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Determination of Catecholamine Content Changes in Mouse Brain Following Chronic Ketogenic Diet

Ryan Adams

Trinity College, ryan.adams@trincoll.edu

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Determination of Catecholamine Content Changes in Mouse Brain Following Chronic Ketogenic Diet

Ryan Adams

Trinity College, Hartford

Research Advisor: Dr. William Church

Second Reader: Hebe Guardiola-Diaz

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Abstract:

This work investigated the effects of the ketogenic diet on catecholamine concentrations in the brains of mice. The ketogenic diet has been shown to modulate the catecholamine concentrations in the cerebrospinal fluid of children as well as altering hippocampal norepinephrine levels in mice. Tissue homogenates of the motor cortex, somatosensory cortex, nucleus accumbens, anterior caudate, posterior caudate and midbrain regions were analyzed using high pressure liquid chromatography to quantify norepinephrine, dopamine, 3, 4-dihydroxyphenylacetic acid (DOPAC), 5-hydroxyindoleacetic acid (5HIAA), homovanillic acid (HVA) and serotonin (5-HT). No significant differences in catecholamine concentration levels were detected in the tissue homogenates. Analysis of metabolite to catecholamine levels indicated a significant difference in the DOPAC/Dopamine in the motor cortex ($p < 0.05$) and midbrain ($p < 0.05$). A significant correlation between neurotransmitter content and beta-Hydroxybutyric acid (BHB) blood levels was seen in several brain regions. While the results of this experiment do not correlate with previous research pertaining to changes in the global CNS catecholamine levels, it is the first to examine catecholamine concentrations in localized brain regions for mice on a chronic KD.

Introduction:

Advancements in identifying the chemical mechanisms underlying behavioral activity have allowed scientists to correlate individual brain nuclei with specific behavioral functions. Targeted stimulation, inhibition or pharmacological intervention to these brain regions can produce noticeable behavioral and metabolic effects. In some instances the exact mechanisms underlying the therapeutic effect are not understood and theoretical models must be established to provide some degree of understanding. The undefined correlation between the noticeable therapeutic effect and changes occurring at the cellular level can be seen in the ketogenic diet.

The ketogenic diet is a high fat, low protein and carbohydrate diet which has been utilized as a non-pharmacological alternative for individuals suffering from drug resistant epilepsy (Szot, 2001; Bough, 2007). A resurgence in research has begun in the past few years concerning the diet's efficacy not only in mitigating epileptic activity, but alleviating several behavioral aspects associated with autism. While the exact mechanism by which the ketogenic diet induces its therapeutic effect remains unclear, recent literature implicates the catecholamine class of neurotransmitters as being directly involved (Weinshenker, 2008; Sharopov, 2012; Dahlin, 2011). In order to examine the effects of the ketogenic diet on catecholamine levels, we examined the concentrations of norepinephrine, dopamine, DOPAC, 5HIAA, HVA and 5HT in the brains of mice on a three week chronic ketogenic diet. The endogenous levels of these neurotransmitters were quantified in the motor cortex, somatosensory cortex, nucleus accumbens, anterior and posterior caudate putamen and substantia nigra utilizing high pressure liquid chromatography in order to identify the mechanisms by which the ketogenic diet mitigates epileptic activity and alleviates autistic behaviors.

Epilepsy and the Ketogenic Diet:

Epilepsy is a neurological disorder characterized by recurrent episodes of convulsive seizures, loss of consciousness, sensory disturbances and abnormal behavior (Connelly 2004). Individuals who suffer from epilepsy may experience these symptoms individually or concurrently in the span of an epileptic episode. Several causes for the development of epileptic episodes have been identified such as photosensitivity, neurodegeneration, and lesions induced by brain trauma or surgery. Although there are many classifications for epilepsy, the majority are induced by uncontrolled electrical discharges from neurons located in the cerebral cortex (Chowdhury, 2013). The magnitude and duration of these electrical discharges directly determines the type of

epileptiform activity present in the individual. These uncontrolled discharges have traditionally been classified as either generalized or focal seizures based upon clinical and electroencephalographic criteria (Alarcon, 2012). Generalized seizures emerge simultaneously from both cerebral hemispheres and affect the majority of the cerebral cortex at once. Individuals suffering from a generalized seizure will experience a loss of consciousness in conjunction with muscular contractions and jerking. Focal seizures are generated in a single hemisphere from a localized region of the cerebral cortex. The effects of focal seizures are determined by the cortical foci from which they originate and have the ability to impair consciousness, awareness and memory.

Therapeutic strategies such as the ketogenic diet have allowed individuals suffering from recurrent seizure activity to greatly reduce its occurrence in everyday life (Marsh, 2006; Bough, 2007; Dahlin, 2005). The ketogenic diet was originally developed in 1921 by scientist and physicians trying to study the effect of ketonaemia on the brain (Wilder, 1921). It was discovered that a high fat, low protein, low carbohydrate diet regime forced the body to metabolize fat as its main energy source. The metabolism of fats leads to the production of acetoacetic acid, 3-hydroxybutyric acid and acetone ketone bodies. When the ketone bodies were present in the circulatory system, they began to elicit a sedative effect, similar to that seen in anesthesia (Sirven, 1999).

One longitudinal study examined the efficacy of the ketogenic diet for alleviating epileptic activity over the course of 3, 6 and 12 months after initiating the diet as well as a 3 to 6 year follow up. The study focused on 150 individuals who exhibited an average of 410 seizures per month and failed to improve on a mean of 6.2 medications. Twelve months after initiating the diet, 7% of the children exhibited a 100% reduction in their seizures. Another 20% of those

individuals had 90% alleviation in their seizures frequency. The alleviation of seizure frequency continued past the 12 month mark. In the 3 to 6 year follow ups, 27% of these same children had few or no seizures (Hemingway, 2001).

Although the exact mechanism by which the ketogenic diet reduces epileptic activity remains unknown, several studies have pointed to changes occurring at the cellular level (Voskuyl, 2001; Cullingford, 2008; Bough 2006; Kim do, 2007). One hypothesis implicates a gradual change in the metabolism of energy based upon lipids as a fuel source. As fats become the primary source of energy in the body, the concentrations of polyunsaturated fatty acids begins to increase. These polyunsaturated fatty acids are believed to possess specific neuroprotective properties. Polyunsaturated fatty acids may bind to neuronal membranes and block the voltage gated sodium and calcium channels, thus reducing the excitability of the neuron (Voskuyl, 2001). They are also believed to reduce inflammation through activation of peroxisome proliferator activated receptors located on the cell membrane (Cullingford, 2008). It is also believed that polyunsaturated fatty acids reduce the production of reactive oxygen species through the activation of mitochondrial uncoupling proteins (Bough, 2006). The ketone bodies themselves significantly decrease reactive oxygen species (ROS) levels in dissociated neurons and in isolated neocortical mitochondria through an inhibition in mitochondrial permeability transition (Kim, 2007). This inhibition of mitochondrial permeability transition (mPT) arises from an increase in the threshold for calcium-induced excitation seen in the presence of ketone bodies. This ultimately leads to a decrease in mitochondrial ROS production during periods of neuronal excitability.

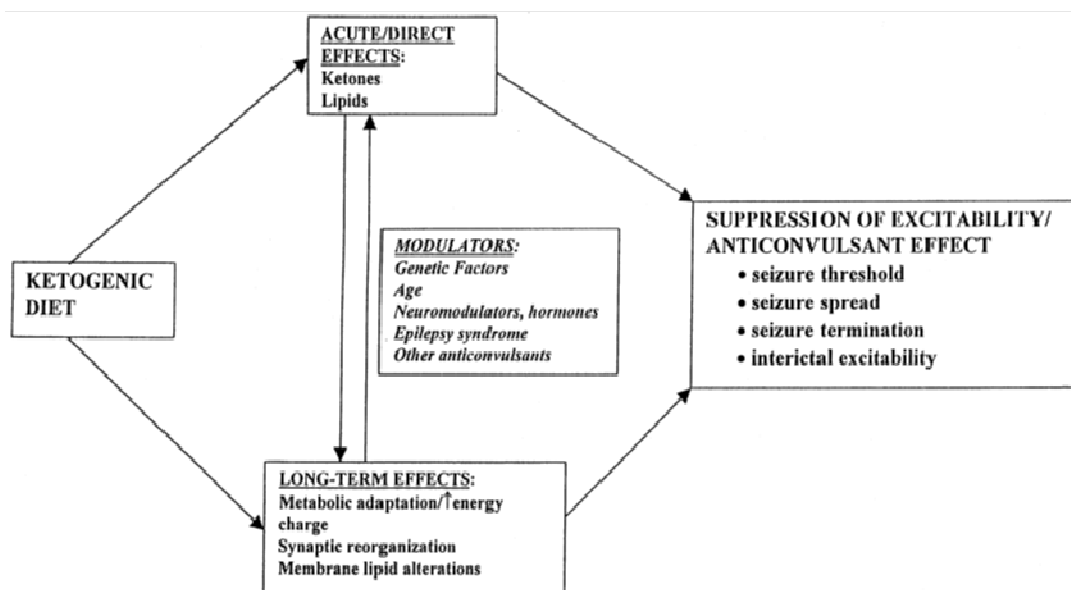


Figure 1: Dynamic model of interacting factors influencing the ability of the ketogenic diet to suppress neuronal hyper excitability and seizures. Acute and long-term factors play a role in alleviating epileptic symptoms, through several modulating factors (Stafstrom, 1999).

The ketogenic diet has recently been shown to alter the concentrations of several key amino acids. Highly significant changes were found in eight amino acids after a 4 month chronic ketogenic diet (Dahlin, 2005). A significant increase in GABA, taurine, serine, and glycine concentrations was seen in the cerebral spinal fluid of 26 children. A significant decrease in asparagine, alanine, tyrosine and phenylalanine was also noted in these individuals. However, the CSF concentrations of aspartate, glutamate, arginine, threonine, citrulline, leucine, isoleucine and valine/methionine all remained unchanged during the diet. It was also seen that higher GABA levels (Pre KD: $3.33 \pm 1.57 \mu\text{mol/L}$ and Post KD: $3.71 \pm 1.66 \mu\text{mol/L}$) were correlated to a greater reduction in epileptic activity. An increase in the inhibitory amino acid glycine (Pre KD: $6.9 \pm 1.4 \mu\text{mol/L}$ and Post KD: $8.9 \pm 2.8 \mu\text{mol/L}$) may also play a role in reducing neuronal excitability and overall epileptic activity. These changes in amino acid levels seen pre and post ketogenic diet indicate that a fluctuation may occur in the production of neurotransmitters in the central nervous system due to a reduction of available precursors. Recent literature indicates that

the ketogenic diet may influence the production of the catecholamine class of neurotransmitters in the central nervous system (Weinshenker, 2008; Sharopov, 2012; Dahlin, 2011).

It was found that norepinephrine functions as an anticonvulsant when there is specific stimulation of the locus ceruleus or if noradrenergic agonists are administered (Weinshenker 2008). It was discovered that mice that were fed a strict ketogenic diet regimen produced two times as much extracellular norepinephrine in the hippocampus than mice just fed a normal diet. It was concluded that norepinephrine contributes to a basal level of inhibition on the epileptic circuits in the brain and allows other anticonvulsant treatments to take effect (Weinshenker 2008).

Several other experiments examined the role of norepinephrine in the efficacy of the ketogenic diet. It was seen that selective damage to the locus ceruleus enhances occasional seizures into self-sustaining status epilepticus (SSSE) in rats (Giorgi, 2004). In order to test whether an intact norepinephrine system was directly involved in the efficacy of the ketogenic diet, dopamine β -hydroxylase knockout ($Dhb^{-/-}$) mice were placed on a chronic two-week diet. The results of this experiment show that these knockout mice do not exhibit an increased resistance to flurothyl seizures when treated with a ketogenic diet (Szot, 2001). These results suggest norepinephrine concentrations mediate the therapeutic effect of the ketogenic diet.

Dopamine has also been implicated in modulating epileptiform activity in the intact hippocampus of newborn mice *in vitro*. One study examined the *in vitro* effects of dopamine agonists and antagonists on the isolated cortico-hippocampal formation of immature (postnatal days 3 and 4) mice using field potential recordings from CA3. In order to induce epileptiform discharges, the extracellular concentration of Mg^{2+} was reduced to 0.2 mM. The results of this experiment showed that dopamine in concentrations less than 0.3 μ M mitigated epileptiform

activity whereas dopamine in excess of 3.0 μM exacerbated epileptiform activity. The mitigation or exacerbation of epileptiform activity was dopamine receptor mediated. Administering a D1 receptor agonist promoted a strong pro-convulsive effect whereas a D1 receptor antagonist canceled the pro-convulsive response. It was also seen that the D2 receptor agonist quinpirole exhibited a weak anticonvulsant effect. However, this anticonvulsant response was inhibited through D1, D2 and D3 receptor antagonists (Sharopov, 2012). These results indicate that the binding affinity of dopamine to specific receptors is critical in modulating seizure susceptibility. It may be possible that the efficacy of the ketogenic diet stems from a modulation of the dopamine binding affinity to specific receptors not only the hippocampus but other brain regions as well.

Recent research examined whether the concentrations of norepinephrine, dopamine and 5-HT were affected in children with epilepsy. Both the monoamines and their metabolites were examined in the cerebrospinal fluid prior and post administration of the ketogenic diet. During treatment with the ketogenic diet, five children had a 100% seizure reduction and another five had a 75—99% reduction. In all, 17 children had a >50% seizure reduction and 9 had a less than 50% reduction (Dahlin, 2011). Statistical analysis of the cerebrospinal fluid indicated that HVA and 5HIAA (metabolites of DA and 5HT, respectively) were significantly reduced during treatment with the ketogenic diet. Homovanillic acid (HVA) concentrations were reduced from 409.8 ± 134.8 nmol/ pre ketogenic diet to 341.7 ± 127.1 nmol/L post ketogenic diet. 5-HIAA concentrations were reduced from 158.3 ± 67.8 nmol/L pre ketogenic diet to 137.3 ± 47.2 nmol/L post ketogenic diet. Although there was evidence pointing to a difference in metabolite concentrations for dopamine and serotonin, no significant difference was seen between the diet and non-diet groups pertaining to the concentrations of dopamine and serotonin. The

concentration of norepinephrine was also unchanged between the diet and non-diet groups. It was believed that the concentration changes observed during treatment were part of the many changes taking place at the cellular level which contributed to the effects of the diet. It may be possible that small changes in the monoamine concentrations may play a role in other mechanisms by which the ketogenic diet exerts its effect (Dahlin, 2011).

Alleviation of Autistic Behavior through the Ketogenic Diet:

While the efficacy of the ketogenic diet has been established for individuals suffering from epileptiform activity, new research has connected the diet to the alleviation of autistic behaviors. Autism is a neurodevelopmental disorder which induced through abnormal biology and chemistry in the central nervous system. This central nervous system dysfunction leads to improper development of interpersonal communication, social and environmental engagement skills in the individual (Myers, 2007). Autistic individuals also exhibit symptoms akin to obsession compulsive like behaviors and rituals. A high level of repetition in daily behaviors and specific motor activities has been seen for individuals afflicted with Autism.

A recent study investigated the efficacy of the ketogenic diet in alleviating autistic behaviors for an isolated population of autistic individuals on the island of Crete. Of the original 30 participants in the study, 18 were able to tolerate the diet for a period of 6 months (4 weeks chronic diet interrupted by 2 weeks non-diet). Participants were rated on the Childhood Autism Rating Scale prior to initiation of the diet. Upon completion of the 6 month routine, significant improvement (> 12 units of the Childhood Autism Rating Scale) was recorded in two patients, average improvement (> 8–12 units) was noted in eight patients, and minor improvement (2–8 units) was seen in the remaining eight patients (Evangelidou, 2003). These results indicate that the

ketogenic diet is capable of inducing changes in the brains metabolism to alleviate the degree of Autistic behaviors present in these individuals.

Several brain regions such as the cerebellum, medial temporal structures and prefrontal cortex have been linked to the noticeable behavioral dysfunctions demonstrated by autistic individuals. It is also believed that dysfunction in one brain region during the early stages of development will likely affect the development and function of related pathways (Dawson, 1998). Functional magnetic resonance imaging has shown an increased volume of the caudate nucleus during maturation in autism ($7.83 \pm 1.03 \text{ cm}^3$), while it decreased in control subjects ($7.61 \pm 0.89 \text{ cm}^3$) (Langen, 2009). Due to the structural abnormalities which are present in autistic individuals, it can be inferred that the neurochemical composition of these brain regions are abnormal when compared to normal individuals (Martineau, 2008; Langen, 2011; Stall, 2012; Kane, 2012).

In order to further understand the mechanism by which the ketogenic diet alleviates autistic behaviors, the differences in catecholamine concentration in diet and non-diet groups must be identified. Recent literature has suggested that norepinephrine, dopamine and 5-HT mediate the behavioral disturbances seen in autism. In order to examine whether these neurotransmitters were altered in autistic individuals, the endogenous levels of dopamine, HVA, DOPAC, 3-methoxytyramine (3MT), norepinephrine, epinephrine, serotonin and 5HIAA were determined from the urine of 156 autistic children between the ages of 2 and 12.5 (Martineau, 2008). The results of this experiment indicate that individuals with autism embody higher levels of HVA and 5-HT in conjunction with decreased levels of dopamine. It was also noted that both norepinephrine and the dopamine metabolite 3-methoxytyramine were increased in individuals who had autism compared to normal individuals.

Dopamine has recently been implicated in mediating repetitive behaviors exhibited by autistic individuals. A significant increase in dopaminergic activity in the basal ganglia region, specifically the caudate nucleus and striatum, has been seen in autistic individuals (Turner, 2006; Hollander, 2005). These regions are innervated by the dopamine producing cells of the substantia nigra through the nigrostriatal pathway. The noticeable decrease in dopamine levels seen in the urine of autistic individuals indicates that there is some sort of dysregulation occurring in its activity at the cellular level or that dopamine is being metabolized faster outside the CNS. A dysfunction of the dopamine-3 receptor gene was noted in the pre-frontal striatal circuitry and led to an increase in impulsivity behaviors in individuals with autism (Staal, 2012).

Another neurotransmitter which has recently been linked to autistic behaviors is serotonin (Kane, 2012). Kane et al, showed that mice depleted of serotonin via the genetic knockout of tryptophan hydroxylase 2 (TPH2) exhibited substantial deficits in numerous validated tests of social interaction and communication as well as highly repetitive and compulsive behaviors. These mice were also seen to have developmental retardation of a number of somatosensory reflexes and developmental milestones which are related to proper central nervous system function.

Taken together, these studies suggest that a global, multi-neurotransmitter dysregulation may be associated with autistic behavior. It is possible that the behavioral, social and communicative dysfunctions noted in autistic individuals result from a combination of multiple catecholamine and monoamine abnormalities. A restoration of normal behavior seen in autistic individuals treated with the ketogenic diet may indicate that the ketogenic diet has an effect on the catecholamine system.

Methods:

Mouse Dietary Treatment:

A cohort of 18, CD1 male mice was utilized in this study (Table 2). Prior to brain extraction at 8 weeks of age, mice were placed on either a three week chronic ketogenic diet (Bioserve, #F3666) or control diet (Constant Nutrition #5001)(Table 1). The mice were deeply anesthetized with isoflurane and decapitated utilizing dissection scissors.

Amino Acid	Ketogenic Diet (g/kg)	Control Diet (g/kg)
Alanine	2.3	14.30
Arginine	3.1	14.10
Aspartic Acid	5.5	28.10
Cystine	0.3	3.10
Glutamic Acid	17.3	43.70
Glycine	2.1	12.10
Histidine	2.3	5.70
Isoleucine	4.7	11.40
Leucine	7.1	18.30
Lysine	6.3	14.10
Methionine	2.2	6.70
Phenylalanine	3.8	10.40
Proline	8.7	14.90
Serine	4.8	11.90
Threonine	3.7	9.10
Tryptophan	1	2.90
Tyrosine	4.8	7.10
Valine	5.5	11.70

Table 1: Amino acid dietary concentrations (g/kg) for mice administered the ketogenic diet and normal diet. Values represent grams of amino acid per kilogram of food.

Brain Dissection Guidelines:

Bilateral tissue punches were acquired from the motor cortex (Figure 1A), somatosensory cortex, nucleus accumbens, anterior caudate (Figure 1B), posterior caudate (Figure 1C) and the midbrain (Figure 1D) utilizing a microtome instrument. A mouse brain atlas was used in conjunction with the microtome to properly identify the desired brain regions

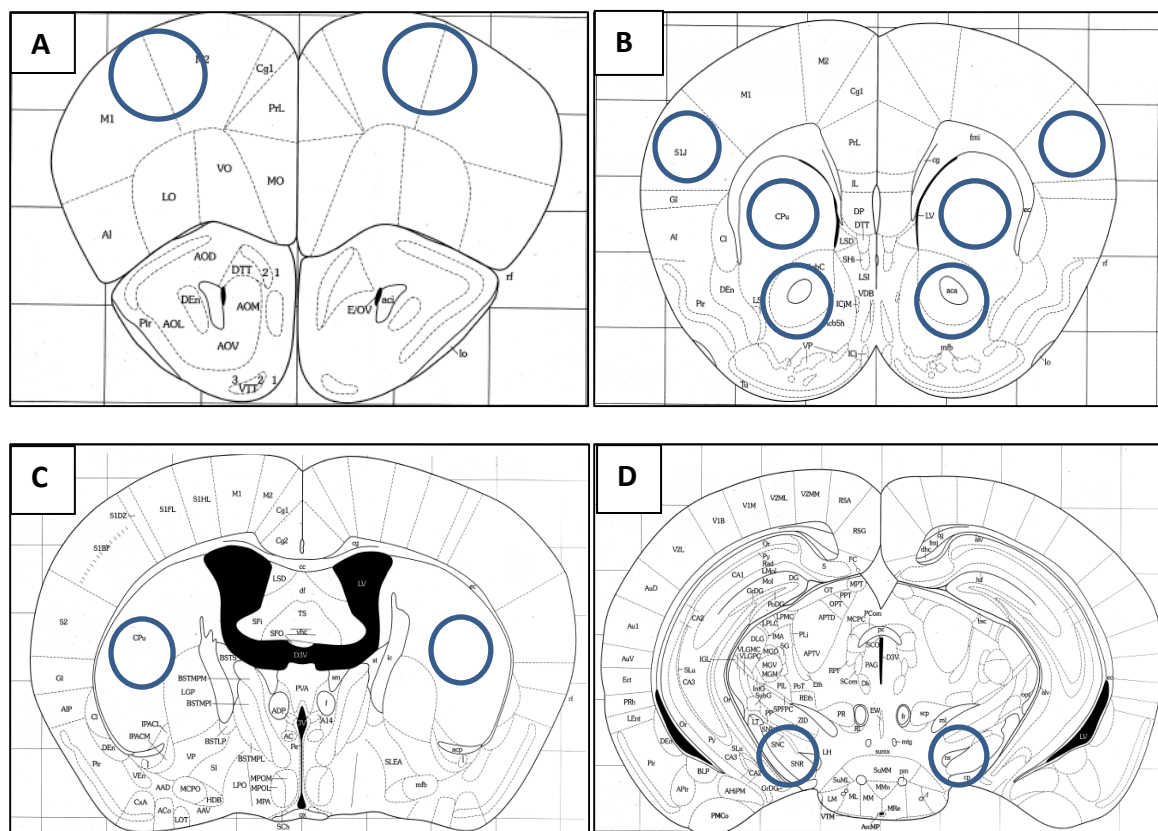


Figure 2: Stereotaxic Depiction of Tissue Punch Locations. (A) Motor Cortex, (B) Somatosensory Cortex, Anterior Caudate and Nucleus Accumbens, (C) Posterior Caudate, (D) Midbrain

Tissue Punch Processing:

Tissue samples were deposited into individual 2mL centrifugal tubes containing an ethanol tissue homogenizing internal standard solution (400 μ L). Tissue punch samples were then sonicated and placed on ice. Subsequent to tissue disruption, samples were centrifuged at 12000xg for a period of 10 minutes. The resulting supernatant was then filtered through a 0.2 μ m nylon disposable syringe into individual centrifuge vials. TH-IS solution was then evaporated off using a centrifuge rotovap at 45°C. Samples were then reconstituted in 1.00 mL of phosphate buffer and stored in a -80° C refrigerator until HPLC analysis.

Protein Determination using UV Spectrophotometry:

Protein content of tissue punches was determined by the Modified Lowry Protein Assay. Pellets were dissolved in 1.0 M NaOH. The samples were then sonicated for 5 seconds in order to dissolve the sample into the NaOH solution. Post pellet disruption, 200 μ L was pipetted out and added to 1000 μ L of MLPAR (Lowry reagent). The samples were then left to stand for 10 minutes before 1M foline (100 μ L) was added into the centrifugal vial. After 30 minutes, 1300 μ L of solution was removed from the centrifuge vial and analyzed at 730nm in order to quantify the protein concentration.

HPLC Solution Preparation:***Tissue Homogenizing Internal Standard (TH-IS) Solution***

Tissue punches (1.0 mm thick 1.0 mm diameter) were homogenized using a solution containing DHBA (0.0107 g) in Antioxidant Solution (100 mL). 10 μ L was extracted from the stock solution and diluted utilizing ethanol (10mL) to produce the final homogenizing solution.

Antioxidant Stock Solution (AOS):

Sodium metabisulfite (1.0015 g), Ethylenediaminetetraacetic acid (0.2089 g) and 6M HCL (1670 μ L) were diluted in a volumetric flask up to 1000 mL with DiH₂O.

Catecholamine Stock Solutions:

Calibration mixtures of the neurochemicals were prepared from stock solutions. Norepinephrine (0.0096g), Dopamine (0.0115g), 3, 4-Dihydroxyphenylacetic acid (0.0116g), 5-Hydroxyindoleacetic acid (0.0103g), Homovanillic acid (0.0131g) and Serotonin (0.0101g) were added to individual containers and dissolved with Antioxidant Stock solution (100mL).

Sodium Phosphate (NaH₂PO₄) Buffer:

Sodium phosphate (10.7982g) was dissolved in 1000mL DiH₂O

Catecholamine Calibration Curve Construction:

A series of five control standards varying in catecholamine stock volumes were utilized to develop a calibration curve which could be used to determine catecholamine concentrations (nanograms) related to the tissue punches. Catecholamine stock solutions (20μL) were diluted with Antioxidant Stock (980μL) to produce a concentration relative to that found in the tissue punches. Individual control standards were prepared from the diluted catecholamine stock solutions (0.5μL, 1.0μL, 2.0μL, 5.0μL and 10.0μL) and diluted with 400μL TH-IS solution. Standards were then rotovaped along with brain samples acquired on the same day.

HPLC Analysis of Brain Tissue:

The individual supernatant samples were analyzed using high pressure liquid chromatography. The samples were then filtered with 0.2 μm disposable nylon syringe filters into autosampler vials for analysis. Brain tissue samples were kept at 4°C while in the autosampler.

The concentrations of 3-hydroxytyramine (Dopamine) and its metabolite, 3, 4-dihydroxyphenylacetic acid (DOPAC) as well as Arterenol (Norepinephrine), 5-hydroxytryptamine (Serotonin), 5-Hydroxyindoleacetic acid (5-HIAA) and Homovanillic acid (HVA) were analyzed through electrochemical and UV detectors. Flow rate was kept constant at 0.5 mL/min throughout the sequence. Electrochemical detectors were set at -150 mv and +300 mv with a range of 2μA and 5μA respectively. A guard cell potential was set at +350 mv. UV detection was obtained with set values for lambda at 245 and 280 nm.

An acetonitrile, phosphate buffer and ion pairing agent mobile phase (MD-TM) was acquired from Thermo Scientific in 2 liter quantities and was used for the duration of the HPLC analysis. EZ start software was utilized to quantify peak retention time and area for compounds depicted on the chromatogram. A specific sequence order was constructed in order to provide reliable HPLC data. The five control standards were run in order to construct a reliable calibration curve. Subsequent to the calibration run, a blank solution (TH-IS only) was utilized to clear residual neurotransmitters from the column. The remainder of the sequence consisted of alternating brain samples with blank runs. The end of the sequence consisted of five control standards interspersed between brain samples in order to test the peak allusion throughout the entire analysis. Catecholamine concentration was quantified using the standard catecholamine curve equations in conjunction with recorded peak areas.

Results:

Trunk Blood Analysis

Post mortem analysis of trunk blood indicated a difference in the blood glucose and beta-Hydroxybutyric acid (BHB) levels between mice fed a control diet and mice fed a three week 8% ketogenic diet (Table 2). Trunk glucose levels are seen to be significantly increased for mice on the control diet (140.5 ± 22.2 mg/dL) then mice administered an 8% ketogenic diet (78.8 ± 18.8 mg/dL). Although there was no significant difference, trunk BHB ketone concentrations are elevated in the ketogenic diet (2.8 ± 1.4 mM) compared to the control diet (0.26 ± 0.1 mM).

CD1 Mouse Brain	Trunk Glucose (mg/dL) *	Trunk BHB (mM)*	Diet
A	142	0.3	CD
B	143	0.3	CD
C	120	0.3	CD
D	127	0.3	CD
E	91	1.9	8% KD
F	81	3.2	8% KD
G	95	2.7	8% KD
H	50	5.3	8% KD
I	183	0.1	CD
J	107	0.2	CD
K	164	0.2	CD
L	162	0.3	CD
M	75	1.3	8% KD
N	73	1.5	8% KD
O	55	4.6	8% KD
P	110	2.2	8% KD
Q	122	0.3	CD
R	135	0.3	CD

Table 2: Data was obtained from Masino Lab (Trinity College). Represents efficacy of the KD through reduction of trunk blood glucose and an increase in trunk beta-Hydroxybutyric acid

Amino Acid Analysis between Control Diet and Ketogenic Diet:

Nutritional analysis between the control diet and ketogenic diet revealed a difference between the grams per kilogram composition of amino acids (Table 1). Mice administered an 8% ketogenic diet ingested less total amino acids (85.5 g/kg) than mice fed a control diet (239.6 g/kg).

Quantification of Catecholamine Concentrations:

To determine whether catecholamine concentrations are altered by a three week chronic ketogenic diet, high pressure liquid chromatography was utilized to quantify the amount of norepinephrine, dopamine, DOPAC, 5-HIAA, HVA and serotonin in bilaterally acquired brain punches. No significant difference was found between the control diet and ketogenic diet for

catecholamine concentrations in the motor cortex, somatosensory cortex, nucleus accumbens, anterior caudate, posterior caudate or the midbrain (Figures 1-6). Analysis of neurotransmitter to metabolite ratio indicated a significant difference for DOPAC/Dopamine (Figure 7) in the motor cortex (Control: 0.36 ± 0.21 , KD; 0.72 ± 0.28) and midbrain (Control: 0.47 ± 0.31 , KD: 0.8 ± 0.46). No significant difference was seen between the ratio of HVA/Dopamine (Figure 8) and 5-HIAA/Dopamine (Figure 9).

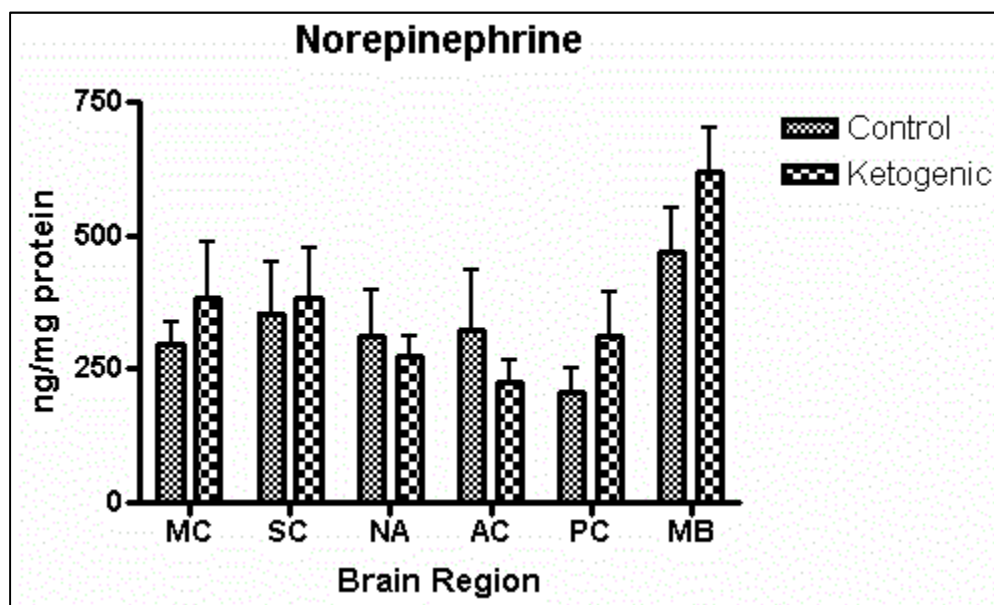


Figure 3: Norepinephrine concentration in the Motor Cortex (MC), Somatosensory Cortex (SC), Nucleus Accumbens (NA), Anterior Caudate (AC), Posterior Caudate (PC) and Midbrain (MB). No significant difference was seen in the catecholamine concentrations across all brain regions.

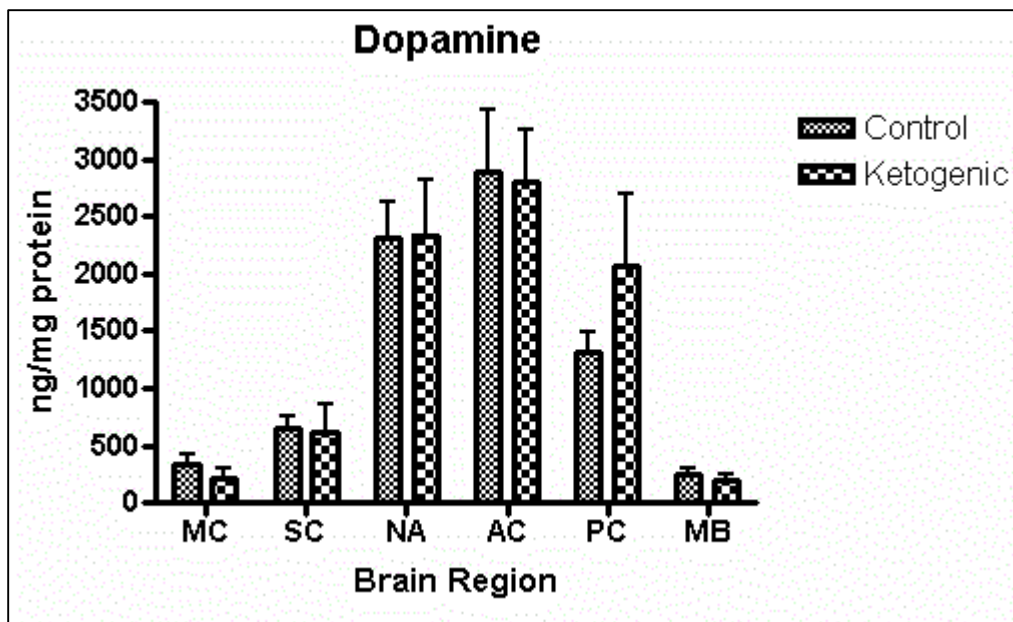


Figure 4: Dopamine concentration in the Motor Cortex (MC), Somatosensory Cortex (SC), Nucleus Accumbens (NA), Anterior Caudate (AC), Posterior Caudate (PC) and Midbrain (MB). No significant difference was seen in the catecholamine concentrations across all brain regions.

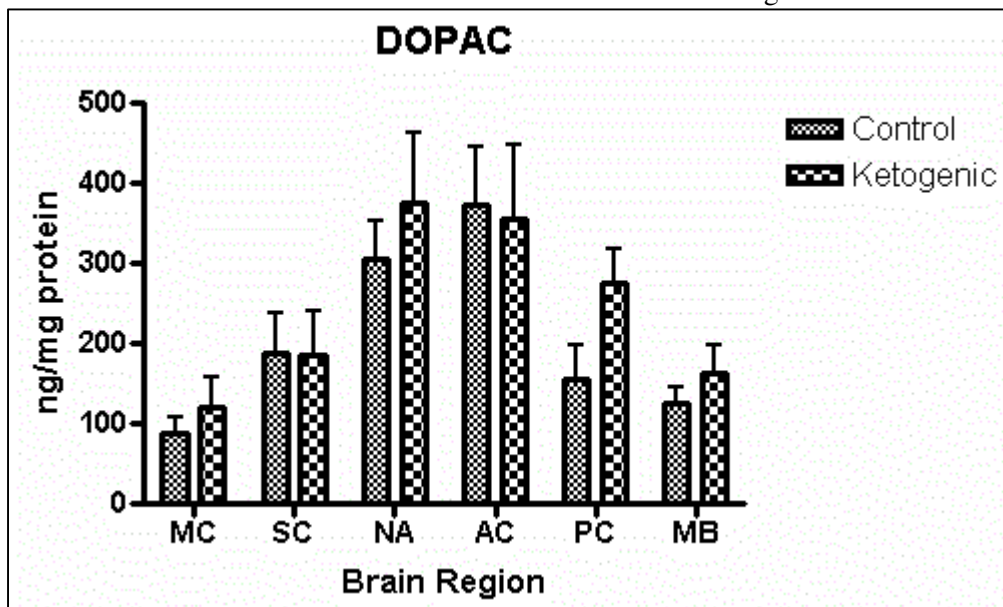


Figure 5: DOPAC concentration in the Motor Cortex (MC), Somatosensory Cortex (SC), Nucleus Accumbens (NA), Anterior Caudate (AC), Posterior Caudate (PC) and Midbrain (MB). No significant difference was seen in the catecholamine concentrations across all brain regions.

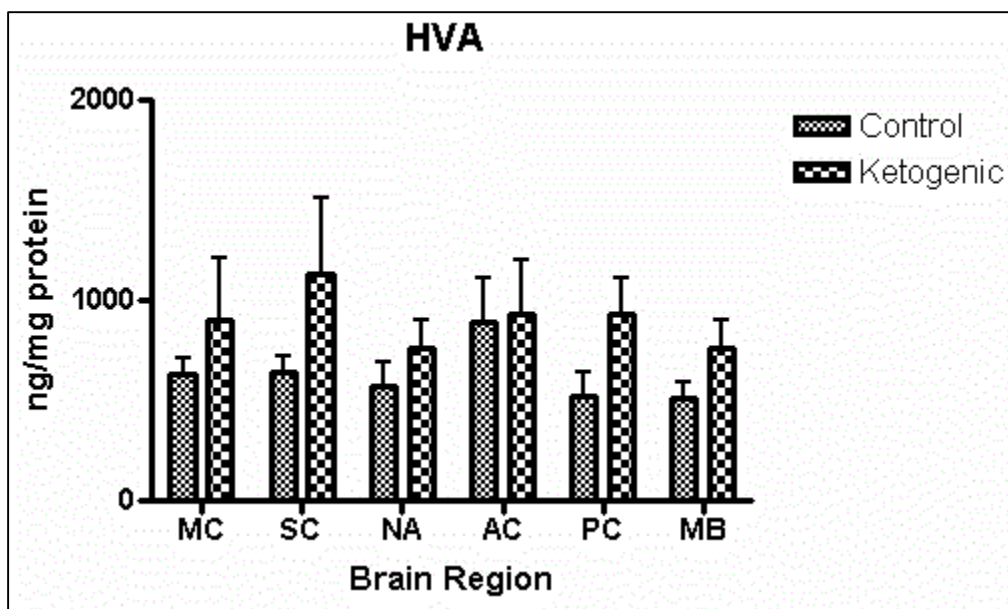


Figure 6: HVA concentration in the Motor Cortex (MC), Somatosensory Cortex (SC), Nucleus Accumbens (NA), Anterior Caudate (AC), Posterior Caudate (PC) and Midbrain (MB). No significant difference was seen in the catecholamine concentrations across all brain regions.

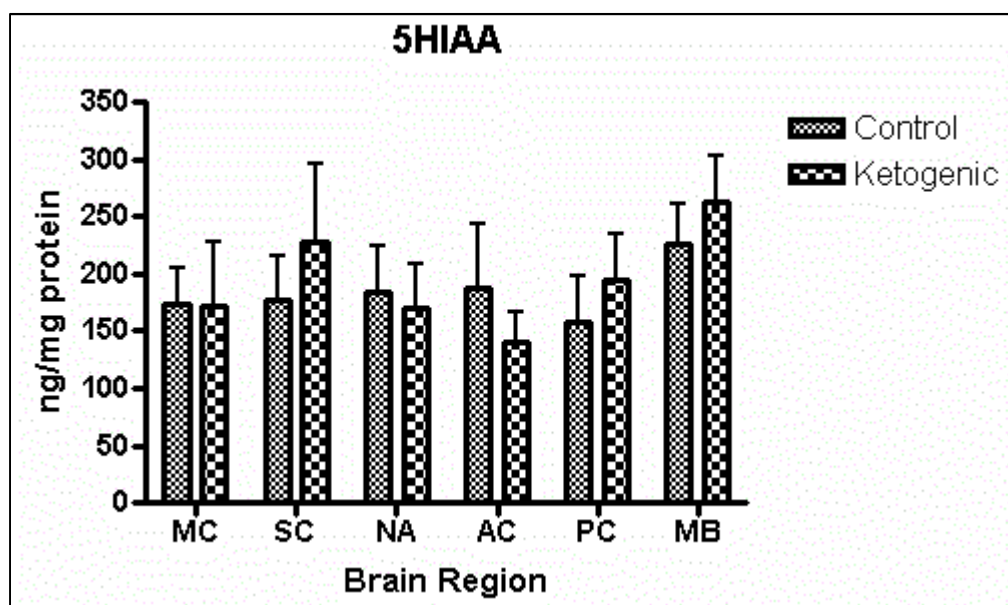


Figure 7: 5HIAA concentration in the Motor Cortex (MC), Somatosensory Cortex (SC), Nucleus Accumbens (NA), Anterior Caudate (AC), Posterior Caudate (PC) and Midbrain (MB). No significant difference was seen in the catecholamine concentrations across all brain regions.

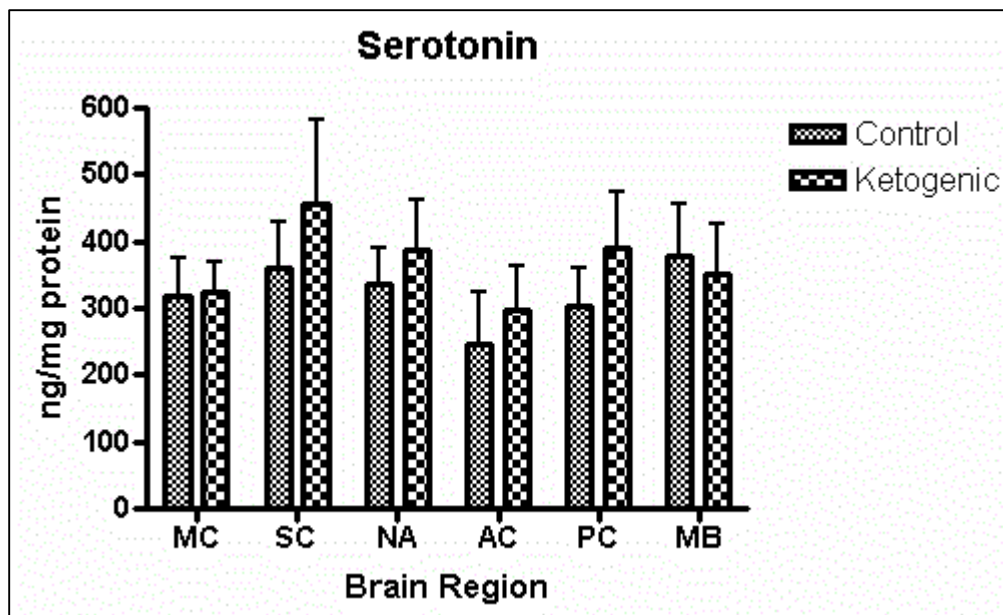


Figure 8: Serotonin (5HT) concentration in the Motor Cortex (MC), Somatosensory Cortex (SC), Nucleus Accumbens (NA), Anterior Caudate (AC), Posterior Caudate (PC) and Midbrain (MB). No significant difference was seen in the catecholamine concentrations across all brain regions.

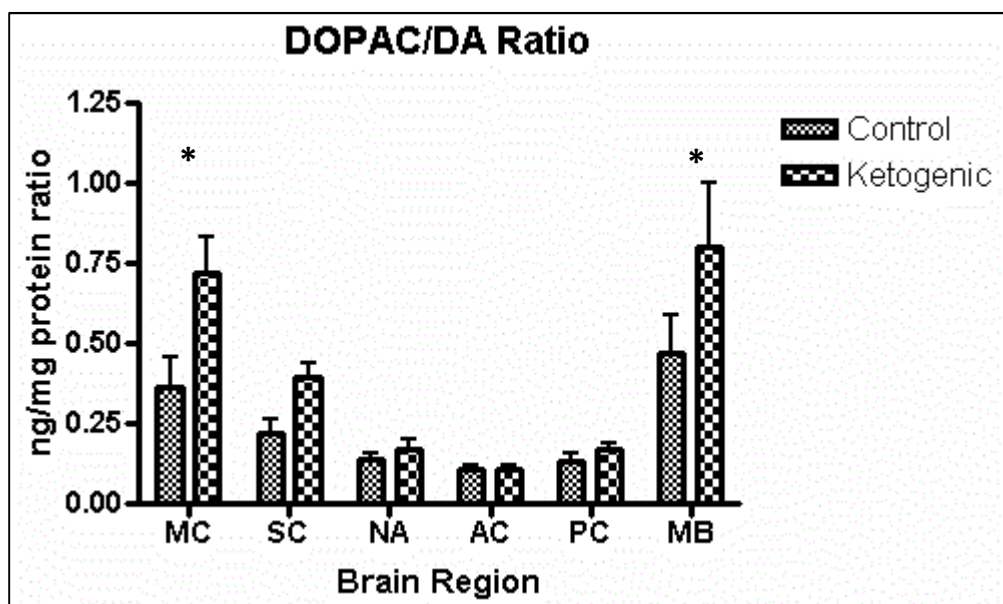


Figure 9: The ng/mg protein ratio for Dopamine and its metabolite DOPAC in the Motor Cortex (MC), Somatosensory Cortex (SC), Nucleus Accumbens (NA), Anterior Caudate (AC), Posterior Caudate (PC) and Midbrain (MB). Significant difference is seen in the Motor Cortex ($p < 0.05$) and Midbrain ($p < 0.05$).

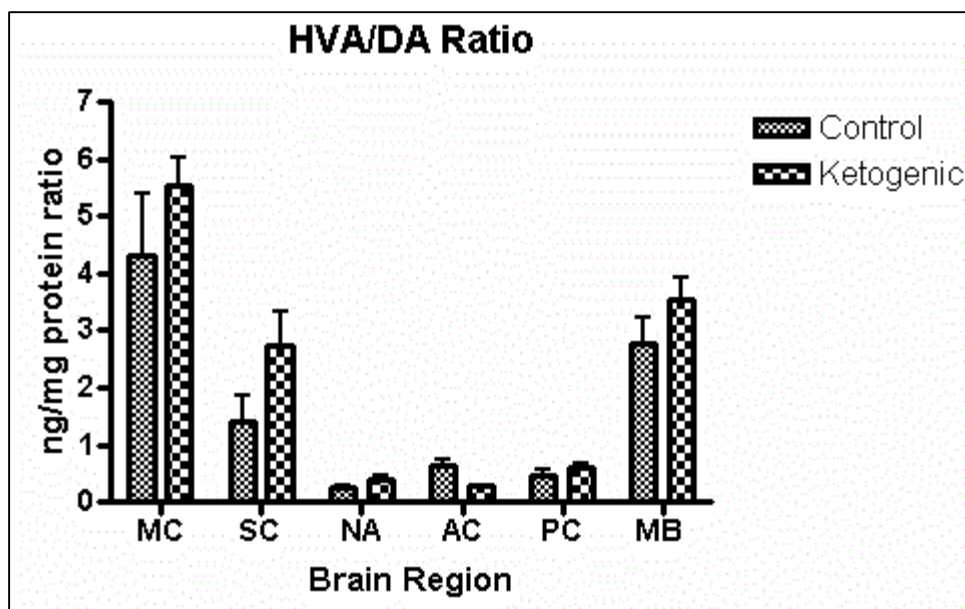


Figure 10: The ng/mg protein ratio for Dopamine and its metabolite HVA in the Motor Cortex (MC), Somatosensory Cortex (SC), Nucleus Accumbens (NA), Anterior Caudate (AC), Posterior Caudate (PC) and Midbrain (MB). No significant difference was seen between the Control and Ketogenic diet ratio across all brain regions.

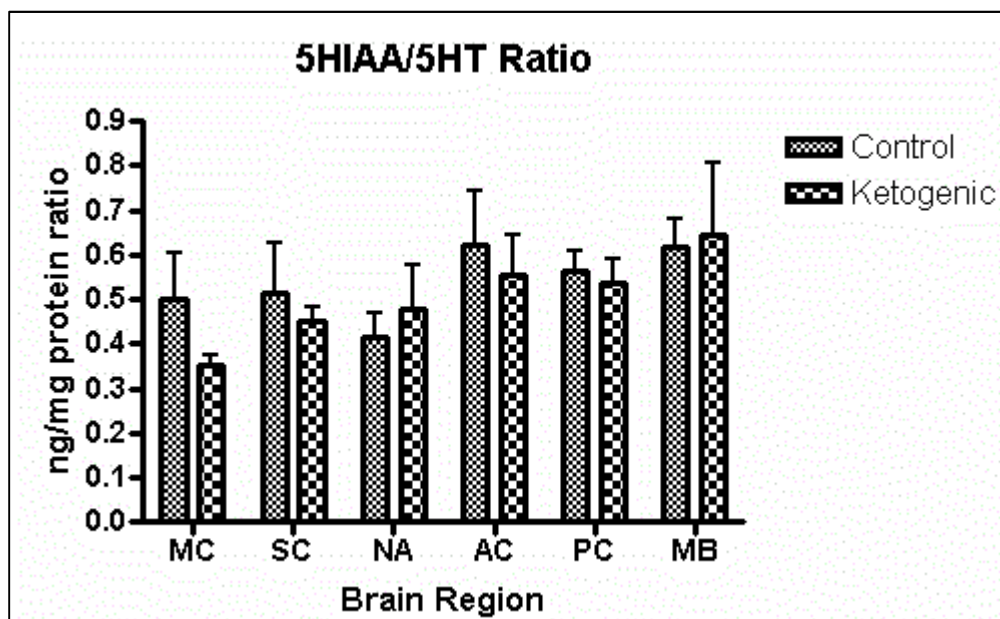


Figure 11: The ng/mg protein ratio for Serotonin (5-HT) and its metabolite 5HIAA in the Motor Cortex (MC), Somatosensory Cortex (SC), Nucleus Accumbens (NA), Anterior Caudate (AC), Posterior Caudate (PC) and Midbrain (MB). No significant difference was seen between the Control and Ketogenic diet ratio across all brain regions.

Correlation between Neurotransmitter Concentration and Blood beta-Hydroxybutyric Acid (BHB) Levels:

Levels of blood beta-Hydroxybutyric acid (BHB) was seen to have a significant correlation to the ng/mg protein concentration for norepinephrine ($p < 0.005$, $R^2 = 0.85$), dopamine (SC: $p < 0.005$, $R^2 = 0.90$) (MB: $p < 0.005$, $R^2 = 0.89$) and serotonin ($p < 0.005$, $R^2 = 0.85$) in several localized brain regions (Figure 12). Higher blood BHB levels were correlated to an increased catecholamine concentration for mice administered an 8% chronic ketogenic diet.

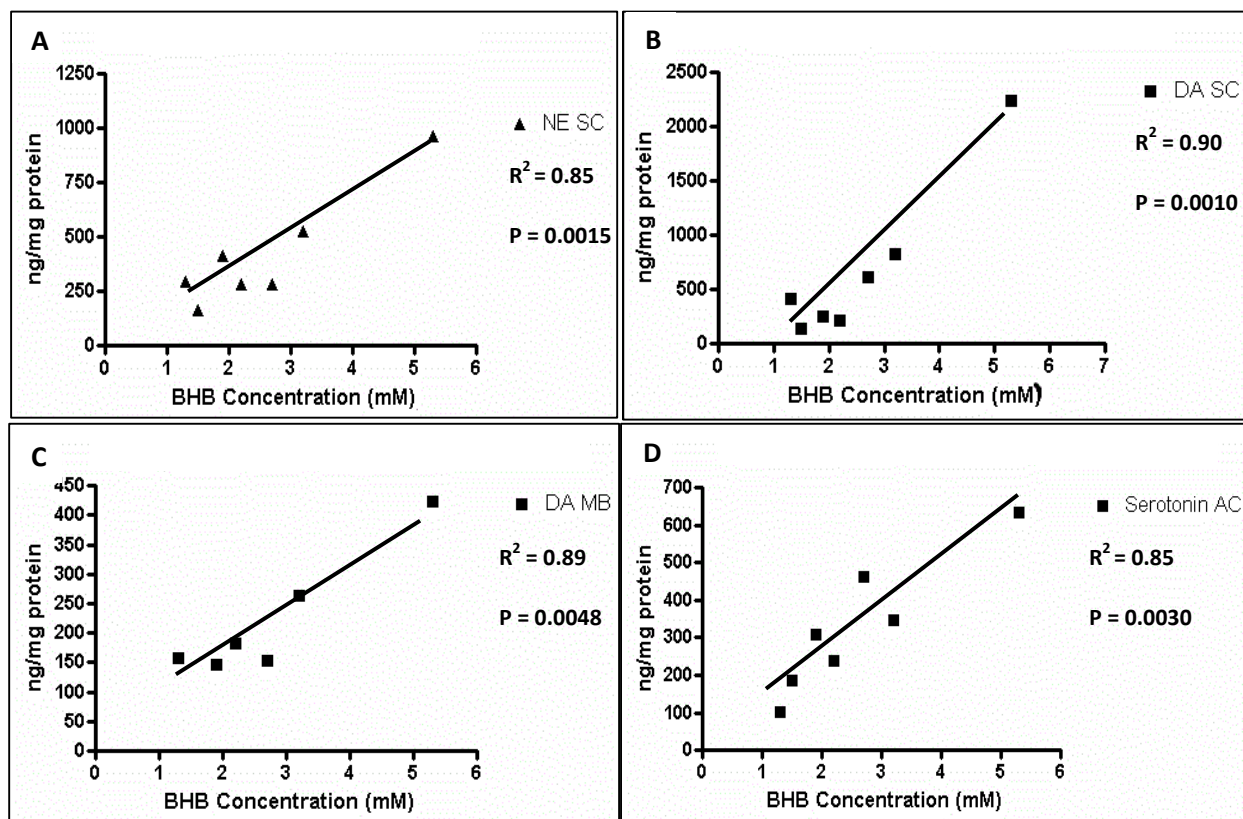


Figure 12: A significant correlation was seen between blood beta-Hydroxybutyric acid (BHB) concentration and the catecholamine ng/mg protein for (A) Somatosensory Cortex Norepinephrine, (B) Somatosensory Cortex Dopamine, (C) Midbrain Dopamine and (D) Anterior Caudate Serotonin.

Discussion:

Motor cortex, somatosensory cortex, nucleus accumbens, anterior caudate, posterior caudate and midbrain tissue samples taken from CD1 strain mice showed no significant difference in catecholamine concentrations between a control diet and an 8% ketogenic diet. The lack of significant difference in this experiment could be the result of the mice strain utilized. The CD1 strain mouse is neither epileptic nor autistic so the therapeutic effect of the ketogenic diet may not be produced.

The findings of this experiment do not correlate with prior research examining catecholamine concentrations on a ketogenic diet. One study found no difference in the concentration of norepinephrine in brain tissue except for a two fold increase in the extracellular fluid in the hippocampus in animals fed the ketogenic diet compared to controls (Weinshenker, 2008). It was also shown that a significant difference was present in the CSF of children on the ketogenic diet for the dopamine metabolite HVA and the serotonin metabolite 5-HIAA (Dahlin, 2011).

The lack of significant difference in catecholamine concentrations for mice on a ketogenic diet could also be the result of the localized brain regions examined. Prior research focused primarily on the cerebrospinal fluid which is a global representation of activity in the central nervous system. For our experiment, we focused on specific regions of the brain in an attempt to correlate local catecholamine concentrations to the therapeutic effect induced by the ketogenic diet (Marsh, 2006; Bough, 2007; Dahlin, 2005; Evangeliou, 2003). It may be possible that the localized brain regions examined exhibit no catecholamine concentration change on the ketogenic diet compared to a control diet.

It was observed that there was a significant increase in dopaminergic neuronal activity, as measured by the DOPAC/DA ratio, in the Motor Cortex and Midbrain for mice fed a ketogenic diet. This increase in dopaminergic activity could be the result of increased blood beta-Hydroxybutyric acid (BHB) levels present during the ketogenic diet. After the depression of excitatory postsynaptic potentials (EPSPs) by 60 min of glucose deprivation, administration of 0.5–10 mM BHB through artificial CSF incubation restored EPSPs in brain slices from postnatal day (PND) 15 rats but not in brain slices from PND 30 or 120 rats (Izumi, 1998). Izumi et al, believed that the primarily lipid based diet for suckling rats produced a state of ketosis in the body which was then switched back to a glucose based metabolism as the rat was ingesting other food sources. This restoration of EPSP's is critical in a lipid based metabolism induced by the ketogenic diet. It may be possible that BHB is increasing dopaminergic activity in the motor cortex and midbrain through physiological changes in the synaptic integration to the neuron.

Ketone bodies, such as BHB, were also shown to reduce the firing rate of spontaneously active substantia nigra pars reticulata (SNr) GABAergic neurons in mouse brain slices (Depaulis, 1994). The reduction in inhibitory synaptic input from the SNr GABAergic neurons might facilitate the depolarization of dopaminergic neurons. This finding is consistent with our results pertaining to the up regulation of dopaminergic activity in the motor cortex and midbrain.

Further research focused on the effects of BHB on calcium influx into the neuron. Beta-Hydroxybutyric acid was seen to elicit a faster influx of calcium into the neuron in the presence of 25mM and 5.5mM glucose (Laeger, 2010). The influx of calcium during an action potential facilitates the release of synaptic vesicles in the pre-synaptic neuron. Due to the high concentration of BHB seen during the ketogenic diet, it may be possible that BHB is functioning to not only restore EPSP's but to facilitate the influx of calcium into the pre-synaptic neuron.

The combination of EPSP restoration, reduction in GABAergic inputs and a facilitation of calcium influx into the neuron may explain the increase in dopaminergic activity in the motor cortex and midbrain indicated by the DOPAC/DA ratio. It may also explain why higher blood BHB levels are significantly correlated to increased ng/mg protein neurotransmitter concentrations.

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