) and the ratio of phosphocreatine/creatine were unchanged as a function of age (Figure 26C).
Figure 26: Levels of Creatine and Phosphocreatine in Wildtype Mouse Cerebral Cortex as a Function of Age. (A) Compared to old wildtype mice, the levels of phosphocreatine were decreased significantly at 3 (p < 0.05) and 6 (p < 0.001) months of age. (B) There were no statistically significant changes in creatine levels and the ratio of phosphocreatine/creatine (C) between different age groups. * p < 0.05 versus control, *** p < 0.001 versus control. Values are mean ± SD. Sample sizes are indicated on respective bars.

We next analyzed the extent to which levels of adenine nucleotides and their energetic ratios were affected by aging. Levels of ATP (Figure 27A), ADP (Figure 27B), and AMP (Figure 27C) were unchanged across the various ages. We did however observe that the ratio of ATP/ADP increased significantly in old animals compared to 3 month (Figure 28A, p < 0.01) and 6 month (Figure 28A, p<0.05) old animals. Adenylate energy charge values (Figure 28B) were not affected by adenosine levels (Figure 29).
Figure 27: Levels of Adenine Nucleotides in Wildtype Mouse Cerebral Cortex as a Function of Age. No statistically significant differences were observed for ATP levels (A), ADP levels (B), and AMP levels (C) as a function of age. Values are mean ± SD. Sample sizes are indicated on respective bars.

Figure 28: Energetic Ratios in Wildtype Mouse Cerebral Cortex as a Function of Age. (A) The ratio of ATP/ADP was decreased significantly at 3 months (p < 0.01) and 6 months (p < 0.05) compared to old mice. (B) We did not observe any statistically significant changes for adenylate energy charge ((ATP + (1/4ADP)) / (ATP + ADP + AMP)) between any age group. * p < 0.05 versus control. ** p <
0.01 versus control. Values are mean ± SD. Sample sizes are indicated on respective bars.

Figure 29: Levels of Adenosine in Wildtype Mouse Cerebral Cortex as a Function of Age. Adenosine levels were not significantly different between any of the age groups. Values are mean ± SD. Sample sizes are indicated on respective bars.

We next examined each energy measurement from the trisomic Ts65Dn mice as a function of age. We hypothesized that energy levels would be increased in aged animals due to compensatory responses. People with DS have an elevated cerebral metabolic rate for glucose and this could possibly facilitate increased energy levels in aged humans and mice prior to or in parallel to dementia. Levels of phosphocreatine were elevated significantly in old Ts65Dn mice compared to three (Figure 30A, p < 0.001) and six month old animals (Figure 30A, p < 0.001) whereas levels of creatine were unchanged (Figure 30B). The ratio of phosphocreatine/creatine was increased significantly in 6 month old mice from the levels observed in both 3 month (Figure 30C, p < 0.05) and old mice (Figure 30C, p < 0.05).
Figure 30: Levels of Creatine and Phosphocreatine in Ts65Dn Mouse Cerebral Cortex as a Function of Age. (A) Compared to old Ts65Dn mice, the levels of phosphocreatine were decreased significantly at 3 (p < 0.001) and 6 (p < 0.001) months of age. (B) There were no statistically significant changes in creatine levels. (C) The ratio of phosphocreatine/creatine was significantly decreased in 3 month old (p < 0.05) and old mice (p < 0.05) compared against 6 month old mice. * p < 0.05 versus control, *** p < 0.001 versus control. Values are mean ± SD. Sample sizes are indicated on respective bars.

We next analyzed the extent to which levels of adenine nucleotides and their energetic ratios were affected by aging in Ts65Dn mice. Levels of ATP were elevated significantly in aged animals compared to 3 month (Figure 31A, p < 0.05) and 6 month old mice (Figure 31A, p < 0.01) whereas the levels of ADP were elevated significantly (p < 0.05) only in 3 month old mice compared to 6 month old mice (Figure 31B). We did not find any significant changes to the levels of AMP across the age groupings (Figure 31C). The ratio of ATP/ADP and...
adenylate energy charge values increased significantly in aged animals compared to 3 month (Figure 32A, p < 0.001; Figure 32B, p < 0.01) and 6 month (Figure 32A, p < 0.001; Figure 32B, p < 0.01) old animals. Adenosine levels did not exhibit age-related differences (Figure 33).

Figure 31: Levels of Adenine Nucleotides in Ts65Dn Mouse Cerebral Cortex as a Function of Age. (A) Compared to old Ts65Dn mice, levels of ATP were significantly lower in 3 month (p < 0.05) and 6 month (p < 0.01) old animals. (B) Levels of ADP are decreased significantly at 6 month compared to 3 month (p < 0.05) trisomic mice. (C) No statistically significant differences were observed for the levels of AMP in Ts65Dn mice. * p < 0.05 versus control, ** p < 0.01 versus control. Values are mean ± SD. Sample sizes are indicated on respective bars.
In our studies we examined the effects of genetically modifying a candidate gene of the purine biosynthetic pathway, GART, on brain energy levels. GART modifications occur in the enzymatic steps preceding production of SAICAR whereas ADSL mutations are performed in the steps proceeding SAICAR (Figure 6). Phosphocreatine and creatine levels were not significantly
different in cerebral cortex (Figure 34A & B, respectively) and cerebellum (Figure 35A & B, respectively). Phosphocreatine/creatine ratios were not significantly different in P8E5_P7293 cerebral cortex (Figure 34C) or cerebellum (Figure 35C), however, were significantly ($p < 0.001$) reduced ratio in cerebellum of P8E5_P7139 mice (Figure 35C).

Figure 34: Effects of GART Genetic Modification on Levels of Creatine and Phosphocreatine in Mouse Cerebral Cortex. No statistically significant changes were observed for levels of phosphocreatine (A), creatine, (B), or phosphocreatine/creatine ratios. Values are mean ± SD. Sample sizes are indicated on respective bars.
Figure 35: Effects of GART Genetic Modification on Levels of Creatine and Phosphocreatine in Mouse Cerebellum. No statistically significant changes were observed for levels of phosphocreatine (A), creatine (B), or phosphocreatine/creatine ratios. Values are mean ± SD. Sample sizes are indicated on respective bars.

The GART gene codes for 3 enzymes, phosphoribosylglycineamide synthase (GARS), phosphoribosylglycineamide transformylase (GART), AIR synthase (a trifunctional protein; Spiegel et al., 2006). Because all of these enzymes catalyze reactions upstream from SAICAR in the purine de novo biosynthetic pathway we hypothesized that altering the GART gene would lead to decreased levels of adenine nucleotides in cortex and cerebellum. The levels of ATP were unchanged in cerebral cortex of genetically modified GART mice.
(Figure 36A) however were increased significantly (p < 0.05) in cerebellum of animals born to founder mouse P7293 (Figure 37A). This finding was unexpected because presumably these transgenic animals should have elevated SAICAR levels and therefore a lesser ability to generate and/or maintain ATP levels. Upon examination of ADP levels from these animals, we noted that these levels were elevated significantly (p < 0.05) as a result of the transgene in cortex from P7139 litters (Figure 36B). There was no change in levels of ADP in cerebellum for either group (Figure 37B, p > 0.05). Levels of AMP were unchanged in cerebral cortex from GART mice (Figure 36C), however were significantly (p < 0.05) reduced from animals born from founder P7293 (Figure 37C). These elevations in ADP and ATP, like in ADSL, may be due to this GART modification having more profound effects developmentally than it does in adult animals with this increase in ATP resulting as a compensatory mechanism to previously diminished nucleotides.
Figure 36: Effects of GART Genetic Modification on Levels of Adenine Nucleotides in Mouse Cerebral Cortex. (A) No statistically significant changes were observed for levels of ATP compared to wildtype founder mice. (B) Levels of ADP were increased significantly (p < 0.05) for P8E5_P7139 transgenic mice compared to its wildtype founder control. (C) Levels of AMP were not altered significantly for any transgenic group compared to its wildtype founder. * p < 0.05 versus control. Values are mean ± SD. Sample sizes are indicated on respective bars.
Figure 37: Effects of GART Genetic Modification on Levels of Adenine Nucleotides in Mouse Cerebellum. (A) Levels of ATP in P8E5_P7293 were increased significantly (p < 0.05) compared to its wildtype control. (B) Levels of ADP were not altered significantly for either transgenic group compared to its respective wildtype control. (C) Levels of AMP were decreased significantly (p < 0.05) in P8E5_P7293 transgenic mice compared to its wildtype control. * p < 0.05 versus control. Values are mean ± SD. Sample sizes are indicated on respective bars.

We calculated ATP/ADP ratios and adenylate energy charge values for GART mice and found that adenylate energy charge was elevated significantly (p < 0.05) in cerebral cortex from wildtype P8E5_P7293 mice (Figure 38B) and in cerebellum ATP/ADP ratios (Figure 39A, p < 0.01), adenylate energy charge (Figure 39B, p < 0.001).
Figure 38: Effects of GART Genetic Modification on Energetic Ratios in Mouse Cerebral Cortex. (A) Compared to wildtype founder mice, ATP/ADP ratios were not significantly changed for either of the transgenic groups. (B) Adenylate energy charge values were increased significantly (p < 0.05) for P8E5_P7293 transgenic mice compared to its wildtype founder mice. * p < 0.05 versus control. Values are mean ± SD. Sample sizes are indicated on respective bars.

Figure 39: Effects of GART Genetic Modification on Energetic Ratios in Mouse Cerebellum. Compared to wildtype founder mice, ATP/ADP ratios (A) and adenylate energy charge values (B) were increased significantly for P8E5_P7293 transgenic mice compared to its wildtype control (p < 0.01 & p < 0.001, respectively). No statistically significant changes were observed for P8E5_P7139 transgenic versus control animals. * p < 0.05 versus control. Values are mean ± SD. Sample sizes are indicated on respective bars.

In P8E5_P7293 transgenic animals we noted significantly lower levels of adenosine compared to wildtype controls in cerebral cortex (Figure 40, p < 0.001)
and cerebellum (Figure 41, p < 0.05). This effect was not observed in P8E5_P7139 mice.

Figure 40: Effects of GART Genetic Modification on Adenosine Levels in Mouse Cerebral Cortex. (A) Compared to the wildtype founder mice, levels of adenosine were decreased significantly (p < 0.001) for P8E5_P7293 compared to its wildtype founder control. *** p < 0.001 versus control. Values are mean ± SD. Sample sizes are indicated on respective bars.

Figure 41: Effects of GART Genetic Modification on Adenosine Levels in Mouse Cerebellum. (A) Compared to the wildtype founder mice, levels of adenosine were decreased significantly (p < 0.05) for P8E5_P7293 compared to its wildtype founder control. * p < 0.05 versus control. Values are mean ± SD. Sample sizes are indicated on respective bars.
Treatment of Rat Cerebral Cortical Neurons with HIV-1 Protein Tat as a Model for HIV-1 Dementia

The HIV-1 protein Tat, but not mutant-Tat missing amino acids 31-61, causes neuronal cell death. To control for possible non-specific effects of Tat, parallel experiments were performed throughout these studies with equivalent concentrations of Tat$_{1-72}$ and mutant-Tat. Primary cultures of mouse cerebral cortical neurons treated for 24 h with concentrations of Tat$_{1-72}$ and mutant-Tat ranging from 10 pM to 400 nM (results for effects of 10 to 400 nM were illustrated in Fig. 42A) showed statistically significant decreases in the percentage of viable neurons with 100 nM (84.0% ± 12.1; p < 0.05) and 400 nM Tat$_{1-72}$ (60.4% ± 8.6; p < 0.001) but not mutant-Tat as determined by trypan blue exclusion (Figure 42B) and MTT assay (data not shown). Because of findings that Tat affects mitochondrial function and MTT assays reflect cell viability and mitochondrial function all subsequent experiments for determination of cell viability used trypan blue exclusion. Time profile studies for Tat-induced neuronal death established that Tat$_{1-72}$ at 100 nM increased significantly (p < 0.01) levels of neuronal cell death to comparable levels at incubations intervals of 24 and 48 h (Figure 42B). Accordingly, all subsequent studies on cell viability used Tat at a concentration of 100 nM and incubations of 24 h.
Figure 42: Tat_{1.72} Concentration- and Time-Dependently Decreases Cortical Neuron Viability. (A) Treatment of neurons with Tat_{1.72}, but not mutant-Tat_{1.72}, at concentrations of 100 and 400 nM significantly reduced (p < 0.05 and p < 0.001, respectively) neuronal viability as determined with trypan blue exclusion (n=12). (B) Tat at a concentration of 100 nM produced time dependent increases in neuronal cell death as determined by trypan blue exclusion assay; statistically significant (p < 0.01) increases were observed with 24 h and 48 h incubations. (n = 5 for control, n = 6 for treatment groups) Results are expressed as the number of non-viable cells relative to total cell number. * p < 0.05, ** p < 0.01, *** p < 0.001 versus untreated controls

Creatine Protection Against Tat_{1.72}-Induced Neuronal Cell Death

To test for creatine protection against Tat_{1.72}-induced neuronal cell death, neurons were treated with Tat_{1.72} (100 nM) for 24 h in the absence or presence of creatine at concentrations of 1, 3, 5, and 20 mM. Creatine, at a concentration of 3 mM, significantly (p < 0.05) reduced levels of Tat-induced neurotoxicity from 12.6 ± 4.5 % to 6.5 ± 2.2 % (Figure 43).
Figure 43: Creatine Concentration-Dependently Protects Against Tat-Induced Neuronal Cell Death. Statistically significant (p < 0.01) increases in neuronal cell death were observed with treatments of 100 nM Tat at 24 h as determined by trypan blue exclusion assay. Statistically significant (p < 0.05) protection against Tat-induced neuronal cell death was observed with 3 mM creatine (n = 4). Results are expressed as the number of non-viable cells relative to total cell number. ** p ≤ 0.01 Tat versus control.

Effects of Tat and Creatine on Levels of Adenine Nucleotides and Phosphocreatine

To determine underlying mechanism(s) responsible for the neurotoxic actions of Tat_{1-72} and the neuroprotective effects of creatine we measured, at a time period (4 h) that precedes the neurotoxic effects, levels of adenine nucleotides, creatine and phosphocreatine. Creatine (3 mM) treatment of neurons for 4 h significantly (p < 0.01) increased levels of ATP from control levels of 10.3 ± 0.3 to 13.6 ± 0.6 nmol/mg protein (Fig. 44A). After treatment with Tat_{1-72} (100 nM, 4h), ATP levels were significantly (p < 0.05) decreased to 7.3 ± 0.6 nmol/mg protein. Co-application of 3 mM creatine with 100 nM Tat_{1-72} for 4 h protected against Tat-induced decreases in ATP levels and returned levels to near control values of 10.0 ± 0.8 nmol/mg protein. Similar, but statistically non-
significant, changes to levels of ADP (Figure 44B) and AMP (Figure 44C) were observed for the same treatment groups. When the data were calculated to determine adenylate energy charges (Figure 45B) or ATP/ADP ratios (Figure 45A), no statistically significant differences were observed between controls and treated cells.

Figure 44: Effects of Tat, Mutant-Tat and Creatine on Levels of Adenine Nucleotides. (A) Compared with controls, creatine (Cr, 3 mM) increased significantly (p < 0.01), and Tat (100 nM) but not mutant-Tat (mTat, 100 nM) decreased significantly (p < 0.05) levels of ATP in cortical neurons. Creatine (3 mM) co-applied with Tat (100 nM) significantly (p < 0.05) protected against Tat-induced decreases in levels of ATP. (B, C) Similar, but statistically non-significant, changes to ADP (B) and AMP (C) were observed for treatments with Tat, mutant-Tat and creatine. (D, E) No statistically significant differences were observed for treatments with Tat, mutant-Tat and creatine on (D) adenylate energy charge or (E) levels of ATP/ADP (n = 5 for control, creatine, Tat, and...
creatine with Tat experiments, n=4 for mTat). * p ≤ 0.05, ** p ≤ 0.01 versus control.

Figure 45: Effects of Tat, Mutant-Tat and Creatine on Energetic Ratios. No statistically significant differences were observed for treatments with Tat, mutant-Tat and creatine on (A) adenylate energy charge or (B) levels of ATP/ADP (n=5 for control, creatine, Tat, and creatine with Tat experiments, n=4 for mTat). * p ≤ 0.05, ** p ≤ 0.01 versus control.

We next determined the effects of Tat and/or creatine on cellular levels of creatine and its associated source of tissue energetics, phosphocreatine. Incubation of cells with 3 mM creatine for 4 h resulted in statistically significant (p < 0.01) increases in cellular levels of creatine from control levels of 62.3 ± 3.2 nmol/mg protein to 86.2 ± 5.0 nmol/mg protein (Figure 46A). Tissue levels of creatine were not significantly changed when cells were treated with 100 nM Tat (64.9 ± 3.4 nmol/mg protein) or with 100 nM mutant-Tat (60.0 ± 4.8 nmol/mg protein). Tat co-applied with creatine resulted in statistically significant (p < 0.001) increases in levels of creatine to 98.0 ± 4.4 nmol/mg protein (Figure 46A). Levels of phosphocreatine were not affected significantly by any of the treatments (Figure 47B). The ratio of phosphocreatine to creatine (Figure 46C) of 1.2 ± 0.1 in controls was decreased significantly to 1.0 ± 0.04 by Tat (p < 0.05),
to $0.8 \pm 0.02$ by creatine ($p < 0.01$) and to $0.7 \pm 0.07$ by creatine plus Tat ($p < 0.001$). Co-application of creatine and Tat decreased significantly ($p < 0.001$) the ratio of phosphocreatine to creatine to a level significantly ($p < 0.05$) less than that observed with Tat alone.

![Bar charts](image)

Figure 46: Effects of Tat, Mutant-Tat and Creatine on Levels of Creatine and Phosphocreatine in Primary Rat Cortical Neurons. (A) Treatment of neurons with creatine, Tat, creatine plus Tat, and mTat did not significantly alter levels of phosphocreatine (B) Treatment of neurons with creatine (3 mM) increased significantly levels of creatine whether applied alone ($p < 0.01$) or concomitantly with Tat ($p < 0.001$). (C) Ratios of phosphocreatine to creatine were decreased significantly following treatment with either creatine ($p < 0.01$) or Tat ($p < 0.05$). * $p < 0.05$ versus control. Values are mean ± SD. Sample sizes are indicated on respective bars.
Creatine Protects Against Tat-Induced Decreases in Mitochondrial Membrane Potential

Because HIV-1 Tat has been shown to affect mitochondrial function (Kruman et al., 1998; Langford et al., 2004; Perry et al., 2005) and creatine has been shown to have neuroprotective actions through mitochondrial mechanisms, we next examined mitochondrial mechanisms related to redox catastrophe. Tat (100 nM, 4h) decreased significantly (p < 0.001) the ratio of JC-1 aggregate to monomer by about 36% (Figure 47). This Tat-induced hypopolarization of mitochondria was blocked completely (p < 0.001) with co-application of creatine (Figure 47). Creatine and mutant-Tat alone had no significant effects on mitochondrial membrane potential.

Figure 47: Effects of Tat, Mutant-Tat and Creatine on Mitochondrial Membrane Potential as Determined with the Fluorescent Dye JC-1. Tat (100 nM), but not mutant-Tat (mTat, 100 nM), decreased significantly (p < 0.001) mitochondrial membrane potential. Creatine (Cr, 3 mM) did not affect mitochondrial membrane potential, but creatine (3 mM) co-applied with Tat (100 nM) significantly (p < 0.001) blocked Tat-induced mitochondrial hypopolarization (n = 4). *** p ≤ 0.001 versus control.
Creatine Protects Against Tat-Induced Increases in the Opening of Mitochondrial Permeability Transition Pores

Treatment of cultured neurons with 100 nM Tat caused a significant (p < 0.01) increase in the number of cells with open mitochondrial permeability transition pores (Figure 48). Co-application of 3 mM creatine with Tat resulted in a statistically significant (p < 0.05) decrease in the number of cells with mitochondrial permeability transition pore opening compared to Tat treatment alone (Figure 48). Mutant-Tat and creatine alone had no significant effects on mitochondrial permeability transition pore opening.

Figure 48: Effects of Tat, Mutant-Tat and Creatine on Opening of Mitochondrial Permeability Transition Pores as Determined with Calcein-AM Fluorescence and Cobalt Chloride Quenching. Tat (100 nM), but not mutant Tat (mTat, 100 nM), increased significantly (p < 0.01) the percentage of cells with open mitochondrial permeability transition pores. Creatine (Cr, 3 mM) did not affect pore opening but creatine (3 mM) when co-applied with Tat (100 nM) significantly (p < 0.05) blocked Tat-induced mitochondrial permeability transition pore opening (n = 5 for control, n = 4 for all treatment groups). ** p < 0.01 versus control

Effects of Tat and Creatine on Levels of Reactive Oxygen Species

Tat (100 nM) increased significantly (p < 0.01) reactive oxygen species to a level similar to that observed with the positive control used, hydrogen peroxide.
Creatine (3 mM) did not significantly reduce Tat-induced increases in levels of reactive oxygen species. Mutant-Tat (100 nM) produced statistically significant (p < 0.05) increases in levels of reactive oxygen species similar to those of cells treated with Tat1-72.

Figure 49: Effects of Tat, Mutant-Tat and Creatine on Formation of Reactive Oxygen Species as Determined with the Fluorescent Dye H$_2$DCFDA. Tat (100 nM), creatine (3 mM) plus Tat (100 nM), mutant-Tat (100 nM) and the positive control hydrogen peroxide (H$_2$O$_2$, 500 μM) significantly increased reactive oxygen species. Creatine (3 mM) did not block Tat-induced increases in levels of reactive oxygen species (n=8 for all groups except creatine where n=9). * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001 versus control.

Ketogenic Diet as a Treatment for Epilepsy

*KD Treatment Induces Rapid Ketonemia but Delayed Seizure Protection*

When effective, KD produces sustained ketonemia and increased seizure resistance to a variety of proconvulsant challenges (Appleton & DeVivo, 1974, Bough & Eagles, 1999, Rho et al., 1999). To determine how long the diet must be administered to achieve an anticonvulsant effect in adolescent rats, we monitored seizure threshold repeatedly in the same cohort of rats over nine weeks (n=16 KD and 11 control rats). Despite a precipitous increase in plasma...
BHB levels and a moderate hypoglycemia that developed within one day of diet initiation, the pentylentetrazole (PTZ) seizure threshold was not consistently elevated above controls until at least 13 days of diet treatment (Figure 50A-D). To determine if the observed decline in seizure threshold in the control group was due to kindling by repeated administration of PTZ (Han et al., 2000), separate groups of seizure-naive rats were fed the KD or control diets for three weeks and then tested once for seizure threshold against either PTZ (n=25) or flurothyl (n=20). In both tests, seizure threshold was elevated compared to controls after the same duration (20-21 days) of KD treatment (Figure 50E,F). These findings suggest that the decline in PTZ seizure threshold over three weeks in control rats was not due to a chemical kindling effect, but instead may reflect a developmental change. We conclude that chronic but not acute ketosis is associated with the anticonvulsant action of the ketogenic diet.
Figure 50: The Anticonvulsant Effect of the KD Develops Slowly. (A) Pentylenetetrazole (PTZ) seizure threshold, (B) plasma levels of β-hydroxybutyrate (BHB), (C) body weight, and (D) plasma levels of glucose were measured repeatedly for 65 days before, during, and after administration of a KD to rats. The bar (D) represents the time during which a KD was administered to the KD group (solid circles). Control (CON) animals were maintained on normal chow, ad libitum throughout the experiment (open squares). On day 31, KD-fed animals were reverted to a control diet (gray circles). Points represent the mean ± standard error of the mean (SEM) of each diet group: KD-fed (n = 16) and control animals (n = 11). * p < 0.05, analysis of variance. Separate cohorts of seizure-naive animals exhibited similar elevations in seizure threshold as evaluated by either PTZ (E; n = 25) or flurothyl (F; n = 20) after 20 to 21 days of diet treatment. Bars represent group mean ± SEM. * p < 0.05, unpaired t test.
Metabolic Genes are Coordinately Upregulated after KD

The updated CDF file (Dai et al., 2005) was used to assign probe pairs to accession numbers for the Affymetrix rat 230A GeneChip. The updated assignments resulted in a reduction of the number of transcripts available on this chip from 15,866 (reported by Affymetrix) to 10,179. Normalization and filtering of the signal intensities resulted in 5518 genes that were reliably expressed (i.e., "present") in either or both treatment groups. Statistical analysis at a 1% false discovery rate yielded 658 differentially-expressed genes in the hippocampus after KD, seven of which were expected to be false positives. Only 60% of these differentially expressed genes were identified using the original CDF file available through Affymetrix. A total of 384 transcripts were induced (297 with known functions), whereas 274 were repressed (199 identified genes) after KD (Fig 51A). A list of all differentially expressed transcripts is available in Table 3 of the Appendix. Differentially expressed genes occurred for transcripts exhibiting a 100-fold range of control expression levels (Figure 51A). Low variability was generally observed across arrays (Figs 51A-B) and resulted in the ability to identify transcripts with as little as a ±16% repression or induction. The mean coefficient of variation (CV) of the expression level was 9%.

Batch searches of the differentially-regulated genes produced a Gene Ontology (GO) biological process for about half of the named genes. Many of the remaining genes could be assigned to functional categories after searches of EntrezGene, Genecards, the Rat Genome Database or PubMed. In all, 462 genes could be assigned to one of eight functional categories (Figure 51C). More
than half of these genes were associated with one of three categories: metabolism (104 genes), signal transduction (101 genes), and transcription (77 genes).

Transcripts in one functional category (metabolism) were coordinately up-regulated after KD, whereas those in the synaptic transmission category were down-regulated (* p < 0.02 in Fig 51C, Fisher's exact tests). Within the metabolism category, three subcategories of transcripts involved in energy (34 genes), lipid (17 genes), and protein (34 genes encoding proteolytic enzymes or proteasomes) metabolism predominated; 19 others were not members of these three sub-categories. Energy metabolism genes accounted for the coordinate induction of this transcript category after KD (p < 0.0001, Fisher's exact test with Bonferroni correction, Fig 52A). All 34 of the transcripts in this category were upregulated. Hierarchical clustering of expression values for differentially-expressed energy metabolism genes showed that both transcripts associated with glycolysis, all six in the tricarboxylic acid (TCA) cycle pathway, and all 21 encoding oxidative phosphorylation enzymes, were up-regulated after KD (Figure 52B). The oxidative phosphorylation transcripts encoded protein subunits of complex I (five subunits of NADH dehydrogenase), complex II (subunits A and D of succinate dehydrogenase), complex IV (cytochrome C oxidase subunit Vial), and five subunits of the F_0-F_1 ATP synthase complex. Transcripts for creatine kinase, glycogen phosphorylase, glucose-6-phosphate dehydrogenase, and acetyl-coA synthase 2 were also upregulated.
If the modest but concerted upregulation of transcripts encoding metabolic proteins were focused in a subpopulation of principal cells rather than distributed throughout the hippocampus, differential expression of these transcripts should be much larger in cells harvested by laser capture microscopy and assayed by quantitative RT-PCR. Based on estimates of the relative amounts of RNA in each neuron population compared with whole hippocampus, and assuming all of the change was concentrated within one cell population, one would predict a log$_2$ ratio for most of the differentially expressed transcripts $>$3, well within the limit of detection for quantitative RT-PCR. Log$_2$ ratios of $>$0.8 can be consistently detected in qRT-PCR (Greene et al., 2005). If on the other hand the modest upregulations observed were distributed evenly over various cell types in hippocampus, qRT-PCR should show no differences. This hypothesis was tested for dentate granule cells and CA1 pyramidal cells by assaying the levels of eight energy metabolism transcripts by qRT-PCR. In this experiment there was no measurable difference in energy transcript levels for neurons isolated from KD or control-fed rats. These findings suggest that the KD caused a widespread, coordinate up-regulation of energy metabolism transcripts across hippocampal cell types.

A coordinated upregulation of 19 proteasome-related transcripts occurred in hippocampus after KD, compared with only four downregulated transcripts. A previous report (Sullivan et al., 2004) showed that proteasome inhibition was associated with reduction in the activity of complex I and II components of oxidative phosphorylation in hippocampal mitochondria, thus
upregulation of proteasome transcripts is consistent with enhanced oxidative phosphorylation.

A total of 39 genes directly associated with ion channels or synaptic transmission changed after KD. Downregulated transcripts (n=23) predominated (Figure 51C, p < 0.01), which included two voltage-dependent calcium channel subunits (γ4 and α1D), the ClCN1 chloride channel, the KCNH3 and KCNE1-like potassium channels, P2X3 and P2X7 purinergic receptors, and synaptotagmins 6 and XI. Upregulated transcripts (n=16) include the glutamate receptor subunits GluR2 and KA1, the KCNN2 potassium channel, the SCN1a type Iα sodium channel subunit, and the SLC1A1 glutamate transporter (EAAC1). No genes encoding GABA_β receptors, metabotropic glutamate receptors, or subunits of NMDA receptors were induced or repressed after KD. Elucidation of the functional consequences of these changes requires further study.
Figure 51: Changes in Gene Expression after KD. (A) Genes induced (gray circles) or repressed (white circles) after KD (1% false discovery rate; n = 658; see Materials and Methods). Each symbol depicts one transcript. Variability (dashed line) is represented by the mean coefficient of variation (CV) determined as in B. (B) The relation between CV and control expression level was calculated from the best fit of the plot of the mean CV versus mean expression for all differentially expressed probe sets (n = 6 arrays). (C) The number of genes induced or repressed in each functional category after KD. Functional categories were assigned to 462 differentially expressed known genes. Each bar represents the total number of genes per category. Two categories were significantly upregulated or downregulated after KD (* p < 0.02, Fisher's exact test). No differences in activities of selected energy metabolism enzymes.

In view of the microarray results, we determined whether a coordinate upregulation in energy transcripts in the hippocampus was paralleled by an increase in metabolic enzyme activities after KD. We found that activities of glycolytic enzymes (hexokinase, glucose-6-phosphate dehydrogenase, and lactate dehydrogenase) and enzymes involved in the TCA cycle (citrate synthase, α-ketoglutarate dehydrogenase, and malate dehydrogenase) were not significantly altered after KD (Table 1). Except for malate dehydrogenase (Figure 52B), transcripts for the other enzymes were unchanged after KD or below
detection level. These data suggest that the modest increases in transcript levels observed (<35%, Figure 52B) were insufficient to cause significant increases in enzyme activities measured *in vitro*. At $\alpha = 0.05$, the power to detect a 25% change in enzyme activity was $\approx 91\%$ for all of these enzyme assays. Small, individually unremarkable increases in the activity of several sequential enzymes in a metabolic pathway can, however, cause a substantial increase in total flux through the pathway. Indeed, the increased glutamate level (Table 1) probably reflects a higher flux through the TCA cycle. The integration of glutamate, glutamine and alanine levels, all of which are energy metabolites, also shows a larger combined level in hippocampus of KD ($17.7 \pm 0.4 \text{ nmoles/mg wet weight}$) rats than controls ($16.3 \pm 0.2$; $p = 0.02$).
Figure 52: Metabolic Genes Changed after KD. (A) The 104 metabolism-related genes in Figure 52C could be further subcategorized into energy metabolism, lipid metabolism, protein (proteolysis and proteasome) metabolism, and a miscellaneous subgroup. Bars indicate the total number of genes per subgroup. Two categories showed coordinate regulation of the genes in the category (* p < 0.0001 for energy metabolism, and p < 0.02 for lipid metabolism, Fisher’s exact test with Bonferroni correction). (B) Relative expression levels of 24 energy metabolism genes that were upregulated by KD. Each row corresponds to one gene. Genes associated with glycolysis, the tricarboxylic acid (TCA) cycle, or oxidative phosphorylation are grouped.

**Increased Number of Mitochondrial Profiles in the Hippocampus after KD**

Ketosis and fasting result in loss of oxidative phosphorylation and mitochondrial function and numbers in muscle (Lecker et al., 2004, Yechoor et al., 2002). We next determined whether the concerted increase in expression of energy metabolism genes in hippocampus (Figure 52) was accompanied by...
mitochondrial biogenesis. By visually scoring electron micrographs taken from the dentate/hilar region of hippocampus, we found significantly more mitochondrial profiles in animals fed a KD compared to controls (Figure 53). Data were remarkably consistent across animals (CV=3% for both KD and CON), indicating a 46% increase in mitochondrial profiles for KD-fed animals versus controls (Figure 53B left panel). In the same micrographs there was no difference in the number of dendritic profiles (Figure 53B right panel). The large majority of mitochondria counted in both control and KD tissue appeared to be located in neuronal processes (dendrites or axon terminals). The finding of increased numbers of mitochondrial profiles was reinforced by our observation that 39 of 42 differentially regulated transcripts encoding mitochondrial proteins were upregulated after KD (Figure 53C). In addition to 23 energy metabolism genes, up-regulated mitochondrial transcripts included two mitochondrial ribosome subunits (L20 and L53), the ANT3 and ANT4 nucleotide transporters, ornithine aminotransferase, acyl-coA synthase 3 and hydroxyacyl-coA dehydrogenase.
Figure 53: Mitochondrial Biogenesis in Hippocampus of Rats Fed a KD. (A) Representative electron micrographs taken from control- (CON) or KD-fed animals (original magnification, X 20,000; calibration bar = 0.5 µm). Arrow indicates a mitochondrial profile. The number of mitochondrial profiles in each micrograph was counted. (B) There were significantly more mitochondrial profiles in the dentate gyrus of KD-fed animals than in control animals. Mitochondrial counts were taken from 4 KD-fed animals representing a total of 2,680 µm² and 5 control rats representing 3,080 µm². Data are presented as mean of averaged counts ± standard error of the mean per 100 µm² (n = 4 or 5). * p < 0.001, t test. By comparison, there was no difference in the density of dendritic profiles in the same micrographs, which guards against differential shrinkage of the tissue in KD versus control brains. (C) A total of 39 transcripts, assayed by microarray, encoding mitochondrial proteins were upregulated after KD (open bar), whereas only one transcript was downregulated (gray bar). ** p < 0.0001, Fisher’s exact test.

Energy Reserves are Increased after KD

We next determined whether the coordinate induction of energy metabolism genes and mitochondrial biogenesis led to increased production of energy metabolites in hippocampus after KD. The β-hydroxybutyrate level was significantly (p < 0.001) elevated. Glycogen was significantly (p = 0.01) decreased after KD as expected from the moderate hypoglycemia, whereas glutamate and glutamine were elevated (Table 1).
Table 1: Levels of Energy Metabolites following Ketogenic Diet.

<table>
<thead>
<tr>
<th>Enzyme or Metabolite</th>
<th>CON (mean ± SD)</th>
<th>n</th>
<th>KD (mean ± SD)</th>
<th>n</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Enzymes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hexokinase</td>
<td>7.20 ± 1.46</td>
<td>10</td>
<td>7.19 ± 0.71</td>
<td>10</td>
<td>0.98</td>
</tr>
<tr>
<td>Glucose 6-phosphate dehydro-</td>
<td>1.13 ± 0.09</td>
<td>9</td>
<td>1.21 ± 0.21</td>
<td>9</td>
<td>0.296</td>
</tr>
<tr>
<td>nase</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>26.0 ± 1.32</td>
<td>6</td>
<td>23.9 ± 2.31</td>
<td>6</td>
<td>0.087</td>
</tr>
<tr>
<td>β-Hydroxybutyrate dehydrogen-</td>
<td>1.22 ± 0.09</td>
<td>10</td>
<td>1.28 ± 0.16</td>
<td>10</td>
<td>0.32</td>
</tr>
<tr>
<td>ase</td>
<td>24.3 ± 2.33</td>
<td>10</td>
<td>26.2 ± 4.89</td>
<td>10</td>
<td>0.28</td>
</tr>
<tr>
<td>Citrate synthase</td>
<td>1.54 ± 0.17</td>
<td>10</td>
<td>1.54 ± 0.24</td>
<td>10</td>
<td>0.97</td>
</tr>
<tr>
<td>α-Ketoglutarate dehydrogen-</td>
<td>98.6 ± 10.3</td>
<td>10</td>
<td>90.8 ± 14.2</td>
<td>10</td>
<td>0.17</td>
</tr>
<tr>
<td>ase</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamate</td>
<td>11.5 ± 0.43</td>
<td>9</td>
<td>12.2 ± 0.48</td>
<td>9</td>
<td>0.03</td>
</tr>
<tr>
<td>Glutamine</td>
<td>4.04 ± 0.24</td>
<td>9</td>
<td>4.53 ± 0.41</td>
<td>9</td>
<td>0.01</td>
</tr>
<tr>
<td>Aspartate</td>
<td>1.98 ± 0.17</td>
<td>9</td>
<td>1.99 ± 0.18</td>
<td>9</td>
<td>0.84</td>
</tr>
<tr>
<td>Glycine</td>
<td>2.83 ± 0.15</td>
<td>9</td>
<td>3.11 ± 0.32</td>
<td>9</td>
<td>0.03</td>
</tr>
<tr>
<td>Taurine</td>
<td>5.70 ± 0.28</td>
<td>9</td>
<td>4.63 ± 0.37</td>
<td>9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.74 ± 0.03</td>
<td>9</td>
<td>0.81 ± 0.07</td>
<td>9</td>
<td>0.01</td>
</tr>
<tr>
<td>Citrate</td>
<td>0.0923 ± 0.011</td>
<td>9</td>
<td>0.0856 ± 0.0127</td>
<td>10</td>
<td>0.24</td>
</tr>
<tr>
<td>Glucose</td>
<td>3.68 ± 0.22</td>
<td>8</td>
<td>2.88 ± 0.19</td>
<td>9</td>
<td>0.01</td>
</tr>
<tr>
<td>Protein/wet weight&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.119 ± 0.004</td>
<td>10</td>
<td>0.119 ± 0.007</td>
<td>10</td>
<td>0.88</td>
</tr>
<tr>
<td>PCR:Cr ratio</td>
<td>1.32 ± 0.038</td>
<td>7</td>
<td>1.48 ± 0.045</td>
<td>9</td>
<td>0.017</td>
</tr>
</tbody>
</table>

Glycogen measurements were performed on rats killed by head-focused, high-energy microwave irradiation to preserve in vivo nucleotide levels. The other analyses were from fresh-frozen tissue. Data were expressed as nmol/mg wet weight (metabolites) or nmol/mg wet weight/min (enzymes). An unpaired Student's t test was used to compare statistical differences of the mean between groups. <sup>a</sup>Mitochondrial cytosolic. <sup>b</sup>Milligram protein/mg hippocampal fresh tissue, reported as a measure of potential protein catabolism or of hydration changes that might have affected analyses. CON = control; SD = standard deviation.

There was a significantly larger phosphocreatine to creatine ratio (Figure 55C, p < 0.05, t-test) in rat hippocampus following three weeks on KD. ATP, ADP and AMP levels were not significantly increased in hippocampus after KD, although ADP level showed a 2-fold trend towards an increase. Other discrete rat brain regions were also assayed for adenine nucleotides, phosphocreatine and creatine and these findings support our published hippocampal data (Bough et al., 2006). There was a significantly (p < 0.01) elevated phosphocreatine to creatine ratio in hypothalamus (Figure 54C). Although levels of ATP were not changed in hippocampus, we observed levels elevated significantly in cerebral...
cortex (p < 0.05), hypothalamus (p < 0.001), and striatum (Figure 55A, p < 0.05). Levels of ADP and AMP were unaltered (Figure 55B & C). Dependent on levels of adenine nucleotides in a particular brain region in relation to each other, alternative measurements of brain energetics can be obtained by comparing either ATP/ADP ratios or the adenylate energy charge values. We observed a significantly (p < 0.001) elevated ratio of ATP to ADP in brain from rats fed a KD in hypothalamus and striatum (Figure 56A). Significantly increased adenine nucleotide energy ratios were demonstrated after KD administration in most regions examined; cerebral cortex (p < 0.01), hypothalamus (p < 0.01), striatum (p < 0.05), and cerebellum (Figure 56B, p < 0.05). Levels of adenosine were quantified for brain regions, excluding hippocampus, and no significant changes were observed although there was a trend toward increased adenosine levels (Figure 57). Because most of the tissue glutamate in the brain is used as an energy source rather than neurotransmitter (Walaas & Fonnum, 1980), our results are consistent with the notion that energy reserves are elevated in rat brain after KD. The magnitude and direction of change of metabolites measured here in hippocampal tissue after the KD are similar to those reported by DeVivo et al. (1978) for whole brain.
Figure 54: Effects of KD on Levels of Creatine and Phosphocreatine in Discrete Rat Brain Regions. (A) Compared with controls, levels of phosphocreatine were not statistically different in any of the tested brain regions following administration of an 8% KD for 3 weeks. (B) We did not observe any statistically significant differences in levels of creatine. (C) Statistically significant increases were observed for the ratio of phosphocreatine/creatine in hippocampus and hypothalamus compared to control rats. * p < 0.05 versus control. Values are mean ± SD. Sample sizes are indicated on respective bars.
Figure 55: Effects of KD Administration on Levels of Adenine Nucleotides in Discrete Rat Brain Regions. (A) Levels of ATP increased significantly in cerebral cortex (p < 0.05), hypothalamus (p < 0.001), and striatum (p < 0.05) after administration of an 8% KD for 3 weeks. (B) We observed statistically non-significant elevations in levels of ADP in cerebral cortex, striatum, and cerebellum compared to control rats. (C) No statistically significant changes were observed for AMP levels in all tested brain regions compared to controls. * p < 0.05 versus control, ** p < 0.01 versus control, *** p < 0.001 versus control. Values are mean ± SD. Sample sizes are indicated on respective bars.
Figure 56: Effects of KD Administration on Energetic Ratios in Discrete Rat Brain Regions. (A) We observed statistically significant increases for the ratio of ATP/ADP after administration of an 8% KD for 3 weeks in hypothalamus and striatum compared to control rats (p < 0.001). (B) Statistically significant increases for the adenylate energy charge were all demonstrated in cerebral cortex and hypothalamus (p < 0.01), striatum and cerebellum (p < 0.05). * p < 0.05 versus control, ** p < 0.01, *** p < 0.001. Values are mean ± SD. Sample sizes are indicated on respective bars.

Figure 57: Effects of KD Administration on Levels of Adenosine in Discrete Rat Brain Regions. Compared with controls, we observed statistically non-significant increases (p < 0.05) for levels of adenosine after administration of an 8% KD for 3 weeks in cerebral cortex, striatum, and cerebellum. Values are mean ± SD. Sample sizes are indicated on respective bars.
To verify our initial findings that brain energy levels in rats fed a KD were significantly elevated we repeated our experiments. Levels of phosphocreatine were elevated significantly in hippocampus (p < 0.05), cerebral cortex (p < 0.05), and striatum (Figure 58A, p < 0.05) following administration of KD. In KD-fed rats levels of creatine were increased significantly (p < 0.05) in cerebral cortex (Figure 58B). No changes to the ratio of phosphocreatine to creatine were noted (Figure 58C). ATP levels were elevated significantly in hippocampus (p < 0.05) and cerebral cortex (Figure 60A, p < 0.01). Cortical levels of ADP were increased (Figure 60B, p < 0.05) whereas AMP levels were not significantly increased (Figure 59C). Upon examination of ATP/ADP ratios and adenylate energy charge values we did not observed any changes (Figure 60A-B). Levels of adenosine were quantified and significant increases were noted in cerebral cortex (Figure 61, p < 0.05), striatum (p < 0.05), and cerebellum (p < 0.05).
Figure 58: Effects of KD Administration on Levels of Creatine and Phosphocreatine in Discrete Rat Brain Regions. (A) Compared with controls, levels of phosphocreatine increased significantly (p < 0.05) in hippocampus, cerebral cortex, and striatum following administration of an 8% KD for 3 weeks. (B) Levels of creatine were elevated significantly (p < 0.05) in cerebral cortex compared to control animals. (C) No statistically significant changes were observed for the phosphocreatine/creatine ratios in all brain regions compared to controls. * p < 0.05 versus control. Values are mean ± SD. Sample sizes are indicated on respective bars.
Figure 59: Effects of KD Administration on Levels of Adenine Nucleotides in Discrete Rat Brain Regions. (A) Compared with controls, levels of ATP increased significantly in both hippocampus (p < 0.05) and cerebral cortex (p < 0.01) following administration of an 8% ketogenic diet for 3 weeks. (B) Levels of ADP were elevated significantly (p < 0.05) in hippocampus compared to control rats. (C) No statistically significant changes were observed for AMP levels in all tested brain regions compared to controls. * p < 0.05 versus control, ** p < 0.01 versus control. Values are mean ± SD. Sample sizes are indicated on respective bars.
Figure 60: Effects of KD Administration on Energetic Ratios in Discrete Rat Brain Regions. (A) Compared with controls, no statistically significant differences were observed for ATP/ADP ratios after administration of an 8% KD for 3 weeks in all tested brain regions. (B) No significant differences were observed for adenylate energy charge in all measured brain regions. * p < 0.05 versus control. Values are mean ± SD. Sample sizes are indicated on respective bars.

Figure 61: Effects of KD Administration on Levels of Adenosine in Discrete Rat Brain Regions. Compared with controls, adenosine levels were increased significantly (p < 0.05) after administration of an 8% KD for 3 weeks in cerebral cortex, striatum, and cerebellum. No statistical difference was observed for hippocampus. * p < 0.05 versus control. Values are mean ± SD. Sample sizes are indicated on respective bars.
Synaptic Transmission is More Resistant to Low Glucose after KD

Synaptic transmission is highly dependent on ATP availability (Attwell & Laughlin, 2001). In view of our findings of a diet-induced modest enhancement in brain energy reserves, we asked whether synaptic transmission in hippocampal slices from KD-fed animals is significantly more resistant to mild metabolic stress compared to controls (Figure 62). Paired stimuli (50 ms delay) were delivered to the medial perforant path and field excitatory postsynaptic potentials (fEPSPs) were recorded from the dentate granule cell layer (Figure 62A). Stimulus intensities required to evoke fEPSPs (30-70 μA) did not differ across experiments or treatment groups. Reducing the bath glucose concentration from 10 mM to 2 mM for 7 to 10 min rapidly and reversibly depressed the slope of the fEPSP by 53 ± 9% in controls, but only by 27 ± 8% in KD-fed animals (Figure 62B, upper panel; p < 0.001 difference between CON and KD, ANOVA). Paired-pulse ratios increased in response to low glucose in slices taken from control and KD rats (Figure 62B, lower panel; p < 0.001, ANOVA), which supports the notion that diminished energy supply leads to an increased failure of transmitter release. The latency to onset of the effect induced by low glucose varied substantially from slice to slice in control rats (Figure 62C), but for each pair of slices studied simultaneously (i.e., one from a KD-fed animal and one from a control-fed animal), the onset of fEPSP reduction after low glucose always occurred more rapidly in control tissue than in KD tissue (Figure 62C, n = 9 slice pairs from 6 pairs of rats). In slices taken from KD-fed animals, the latency to 25% inhibition of the fEPSP was 46 ± 10% longer than that of control slices (Figure 62C, p <
0.002, paired t-test). This difference, however, waned after slices were incubated in normal glucose-containing artificial CSF at room temperature for greater than 3.5 h (data not shown), as expected if brain metabolism could revert rapidly when excess glucose is supplied. Collectively, these findings support the notion that KD-induced enhancement in energy reserves can maintain synaptic transmission in the dentate gyrus for longer periods of time under metabolic stress.
Figure 62: Synaptic Transmission is More Resistant to Metabolic Challenge in Slices taken from KD–Fed Animals than those from Control-Fed Animals. (A) Representative paired-pulse evoked responses from control (top set of traces) and KD-fed (bottom set of traces) animals before (baseline) and 10 minutes after perfusion with low glucose (2 mM). Responses are the average of five traces. Symbols denote stimulus artifacts. (B) Average response of hippocampal slices prepared from control (open squares; n = 5 rats, 9 slices) and KD tissue (filled circles; n = 5 rats, 9 slices) to challenge with low (2 mM) glucose. (Top) Symbols represent the mean slope of the field excitatory postsynaptic potential (fEPSP) slope ± standard error of the mean (SEM). The peak reduction in fEPSP slope (averaged over measurements during the period shown by the bar) after challenge with low glucose was significantly greater in control animals (** p < 0.001, analysis of variance [ANOVA]). (Bottom) Paired-pulse responses during low glucose (** p < 0.001 compared with control period, ANOVA). (C) Failure of
synaptic transmission during low glucose was delayed in slices taken from KD-fed animals compared with control slices. Mean latencies to 25% reduction in synaptic efficacy are shown for experimentally matched slices prepared from control (open squares) and KD (filled circles) rats. Bars represent the mean.

**Exogenously Applied Adenosine Decreases fEPSP Amplitude after KD.**

Adenosine is the brain’s endogenous anticonvulsant and an endogenous neuromodulator that is released during seizures, ischemia, and hypoxia (Berman et al., 2000; Fredholm et al., 1984; Olsson et al., 2004). It exerts both antiepileptic and neuroprotective effects (Ribeiro et al., 2003) that are mediated by adenosine receptors, of which the inhibitory A1 receptor subtype (A1R) is the most abundant in seizure prone regions, such as hippocampus (Fredholm et al., 2001, 2005b). Since our previous experiments demonstrated that KD-fed brain has elevated energy metabolite and adenosine levels as well as a greater latency to low glucose levels, we examined in collaboration with Dr. Susan Masino at Trinity College, the effects of adenosine (50 μM) application to hippocampal slices on fEPSP amplitude from animals fed either a control or KD. We hypothesized that KD-fed animals would have reduced excitability in response to adenosine. We found that indeed KD fed brain has a reduction in excitability to exogenously applied adenosine (Figure 63). Collectively, these findings support the notion that adenosine has a greater propensity to inhibit excitability in this tissue and these effects, although still undetermined in our model system, are likely to occur through the A1R because this adenosine receptor has a strong inhibitory effect on excitatory synaptic transmission (Cunha, 2005).
Figure 63: Effect of KD on fEPSP Amplitude in Hippocampus. Application of adenosine (50 μM) differentially inhibited the baseline fEPSP in hippocampal slices obtained from KD-fed versus control animals (p < 0.05). The control slices showed greater inhibition than the slices from KD animals. Error bars are SD.
There is considerable evidence suggesting that impaired energy metabolism, along with mitochondrial dysfunction plays a fundamental role in the pathogenesis and progression of neurodegenerative disorders as a primary and/or secondary mechanism in the neuronal death cascade (Beal, 2005). This not only comprises neurodegenerative disorders, but furthermore acute and chronic conditions of the central and peripheral nervous systems. Although other means may exacerbate bioenergetic dysfunction, for instance changed transcription and protein aggregation; blighted energy metabolism may elicit pro-apoptotic signaling, oxidative damage, excitotoxicity, and hinder nuclear and mitochondrial DNA repair. These pathologic messages can interact and potentiate each other and result in an unrelenting cycle of energy depletion. Energy is important to the biological and molecular regulation of multiple cellular functions and therefore diminished energy levels threaten cellular homeostasis and integrity.

While the notion of neuroprotection was first considered by Greek physicians using hypothermia to treat stroke, a modern-day therapeutic strategy inducing sustained ATP levels may have both direct and indirect importance in ameliorating the severity of many of the pathogenic mechanisms associated with
neurological disorders. Indeed, if neuronal dysfunction and loss are caused by diminished energy stores, then therapeutic regimens that buffer intracellular energy levels may hinder progression or prevent the neurodegenerative process (Klein & Ferrante, 2007). A major store of energy in the brain is ATP, which is closely tied to creatine and phosphocreatine levels within cells. Creatine is shuttled across membranes via a creatine transporter, CreaT (Snow & Murphy, 2001) and creatine kinase catalyzes the reversible transfer of a phosphoryl group from phosphocreatine to ADP, generating ATP. Creatine counterbalances energy depletion by forming phosphocreatine, providing a spatial energy buffer to re-phosphorylate ADP to ATP at sites of energy utilization and forming phosphocreatine and ADP from creatine and ATP at locations of high-energy phosphate fabrication (Tombes & Shapiro, 1985). Therefore, increasing creatine levels may replenish energy stores and hence improve neuronal function. Moreover, creatine is implicated in regulating glycolysis, stabilizing the mitochondrial variety of creatine kinase, and thus inhibiting the mitochondrial permeability transition pore (O’Gorman et al., 1997).

Cholesterol Fed Rabbits as a Model for Alzheimer’s Disease

Alzheimer’s disease (AD) is the most common form of degenerative dementia, characterized by profound memory impairment, reduced self-care, behavioral and psychological symptoms, and emotional troubles (Mimura, 2008). Previous reports have demonstrated decreased energy levels in probable AD (Pettegrew et al., 1994) and this led to our hypothesis that energy levels in cholesterol-fed rabbit cortex will be reduced. Following a 12 week dietary
administration of a 2% cholesterol diet we observed a trend toward lower levels of phosphocreatine and this is not surprising since hypercholesterolemia-induced ischemic myocardia in swine reduced levels of phosphocreatine by 50% (Skinner et al., 1973). As well, patients with AD have reduced levels of phosphocreatine (Pettegrew et al., 1994). Significantly lower creatine levels were observed and may be due to content of the diet and/or availability of creatine to the tissue. Most notably, we indeed observed that levels of ATP, ADP and AMP were decreased significantly when compared to standard chow fed rabbit. These findings support studies demonstrating that excessive cholesterol intake is a risk factor for AD as well as genetic analyses that have shown reduced expression of many genes encoding ETC proteins. An alteration to the oxidative phosphorylation process does reduce the capacity of ATP production and will promote generation of reactive oxygen species that in turn will further hinder function of the ETC. This process may hold true in cholesterol fed rabbit as a model for AD however we did not perform studies to measure ETC protein levels and mitochondrial reactive oxygen species production. Further studies examining these critical factors that strongly influence cellular energy production are necessary to determine if decreased levels of adenine nucleotides are responsible for the pathology observed in this model system. As well, performing these studies in young animals, before AD pathology manifestation, may provide information as to whether diminished cerebral energy metabolism precedes AD pathology or if changes to energy levels are merely a consequence of the disease.
Upon examination of adenylate energy charge one can readily appreciate the vast energetic difference between traditionally sacrificed and head-focused microwave-irradiated animals. The energetic charge values for these particular experiments were incredibly low and are caused by a rapid post-mortem dephosphorylation of adenine nucleotides when brain enzymes are not snap-inactivated. However, these diminished levels are present in both treatment and control groups and therefore allow comparisons between groups. A worthy experiment would be to kill these animals with head-focused microwave irradiation to better determine adenine nucleotide levels. There are no accurate brain energy metabolite measurements in the literature for normal or cholesterol-fed rabbits to date. The major obstacle for this specific study is to acquire appropriate modifications to a microwave irradiation unit so that it can house an animal as large as a rabbit; to date, the largest commercially available microwave arms can only hold a two pound animal.

Adenine nucleotides were decreased significantly and therefore we hypothesize that cortical adenosine levels will be increased in these animals. From these rabbits, we did not perform adenosine measurements as the total amount of tissue available for this study was limited. In chronic neurodegenerative disorders, such as AD, the density of adenosine A1 receptors (A1R) is reduced (Deckert et al., 1998; Angulo et al., 2003; Ulas et al., 1993) and this is likely due to receptor internalization following constant high-level stimulation. Likewise, several studies have shown that short periods of brain ischemia, which also trigger a robust increase in the extracellular levels of.
adenosine (Kobayashi & Millhorn, 1999), produce a long-lasting decrease in the density of A₁Rs in several brain regions (Nagasawa et al., 1994).

A potentially modifiable vascular risk factor for AD is hypercholesterolemia and dietary fat intake (Sjogren et al., 2006). Hypercholesterolemia is a precursor to atherosclerosis, cardiovascular disease and diabetes and lofty serum cholesterol levels have created a major market for lipid-lowering drugs prescribed to persons at risk of cardiovascular or cerebrovascular conditions. One of these classes of drugs that lower cholesterol levels, statins, have been suggested as useful in both prevention and treatment of AD (Wolozin et al., 2000; Zandi et al., 2005). The mechanism by which statins may provide a benefit against cerebrovascular disease including AD remains speculative and is likely multifactorial.

**ADSL Transgenic Mice as a Model for Autism**

A mere 30 years ago, there was an overall lack of any biological understanding of autism spectrum disorders; we now know that defined mutations and genetic syndromes account for approximately 10-20% of cases. However, no single identified cause accounts for greater than 1-2% of cases. A genetic mutation that fits this criterion is adenylosuccinate lyase (ADSL) deficiency. ADSL deficiency is a defect in purine metabolism and causes severe neurological and physiological symptoms. The deficiency was first described in 1984 as three patients with severe psychomotor delay and features of autism were found to have elevated levels of succinylpurines in their cerebrospinal fluid,
We examined the effects of specific mutations to the ADSL gene in mice on brain energy levels from either cortex or cerebellum in collaboration with Dr. David Patterson's group at the University of Denver, Eleanor Roosevelt Institute. The majority of genetically modified animals from discrete founder mice did not demonstrate significant differences from wildtype mice on measurements of phosphocreatine, creatine, adenine nucleotides, energetic ratios, SAICAR, and adenylosuccinate. However some of these groups did have alterations to energetic systems in these brain regions. Of the eight transgenic groups, only half of these groups showed significant differences on any of our biochemical measurements; D87E_M347, ADSL_P9383, ADSL_P9365, and R426H_M1036. There may have been important differences between some of the groups that we did not observe any change from wildtype control, as our sample sizes were too small to properly evaluate statistically in our preliminary experiments. These groups from our preliminary studies, with sample sizes less than three, had to be "forced" to ignore statistical assumptions in order to generate a p value that may or may not represent proper analyses for these groups. The reason for our small sample sizes is that ADSL animals are difficult to generate and we only had access to limited numbers of these animals as they are not available commercially. Luckily, our second set of received animals had a larger sample size thus allowing for proper statistical analyses to be performed. In addition, three of the five preliminary groups were present with proper sample size in
these experiments and represented the only groups where differences were noted in the preliminary studies.

From our preliminary experiments on the ADSL deficient D87E_M347 group, we observed levels of phosphocreatine and creatine that were decreased significantly from control animals though we did not see any significant changes to these levels in our second set of animals. This may be an example where an incorrect usage of statistics generated a significant result that may not hold true with proper sample size.

The other ADSL deficient group that showed significant differences from preliminary studies was ADSL_P9383; we observed that levels of phosphocreatine, ATP, ADP, SAICAR were decreased significantly as well as the ratios of ATP/ADP and adenylate energy charge. Findings from the second set of ADSL_P9383 animals agree well with the preliminary studies in that we found levels of phosphocreatine, ATP, ADP and the ratio of ATP/ADP to be decreased significantly from wildtype control mice. Thus, these data provide an example of diminished high-energy brain metabolites as a result of ADSL deficiency presumably due to a reduced flux through the purine biosynthetic pathway.

Typically, tissues that are deficient for ADSL maintain normal concentrations of adenine nucleotides and have elevated levels of SAICAR and adenylosuccinate (Van Den Bergh, 1993a) and our data do not support findings in humans. One potential trouble with mouse models to study purine metabolism is that the purine pathway is dissimilar in mice and humans. Humans are missing the enzyme uricase (Becker & Roessler, 1995), hence the end product of purine metabolism
in humans is urate. Accumulation of urate in human provides a significant antioxidant capacity in human plasma and thus help to protect against oxidative stress (Rosell et al., 1999). It is possible that this difference in the purinergic pathways between species accounts for our discrepant findings. These data also suggest that SAICAR levels are decreased significantly in these ADSL deficient mice however this, as well as all other SAICAR measurements, should be interpreted with caution.

Normally, SAICAR is extremely difficult to measure in normal brain as its levels are very low. We measured what we believe to be SAICAR via HPLC, based on retention times and sample spiking. During our measurements we collected HPLC fractions that were to contain our compound of interest and performed mass spectrometry on half of the collected samples to verify that indeed the compound was SAICAR, the remaining samples were lyophilized and then analyzed by Dr. Erin Spiegel via Bratton-Marshall assay. All verification experiments were unable to conclusively demonstrate that the HPLC peak was SAICAR and not a separate compound with an identical retention time. Assuming that our HPLC analyses were correct, we did not expect levels of SAICAR to be lowered significantly in ADSL deficient animals but taken together with diminished adenine nucleotides and phosphocreatine levels it appears that this specific ADSL mutation leads to an overall repression of the purine synthetic pathway. Early work performed by our collaborator, Dr. Patterson, found that intermediate levels of AICAR lead to retrograde flux through the pathway and accumulation of SAICAR (Sabina et al., 1985). Higher levels of AICAR result in
the pyrophosphorylation of AICAR at the expense of phosphoribosylpyrophosphate (PRPP) and a reduction of flux through the de novo pathway (Sabina et al., 1985).

The group R426H_M1036 is also ADSL deficient due to swapping arginine at position 426 to histidine. These mice displayed multiple energetic alterations when compared to wildtype mice. Levels of ATP and ADP, as well as these animals’ adenylate energy charge were increased significantly in R426H_M1036 mice whereas the levels of AMP were decreased significantly in ADSL deficient mice. As well, adenylosuccinate levels were reduced significantly as well. These findings were surprising to observe in mice with the R426H mutation as this specific mutation has been commonly reported to cause moderate to severe autism in humans (see Table 5 in the Appendices). Typically, tissues that are deficient for ADSL maintain normal concentrations of adenine nucleotides and have elevated levels of SAICAR and adenylosuccinate (Van Den Bergh, 1993a); however we did not observe any of the expected levels. A lack of another purine could lead to the phenotype of ADSL deficiency apart from the compounds we measured. For example inosine, a nucleoside that can stimulate extensive axon outgrowth in goldfish and rats (Benowitz et al., 1999; Benowitz et al., 1998; Petrausch et al., 2000) or one of its metabolites may be altered. It is possible that exchanging an arginine for a histidine residue at position 426 does not produce the same phenotype in rodents as it does in human or it may be that this modification has important developmental consequences that are undetectable with energetic measurements by adulthood as homeostatic mechanisms may
have corrected adenine nucleotide, SAICAR, and adenylosuccinate levels to normal. Performing these energetic measurements on very young or embryonic mice may provide insight for whether our observed levels hold true during development or if there are critical developmental events that transiently occur.

Cerebral tissues from all other ADSL deficient groups did not demonstrate alterations to adenine nucleotide, SAICAR and adenylosuccinate levels. These data supporting the null hypothesis for adenine nucleotides are in agreement with human ADSL deficiency studies (Van den Bergh, 1993a, Van den Berghe et al., 1993a, Van den Berghe et al., 1993b). Experiments on cultured fibroblasts from ADSL deficient patients demonstrated that the rate of de novo purine synthesis and nucleotide concentrations is not significantly different from individuals without the deficiency and that flux through the two ADSL catalyzed steps still occurs in these cells (Van den Berghe et al., 1993a; Van den Berghe et al., 1993b).

Furthermore, these fibroblasts displayed standard growth rates and levels of ATP in the presence of purine bases (Van den Bergh et al., 1993a). Mammalian cells can synthesize purines via the adenine salvage pathway when cultured in the company of purine bases and this is adequate to sustain synthesis of adenine nucleotides in fibroblasts (Van den Bergh et al., 1993b). Normal physiological levels of adenine nucleotides were observed in liver, kidney, and muscle of ADSL deficient patients (Van den Berghe & Jaeken, 1986). One explanation is that the ADSL deficiency is overcome in these tissues by a contribution of purines from non-affected cells (e.g. erythrocytes, granulocytes) through purine salvage enzymes (Van den Berghe et al., 1997). Taken together these studies indicate
that ADSL deficiency causes negligible changes in specific tissues when
unaffected cell-types are capable of contributing purines.

Ts65Dn and GART Transgenic Mice as Models for Down Syndrome

Down syndrome (DS), or trisomy 21, is the most common genetic basis of
considerable intellectual disability that occurs in approximately one in 733 live
births (Centers for Disease Control and Prevention, 2006) with complete trisomy
of the chromosome in roughly 95% of cases. DS can also be caused by partial
trisomy of chromosome 21 (HSA21) but this occurs less frequently. Increases to
levels of enzymes, resulting from trisomy, are of particular interest in the de novo
purine metabolic pathway. There are many mitochondrial metabolism genes
located on HSA21 and of these genes a large proportion are trisomic in the
Ts65Dn mouse. With resultant increases to metabolic enzymes, it is extremely
difficult to predict how these changes will ultimately affect energy and
mitochondrial reactive oxygen species production. These mitochondrial protein
alterations could lead to two possible outcomes; increased ETC proteins could
facilitate either increased or decreased energy production. We hypothesize the
latter outcome to be more probable since evolutionary processes have likely
selected for the most advantageous arrangement and levels of metabolic
proteins. Any change to the mitochondria’s homeostatic environment could
potentially offset the proton gradient, generation and control of reactive oxygen
species, and ultimately energy production. For example, even though metabolism
genomes are elevated in the Ts65Dn mouse, adenine nucleotide levels are
decreased. We observed diminished levels of ATP, ADP along with the ratio of
ATP/ADP and adenylate energy charge in mice either three or six months of age. We also observed that phosphocreatine levels decreased significantly. These results taken together suggest that trisomy of mitochondrial metabolism genes on chromosome 21 is sufficient to decrease brain energy.

A second model system used in our experiments was the GART transgenic mouse that expressed a GART gene that solely fabricates the GARS-AIRS-GART protein but not the single GARS protein. Since the purine pathway differs in humans and mice it is difficult to predict whether increases to an enzyme responsible for catalyzing three steps in the biosynthetic pathway would increase, decrease, or not affect energy levels. This model system is less complex than the Ts65Dn model as only a single gene was modified and therefore there are fewer modifications to the de novo purine biosynthetic pathway. We hypothesized that increasing GART would lead to a greater flux through the purine pathway; however it is possible that endproduct inhibition could restrain the pathway. In our experiments on these transgenic mice from two separate founders we observed an increase to high energy adenine nucleotides such as ATP and ADP, as well as elevations to the ratio of ATP/ADP and adenylate energy charge meanwhile we observed diminished levels of AMP and adenosine in these animals. Taken together it appears as though expression of human GART in mice results in increased energy levels without endproduct inhibition. From these studies it is impossible to determine if overexpression of mouse GART will cause these same effects and if it does cause the same effect why the effect is not observed in Ts65Dn mice. One explanation for why trisomy
of GART does not increase energy levels in mouse is that factors decreasing energy outweigh the effects of GART alone.

In addition to our metabolite measurements in three and six month old animals we also examined old mice, between the ages of 12 and 18 months. In agreement with increases to the cerebral metabolic rate of glucose data commonly reported in the literature, we found that levels of ATP were elevated significantly in Ts65Dn mice compared to wildtype controls. Furthermore, when comparing across age groupings of our trisomic mice we found that levels of phosphocreatine and ATP as well as the ratio of ATP/ADP and adenylate energy charge were increased significantly in old animals. These data suggest that the cerebral brain energy increases in our model system for DS with age and could potentially indicate a compensatory response in these mice prior to the progression of AD pathology. However, compensatory responses may eventually become pathological or unsuccessful to offset progressive degeneration (Mesulam, 1999). Prior to energy metabolite measurements, behavioral tests should be performed on these animals to determine whether or not these mice have developed AD and this will determine if increased energy levels is truly a compensatory response to dementia in this model system. Attaining a better understanding for the relationship between energy levels and dementia may provide insight for alternative therapeutic approaches to impede AD onset.
Treatment of Rat Cerebral Cortical Neurons with HIV-1 Protein Tat as a Model for HIV-1 Dementia

The advent of effective highly active anti-retroviral therapeutics has resulted in increased longevity of HIV-1 infected individuals, and an increased prevalence of HIV-1 associated dementia (Bouwman et al., 1998). This increased prevalence has renewed efforts not only to determine mechanisms underlying this neurological disorder that is the most common cause of dementia in people less than 60 years of age, but also to identify effective therapeutic strategies. Implicated in the neuropathogenesis of HIV-1 associated dementia is the non-structural protein Tat (Sabatier et al., 1991; Nath & Geiger, 1998; Perry et al., 2005). Our study was focused to determine the extent to which HIV-1 protein Tat-induced neurotoxicity involved mitochondrial bioenergetic crisis and/or redox catastrophe, as well as the extent to which and the mechanisms by which creatine protected against this neurotoxicity. The major findings of the present study are that creatine protected against HIV-1 Tat-induced neuronal cell death through mitochondrial mechanisms including blocking decreases in ATP levels, depolarization of mitochondrial membrane potentials, and mitochondrial permeability pore opening. These findings are important because it provides further evidence for the involvement of mitochondria in HIV-1 Tat-induced neuronal cell death and identifies creatine as a potential therapeutic intervention. HIV-1 Tat of shorter and longer sequences including amino acids 31-61, 1-72 and 1-86 are neuroexcitatory and neurotoxic (Chauhan et al., 2003; Kruman et al., 1998; Bonavia et al., 2001; Bonavia et al., 2001; Nath et al., 1996;
New et al., 1997; Shi et al., 1998). Tat depolarizes and kills neurons by activating cell surface NMDA and non-NMDA glutamate receptors (Chandra et al., 2005; Cheng et al., 1998; Haughey et al., 2001; Magnuson et al., 1995; Self et al., 2003; Song et al., 2003; Song et al., 2003). Tat-induced excitotoxicity may be further enhanced because Tat has been shown to decrease glutamate uptake in astrocytes (Zhou et al., 2004) and this may increase further activation of glutamate receptors on nearby neurons. Intracellularly, endoplasmic reticulum and mitochondria both contribute to Tat-induced neurotoxicity even though, for example, Tat\textsubscript{1-72} is internalized by cells to only a limited extent (Ma & Nath, 1997). Increased levels of intracellular calcium through activation of excitatory amino acid receptors and IP\textsubscript{3}-regulated stores of endoplasmic reticulum calcium clearly contribute to Tat-induced neurotoxicity (Haughey et al., 1999; Mayne et al., 2000; Haughey et al., 2001; Self et al., 2003; Fotheringham et al., 2004). Mitochondrial dysfunction has also been shown to be involved in Tat-induced neuronal cell death, and the implicated mechanisms include alterations in bioenergetics (Perry et al., 2005), mitochondrial membrane potential (Perry et al., 2005; Aksenov et al., 2006), reactive oxygen species (Perry et al., 2005; Aksenov et al., 2006; Wallace et al., 2006), mitochondrial permeability transition pore opening (Kruman et al., 1998), and caspase activation (Kruman et al., 1998; Singh et al., 2005). Moreover, the neurotoxic properties of Tat are exaggerated by glutamate and drugs of abuse (Haughey et al., 2001; Aksenov et al., 2006).

Our findings that exogenous Tat treatment resulted in mitochondrial dysfunction through hypopolarization of mitochondrial membrane potential, production of
reactive oxygen species, opening of the mitochondrial permeability transition pores and decreasing cellular bioenergetics help elucidate further the mechanisms by which Tat causes neurotoxicity.

Tat_{1-72} with amino acids 31 to 61 deleted (mutant Tat_{1-72}), is not neurotoxic when applied directly to neurons or to hippocampal slices and consequently has been used by others and us as a control peptide for the actions of Tat (Gurwell et al., 2001; Prendergast et al., 2002; Self et al., 2004). However, recent results of others and us indicate that mutant Tat_{1-72} is biologically active; it can increase cytokine and chemokine expression (Flora et al., 2003; Pu et al., 2003; Toborek et al., 2003). Indeed, we showed recently that Tat_{1-72} and to a greater extent mutant Tat_{1-72} increased the release of TNF-α from monocytes, macrophages and microglia, and that media from Tat-stimulated monocytes caused neurotoxicity that was TNF-α mediated (Buscemi et al., 2007). Therefore, while mutant Tat_{1-72} was without effect in most of the current studies reported here, it was not totally surprising when we observed increased levels of reactive oxygen species following treatment of neurons with mutant Tat_{1-72}.

Tat has been shown to induce mitochondrial permeability transition pore opening in lymphoid cells (Macho et al., 1999) and cyclosporine A, a known inhibitor of mitochondrial permeability transition pore opening, inhibits Tat-induced neuronal apoptosis (Kruman et al., 1998). Here, we showed directly that Tat_{1-72} opened mitochondrial permeability transition pores in neurons. Opening of the mitochondrial permeability transition pore results in rapid decreases in proton gradients across the inner mitochondrial membrane and this may explain...
reductions in mitochondrial membrane potential and cellular levels of ATP; all of which may result in the release of pro-apoptotic factors such as cytochrome c, apoptosis inducing factor, and caspases (Zoratti & Szabo, 1995). Creatine was shown in the current study to block mitochondrial permeability transition pore opening and neurotoxicity and further studies are warranted to determine the extent to which creatine prevents the release of implicated pro-apoptotic factors. The present data and results from other studies have shown that Tat increases oxidative stress in neurons (Kruman et al., 1998; Langford et al., 2004; Perry et al., 2005). Moreover, oxidative stress has been demonstrated in the brain and CSF of patients with HIV-1 associated dementia (Chauhan et al., 2003; Turchan et al., 2003). However, although creatine can act as a direct antioxidant by quenching superoxide anions, hydrogen peroxide, and peroxynitrite (Lawler et al., 2002), here creatine did not protect against Tat-induced increases in reactive oxygen species. With the caveat that protection may have been observed under different conditions, creatine neuroprotection did not appear to be due to the antioxidant actions of creatine.

In this study, Tat decreased significantly levels of intracellular ATP. These decreases may have been due to the hypopolarization of mitochondrial membrane potentials observed, loss of proton gradients across mitochondrial membranes, reduced ATP synthase activity, and subsequent depletion of intracellular levels of ATP. However, the inability of Tat to induce changes in either the adenylate energy charge or ATP/ADP ratio is indicative of only mild effects of Tat on cellular bioenergetics as they relate to adenosine nucleotides.
and suggest that the role of the creatine/creatine phosphate system as a high-energy phosphate donor could only play a minor role in the neuroprotective properties of creatine in this model.

The most robust neuroprotective mechanisms through which creatine appears to function in our model was through stabilization of mitochondrial membrane potential and keeping closed permeability transition pores. In this manner, creatine would prevent the redox catastrophe and resulting neurotoxicity. The readily available dietary supplement creatine might prove useful in preventing or lessening the severity of HIV-1 associated dementia.

Ketogenic Diet as a Treatment for Epilepsy

The molecular mechanisms underlying regulation of energy metabolism in the brain are not as well defined as those described for muscle. Indeed, chronic ketosis with caloric restriction appears to affect metabolism quite differently in skeletal muscle and brain. In muscle, ketonemia and/or fasting is accompanied by down-regulation of oxidative phosphorylation, fewer mitochondria, less efficient mitochondrial respiration, and a fall in glutamate and glutamine levels (Hammarqvist et al., 2005; Iossa et al., 2003; Lecker et al., 2004; Sparks et al., 2005). By comparison, in the hippocampus chronic ketosis with caloric restriction results in upregulation of transcripts encoding oxidative phosphorylation and other mitochondrial proteins, mitochondrial biogenesis, elevated phosphocreatine to creatine ratio, and elevated glutamate and glutamine levels. Some of the energy transcripts were reported in a previous microarray study of KD (Noh et al., 2004), but their use of only one sample each in KD and control groups makes
interpretation difficult. In muscle PGC1α, γ and Δ are powerful regulators of mitochondrial biogenesis and are downregulated during high fat diets (Mootha et al., 2003; Sparks et al., 2005). However, transcript levels for these proteins in both control and KD rats were below detection level in whole hippocampus and also in dentate granule cells, CA1 and CA3 pyramidal cells harvested by laser capture microscopy (Borges et al., 2007). Our results suggest significant differences in how brain and muscle respond to high fat diets.

The ketogenic diet (KD) is a high-fat, calorie-restricted diet used to treat childhood epilepsies that do not respond to available drugs. Despite its clinical use for nearly 100 years, how the KD controls seizures remains unknown. Transcriptional profiling of rat hippocampus after KD showed a concerted upregulation of numerous transcripts encoding energy metabolism proteins and mitochondrial proteins. The most striking finding of this study was a 46% increase in the density of mitochondrial profiles in the dentate gyrus of KD-fed rats, most of which were in neuronal processes. The increased phosphocreatine to creatine ratio and increased level of amino acid alternative energy sources, together with the improved resistance to low glucose, are consistent with an increased capacity to sustain ATP production in hippocampus in the face of increased physiological need. These findings collectively point to an enhanced energy production capacity in the hippocampus of rats fed a ketogenic diet.

Limitations

We studied the effects of a KD on the hippocampus of non-epileptic, adolescent rats, whereas this dietary treatment is often used in children with
epilepsy originating outside the hippocampus. The validity of this model should therefore be carefully considered, and several points are relevant. First, the KD is effective in a variety of epilepsies including temporal lobe epilepsy (TLE), which involves the hippocampus (Freeman et al., 1998). Second, the rat model faithfully reproduces at least four key aspects of KD treatment observed clinically – maintained ketonemia, maintained hypoglycemia, reduced weight gain, and increased resistance to seizures. Third, hippocampal sclerosis may coexist with non-temporal focal epilepsy (Pan et al. 2004) or cortical dysplasia, a more common cause of childhood epilepsy (Bocti et al., 2003). Moreover, Lundberg et al. (1999) reported a high frequency of hippocampal asymmetry or sclerosis in benign childhood epilepsy with centrotemporal spikes, a common cause of childhood epilepsy. Therefore, although not involved in all childhood epilepsies, the hippocampus is a relevant structure. Finally, previous in vivo electrophysiological studies showed a robust anticonvulsant effect of the KD within the DG of the hippocampus in a rat model (Bough et al., 2003). KD-fed (and calorie-restricted) animals required greater stimulus intensities to evoke the same network excitability as compared to ad lib-fed controls. Enhanced paired-pulse inhibition was observed, consistent with enhanced functional, fast GABAergic inhibition, and electrographic seizure threshold was elevated in a kindling-like protocol. Thus, we decided to focus on a well-defined region of the brain, known to exhibit these KD-induced effects, and known to be importantly involved in the development and maintenance of epileptic phenotypes.

We and others (Appleton & DeVivo, 1974) found that an elevation in
seizure threshold required two weeks or more of the KD to fully develop in rats; this effect waned slowly upon reversion to a high carbohydrate diet. By contrast, Freeman and Vining (1999) reported that in five children with Lennox-Gastaut syndrome who were experiencing more than 20 myoclonic or atonic seizures per day, seizure frequency was drastically reduced one or two days after initiation of the KD. Aside from this report there are only anecdotal comments about how rapidly the KD becomes effective in epileptic patients, although typical practice is to assess efficacy after several weeks on the diet. The speed with which the anticonvulsant effect of the KD develops might well depend on age, physiological status, and how the effect is measured (seizure threshold vs. seizure frequency).

The Affymetrix GeneChip has been the most popular microarray platform, yet over the past few years there has been a growing gulf between the historical assignment by Affymetrix of oligonucleotide sequences to genes, and modern assignments. Dai et al. (2005) resolved this problem by reassigning sequences to transcripts based on the most up-to-date Unigene build. These assignments significantly simplify analysis of microarray experiments as described in Methods, and Results. Several reviews have highlighted issues of particular importance to microarray studies of the brain (Cao & Dulac, 2001; Luo & Geschwind, 2001; Mirnics & Pevsner, 2004). Chief among these are cellular heterogeneity within a brain region, low expression levels of many transcripts in neurons, and the short supply of neuron-specific genes on most cDNA microarrays. Beyond the technical challenges of identifying differentially expressed low abundance genes in identified neurons that are relevant to
epilepsy, a number of interpretational pitfalls must be recognized. Microarrays report the relative expression of mRNAs and thus have the major limitation of not directly measuring protein levels themselves. Moreover, post-translational processing that alters protein function is hidden from microarray studies. Experience has shown that even subtle alterations in a cell’s environment can change gene expression, so model-specific effects are also expected. The consequence of these limitations is that, from the standpoint of changes in protein function, both false positive and especially false negative signals are expected to be common in microarray studies of RNA expression. Experimental designs must include strategies (e.g., replication, follow-up confirmation of signals) to minimize the impact of these deficiencies.

An Energy Preservation Hypothesis for the Anticonvulsant Effect of the KD

Several mechanisms for the anticonvulsant action of the KD have been proposed, including acidosis, enhanced GABA production, change in electrolyte balance or energy metabolism, activation by free-fatty acids (FFAs) of potassium or other channels, or dehydration (Schwartzkroin, 1999). One of the oldest theories stems from the idea that an increased production of ATP should enhance neuronal stability by stabilizing the resting membrane potential, perhaps via enhanced operation of the Na⁺/K⁺ ATPase (DeVivo et al., 1978).

Taken together, our findings suggest a modification of the energetic hypothesis for the anticonvulsant effect of the ketogenic diet (Figure 64, * denotes findings in the present study). We propose that chronic but not acute ketosis activates a genetic program that leads to mitochondrial biogenesis in the
hippocampus, which results in enhanced energy stores. The half-life of liver mitochondria is three to four days (Lipsky & Pedersen, 1981). If that of brain mitochondria were similar, five half-lives (15-20 days) would be approximately the time required to achieve an anticonvulsant action of the KD, consistent with the model presented in Figure 64. We suggest that mitochondrial biogenesis increases ATP production capacity, with excess high-energy phosphates stored as phosphocreatine. Glutamate and glutamine formed from the ketone-boosted TCA cycle provide an important second energy store that, in concert with phosphocreatine, can be drawn upon to sustain ATP levels in times of need (i.e., during hyperexcitability leading to a seizure). The ability to sustain ATP levels during metabolic or physiological stress should allow neurons to fuel Na⁺/K⁺ ATPase and other transporters that stabilize membrane potential in neurons and thus maintain ionic homeostasis for longer periods of time. Under low glucose conditions, transmitter release from perforant path terminals could be maintained about 60% longer in hippocampal slices taken from KD compared to control-fed rats, a likely consequence of enhanced energy stores.

The enhanced resistance to metabolic stress could thus elevate seizure threshold, if seizure initiation results from a crescendo barrage of neuronal impulses that eventually causes ATP levels to decline, leading to sustained membrane depolarization and runaway neuronal firing. The Na⁺/K⁺ ATPase inhibitor, ouabain, lowers seizure threshold to kainate (Brines et al., 1995), as expected if oxidative metabolism opposes seizure initiation. It is noteworthy that the KD increases seizure threshold, but cannot terminate a breakthrough seizure.
and may actually provide a greater energy supply that exacerbates spread of seizures once initiated (Bough et al., 2000b), consistent with our model. Although the degree of glutamate and phosphocreatine elevation is modest in KD-fed rat hippocampi, a similar elevation in PCr/Cr ratio has been observed previously in total brain after KD (DeVivo & Leckie, 1978). Both glutamate and phosphocreatine are present in approximately 10-fold higher concentrations than ATP, befitting their role as energy buffers. Thus, small absolute adjustments should be effective. Phosphocreatine opposes an acute activity-dependent fall in ATP levels by donating its phosphoryl group to ATP (Rango et al., 1997).

There is an established role for diminished ATP production capacity in both patients with epilepsy (Antozzi et al., 1995; Kunz et al., 2000) and pilocarpine-treated rats (Kudin et al., 2002). In a recent study of human epileptic tissue, the rate of recovery of the resting membrane potential following an evoked stimulus train was positively correlated with the PCr/ATP ratio, but inversely correlated with granule cell bursting (Williamson et al., 2005). It is interesting to speculate whether GABAergic inhibitory interneurons, which tend to have a greater energetic requirement to maintain high-frequency modes of firing (Atwell & Laughlin, 2001), are particularly advantaged by improved energy levels associated with the KD. Electrophysiological evidence collected in vivo showed that KD decreases hippocampal network excitability in part by enhancing GABAergic inhibition (Bough et al., 2003). Enhanced cellular metabolism might be expected to prolong activation of inhibitory interneurons, diminish network excitability, and thus improve seizure control. A critical test of this hypothesis will
require the development of a method for selectively interrupting mitochondrial biogenesis in the brain.

Whereas glycogen was elevated in total brain (DeVivo & Leckie, 1978), we observed reduced levels of glycogen in hippocampus. It is doubtful, however, that reduced glycogen level plays a particularly important role in seizure resistance because hypoglycemia can rapidly reduce levels of brain glycogen (Choi et al., 2003; Garriga & Cusso, 1992), and KD-induced hypoglycemia occurred well before the anticonvulsant effect appeared. It is thus unlikely that glycogen, the largest energy store in brain, is regulating seizure threshold directly.

![Diagram](https://via.placeholder.com/150)

**Figure 64: Hypothesis for Anticonvulsant Effect of KD.** Chronic ketosis in the brain is proposed to trigger mitochondrial biogenesis and associated induction of transcripts encoding proteins in the oxidative phosphorylation and tricarboxylic acid (TCA) pathways. Mitochondrial biogenesis increases ATP production capacity, but under resting conditions, excess ATP is converted to phosphocreatine, leading to increased phosphocreatine/creatine ratios. Ketones also serve as a substrate for glutamate synthesis via the TCA cycle, and some glutamate is reversibly converted to glutamine. Both glutamate and PCr act as energy buffers that can be drawn on to synthesize ATP when needed to fuel
Na⁺/K⁺-ATPase and other pumps, which serve to stabilize neuronal membrane potential. The resulting enhanced resistance of hippocampal tissue to metabolic stresses accompanying hyperexcitability results in an elevated seizure threshold. Asterisks indicate results observed in this study.

Relevance to Childhood Epilepsies

We studied the effects of a KD in the hippocampus of nonepileptic, adolescent rats. Because this dietary treatment is often used in children with epilepsy originating outside the hippocampus, the validity of this model should be considered carefully and several points are relevant. First, the KD is effective in adolescents and adults, if tolerated (Bough et al., 1999b; Sullivan et al., 2004). Second, the KD has efficacy in a variety of epilepsies including temporal lobe epilepsy, which involves the hippocampus. Third, the rat model faithfully reproduces at least four key aspects of KD treatment observed clinically; maintained ketonemia, maintained hypoglycemia, reduced weight gain, and increased resistance to seizures. Fourth, hippocampal sclerosis may coexist with nontemporal focal epilepsy (Yudkoff et al., 2005) or cortical dysplasia, a more common cause of childhood epilepsy (Bocti et al., 2003). Moreover, Lundberg and colleagues (Blumcke et al., 1999) reported a high frequency of hippocampal asymmetry or sclerosis in benign childhood epilepsy with centrotemporal spikes, a common cause of childhood epilepsy. Therefore, although not involved in all childhood epilepsies, the hippocampus is a relevant structure. Finally, previous in vivo electrophysiological studies showed a robust anticonvulsant effect of the KD within the dentate gyrus of the hippocampus in a rat model (Sparks et al., 2005). KD-fed (and calorie-restricted) animals required greater stimulus intensities to
evoke the same network excitability compared with ad libitum–fed control animals. Enhanced paired-pulse inhibition was observed, consistent with enhanced functional fast GABAergic inhibition, and electrographic seizure threshold was elevated in a kindling-like protocol. For these reasons, we decided to focus on a well-defined region of the brain, known to exhibit these KD induced effects and known to be involved importantly in the development and maintenance of epileptic phenotypes. We and others (Appleton & DeVivo, 1974) found that an elevation in seizure threshold required two or more weeks of the KD to fully develop in rats; this effect waned slowly following reversion to control or even high carbohydrate diet (Appleton & DeVivo, 1974). By contrast, Freeman and colleagues (Prins et al., 2005) reported that in five children with Lennox–Gastaut syndrome who were experiencing more than 20 myoclonic or atonic seizures per day, seizure frequency was drastically reduced one or two days after initiation.

Implications for Neuroprotection

Mitochondrial dysfunction contributes to reperfusion injury, congestive heart disease, type 2 diabetes, and neurodegenerative disorders. Adaptive responses that induce mitochondrial biogenesis and enhance oxidative phosphorylation could therefore limit the progression of these disorders. The notion that mitochondrial biogenesis plays a role in neuronal survival in epilepsy is supported by the observation that surviving dentate hilar neurons in humans with epilepsy contain more mitochondria than normal (Blumcke et al., 1999). Several studies have shown that the KD can be neuroprotective. In mice treated...
with the KD for several weeks (Noh et al., 2003) reported that kainate-induced status epilepticus caused less hippocampal cell death and less caspase-3 activation than control-fed mice. Whereas KD-fed mice exhibited a delay in seizure onset (increased seizure resistance), seizure severity was comparable to controls. In rats, the KD is neuroprotective in models of controlled cortical injury (Prins et al., 2005) or hypoglycemia (Yamada et al., 2005), but, unlike mice, not status epilepticus produced by either kainate (Muller-Schwarze et al., 1999) or lithium-pilocarpine (Zhao et al., 2004).

Proteasome inhibition leading to mitochondrial dysfunction appears to contribute to several neurodegenerative disorders (Jana et al., 2001; Sullivan et al., 2004). Mitochondrial biogenesis, a coordinate increase in 19 proteasome transcripts, increased production of the UCP2 uncoupling protein, reduced reactive oxygen species generation, enhanced respiration rate of isolated mitochondria (Andrews et al., 2005; Sullivan et al., 2004), and enhanced alternative energy stores, all point to a myriad of potential neuroprotective mechanisms induced after KD. The enhanced ability to maintain energy levels was demonstrated by our biochemical analyses, we observed elevated phosphocreatine levels ATP levels, ATP/ADP ratio, adenylate energy charge should improve calcium homeostasis and limit synaptic dysfunction after metabolic challenges (Yamada et al., 2005). The strong trend towards higher ADP levels may additionally protect against seizure-induced neuron death by inhibiting opening of the mitochondrial transition pore (Gizatullina et al., 2005), and/or facilitating opening of \( K_{\text{ATP}} \) channels, which should reduce neuronal
excitability (Babenko, 2005).

The purine, adenosine, was demonstrated to have a non-statistically significant trend toward increases levels following a KD in our preliminary studies however we subsequently observed that levels were increased significantly in KD fed rat (Figure 61). During epilepsy, adenosine kinase (ADK) is upregulated. For example, kainic acid-induced status epilepticus led to a significant increase in ADK activity and spontaneous seizures (Gouder et al., 2004). Pharmacoresistant seizures in this mouse model (Gouder et al., 2003) could be suppressed by application of the ADK inhibitor 5-iodotubercidin (3.1 mg/kg, i.p.). These results suggest that upregulation of ADK in epilepsy is implicated in epileptogenesis through a reduced adenosine tone at the A1R. ADK was also upregulated in kindled rats and in the rat pilocarpine model of epilepsy (Boison, 2008). Upregulation of ADK in epilepsy is paralleled by downregulation of A1Rs (Rebola et al., 2003; Rebola et al., 2005).

Cellular energy charge is a primary consideration that defines the status of metabolism. The intracellular concentration of ATP is much greater than that of adenosine. Thus, minor alterations to the concentration of ATP will cause several-fold changes to the intracellular concentration of adenosine (Cunha, 2001). In fact, there is a tight relation between the levels of adenosine and the energy charge as it is expected that adenosine levels are amended by injurious stimuli. As a result, stimuli varying from elevated neuronal firing to hypoxia or ischemia can cause extracellular levels of adenosine increases (Cunha, 2005). A study conducted by our collaborator Dr. Susan Masino found that exogenous...
application of adenosine to KD fed hippocampal tissue resulted in less inhibition to fEPSPs and this suggests that tonic adenosine levels are raised. Together with our biochemical analyses it is likely that these elevated levels of adenosine are providing a neuroprotective effect through the A₁R.

We conclude that diet can dramatically affect neuronal function within hippocampus. In response to a high-fat, calorie-restricted diet, the hippocampus responds by inducing mitochondrial biogenesis, enhancing metabolic gene expression, and increasing energy reserves. We also observed increased energy reserves in other brain regions in seizure naïve rat. Our findings support an energy preservation hypothesis for the anticonvulsant effects of the KD, which might be particularly important for GABAergic interneurons. Because the enhanced ability of neurons to manage metabolic challenges after KD likely improves neuronal survival as well as function under stressful conditions, the benefits of dietary therapies such as KD might also be extended to the treatment of other neurodegenerative disorders such as Alzheimer’s or Parkinson’s diseases.
### Table 2: Dietary Constituents for Standard Rodent Chow (2018 Harlan-Teklad)

#### Proximate Analysis

<table>
<thead>
<tr>
<th>Component</th>
<th>%</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Protein</td>
<td>%</td>
<td>18.40</td>
</tr>
<tr>
<td>Crude Oil</td>
<td>%</td>
<td>6.00</td>
</tr>
<tr>
<td>Crude Fiber</td>
<td>%</td>
<td>2.50</td>
</tr>
<tr>
<td>Ash</td>
<td>%</td>
<td>5.90</td>
</tr>
<tr>
<td>NFE</td>
<td>%</td>
<td>55.80</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>%</td>
<td>57.33</td>
</tr>
<tr>
<td>Starch</td>
<td>%</td>
<td>41.24</td>
</tr>
<tr>
<td>Sugar</td>
<td>%</td>
<td>4.93</td>
</tr>
</tbody>
</table>

#### Fatty Acids

<table>
<thead>
<tr>
<th>Component</th>
<th>%</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Oil</td>
<td>%</td>
<td>6.00</td>
</tr>
<tr>
<td>Crude Protein</td>
<td>%</td>
<td>18.40</td>
</tr>
<tr>
<td>Crude Oil</td>
<td>%</td>
<td>6.00</td>
</tr>
<tr>
<td>Crude Fiber</td>
<td>%</td>
<td>2.50</td>
</tr>
<tr>
<td>Ash</td>
<td>%</td>
<td>5.90</td>
</tr>
<tr>
<td>NFE</td>
<td>%</td>
<td>55.80</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>%</td>
<td>57.33</td>
</tr>
<tr>
<td>Starch</td>
<td>%</td>
<td>41.24</td>
</tr>
<tr>
<td>Sugar</td>
<td>%</td>
<td>4.93</td>
</tr>
</tbody>
</table>

#### Amino Acids

<table>
<thead>
<tr>
<th>Component</th>
<th>%</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic Acid</td>
<td>%</td>
<td>1.42</td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>%</td>
<td>7.70</td>
</tr>
<tr>
<td>Asparagine</td>
<td>%</td>
<td>1.98</td>
</tr>
<tr>
<td>Glycine</td>
<td>%</td>
<td>0.79</td>
</tr>
<tr>
<td>Threonine</td>
<td>%</td>
<td>0.67</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>%</td>
<td>1.55</td>
</tr>
<tr>
<td>Lysine</td>
<td>%</td>
<td>0.97</td>
</tr>
<tr>
<td>Leucine</td>
<td>%</td>
<td>1.54</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>%</td>
<td>0.95</td>
</tr>
<tr>
<td>Valine</td>
<td>%</td>
<td>0.95</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>%</td>
<td>0.59</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>%</td>
<td>0.61</td>
</tr>
<tr>
<td>Phe + Tyr</td>
<td>%</td>
<td>1.60</td>
</tr>
<tr>
<td>Methionine</td>
<td>%</td>
<td>0.36</td>
</tr>
<tr>
<td>Cysteine</td>
<td>%</td>
<td>0.33</td>
</tr>
<tr>
<td>Met + Cyst</td>
<td>%</td>
<td>0.69</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>%</td>
<td>0.97</td>
</tr>
<tr>
<td>Histidine</td>
<td>%</td>
<td>0.47</td>
</tr>
<tr>
<td>Arginine</td>
<td>%</td>
<td>1.06</td>
</tr>
<tr>
<td>Cysteine</td>
<td>%</td>
<td>0.37</td>
</tr>
<tr>
<td>Available Lysine</td>
<td>%</td>
<td>0.83</td>
</tr>
</tbody>
</table>

#### Minerals

<table>
<thead>
<tr>
<th>Component</th>
<th>%</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium</td>
<td>%</td>
<td>0.61</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>%</td>
<td>0.55</td>
</tr>
<tr>
<td>Potassium</td>
<td>%</td>
<td>0.23</td>
</tr>
<tr>
<td>Iodine</td>
<td>%</td>
<td>0.07</td>
</tr>
<tr>
<td>Copper</td>
<td>mg/kg</td>
<td>2.21</td>
</tr>
<tr>
<td>Zinc</td>
<td>mg/kg</td>
<td>17.00</td>
</tr>
<tr>
<td>Manganese</td>
<td>mg/kg</td>
<td>118.00</td>
</tr>
<tr>
<td>Iron</td>
<td>mg/kg</td>
<td>22.90</td>
</tr>
<tr>
<td>Selenium</td>
<td>mg/kg</td>
<td>0.20</td>
</tr>
<tr>
<td>Cobalt</td>
<td>mg/kg</td>
<td>0.63</td>
</tr>
<tr>
<td>Chromium</td>
<td>mg/kg</td>
<td>0.53</td>
</tr>
</tbody>
</table>

#### Vitamins

<table>
<thead>
<tr>
<th>Component</th>
<th>%</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thiamine</td>
<td>mg/kg</td>
<td>11.55</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>mg/kg</td>
<td>14.00</td>
</tr>
<tr>
<td>Niacin</td>
<td>mg/kg</td>
<td>38.39</td>
</tr>
<tr>
<td>Pyridoxine</td>
<td>mg/kg</td>
<td>18.90</td>
</tr>
<tr>
<td>Pantothenicacid</td>
<td>mg/kg</td>
<td>33.00</td>
</tr>
<tr>
<td>Folate</td>
<td>mg/kg</td>
<td>3.34</td>
</tr>
<tr>
<td>Biotin</td>
<td>mg/kg</td>
<td>0.30</td>
</tr>
<tr>
<td>Vitamin B12</td>
<td>mg/kg</td>
<td>0.06</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>mg/kg</td>
<td>11.20</td>
</tr>
<tr>
<td>Chlorine</td>
<td>mg/kg</td>
<td>2.64</td>
</tr>
<tr>
<td>Inositol</td>
<td>mg/kg</td>
<td>14.55</td>
</tr>
</tbody>
</table>

**Saturated Fatty Acids**

- C:16:0 Palmitic: g/kg
- C:18:0 Stearic: g/kg
- C:20:0 Arachidonic: g/kg
- C:22:0 Dihomo-γ-linolenic: g/kg

**Monounsaturated Fatty Acids**

- C:16:1 Palmitoleic: g/kg
- C:18:1 Oleic: g/kg
- C:20:1 Elaidic: g/kg
- C:22:1 Elaidic: g/kg

**Polyunsaturated Fatty Acids**

- C:18:2 Linoleic: g/kg
- C:20:2 Eicosadienonic: g/kg
- C:20:3 Docosatrienonic: g/kg
- C:20:4 Docosahexanonic: g/kg

**Vitamins**

- Vitamin A: Retinol: mg/kg
- Vitamin D: Ergocalciferol: mg/kg
- Vitamin E: γ-Tocopherol: mg/kg
- Vitamin K: Menadione: mg/kg
- Vitamin B6: Pyridoxine: mg/kg
- Vitamin B12: Cyanocobalamine: mg/kg
- Folate: mg/kg
- Biotin: mg/kg
- Vitamin C: mg/kg
- Chlorine: mg/kg
- Inositol: mg/kg
Table 3: Dietary Constituents for Bio-Serv Ketogenic Diet (F3666 Bio-Serv).

<table>
<thead>
<tr>
<th>PROXIMATE PROFILE (%)</th>
<th>CALORIC PROFILE (Kcal/gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein 8.36</td>
<td>Protein 0.334</td>
</tr>
<tr>
<td>Carbohydrates 0.76</td>
<td>Fat 7.092</td>
</tr>
<tr>
<td>Fat 78.80</td>
<td>Carbohydrate 0.030</td>
</tr>
<tr>
<td>Ash 3.80</td>
<td>Total 7.456</td>
</tr>
<tr>
<td>Moisture &lt;5.00</td>
<td></td>
</tr>
</tbody>
</table>

AMINO ACID PROFILE (gm/kg)

| Alanine     | 2.26  |
| Arginine    | 3.01  |
| Aspartic Acid | 5.18  |
| Cystine     | 0.25  |
| Glutamic Acid | 16.30 |
| Glycine     | 2.01  |
| Histidin    | 2.26  |
| Isoleucin   | 4.43  |
| Leucin      | 6.69  |
| Lysolecine  | 5.04  |
| Methionine  | 0.43  |
| Phenylalanin | 3.68  |
| Proline     | 8.19  |
| Serine      | 4.60  |
| Threonine   | 3.60  |
| Tryptophan  | 1.25  |
| Tyrosine    | 4.60  |
| Valine      | 5.28  |

CARBOHYDRATE PROFILE (gm/kg)

| Monosaccharides | 7.60 |
| Disaccharides   | 0    |

FATTY ACID PROFILE (gm/kg)

| C4 Butanoic   | 4.59 |
| C6 Hexanoic   | 3.19 |
| C8 Octanoic   | 2.99 |
| C10 Decanoic  | 4.39 |
| C10:1 Decenoic | 0.80 |
| C12 Lauric    | 6.41 |
| C12-1 cis-9-Dodecenoic | 0.40 |
| C14 Myristic  | 24.43 |
| C14-1 cis-9-Tetradecenoic | 6.14 |
| C15 Pentadecanoic | 0.48 |
| Palmitic      | 189.03 |
| cis-9-Hexadecenoic | 28.18 |
| Heptadecanoic | 2.38 |
| Heptadecenoic | 1.43 |
| Stearin       | 84.79 |
| Oleic         | 298.34 |
| Linoleic      | 119.44 |
| Linolenic     | 6.33  |
| Eicosanoic    | 1.18  |
| cis-11-Eicosanoic | 3.80 |

MINERAL PROFILE (gm/kg)

| Aluminum | 0.00 |
| Calcium  | 5.60 |
| Chlorine | 1.68 |
| Copper   | 6.51 |
| Chromium | 3.85* |
| Iodine   | 0.22* |
| Iron     | 40.00 |
| Magnesium| 0.55 |
| Manganese|       |
| Manganese | 70.00 |
| Phosphorus| 4.32 |
| Potassium | 4.09 |
| Selenium | 0.12 |
| Sodium   | 1.13  |
| Sulfur   | 0.37  |
| Zinc     | 30.00 |

VITAMIN PROFILE (mg/kg)

| Ascorbic Acid        | --   |
| Biotin               | 0.42 |
| Cal Pantothenate     | 40.00|
| Choline              | 0.00 |
| Folic Acid           | 4.18 |
| Inositol             | --   |
| Menadione            | 2.09 |
| Niacin               | 60.00|
| Pyridoxine           | 14.63|
| Riboflavin           | 12.54|
| Thiamine             | 12.34|
| Vitamin A (IU/kg)    | 10450|
| Vitamin D₃ (IU/kg)   | 2090 |
| Vitamin B₁₂          | 0.02 |

164

Produced with permission of the copyright owner. Further reproduction prohibited without permission.
<table>
<thead>
<tr>
<th>Accession</th>
<th>Symbol</th>
<th>Log2 ratio (KD/Control)</th>
<th>Gene Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>CB584892</td>
<td>Acp1</td>
<td>0.34</td>
<td>Aspartylglucosaminidase</td>
</tr>
<tr>
<td>AA988365</td>
<td>Crmp1</td>
<td>0.35</td>
<td>Collapsin response mediator protein 1</td>
</tr>
<tr>
<td>J03367</td>
<td>Cyc2</td>
<td>0.53</td>
<td>Cytochrome P450, subfamily IC (mephenytoin 4-hydroxylase)</td>
</tr>
<tr>
<td>A104511</td>
<td>Dhrs_1</td>
<td>0.53</td>
<td>Dehydrogenase E1 and transketolase domain containing 1</td>
</tr>
<tr>
<td>A141270</td>
<td></td>
<td>0.38</td>
<td>Dehydrogenase/reductase (SDR family) X chromosome</td>
</tr>
<tr>
<td>AA943554</td>
<td>Gasm3</td>
<td>0.25</td>
<td>EPM2A (fakor) interacting protein 1</td>
</tr>
<tr>
<td>CB869436</td>
<td>Hamp</td>
<td>-0.55</td>
<td>Glutathione S-transferase, mu type 3</td>
</tr>
<tr>
<td>U06273</td>
<td>Ugp2b</td>
<td>-0.74</td>
<td>Heparin</td>
</tr>
<tr>
<td>Accession</td>
<td>Symbol</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>-----------</td>
<td>--------</td>
<td>-------------</td>
<td></td>
</tr>
<tr>
<td>CB325790</td>
<td>Psme2</td>
<td>Protease (prosome, macropain) 28 subunit, beta</td>
<td></td>
</tr>
<tr>
<td>CR465648</td>
<td>Psmd7_pr</td>
<td>Proteasome (prosome, macropain) 26S subunit, non-ATPase, 7</td>
<td></td>
</tr>
<tr>
<td>AA071204</td>
<td>Psma2</td>
<td>Proteasome (prosome, macropain) subunit, alpha type 2</td>
<td></td>
</tr>
<tr>
<td>BG007906</td>
<td>Psma5</td>
<td>Proteasome (prosome, macropain) subunit, alpha type 5</td>
<td></td>
</tr>
<tr>
<td>CX455954</td>
<td>Psmb1</td>
<td>Proteasome (prosome, macropain) subunit, beta type 1</td>
<td></td>
</tr>
<tr>
<td>AI172162</td>
<td>Psmb4</td>
<td>Proteasome (prosome, macropain) subunit, beta type 4</td>
<td></td>
</tr>
<tr>
<td>BI755970</td>
<td>Rba600</td>
<td>Retinoblastoma-associated factor 600</td>
<td></td>
</tr>
<tr>
<td>CB334548</td>
<td>Rips2</td>
<td>Ribophorin II</td>
<td></td>
</tr>
<tr>
<td>AI177765</td>
<td>Ref146_pr</td>
<td>Ring finger protein 146</td>
<td></td>
</tr>
<tr>
<td>A011368</td>
<td>—</td>
<td>Ring finger protein 44</td>
<td></td>
</tr>
<tr>
<td>CR1466026</td>
<td>—</td>
<td>Ring finger protein 8</td>
<td></td>
</tr>
<tr>
<td>CF975601</td>
<td>Skp1a</td>
<td>S-phase kinase-associated protein 1A</td>
<td></td>
</tr>
<tr>
<td>CR442274</td>
<td>Pbox7_pr</td>
<td>strongly similar to XP_576203.1 F-box only protein 7</td>
<td></td>
</tr>
<tr>
<td>A253460</td>
<td>—</td>
<td>Transcribed box</td>
<td></td>
</tr>
<tr>
<td>BM391877</td>
<td>Prss15</td>
<td>Transcribed box</td>
<td></td>
</tr>
<tr>
<td>CB612042</td>
<td>Ubch1</td>
<td>Ubiquitin carboxy-terminal hydrolase L1</td>
<td></td>
</tr>
<tr>
<td>CB327486</td>
<td>—</td>
<td>Ubiquitin specific protease 24</td>
<td></td>
</tr>
<tr>
<td>A412007</td>
<td>Usp5_pr</td>
<td>Ubiquitin-specific protease 5 (isopeptidase T)</td>
<td></td>
</tr>
<tr>
<td>A117722</td>
<td>—</td>
<td>Ubiquitin-conjugating enzyme E2E 2 (UBC4/5 homolog, yeast)</td>
<td></td>
</tr>
<tr>
<td>BC086980</td>
<td>Ube2g1</td>
<td>Ubiquitin-conjugating enzyme E2G 1 (UBC7 homolog, C. elegans)</td>
<td></td>
</tr>
<tr>
<td>CN542760</td>
<td>Vcp</td>
<td>Valosin-containing protein</td>
<td></td>
</tr>
<tr>
<td>CB3357665</td>
<td>Pick2</td>
<td>WW domain binding protein 4</td>
<td></td>
</tr>
</tbody>
</table>

Table 5: List of ADSL Mutations.

<table>
<thead>
<tr>
<th>Region</th>
<th>Base Change</th>
<th>Amino Acid Change</th>
<th>Prediction Size</th>
<th>Number of Histone Polymers</th>
<th>Number of Compound Heterozygotes</th>
<th>SA/AC Ratio</th>
<th>Marital Retardation</th>
<th>Country of Origin</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1</td>
<td>c.34A&gt;T</td>
<td>M3I</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1.000</td>
<td>Yes</td>
<td>USA</td>
<td>Rana et al., 2003</td>
</tr>
<tr>
<td></td>
<td>c.100C&gt;T</td>
<td>A33V</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1.000</td>
<td>Yes</td>
<td>USA</td>
<td>Rana et al., 2003</td>
</tr>
<tr>
<td></td>
<td>c.165C&gt;T</td>
<td>M55L</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1.000</td>
<td>Yes</td>
<td>USA</td>
<td>Rana et al., 2003</td>
</tr>
<tr>
<td></td>
<td>c.247T&gt;C</td>
<td>Y82H</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1.000</td>
<td>Yes</td>
<td>USA</td>
<td>Rana et al., 2003</td>
</tr>
<tr>
<td></td>
<td>c.423C&gt;T</td>
<td>R141W</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1.000</td>
<td>Yes</td>
<td>USA</td>
<td>Rana et al., 2003</td>
</tr>
<tr>
<td></td>
<td>c.519A&gt;G</td>
<td>R173G</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1.000</td>
<td>Yes</td>
<td>USA</td>
<td>Rana et al., 2003</td>
</tr>
<tr>
<td></td>
<td>c.602C&gt;T</td>
<td>R194C</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1.000</td>
<td>Yes</td>
<td>USA</td>
<td>Rana et al., 2003</td>
</tr>
<tr>
<td></td>
<td>c.629A&gt;C</td>
<td>K210R</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1.000</td>
<td>Yes</td>
<td>USA</td>
<td>Rana et al., 2003</td>
</tr>
<tr>
<td></td>
<td>c.735A&gt;G</td>
<td>K245E</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1.000</td>
<td>Yes</td>
<td>USA</td>
<td>Rana et al., 2003</td>
</tr>
<tr>
<td></td>
<td>c.904G&gt;A</td>
<td>D304H</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1.000</td>
<td>Yes</td>
<td>USA</td>
<td>Rana et al., 2003</td>
</tr>
<tr>
<td>E12</td>
<td>c.123G&gt;T</td>
<td>D41K</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1.000</td>
<td>Yes</td>
<td>USA</td>
<td>Rana et al., 2003</td>
</tr>
<tr>
<td></td>
<td>c.1271G&gt;A</td>
<td>R423H</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1.000</td>
<td>Yes</td>
<td>USA</td>
<td>Rana et al., 2003</td>
</tr>
</tbody>
</table>

Base and amino acid numbers corresponding to full length nucleotide sequence of human ADSL cDNA deposited in the EMBL database under accession number X5687 by E.A. Fon. * Denotes siblings, ** Denotes splice site.
Table 6: Genes on Chromosome 21 Important for Mitochondrial Metabolism

<table>
<thead>
<tr>
<th>Gene</th>
<th>In Ts65Dn</th>
<th>Mitochondrial</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Mitochondrial ribosomal protein L39</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>2. Mitochondrial coupling factor 6 (ATP synthase F0 subunit 6)</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>3. Nuclear respiratory factor</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>4. APP</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>5. Bach1</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>6. GART</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>7. SOD1</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>8. NADPH quinine reductase-like</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>9. ATP synthase OSCP unit</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>10. Mitochondrial ribosomal protein S6</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>11. Calcipressin 1</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>12. Carbonyl reductase 1 (NADPH dependent oxidoreductase)</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>13. Carbonyl reductase 3 (NADPH dependent oxidoreductase)</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>14. Thioredoxin-like protein (SH3BGR)</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>15. Mitochondrial NADH oxidoreductase</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>16. CBS</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>17. AMP protein kinase-like (SNF1LK)</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>18. C21orf2 mitochondrial protein</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>

All 18 genes listed are involved in mitochondrial metabolism and are located on chromosome 21 with all of these genes expressed in the mouse and the first 14 trisomic in the Ts65Dn mouse (Akeson et al., 2001; Gardiner et al., 2002; Gitton et al., 2002; Reymond et al., 2002a; Reymond et al., 2002b).
REFERENCES


Barborka, C.J. (1930). Epilepsy in adults - Results of treatment by ketogenic diet in 100 hundred cases. Arch Neurol 38, 905-914.


Produced with permission of the copyright owner. Further reproduction prohibited without permission.


Produced with permission of the copyright owner. Further reproduction prohibited without permission.


Produced with permission of the copyright owner. Further reproduction prohibited without permission.
Hirai, K., Aliev, G., Nunomura, A., Fujioka, H., Russell, R.L., Atwood, C.,
Johnson, A.B., Kress, Y., Vinters, H.V., Tabaton, M., Shimohama, S.,
Cash, A.D., Siedlak, S.L., Harris, P.L.R., Jones, P.K., Petersen, R.B.,

Hofman, F.M., Dohadwala, M.M., Wright, A.D., Hinton, D.R., Walker S. M.
(1994). Exogenous tat protein activates central nervous system-derived

Holtzman, D., Khait, I., Mulkern, R., Allred, E., Rand, T., Jensen, F., Kraft, R.
(1999). In vivo development of brain phosphocreatine in normal and

Holtzman, D., Meyers, R., O'Gorman, E., Khait, I., Wallimann, T., Allred,El,
Jensen F. (1997). In vivo brain phosphocreatine and ATP regulation in

Holtzman, D.M., Santucci, D., Kilbridge, J., Chua-Couzens, J., Fontana, D.J.,
Daniels, S.E., Johnson, R.M., Chen, K., Sun, Y., Carlson, E., Alleva, E.,
Epstein, C.J., Mobley, W.C. (1996). Developmental abnormalities and
age-related neurodegeneration in a mouse model of Down syndrome.
Proc Natl Acad Sci USA 93, 13333-13338.

Hulstaert, F., Blennow, K., Ivanoiu, A., Schoonderwaldt, H.C., Riemenschneider,
M., De Deyn, P.P., Bancher, C., Cras, P., Wiltfang, J., Mehta, P.D., Iqbal,
discrimination of AD patients using beta-amyloid (1-42) and tau levels in
CSF. Neurology 52, 1555-1562.

learning in Ts65Dn mice that model Down syndrome and Alzheimer's

syndrome, have deficits in context discrimination learning suggesting
impaired hippocampal function. Behav Brain Res 118, 53-60.

Iannello, R.C., Crack, P.J., de Haan, J.B., Kola, I. (1999). Oxidative stress and
neural dysfunction in Down syndrome. J Neural Transm Suppl 57, 257-
267.

Ide, T., Steinke, J., Cahill, G.F. Jr. (1969). Metabolic interactions of glucose,
lactate, and beta-hydroxybutyrate in rat brain slices. Am J Physiol 217,
784-792.


produced with permission of the copyright owner. Further reproduction prohibited without permission.


193


Petronilli, V., Miotto, G., Canton, M., Brini, M., Colonna, R., Bernardi, P., Di Lisa, F. (1999). Transient and long-lasting openings of the mitochondrial permeability transition pore can be monitored directly in intact cells by changes in mitochondrial calcein fluorescence. Biophys J 76, 725-734.


Produced with permission of the copyright owner. Further reproduction prohibited without permission.


