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An Initial Analysis of a Long-Term Ketogenic Diet's Impact on Motor Behavior, Brain Purine Systems, and Nigral Dopamine Neurons in a New Genetic Rodent Model of Parkinson's Disease

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AN INITIAL ANALYSIS OF A LONG-TERM KETOGENIC DIET'S IMPACT ON MOTOR
BEHAVIOR, BRAIN PURINE SYSTEMS, AND NIGRAL DOPAMINE NEURONS IN A
NEW GENETIC RODENT MODEL OF PARKINSON'S DISEASE

BY

Jacob G. Rubin

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An Initial Analysis of a Long-Term Ketogenic Diet's Impact on Motor Behavior, Brain Purine Systems, and Nigral Dopamine Neurons in a New Genetic Rodent Model of Parkinson's Disease

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ABSTRACT

A growing body of research suggests that dopaminergic cell death seen in Parkinson's disease is caused by mitochondrial dysfunction. Oxidative stress, with subsequent generation of reactive oxygen species, is the hallmark biochemical product of mitochondrial dysfunction. The ketogenic diet has been found to enhance mitochondrial energy production, protect against reactive oxygen species-generated cell death, and increase adenosine, a purine that modulates dopamine activity. The current study evaluates the effects of a long-term (5-month) ketogenic diet on behavioral, neurochemical, and neuroanatomical measures in PINK1-KO rats, a new animal model of Parkinson's disease. Both wild-type and PINK1-KO animals fed a ketogenic diet exhibited significantly higher blood beta-hydroxybutyrate levels. PINK1-KO animals fed a normal diet experienced a decrease in stride length and an increase in stride frequency over time which was absent in PINK1-KO animals fed a ketogenic diet. Animals fed the ketogenic diet had decreased tissue content of both adenosine and inosine in the nucleus accumbens, posterior caudate, hippocampus, and substantia nigra. Finally, immunohistochemical staining for tyrosine hydroxylase-positive cells in the substantia nigra suggest a ketogenic diet-induced protection of dopaminergic cell death. The results of the present study indicate that a long-term ketogenic diet may positively impact both motor and neuroanatomical correlates and alter neurochemical systems in a genetic rodent model of Parkinson's disease.

INTRODUCTION

Etiology, Symptomology, & Current Treatment

Parkinson's disease (PD) is a neurological condition which causes motor impairments such as bradykinesia, muscular rigidity, postural instability, and resting tremors. The National Institute of Health (NIH) estimates that 500,000 people in the United States suffer from PD; prevalence of PD is expected to double by the year 2030 as the average age of our population grows older (NIH, n.d.; Dorsey et al., 2007). PD symptoms have been attributed to the progressive loss of dopamine (DA) cells in the substantia nigra (SN), but often do not manifest until 60-70% of these cells are already lost (for review see - Mhyre et al., 2012). These symptoms do not typically present until later in life with only 10% of diagnoses occurring before the age of 50 ("Parkinson's Disease: Hope Through Research," NIH). According to the NIH, the average age of symptom onset is 60 while other sources report that the mean age at diagnosis is 70.5 (NIH, n.d.; Van Den Eeden et al., 2003). Furthermore, incidence of PD increases by more than 350% in people over the age of 50 (Van Den Eeden et al., 2003). The most common therapy for patients suffering from PD is levodopa (L-DOPA), the precursor to DA, which can reduce the motor deficits to an extent. Still, treatments which attenuate or prevent dopaminergic (DAergic) cell death have yet to be discovered.

Neuroanatomy of PD

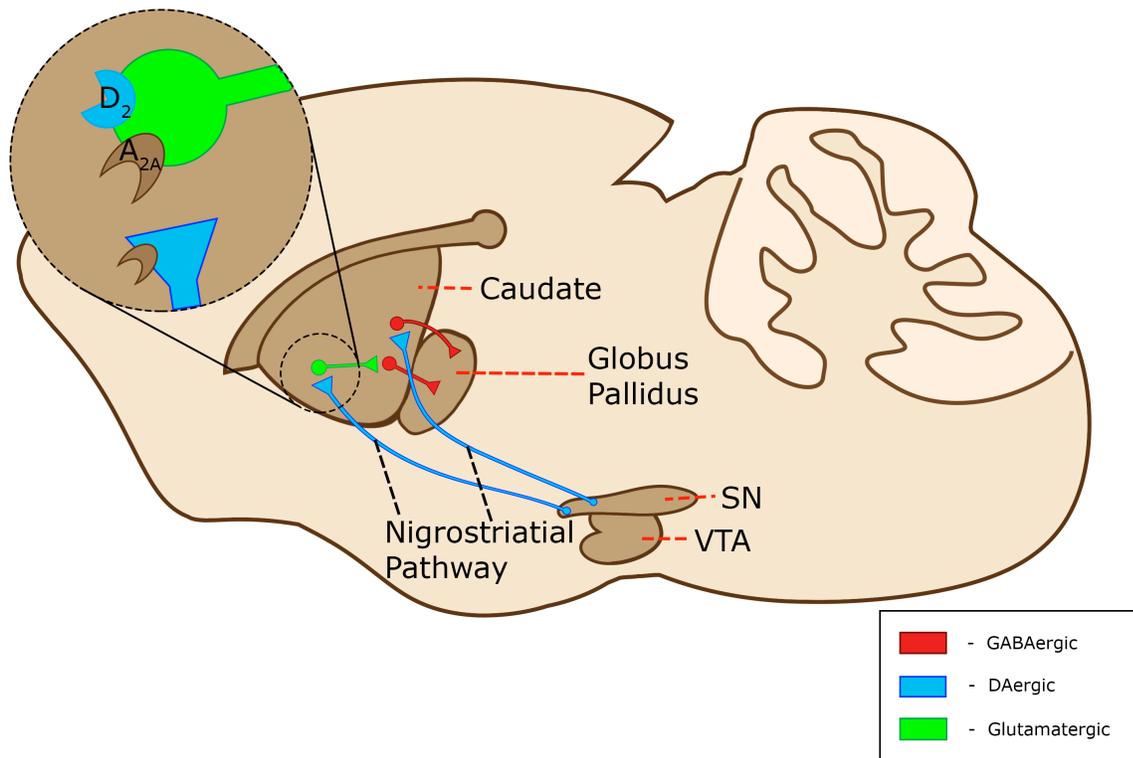


Fig. 1 The figure above displays the nigrostriatal pathway in the rat brain, relevant neuronal pathways, and relative dopamine D₂ and adenosine A_{2A} receptor localization in the basal ganglia. Graphic created by Tom Naragon '17.

The motor deficits associated with PD have been attributed to the loss of DAergic neurons of the basal ganglia, particularly in the SN (Parkinson, 2002). The basal ganglia include the dorsal striatum (caudate nucleus and putamen), ventral striatum (nucleus accumbens and olfactory tubercle), globus pallidus internal and external (GPi and GPe), SN, and the subthalamic nucleus (STN) (Purves et al., 2001; Fig 2). An expansive discussion of each of these components is beyond the scope of the current paper but those with direct relevance to motor function and PD will be discussed. The SN is the main source of DA in the brain and provides the neurotransmitter to the striatum via the nigrostriatal pathway (Beckstead et al., 1979; Fig 1). The nigrostriatal pathway is the network most affected by Parkinsonian pathology (Dauer

and Przedborski, 2003). Interestingly, the striatum projects only to other components of the basal ganglia yet it receives input from a variety of other brain regions; a full anatomical description is beyond the scope of this thesis.

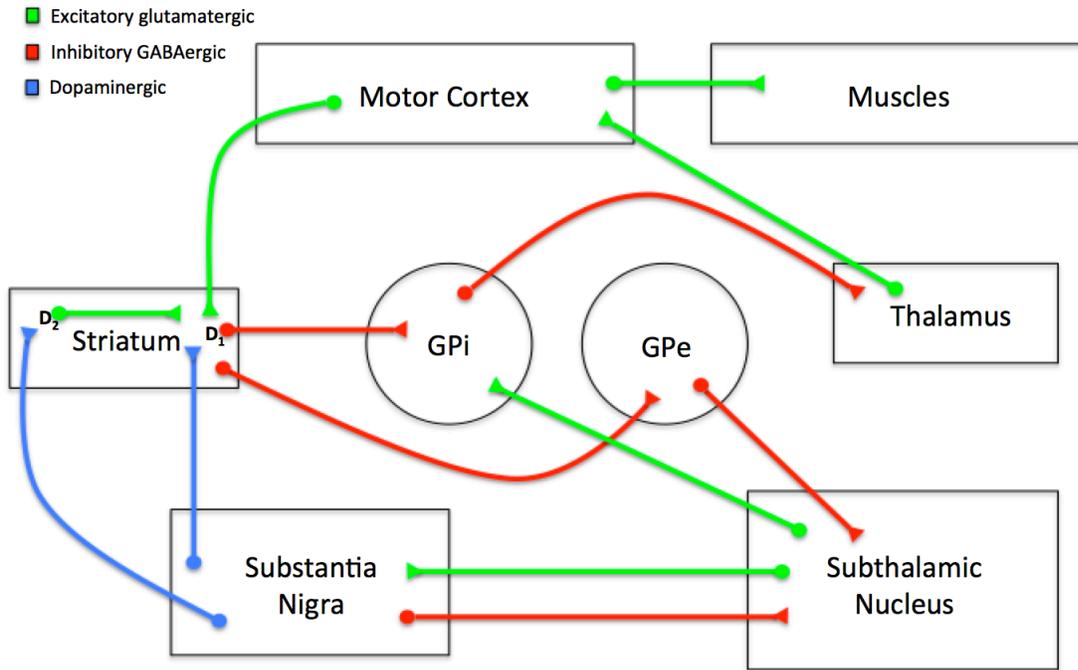


Fig. 2 The figure above depicts the neuronal pathways of the basal ganglia which play a crucial role in the facilitation of smooth motor movement in Parkinson’s disease.

In the context of movement, the structures of the basal ganglia play key roles in allowing the initiation and inhibition of movement (Purves et al., 2001). The thalamus is the main target for these effects due to thalamocortical projections which facilitate activation of the motor cortex. The GPI projects GABAergic neurons to the thalamus which inhibit the thalamus from activating the motor cortex (Purves et al., 2001). When a movement is made, glutamatergic projections from the motor cortex activate striatal GABAergic neurons. These striatopallidal GABAergic cells inhibit the GABAergic neurons of the GPI and thus allow for increased

thalamic activation. The GPe also receives inhibitory input from the striatum and sends its own GABAergic projections to the STN (Purves et al., 2001). The STN sends excitatory glutamatergic projections to the SN as well as the GPi. In the SN, glutamatergic neurons originating from the STN synapse at DAergic neurons which project to the striatum (Purves et al., 2001). In the GPi, glutamatergic projections from the STN augment firing of GABAergic projections to the thalamus which reduces thalamocortical facilitation of the motor cortex. DAergic neurons of the nigrostriatal pathway act on two different dopamine receptors; D₁ and D₂. Agonistic action at D₁ receptors located on striatopallidal GABAergic neurons augment inhibition of the GPi. D₂ receptors, however, are localized to striatal glutamatergic neurons which synapse with striatal GABAergic projections to the GPe. Activation of D₂ receptors enhances inhibition of the GPe which sends GABAergic projections to the glutamatergic neurons of the STN (Purves et al., 2001). It is also important to note the presence of circuitry which serves as self-modulating feedback inhibition between the SN and the STN via nigral GABAergic neurons (Purves et al., 2001).

In PD, the death of nigrostriatal DAergic cells would reduce the ability of the GPi to attenuate excitatory thalamocortical projections. This effect would be compounded by decreases in the STN's ability to augment GPi inhibition of thalamocortical projections that would result from reduction of the SN's indirect control of the STN by way of the striatopallidal GABAergic projections to the GPe. Disinhibition of thalamocortical projections to motor areas may explain the jerky and unwanted movements associated with PD.

Modeling PD

In order to study PD, models that induce the death of nigrostriatal DAergic neurons have been developed. Several animal models of PD use the administration of toxins specifically

designed to produce selective death of DAergic cells. 6-hydroxydopamine (6-OHDA) was one of the earliest toxin-induced models used to model PD and causes neurotoxicity by generating oxidative products when it breaks down extracellularly through auto-oxidation (Hanrott et al., 2006). The reactive oxygen species (ROS) produced cause oxidative stress which has been observed to lead to mitochondrial dysfunction, nuclear fragmentation, activation of the apoptotic caspase pathways, and selective DAergic cell death (Hanrott et al., 2006).

Another toxin-induced model was discovered by inhibiting activity of complex I of the mitochondrial electron transport chain using 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and was found to produce selective DAergic cell death (Langston et al., 1983). The mitochondrial dysfunction created by MPTP leads to the generation of ROS, like in the 6-OHDA model, which can result in the activation of programmed cell death (PCD) mechanisms. Evidence of elevated activation of mitochondrial PCD mechanisms in nigral DAergic cells has been observed in PD which further strengthens the link between mitochondrial dysfunction and neurodegeneration in PD (Tatton, 2000; Hartmann et al., 2001). Furthermore, mitochondrial energy failure has been proposed to disrupt vesicular sequestration of DA (Dauer and Przedborski, 2003), which results in extravesicular auto-oxidation of DA into DA-quinone, which reacts with cysteine residues and destroys proteins (Graham, 1978).

These toxin-induced models selectively produce DAergic cell death because these cells are particularly susceptible to the generation of ROS (Dauer and Przedborski, 2003). This proclivity for creating ROS is a direct result of enzymatic metabolism of DA, which produces hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\cdot-}$) radical, and auto-oxidation of cytosolic DA (Graham, 1978).

The major drawback to acute toxin-induced models of PD is that they do not accurately mimic the progressive loss of nigrostriatal DAergic neurons associated with human manifestations of the disease. However, a recent genetic model developed by Sage Laboratories has been able to address this challenge. By inhibiting the synthesis of phosphatase and tensin (PTEN)-induced kinase 1 (PINK1) the PINK1-knockout (PINK1-KO) model induces mitochondrial dysfunction and progressive loss of DAergic cells in the SN (Villeneuve et al., 2014). In healthy cells, decreases in mitochondrial membrane potential trigger the accumulation of PINK1 on the outer mitochondrial membrane (Imai, 2012). PINK1 recruits another protein, Parkin, from the cytosol which triggers the degradation and clearance of the damaged mitochondria. Without PINK1, dysfunctional mitochondria are not disposed of and produce toxic levels of ROS. Furthermore, it has been reported that PINK1-KO rats display hind limb dragging associated with their mutation (Dave et al., 2014). The PINK1-KO model presents a unique opportunity to study the progressive loss of DAergic neurons and observe Parkinson's-like motor deficits. Again, mitochondrial dysfunction is a well-established means for inducing DAergic cell death, making the PINK1-KO a useful model of human PD.

Purine Involvement in PD

While L-DOPA may be the most common treatment for PD, its long-term use has frequently been associated with decreased efficacy ("off" periods) and dyskinesias (Ahlskog and Muentner, 2001). As a result, the need for non-dopaminergic (non-DAergic) therapies to address PD symptoms is necessary.

Several members of the purine neurochemical family like guanosine and uric acid have been linked to PD. Recent *in vitro* work has revealed that guanosine is able to activate cell

survival pathways in the presence of 6-OHDA (Giuliani et al., 2014). A large body of literature has been published which points to uric acid as a potential biomarker for disease progression in PD (Cipriani et al., 2010; Gao et al., 2016). Uric acid has also been shown to be neuroprotective against the free radical peroxynitrite, which has been associated with the pathology of several inflammatory CNS diseases (Scott and Hooper, 2001). Additionally, reduced levels of uric acid have been reported in postmortem SN tissue (Church and Ward, 1994). The most prominent findings however, are those concerning the purine adenosine. The neuroprotective properties of adenosine are well studied. Although adenosine A₁ receptors are not directly linked to PD pathology, they are widely expressed throughout the CNS in glia and neurons (Dixon et al., 1996). A₁ receptors can exert neuroprotective effects by reducing the influx of presynaptic calcium and inhibiting glutamate release (Masino et al., 2002). Further research has indicated that this effect may in fact be one of feedback inhibition of hyper-excitatory firing, which may otherwise deplete energy and result in neural death (Lovatt et al., 2012).

A large body of research has revealed significant expression of adenosine A_{2A} receptors in the basal ganglia (Rivkees et al., 1995; Rosin et al., 1998; Hettinger et al., 2001; Rebola et al., 2005; Morelli et al., 2010). In striatopallidal neurons, activation of A_{2A} receptors increases the secretion of GABA in the globus pallidus (Ochi et al., 2000). Excess GABA in the globus pallidus suppresses the firing of pallidal GABAergic projections to the STN. A reduction in the inhibitory action of these projections leads to increased activation of glutamatergic neurons in the STN, which form synapses in both the globus pallidus and the SN. In a slightly more macroscopic perspective, highly active GABAergic projections of the basal ganglia attenuate the thalamocortical impact on the motor cortex and, ultimately, reduce the ability to elicit smooth motor function, as seen in patients with PD (Hauser RA and Schwarzschild MA, 2005).

In light of the localization and function of A_{2A} receptors, the purinergic neurochemical system represents a unique non-dopaminergic target for modulating the striatal signaling altered in PD. Numerous studies have explored the effects of antagonistic action at A_{2A} receptors in animal models of PD. A 1974 paper induced a unilateral lesion of the nigrostriatal DA pathway using 6-hydroxydopamine (6-OHDA) (Fuxe and Ungerstedt, 1974). Fuxe and Ungerstedt (1974) found that 6-OHDA-lesioned rats treated with L-DOPA displayed significant improvements in turning behavior following application of the A_{2A} antagonists theophyllamine and caffeine. Later studies confirmed this finding using other A_{2A} antagonists such as 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine (SCH 58261), (E)-8-(3,4-Dimethoxystyryl)-1,3-diethyl-7-methylxanthine, 8-[(1E)-2-(3,4-Dimethoxyphenyl)ethenyl]-1,3-diethyl-3,7-dihydro-7-methyl-1H-purine-2,6-dione (KW-6002 or istradefylline), and (E)-phosphoric acid mono-[3-[8-[2-(3-methoxyphenyl)vinyl]-7-methyl-2,6-dioxo-1-prop-2-ynyl-1,2,6,7-tetrahydropurin-3-yl]propyl] (MSX-3), all of which improved turning behavior in animals treated with L-DOPA or DA receptor agonists (Fenu et al., 1997; Koga et al., 2000; Strömberg et al., 2000a; Pinna et al., 2016). Furthermore, repeated treatment with A_{2A} antagonists failed to exhibit any reduced efficacy of motor stimulation suggesting that, A_{2A} antagonism ameliorates tolerance issues associated with L-DOPA treatments alone (Pinna et al., 2001).

Antagonism of the A_{2A} receptor has also been studied in the MPTP model of PD. It is important to note that rodent models are more resistant to this toxin and most successful reproduction of Parkinsonian syndrome with MPTP has been achieved in non-human primates (Bové et al., 2005). Since translation to non-human primates is an essential step in proof-of-concept for A_{2A} receptor antagonism in human PD, studies have used monkeys treated with

MPTP to test the efficacy of targeting A_{2A} receptors (Kanda et al., 2000). The A_{2A} antagonist KW-6002 significantly improved motor dysfunction induced by MPTP toxicity (Kanda et al., 2000).

Recently, a dual target therapy combining antagonists of the metabotropic glutamate receptor 5 (mGluR5) and the adenosine A_{2A} receptor was tested in rats treated with 6-OHDA (Fuzzati-Armentero et al., 2015). While a combination of the mGluR5 antagonist, 2-methyl-6-(phenylethynyl) pyridine (MPEP), and the A_{2A} antagonist, MSX-3, better protected DA cells in the SN from toxin-induced death, the MSX-3 alone also significantly reduced the toxin-induced cell death in the SN (Fuzzati-Armentero et al., 2015). Behaviorally, MSX-3 administered alone was found to significantly potentiate L-DOPA-induced turning behavior. Again, combination of MSX-3 with MPEP increased this effect and required lower concentrations than individual administration (Fuzzati-Armentero et al., 2015). Another recent study combined an A_{2A} antagonist with a serotonin 1A/1B receptor agonist (Pinna et al., 2016). Pinna and colleagues found that in MPTP-treated monkeys the combination of both drugs reduced the dose of L-DOPA necessary for therapeutic effects. Additionally, administration of all three drugs significantly prevented dyskinetic-like behavior (Pinna et al., 2016). Still, antagonists of the A_{2A} receptor are the frontrunners in non-dopaminergic therapies for PD with the first adenosine A_{2A} antagonist, istradefylline, being recently approved in Japan after successful phase-3 trials (Kondo and Mizuno, 2015).

Purines and the Ketogenic Diet

The ketogenic diet (KD) is a high fat, low carbohydrate eating regimen and is already well established as a successful alternative treatment for medically refractory epilepsy (Freeman and Vining, 1998; Hemingway et al., 2001). The calorie restriction of the KD induces a state of

ketosis by breaking down fatty acids to ketone bodies in the liver (Paoli et al., 2013). These ketone bodies replace glucose as the body's main driver of energy production and have been shown to result in enhanced production of energy substrates (DeVivo et al., 1978). More specifically, elevated fatty acid levels by a KD eventually reach a threshold which surpasses the capacity of the tricarboxylic acid (TCA) cycle and leads to enzymatic conversion of acetyl-CoAs into acetoacetate (ACA) and β -hydroxybutyrate (β H β). Both ACA and β H β are ketone bodies which travel through the blood, cross the blood-brain barrier, and enter the cells of the central nervous system. These ketone bodies are then transported into mitochondria where they are converted into acetyl-CoA and subsequently into ATP via the Krebs cycle (Masino and Rho, 2012). As an energy substrate ketone bodies have been found to improve ATP production and protect against ROS-induced damage (Kashiwaya et al., 2000; Kim et al., 2007; Maalouf et al., 2007; Veech, 2014).

The past decade has seen major strides in discovering the underlying processes which explain the KD's efficacy in epilepsy. Currently, the most compelling theory points to inhibitory action at adenosine A₁ receptors (Masino et al., 2011). As such, research has pressed on, investigating the purinergic system's role in the anticonvulsant effects of the diet. One hypothesis proposes that the KD may in fact be increasing ATP levels which may be rapidly broken down into adenosine in the extracellular space (Masino and Geiger, 2008). By mimicking the low glucose environment of a KD, a study conducted by Kawamura et al. (2010) showed that reduction of extracellular glucose results in the release of ATP via pannexin-1 hemichannels in hippocampal CA3 pyramidal neurons. Dephosphorylation of this extracellular ATP yields adenosine, which acts on A₁ receptors coupled to ATP-sensitive K⁺ channels to reduce neuronal excitability (Kawamura et al., 2010).

In addition to its application in epilepsy, the diet has also been proposed to have therapeutic benefits in other neurological conditions in which deficits in mitochondrial energy metabolism play a role (Baranano and Hartman, 2008; Stafstrom and Rho, 2012). The profundity of the KD's effects on mitochondrial energy metabolism was demonstrated by work using microarrays, which revealed an upregulation of 34 transcripts encoding energy metabolism enzymes (Bough et al., 2006). Furthermore, increased mitochondrial profiles were observed in hippocampal slices of these KD-fed rats. Stimulation of synaptic transmission in these slices exhibited high resistance to a low glucose environment, which suggests an increase in energy reserves (Bough et al., 2006).

While glucose restriction and ketone body-driven energy metabolism enhances production of energy substrates, ketone bodies specifically have been reported to have neuroprotective properties. ACA and β HBA are both able to decrease the mitochondrial output of destructive ROS by increasing NADH oxidation in the mitochondrial respiratory chain (Maalouf et al., 2007). The same study found that glutathione, an endogenous free radical, was also reduced. This may be explained by a study which reported that elevated glutathione peroxidase activity was observed in the hippocampus of rats fed a KD (Ziegler et al., 2003). Ketone bodies also reduce mitochondrial membrane permeability induced by oxidative stressors such as, H_2O_2 and diamide (Kim et al., 2007). The same study affirmed the ability of ketone bodies to decrease ROS levels and provides substantial evidence of their capacity to prevent mitochondrial permeability and oxidative damage in neurons.

The Metabolic Theory: Potential Effects of a Ketogenic Diet on PD

It is well documented that the DAergic neurodegeneration seen in PD is the result of mitochondrial dysfunction (Parker et al., 1989; Schapira et al., 1990; Hattori et al., 1991; Perier

et al., 2012). This is supported by studies which report that DAergic neurons are highly sensitive to free radicals, which are natural byproducts of energy metabolism (Miyazaki and Asanuma, 2008). The KD has been shown to improve mitochondrial energy production and the ketone body β HBA is a more efficient energy substrate in terms of ATP generation (DeVivo et al., 1978; Veech, 2014). In light of the growing body of research highlighting the role of mitochondrial dysfunction in PD, as well as literature which reports KD-induced enhancements in mitochondrial energy production, the KD could present a possible therapeutic benefit for PD.

The purpose of the current study is to evaluate the KD's ability to attenuate the progressive cell death seen in PINK1-KO rats. This model presents a unique opportunity to target progressive loss of DAergic neurons, test the therapeutic efficacy of KD-driven improvements in mitochondrial function, and measure the KD's impact on Parkinsonian motor deficits. Behavioral data on gait alterations of the animals was recorded using the DigiGait motorized treadmill system. Staining for tyrosine hydroxylase (TH) will be conducted on nigral tissue to assess DAergic cell death. A reverse-phase high performance liquid chromatography (HPLC) system will be used to quantify purine levels in 7 discrete brain regions. Blood testing for glucose and β HBA will be used to establish each animal's level of ketosis. It was hypothesized that the KD would enhance mitochondrial energy metabolism such that an attenuation in DAergic cell death would be observed in the KD-fed PINK1-KO rats. It was expected that PINK1-KO rats on the normal diet (ND) would exhibit increased gait abnormalities compared to KD-fed PINK1-KO rats, and in control rats fed either diet.

MATERIALS AND METHODS

Chemicals

Adenosine, guanosine, inosine, xanthine, hypoxanthine, guanine, uric acid, sodium chloride, potassium phosphate monobasic, and sodium 1-pentane sulfonate were acquired from Sigma Aldrich (St. Louis, MO, USA). Potassium phosphate monobasic, HPLC-grade acetonitrile, and 85% phosphoric acid were acquired from ThermoFisher Scientific (Waltham, MA, USA). Potassium chloride, and sodium phosphate dibasic heptahydrate were acquired from J.T. Baker Analytical (Center Valley, PA, USA).

Animal Handling and Diet Administration

All animal care, use, and surgical procedures were approved by the Institutional Animal Care, the Use Committee of Trinity College, and are in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. A total of 24 PINK1 $+/+$ and $-/-$ rats from SAGE Laboratories (St. Louis, MO, USA) were housed in pairs in plastic cages with stainless steel tops and were exposed to a standard 12hr light: 12hr dark cycle. Temperature and humidity were kept constant electronically and food and water were provided *ad libitum*, KD was changed daily. All animals received the control diet until 3 months of age at which point KD and control groups were established. The KD used was the AIN-76A Modified diet which is 8.6% protein, 75.1% fat, 3.2% carbohydrate and contains added mineral and vitamin mixes (BioServ, Flemington, NJ, USA). Rats were given a 5-day acclimation period upon arrival before they were weighed for the first time. Weight gain was continuously monitored every 2 weeks. Once during each of the 3 months prior to sacrifice, blood levels of β HB and glucose were tested using ketone and glucose test strips and digital meters (Abbott Inc., Alameda, CA,

USA). At 8 months old animals were deeply anesthetized using isoflurane (Henry Schein Animal Health, Dublin, OH, USA) before and during sacrifices. Animals were perfused using 1x phosphate buffered saline (PBS) solution (for details see appendix A). The brain of each animal was rapidly harvested and stored in a solution of dry ice and ethanol until tissue was processed approximately 1 hour after sacrifice.

DigiGait Behavioral Analysis

Animals undergoing gait analysis were put on a motorized treadmill tilted at a 13° angle and walked downhill at a speed of 18 cm/s. Video footage was recorded from beneath the treadmill and the area of advancing and retreating paws was analyzed using the DigiGait software. These data were used to evaluate balance, coordination, and gait abnormalities.

Tissue Processing

Brains were sectioned on a Leica SM 2000 R Microtome stage controlled by a Physitemp BFS-3TC temperature regulator set to -20°C (Physitemp Instruments, Clifton, NJ). Brain slices were taken coronally at 1.5mm thickness and bilateral tissue punches were taken from the following regions: motor cortex (MC), somatosensory cortex (SC), nucleus accumbens (NA), anterior caudate (AC), posterior caudate (PC), hippocampus (HC), and substantia nigra (SN) (Fig 3). See appendix B for detailed procedures and protocols.

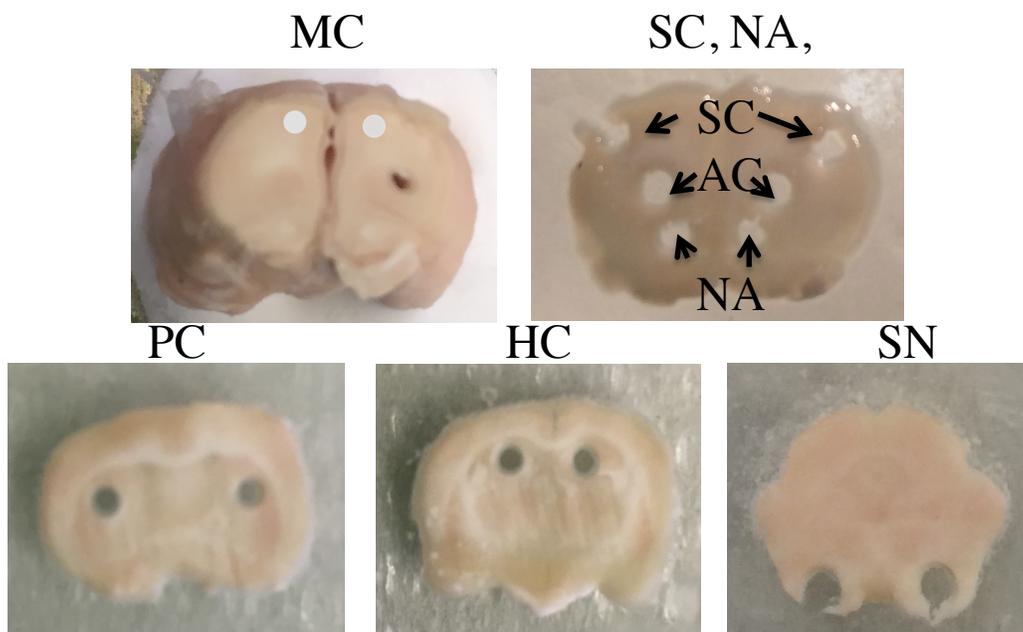


Fig. 3 The above figure depicts the slices and punch site locations of the following brain regions: motor cortex (MC), somatosensory cortex (SC), nucleus accumbens (NA), anterior caudate (AC), posterior caudate (PC), hippocampus (HC), and substantia nigra (SN).

Rat brain regions were identified using a rat stereotaxic atlas (Pellegrino et al., 1979). Tissue punches were transferred to labeled microcentrifuge tubes containing 400 μ L ethanol and sonicated using the Tekmar Sonic Disruptor. Samples were centrifuged for 30 min at 12,400 rpm using the Fisher Scientific Microcentrifuge 235c at 4°C. Supernatants were transferred to separate microcentrifuge tubes and rotovapped at 45°C for 30 min. The amount of protein in each tissue punch was evaluated using standard Modified Lowry Protein Assay protocol from the protein pellets left in the original centrifuge tubes. Neurotransmitter pellets were resuspended in 1mL of DiH₂O and 7 μ L of an 8 μ g/L DHBA solution was added as an internal standard. Fully processed samples were stored at -80°C until analyzed by HPLC.

HPLC Analysis

Purines were quantified using a modified version of a previously described method (Burdett et al., 2013). Two mobile phases were used for reverse-phase HPLC analysis. Mobile phase A (MP-A) contained 0.52 mM sodium 1-pentane sulfonate, 0.20 M potassium phosphate monobasic and was pH adjusted to 3.5 using 85% phosphoric acid (Baker Analyzed; Phillipsburg, NJ, USA). Mobile phase B (MP-B) contained identical concentrations as MP-A plus an addition of 10% acetonitrile (HPLC-Grade, Fisher Scientific, Pittsburgh, PA, USA).

The gradient parameters were as follows: 0-6 min of 100% MP-A, 6-15.5 min ramp to 45% A - 55% B (Fig 4). This gradient was held until 20 min and was followed by a 15 min equilibration period during which the system was rinsed with 100% MP-A before the next injection was made (Fig 4).

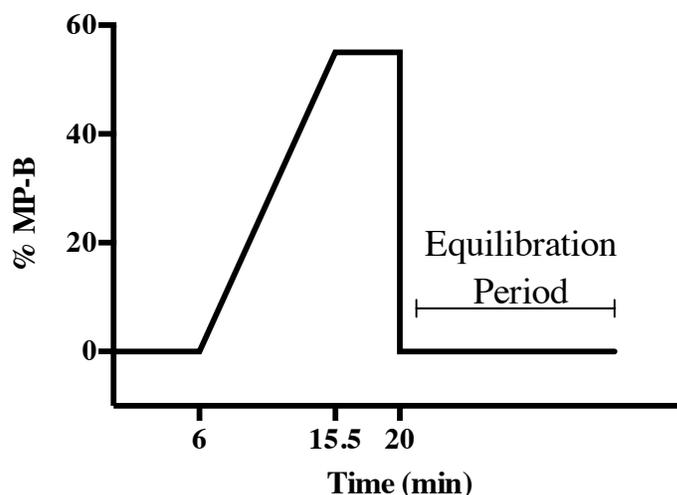


Fig. 4 The figure above is a graphical representation of the mobile phase (MP) gradient parameters used for reverse-phase high performance liquid chromatography (HPLC) analysis of purine levels. Mobile phase A (MP-A) was a 0.52 mM sodium 1-pentane sulfonate, 0.20 M potassium phosphate monobasic solution and was pH adjusted to 3.5 using 85% phosphoric acid. Mobile phase B (MP-B) was created from MP-A but was 10% acetonitrile.

A Hitachi L 2130 HPLC pump was used at a flow rate of 0.7mL/min and separation was carried out at 22°C on a 150 x 3.00mm LUNA 5 μ m C18 analytical column (Phenomenex, Torrence, CA, USA). A sample volume of 100 μ L was manually injected into the HPLC system. Dual electrochemical detection (ESA Coulochem III; E₁ = -0.15V; E₂ = +0.70V; Thermo Scientific, Sunnyvale, CA, USA) and UV detection (λ_1 = 254nm; BioAnalytical Systems, West Lafayette, IN, USA) were used to quantify purine levels. Chromatographic data was collected, stored, and analyzed using EZ Chrom chromatography software (Thermo Scientific).

TH-Staining

The Vectastain® ABC Kit (Vector Labs, Burlingame, CA, USA), and anti-TH antibody (Santa Cruz Biotechnology, Dallas, TX, USA) were used for immunohistochemical staining (for details see appendix C).

Protein Assays

Protein assays were conducted using the standard Modified Lowry Protein assay method (for details see appendix C).

Data Collection, Quantification, and Analysis

Calibration standards were run prior to brain sample injections and a standard calibration curve was used to quantify purine levels in the brain regions of interest. Data was compiled using Excel files and statistically analyzed using GraphPad Prism version 6.0 (GraphPad Software, Inc., San Diego, CA, USA). Statistical significance between groups was determined by two-way ANOVA with post-hoc comparisons.

Experimental Groups

A total of 24 Long-Evans Hooded rats, 12 wild-type (WT) and 12 PINK1-KO (KO), were included in the study. Animals arrived in groups of 4, 2 WT and 2 KO, on a monthly basis and were assigned letters A-X for identification. 4 study groups of 6 animals each were established as follows: WT fed a normal diet (WT/ND), WT fed a ketogenic diet (WT/KD), KO fed a normal diet (KO/ND), and KO fed a ketogenic diet (KO/KD). One animal from the KO/KD group died prematurely due to an unforeseen hypersensitivity to isoflurane anesthetic administered when trimming toenails prior to DigiGait behavioral testing. The use of isoflurane for toenail trimming was discontinued immediately and no other animals were lost prematurely for any reason.

Brains from one animal from each study group, 4 brains total, were sent to the University of Hartford in order to conduct TH-staining of the SN to assess DAergic cell death.

A gravity-driven perfusion apparatus was used during surgical dissection of animals. Initially, an 8L container was filled with 1x PBS and placed on a shelf approximately 2 feet above animals during dissection. Animals 'C' and 'D', from the KO/ND group, were not successfully perfused and cerebral blood could be observed following dissection. Furthermore, lesions were observed in the following animals: D, F, H, I, L, and K. Animals D, F, and H exhibited either asymmetrically enlarged or exploded ventricles which were attributed to the excessive flow rate of the gravity-driven perfusion apparatus. Rats I-X did not show asymmetrically enlarged or exploded ventricles, likely due to the decrease in volume of 1x PBS in the perfusion container after perfusions were conducted for rats A-H. Available images of the observed lesions can be found in appendix D. Final calculations of purine levels in rats A-H in all brain regions were, in most cases, more than 250% higher than those in rats I-X. This

disparity is likely due to perfusion-induced dispersion of purines away from their regions of origin and, as such, data from animals A-H were excluded from neurochemical analysis. At this point each study group consisted of 3 animals.

Finally, two samples for neurochemical analysis by HPLC were inadvertently lost during the tissue sample preparation process. The posterior caudate tissue from animal 'R' came loose in the microcentrifuge. Tissue from the substantia nigra of rat 'V' was not collected after excessive slicing of brain tissue caused the region to be missed entirely.

RESULTS

Chronic KD does not prevent weight gain

A 5-month chronic KD did not prevent weight gain in either the WT or KO animals (Fig 5). Unpaired t-tests showed no significant differences (for all comparisons $p > 0.05$) in starting weight between any of the study groups (WT/ND: $334\text{g} \pm 11.88$, $n = 6$; WT/KD: $374\text{g} \pm 25.19$, $n = 6$, $t = 1.57$; KO/ND: $366\text{g} \pm 9.39$, $n = 6$, $t = 3.32$; KO/KD: $362\text{g} \pm 11.75$, $n = 5$, $t = 2.36$).

Unpaired t-tests also showed no significant differences in final weight between any of the study groups (WT/ND: $518\text{g} \pm 21.55$, $n = 6$; WT/KD: $593\text{g} \pm 48.20$, $n = 6$, $t = 1.55$; KO/ND: $575\text{g} \pm 15.60$, $n = 6$, $t = 3.65$; KO/KD: $536\text{g} \pm 54.62$, $n = 5$, $t = 0.34$). Furthermore, after 5 months on the KD, both WT and KO animals were overtly indistinguishable from normal diet-fed animals.

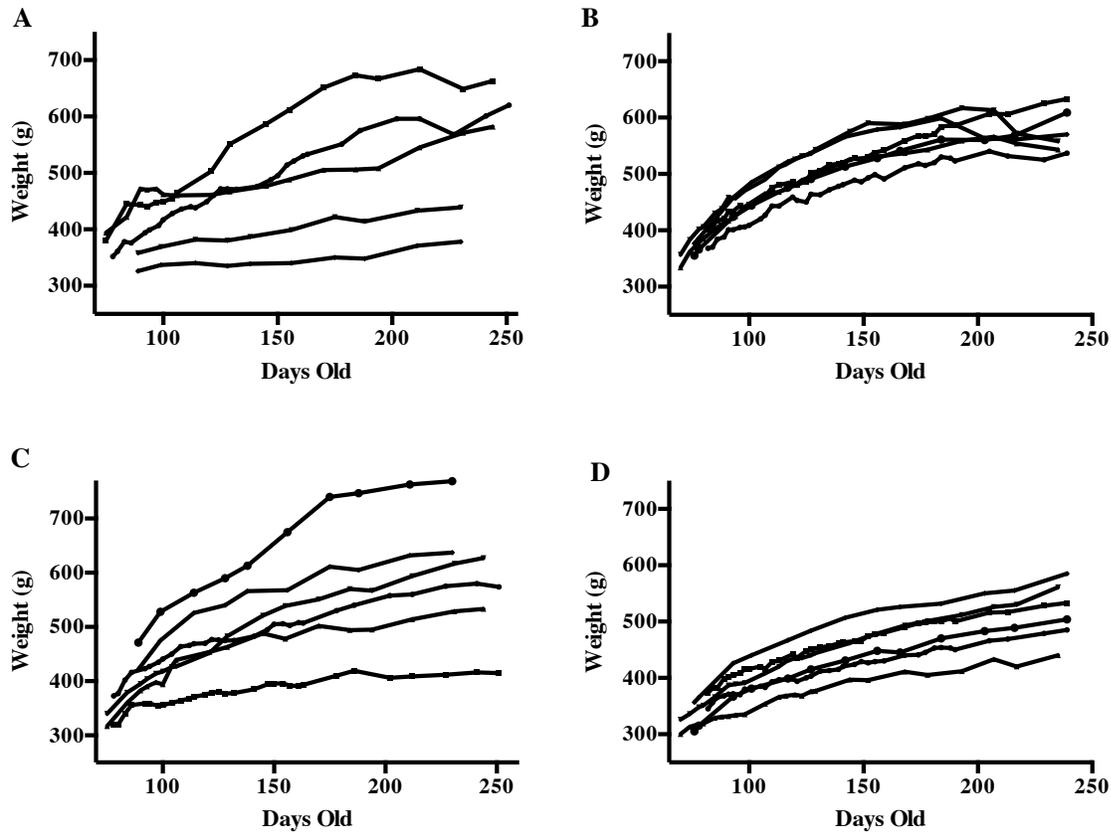


Fig. 5 Chronic KD does not prevent weight gain in wild-type (WT) and knock-out (KO) animals fed a normal diet (ND) or ketogenic diet (KD). **A**) KO/KD animals (n = 5) **B**) KO/ND animals (n = 6) **C**) WT/KD animals (n = 6) **D**) or WT/ND animals (n = 6).

KD alters β HB but not glucose levels

Animal age at the time of blood tests did not have any affect on the results of either β HB or glucose testing ($F = 0.51$, $DFn = 2$, $p > 0.05$ and $F = 1.30$, $DFn = 2$, $p > 0.05$, respectively). Data from all three months were then combined to compare the effect of the KD on blood β HB and glucose levels. As seen in figure 6, animals fed the KD showed increased blood β HB levels when compared to animals fed a normal diet. WT animals on the KD showed an increase of 157% over WT animals on the normal diet ($0.94 \text{ mmol/L} \pm 0.05$, $n = 15$ vs. $0.60 \text{ mmol/L} \pm 0.04$, $n = 13$, $p < 0.05$). PINK1-KO animals fed a KD showed an increase of 167% in blood β HB when

compared to KO animals on the normal diet ($0.97 \text{ mmol/L} \pm 0.06$, $n = 12$ vs. $0.58 \text{ mmol/L} \pm 0.05$, $n = 13$, $p < 0.01$). Blood glucose levels were not affected by the KD (Fig 7).

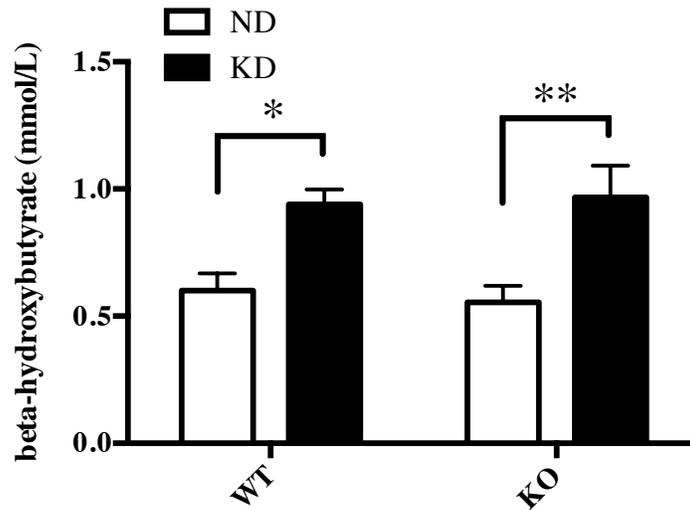


Fig. 6 Blood beta-hydroxybutyrate (β HB) levels in wild-type (WT) and knock-out (KO) animals fed a normal diet (ND) or ketogenic diet (KD). The WT/KD group had significantly higher β HB than WT/ND (* $p < 0.05$). KO/KD animals had significantly higher β HB than KO/ND groups (** $p < 0.01$). Data from months 6, 7, and 8 were combined and compared by study group ($n = 12-15$ data points per group).

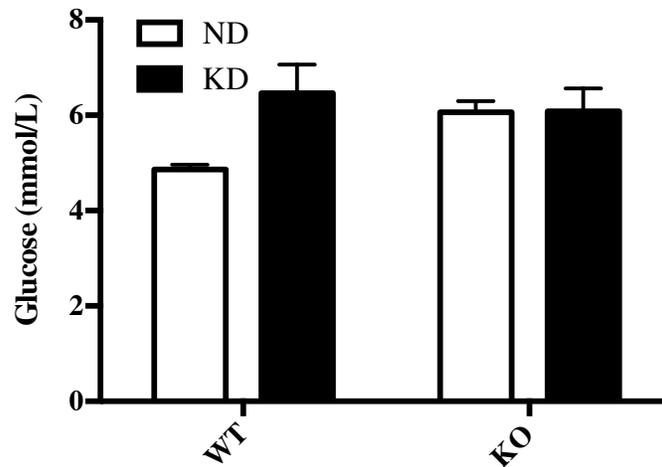


Fig. 7 Blood glucose levels in wild-type (WT) and knock-out (KO) animals fed a normal diet (ND) or ketogenic diet (KD). No significant differences were seen between any of the experimental groups. Data from months 6, 7, and 8 were combined and compared by study group (n = 12-15 data points per group).

KD increases stride length and decreases stride frequency

At 3 months of age there was no statistically significant difference in stride length or stride frequency between any of the experimental groups (Fig 8a and 10a). At 8 months, KO animals on the normal diet showed significantly shorter stride length than WT animals on the normal diet ($8.33 \text{ cm} \pm 0.05$, n = 6 vs. $10.55 \text{ cm} \pm 0.05$, n = 2, $p < 0.05$) and WTs on the KD ($12.21 \text{ cm} \pm 0.40$, n = 8, $p < 0.01$; Fig 8b). Interestingly, KD-fed wild type animals had longer stride length than normal diet-fed WTs ($12.21 \text{ cm} \pm 0.40$, n = 8 vs. $10.55 \text{ cm} \pm 0.05$, n = 2, $p < 0.01$) at 8 months. Figure 9 shows that the stride length of the KO/ND animals gradually decreased over time, ultimately showing a 16.5% decrease from 3 month values, whereas all other groups remained equal to or greater than their respective 3 month values.

No significant differences in stride frequency were seen between any of the groups at 3 months (Fig 10a). However, at 8 months KO animals fed a normal diet showed a significant increase in stride frequency ($2.18 \text{ steps/s} \pm 0.17$, n = 6) compared to WT animals fed a normal

diet ($1.7 \text{ steps/s} \pm 0.0$, $n = 2$, $p < 0.05$) or KD ($1.49 \text{ steps/s} \pm 0.05$, $n = 8$, $p < 0.01$; Fig 10b). KD-fed WT animals showed significantly lower stride frequency than normal diet-fed WTs ($1.49 \text{ steps/s} \pm 0.05$, $n = 2$, $p < 0.01$). Overtime, stride frequency of the KO animals fed a normal diet increased by 18% over the 3 month value (Fig 11).

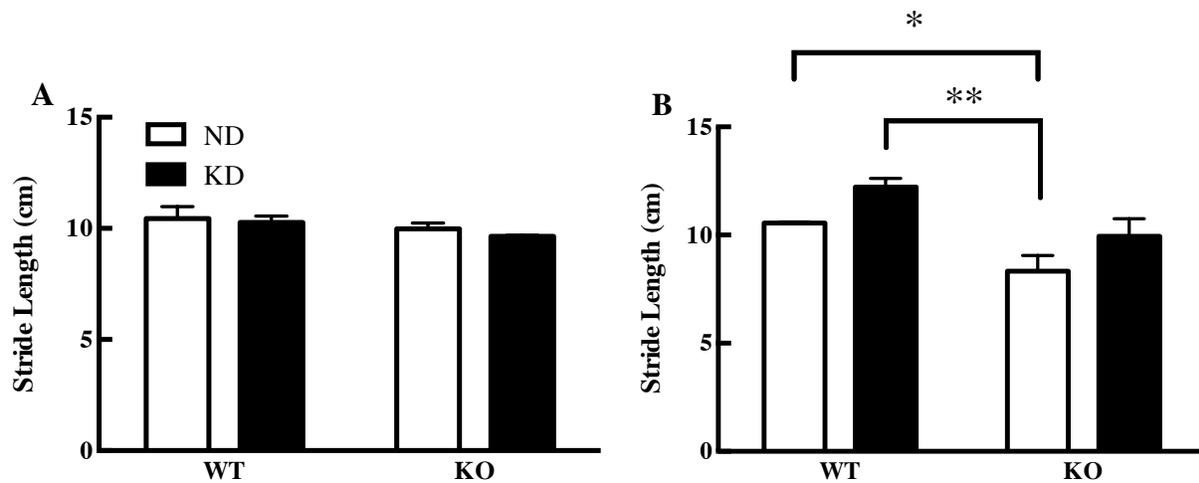


Fig. 8 Effects of the ketogenic diet (KD) on stride length in wild-type (WT) and knock-out (KO) animals fed a normal diet (ND) or KD. **A)** 3 months **B)** and 8 months. KO/ND animals showed a significant reduction in stride length at 8 months compared to WT animals on the ND (* $p < 0.05$). WT/KD animals had significantly higher stride length than KO/ND animals at 8 months (** $p < 0.01$). For all experimental groups $n = 2-4$.

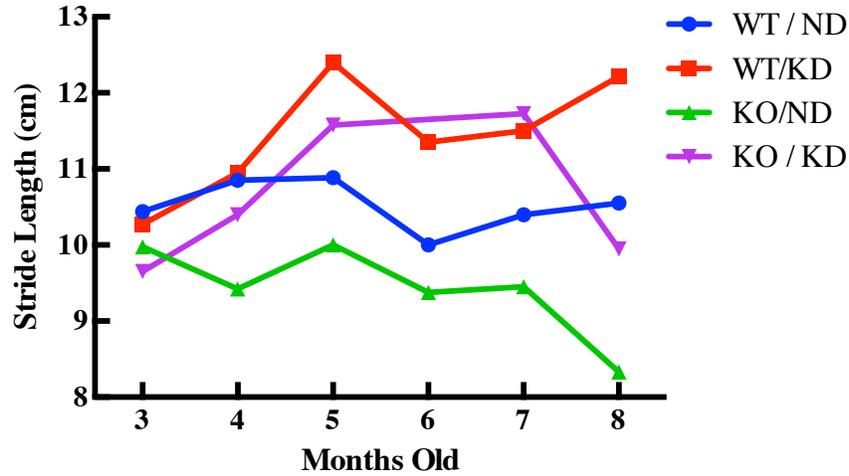


Fig. 9 Changes in stride length in wild-type (WT) and knock-out (KO) animals fed a normal diet (ND) or ketogenic diet (KD). KO/ND animals showed a decrease in stride length at 8 months compared to 3 months. KO/KD animals showed a similar stride length at 3 and 8 months. For all experimental groups n = 2-4.

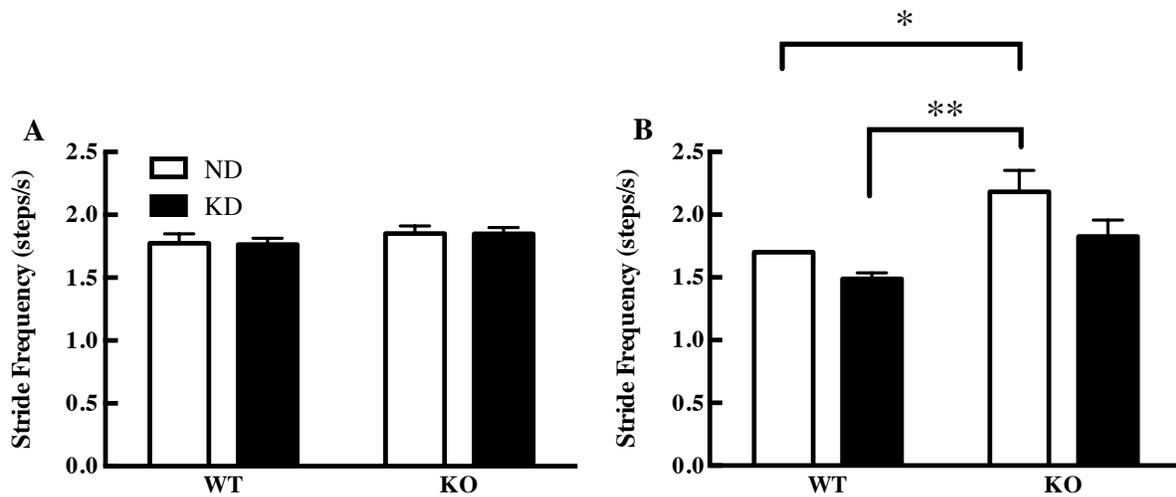


Fig. 10 Effects of the ketogenic diet (KD) on stride frequency in wild-type (WT) and knock-out (KO) animals fed a normal diet (ND) or KD. **A)** 3 months **B)** and 8 months. KO/ND animals showed a significant increase in stride frequency at 8 months compared to WT animals fed a ND (* p<0.05). WT/KD animals had significantly lower stride frequency than the WT/ND group at 8 months (** p<0.01). For all experimental groups n = 2-4.

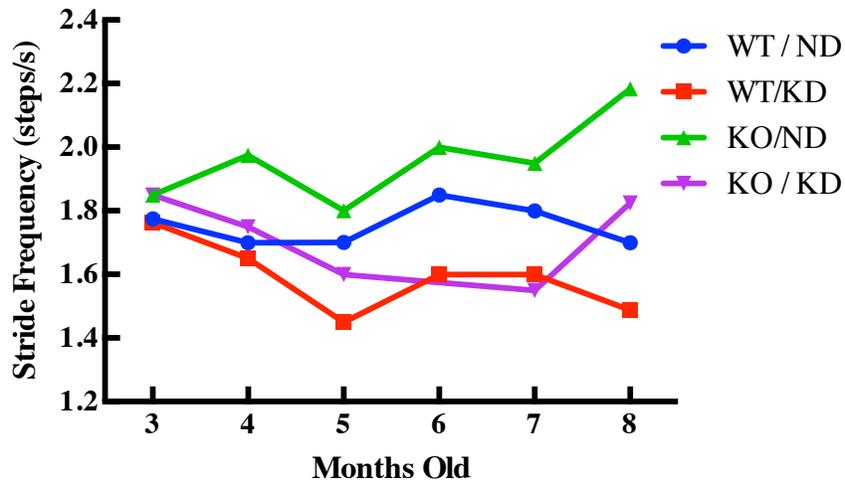


Fig. 11 Changes in stride frequency between 3 and 8 months in wild-type (WT) and knock-out (KO) animals fed a normal diet (ND) or ketogenic diet (KD). Stride frequency of KO/ND animals increased at 8 months compared to 3 months. KO/KD animals showed a similar stride frequency at 3 and 8 months. For all experimental groups n = 2-4.

KD changes purine neurochemistry

The KD selectively reduced adenosine and inosine levels in the brains of both WT and KO animals (Fig 12 and 13). In the nucleus accumbens, adenosine was 57% lower in KD-fed animals compared to WT animals fed a normal diet (WT/KD: $0.94 \mu\text{g}/\text{mg protein} \pm 0.20$, n = 3; KO/KD: $0.94 \mu\text{g}/\text{mg protein} \pm 0.17$, n = 3 vs. WT/ND: $2.18 \mu\text{g}/\text{mg protein} \pm 0.29$, n = 3, $p < 0.05$; Fig 12a). In the posterior caudate adenosine was 60% lower in the KD-fed WT animals and 57% lower in the KD-fed KOs compared to normal diet-fed WT animals (WT/KD: $0.99 \mu\text{g}/\text{mg protein} \pm 0.31$, n = 3; KO/KD: $1.04 \mu\text{g}/\text{mg protein} \pm 0.10$, n = 3 vs. WT/ND: $2.45 \mu\text{g}/\text{mg protein} \pm 0.42$, n = 2, $p < 0.01$ and $p < 0.05$; Fig 12b).

Inosine, a metabolite of adenosine, was non-significantly reduced in the nucleus accumbens of KD-fed WT animals and significantly reduced in the KD-fed KOs compared to the normal diet-fed WT animals (KO/KD: $0.42 \mu\text{g}/\text{mg protein} \pm 0.02$, n = 3 vs. WT/ND: $0.81 \mu\text{g}/\text{mg}$

protein \pm 0.04, $n = 3$, $p < 0.05$; Fig 13a). In the hippocampus, KD-fed WT and KD-fed KO animals had significantly lower inosine levels (42% and 43%, respectively) than those of KOs fed a normal diet (WT/KD: $0.60 \mu\text{g}/\text{mg}$ protein \pm 0.10, $n = 3$; KO/KD: $0.59 \mu\text{g}/\text{mg}$ protein \pm 0.11, $n = 3$ vs. KO/ND: $1.03 \mu\text{g}/\text{mg}$ protein \pm 0.23, $n = 3$, $p < 0.05$; Fig 13b). Finally, in the substantia nigra, KD-fed KOs showed significantly less inosine (36%) than normal diet-fed WT animals (KO/KD: $0.74 \mu\text{g}/\text{mg}$ protein \pm 0.15, $n = 3$ vs. WT/ND: $1.16 \mu\text{g}/\text{mg}$ protein \pm 0.15, $n = 3$, $p < 0.05$; Fig 13c).

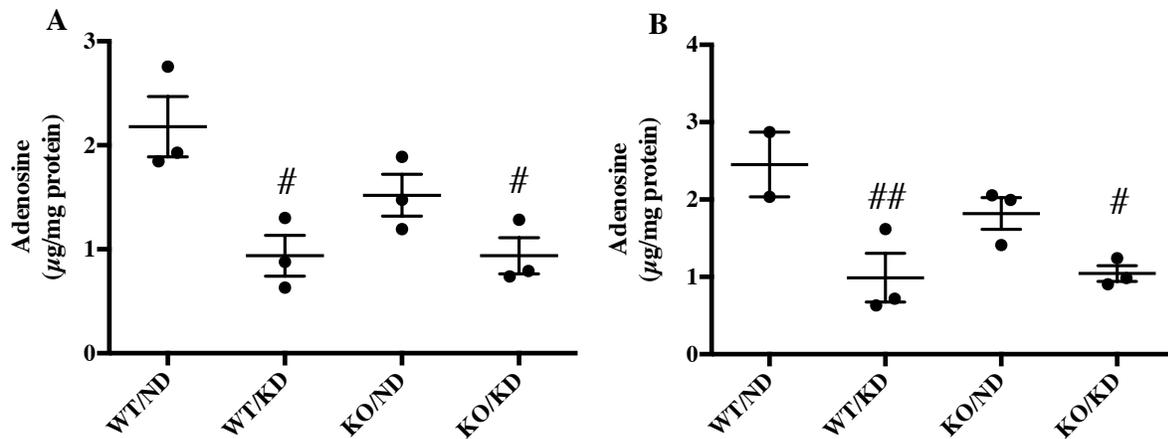


Fig. 12 Adenosine levels in in wild-type (WT) and knock-out (KO) animals fed a normal diet (ND) or ketogenic diet (KD) **A**) nucleus accumbens **B**) and the posterior caudate. Both KD-fed groups showed significantly lower adenosine levels in the nucleus accumbens (**A**) compared to the WT/ND group (# $p < 0.05$). The WT/KD and KO/KD groups also showed significantly lower adenosine levels in the posterior caudate (**B**) compared to WT/ND animals (## $p < 0.01$ and # $p < 0.05$, respectively). For all study groups $n = 2-3$.

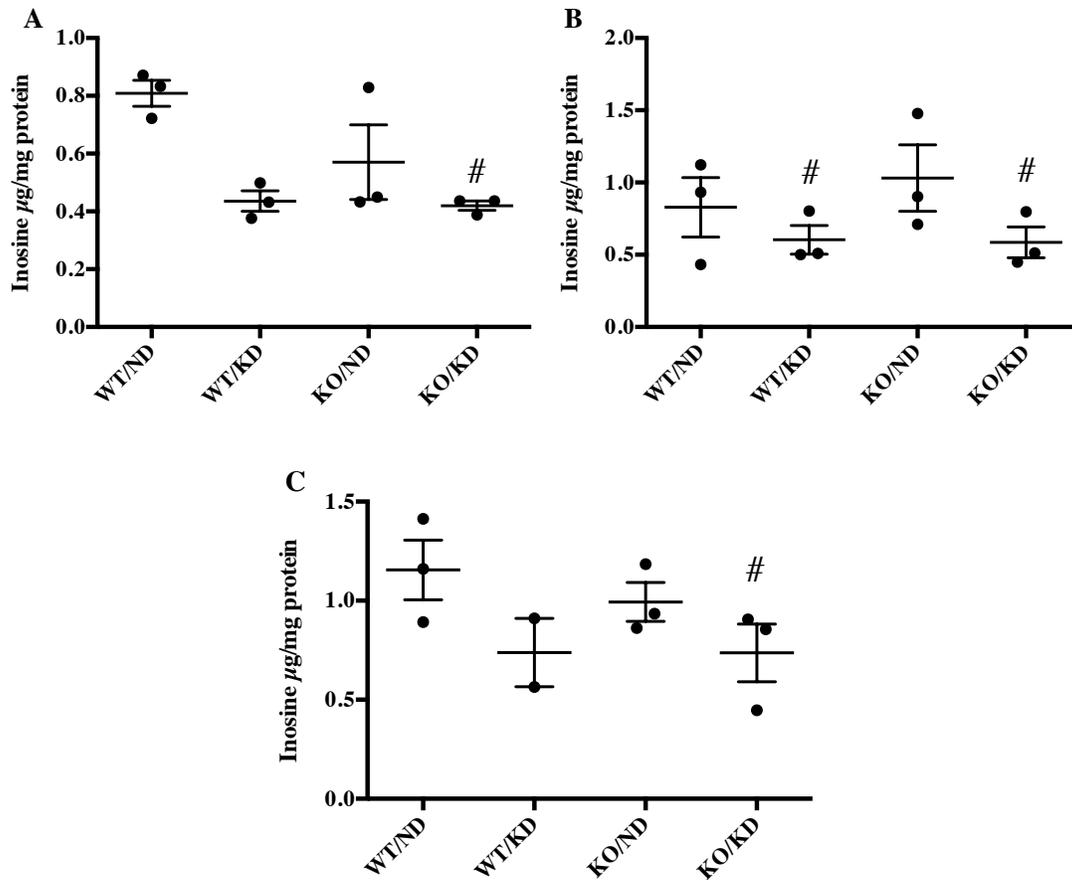


Fig. 13 Inosine levels in wild-type (WT) and knock-out (KO) animals fed a normal diet (ND) or ketogenic diet (KD). **A)** nucleus accumbens **B)** hippocampus **C)** and the substantia nigra. KO/KD animals showed significantly lower inosine levels than WT/ND animals in both the nucleus accumbens (**A**) and substantia nigra (**C**) (# $p < 0.05$). KD-fed WT and KO animals showed significantly less inosine in the hippocampus (**B**) than KO/ND animals (# $p < 0.05$). For all study groups $n = 2-3$.

KD appears to protect nigral dopamine cells

Immunohistochemical staining for TH-positive cells in the SN revealed that a KO animal fed a normal diet had noticeably fewer dopamine cells than a WT animal fed the KD (Fig 14).

The KD appeared to prevent this nigral TH-positive cell loss in KO animals.

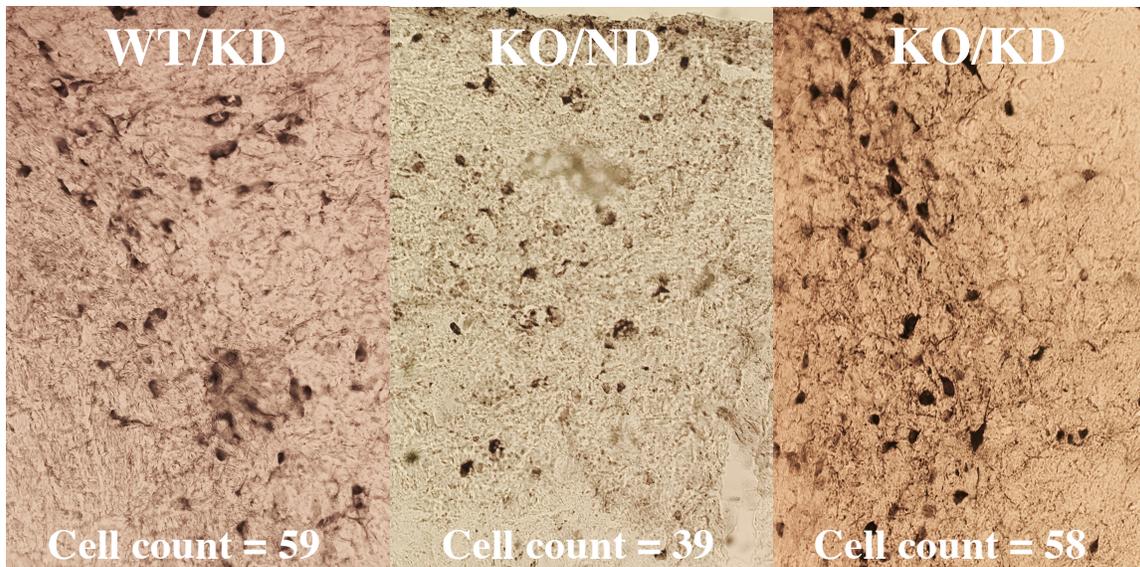


Fig. 14 Tyrosine hydroxylase (TH)-positive cells in the substantia nigra of a wild-type (WT) and knock-out (KO) animal fed a normal diet (ND) or ketogenic diet (KD). A KO animal fed the ND had observably less TH-positive cells in the substantia nigra than either KD-fed animals. For all groups n = 1.

DISCUSSION

Chronic KD does not prevent weight gain

The present study found that rats fed the KD for 5 months gained weight similarly to rats fed a normal diet. Several interesting differences between the weights of KD- and ND-fed animals were observed. Weight gain plots of KD-fed animals were more spread out than those of ND-fed animals. At the end of the 5-month diet, the final weights of KO/KD and WT/KD animals had standard deviations of 122g and 118g compared to 38g and 52g in KO/ND and WT/ND, respectively. Taken together, these data suggest that there may be greater weight-gain variability in KD-fed animals than in ND-fed animals.

A large number of studies on the effects of prolonged KD in animal models have examined KD regimens persisting for up to 11 weeks (DeVivo et al., 1978; Al-Mudallal et al., 1995, 1996; Hori et al., 1997; Cheng et al., 2009; Ruskin et al., 2013; Church et al., 2014). Administration of a KD for 20 days in Sprague-Dawley rats did not result in as much variance as we observed following a 5-month KD (DeVivo et al., 1978). Greater weight gain in KD-fed animals compared to control-fed animals has been reported after a slightly longer (5 week) KD regimen, although this difference was not statistically significant (Hori et al., 1997). Interesting results from a 5-6 week KD study, which used slightly higher protein content (10.4%) than the present study, described that animals were restricted to 10g of the diet per day in order to avoid excessive weight gain (Al-Mudallal et al., 1996). In comparison, our KD was only 8.6% protein and while the KD patties provided to our animals were observably equivalent in size they were not weighed. During daily replacement of KD patties, complete consumption of the previous day's patty was never observed.

KD alters β HB but not glucose levels

Since animals on the KD were not provided any external sources of carbohydrates, tolerated the diet, and gained weight it was deduced that they were converting fatty acids to ketone bodies to serve as their primary energy substrate. β HB and glucose blood levels were measured as an indicator of the conversion to ketosis. KD-fed KO animals had significantly higher blood β HB levels than their ND-fed counterparts. Interestingly, no significant differences in blood glucose levels were seen between either genotype or diet group. Other research reports elevated blood and cerebral β HB levels in KD-fed versus control-fed animals with no change in blood or cerebral glucose levels (Al-Mudallal et al., 1995). Under normal conditions, rat blood β HB levels have been reported to be between 0.20 – 0.30 mmol/L and glucose levels are between 4.00 - 12.17 mmol/L (Leino et al., 2001; Veech, 2004; Paoli et al., 2011). At 8 months of age, normal diet-fed WT animals had mean β HB and glucose levels of 0.60 and 4.86 mmol/L, respectively. KO animals fed the normal diet showed similar β HB levels (0.58 mmol/L) but slightly higher blood glucose levels (6.07 mmol/L) at 8 months. While β HB levels in our normal diet fed animals appear to be slightly higher than previous reports, blood glucose levels were well within the range reported by previous studies. After a 35-day KD, Leino et al. (2001) reported that rat plasma β HB and glucose levels were 1.0 and 11.5 mmol/L, respectively. In comparison, our KD-fed WT and KO animals had β HB levels of 0.94 and 0.97 mmol/L and glucose levels of 6.46 and 6.08 mmol/L, respectively. While β HB levels reported in the current study are nearly identical to previous reports, glucose levels in KD-fed animals are much lower. This could be a result of a shift in energy substrate from glucose to ketone bodies but, since blood data was only collected during the final three months and no initial level prior to KD administration was established no firm conclusion could be drawn regarding a change in energy

substrate. Still, the fact that no significant differences in blood glucose levels were observed between KD- and ND-fed groups suggests that perhaps a 5-month KD provides animals with ample time to compensate for the KD-induced glucose shortage. Indeed, it has been reported that glucose levels in KD-fed animals do not significantly differ from pre-diet levels after a 1-month KD regimen (Leino et al., 2001).

The neuroprotective properties of β HB are well established in Parkinsonian models. In an MPTP mouse model of PD, infusion of β HB protected against both the degeneration of DAergic neurons and the motor deficits associated with the MPTP toxin (Tieu et al., 2003). β HB has also been found to reduce the production of free radicals that induce lipid peroxidation of cell membrane and cause cell death (Maalouf et al., 2007).

Reports suggest that between 50% and 80% of Parkinson's patients have an atypical glucose tolerance (Lipman et al., 1974; Sandyk, 1993). This impaired glucose intolerance is a hyperglycemic state which often precedes diabetes. Furthermore, many of these glucose intolerant patients with PD fulfill criteria for diabetes (Lipman et al., 1974). Glucose consumption leads to insulin secretion and catecholamines have been suggested as modulators of this process (Buse et al., 1970). This glucose intolerance is made worse by L-DOPA therapies and it has been proposed that diabetes in Parkinson's disease may increase the severity of motor dysfunction (Sandyk, 1993). Individuals with glucose intolerance can exhibit fasting glucose levels that can be slightly elevated which may explain the slightly higher levels observed in our KD-fed animals compared to those fed the normal diet (American Diabetes Association, 2005).

KD increases stride length and decreases stride frequency

In humans, PD progression is associated with a decrease in stride length and shuffling gait (Morris et al., 1994). While patients are often able to compensate for these changes when walking at a pace of their selection, gait hypokinesia becomes apparent when walking speed is kept constant (Morris et al., 1994). Our data on PINK1-KO-induced gait changes align with these findings in humans as stride length in KO/ND animals was over 20% shorter than WT/ND animals at 8 months. In contrast, KO/KD animals were observed to have a mean stride length that was only 6% shorter than WT/ND animals. Interestingly, WT animals fed the KD exhibited a 16% increase in stride length between 3 and 8 months. Research examining the effects of the KD on the R6/2 1J mouse model of Huntington's disease, which found evidence of KD-induced improvements in locomotor coordination in male mice (Ruskin et al., 2011). Our results indicate that KD-fed KO animals had similar stride length at both 3 and 8 months while KO animals fed the normal diet exhibited a steady decrease in stride length representing a loss of 16.5% over the course of the experiment.

In parallel with a decrease in stride length, KO/ND animals showed a significant increase in stride frequency at 8 months of age compared to WT/ND animals at the same time point. This change in KO/ND animals amounted to a 15% increase over time, whereas KO/KD animals exhibited nearly identical stride frequency at both 3 and 8 months. WT/KD animals exhibited a 15% reduction in stride frequency between 3 and 8 months of age. As one might expect, the relationship between stride length and stride frequency is approximately inversely proportional. Taken together these results suggest a KD-induced attenuation of PINK1-KO-induced motor deficits.

Hind limb dragging has been reported in 30% of PINK1-KO rats (Dave et al., 2014). Three of the 12 KO animals in the current study were observed to exhibit hind limb dragging, a 25% prevalence rate. All three of these animals were fed the ND which may suggest that the KD prevented the development of this impairment. The DigiGait video footage captured hind limb dragging from below these animals while they walked and allowed for interesting observations to be made. In all three hind limb draggers an equinovarus-like folding of the toes under the pad of the dragged paw was observed. The current study is the first to report this feature of the hind limb dragging in PINK1-KO animals. Our initial results suggest that the KD prevented the development of Parkinson's-like gait changes in PINK1-KO animals.

KD changes purine neurochemistry

The KD is well known for its anti-epileptic effects (Hori et al., 1997; Dahlin et al., 2005, 2012; Baranano and Hartman, 2008; Masino et al., 2012). It has been suggested that a KD-induced increase in extracellular adenosine acts on A_1 receptors which reduce neuronal excitability (Masino and Geiger, 2008; Masino et al., 2011, 2012). Furthermore, adenosine is well-known for its modulatory influence over dopaminergic transmission in the basal ganglia via A_{2A} receptors (Hettinger et al., 2001; Rebola et al., 2005; Morelli et al., 2010, 2012). Antagonism of A_{2A} receptors on nigrostriatal DAergic neurons leads to an augmentation of inhibitory GABAergic activity, enhanced regulation of thalamocortical-facilitated motor function, and makes smooth motor movements possible (Ochi et al., 2000; Hauser RA and Schwarzschild MA, 2005). In light of the modulatory role of adenosinergic A_{2A} receptors on DAergic transmission and smooth motor movement, research has focused on targeting A_{2A} receptors for potential therapeutic applications in PD (Pinna et al., 1996, 2001, 2016; Fenu et al.,

1997; Koga et al., 2000; Strömberg et al., 2000b). This extensive support of A_{2A} receptor antagonism in PD contributed to the recent approval of istradefylline, an A_{2A} antagonist, as an additional therapy for PD (Kondo and Mizuno, 2015).

In the current study, a 5-month KD appeared to have a depressive effect on purine levels in both KO and WT animals. In the nucleus accumbens adenosine levels fell by 57% in both WT and KO animals fed a KD compared to WT animals fed a normal diet. In comparison KO/ND animals exhibited only a 30% decrease in adenosine in the nucleus accumbens compared to WT/ND animals. Additionally, adenosine was lower in the posterior caudate of KD-fed WT and KO animals by 60% and 58%, respectively, compared to WT/ND animals; a 26% decrease was observed in KO/ND animals. A similar trend was seen with inosine, a downstream metabolite of adenosine. In the nucleus accumbens KD-fed WT and KO animals had 46% and 48% less inosine, respectively, than WT/ND animals while KO/ND animals showed only a 30% decrease. In the hippocampus KO/ND animals showed a 19% increase in inosine compared to WT/ND animals while KD-fed WT and KO animals were observed to have decreases of 28% and 29%, respectively. The substantia nigra also showed a depression of inosine levels by 36% in both KD-fed groups and 15% in KO animals fed a normal diet. One potential explanation for these decreases could be that, due to the prolonged length of the diet, compensatory mechanisms become hyperactive and reduce purine levels.

Precise quantification of purines in the brain is very difficult because even the slightest ischemic event can lead to rapid degradation of ATP and subsequent increase in adenosine levels (Delaney and Geiger, 1996). In order to obtain precise cerebral purine levels it is important to inactivate the enzymes involved in the purine metabolic pathway. One such method uses high-energy focused microwave systems which not only kill experimental animals but simultaneously

denature proteins and halt enzyme function (Delaney and Geiger, 1996). This microwave method has been compared to more traditional decapitation methods and found significant differences in adenosine content in regions evaluated in the present study (Delaney and Geiger, 1996). Using 10kW microwave irradiation adenosine levels in the cortex, striatum, and hippocampus were between 50 and 75 times lower than those of animals sacrificed using decapitation. In comparison, adenosine levels in the cortex of animals sacrificed by decapitation were $0.022 \mu\text{g}/\text{mg}$ protein versus $3.46 \mu\text{g}/\text{mg}$ protein (combined motor and somatosensory cortex values) detected in our study. Our adenosine levels may be higher due to the perfusion procedure conducted on all animals prior to decapitation. Clearance of blood in the brain creates the kind of ischemic environment which leads to massive increases in adenosine up to 100-fold (Chu et al., 2013).

KD appears to protect nigral dopamine cells

Immunohistochemical staining for DA cells in the SN showed that a KD-fed KO animal had nearly 150% more DA neurons than a KO animal fed a normal diet. Previous studies have also reported the KD's protection of nigral DAergic neurons in toxin-induced models of PD (Cheng et al., 2009; Yang and Cheng, 2010). Studies evaluating the PINK1-KO confirm that KO animals do, in fact, exhibit nigral DA cell depletion (Dave et al., 2014; Villeneuve et al., 2014). Due to our small sample size, only one animal from each study group was stained for TH-positive cells and, as such, the present findings require additional animals to determine the statistical power of the observed effects. Still, cell counts from representative images of three of the four study groups (WT/ND group not shown due to technical difficulties in staining

procedure) suggest that the KD is able to prevent DAergic cell death in the PINK1-KO model of PD.

Methodological Considerations

As discussed previously (see methods), unforeseen errors in the perfusion of 8 animals led to removal of 3 rats from each group for purine quantification by HPLC. Additionally, the method of sacrifice likely resulted in inflated adenosine levels. In the event of a follow-up study the following recommendations are proposed:

- That a pressure-driven, rather than gravity-driven perfusion apparatus be used to perfuse cerebral vasculature
- Animals from each study group be sacrificed using microwave irradiation, rather than perfusion and decapitation, for quantification of purines by HPLC
- That a greater number of animals be included in the study

Conclusions

This study reports that a 5-month KD exhibits therapeutic potential to ameliorate gait changes and DAergic cell death induced by the PINK1-KO. Furthermore, neurochemical analysis suggests that a prolonged KD may have a depressive effect on purines in discrete regions of the striatum, basal ganglia, and the hippocampus. The findings of the current thesis provide initial evidence of the beneficial effects of the KD in the PINK1-KO model of PD and are deserving of a follow-up study with a larger sample size to reaffirm these promising results.

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APPENDIX – A. Solution Preparation

3x PBS (1L):

24.0g NaCl

8.01g Na₂HPO₄ • 7H₂O

0.6g KCl

0.74g KH₂PO₄

The above solutes were dissolved in 1L of DiH₂O and pH adjusted to 7.45 using 10M NaOH.

The solution was then vacuum filtered.

Note: 1x PBS used for animal perfusion was created by diluting 300mL of 3x PBS to 900mL using DiH₂O.

Stock Purine Megamix (PurM):

10μg of the following chemicals were dissolved in 100mL of DiH₂O: Adenosine, guanosine, inosine, xanthine, hypoxanthine, guanine, and uric acid.

Stock DHBA Internal Standard Solution (DISS):

Two concentration levels of DISS were used for different groups of animals over the course of the study (see appendix C for details).

8μg/mL DISS was created by diluting 4μg of DHBA to 500mL using DiH₂O.

16μg/mL DISS was created by diluting 8μg of DHBA to 500mL using DiH₂O.

Preparation of Mobile Phase (MP) Solutions:

MP-A (1L)

0.1g sodium 1-pentane sulfonate

27.2g potassium phosphate monobasic

The quantities of the two solutes above were dissolved in 1L with $\text{D}_2\text{H}_2\text{O}$, pH adjusted to 3.5 using 85% phosphoric acid, vacuum filtered for HPLC, then degassed for 45 min.

MP-B (250mL)

25mL of HPLC-grade acetonitrile was diluted to 250mL using MP-A and degassed for 15-20min.

APPENDIX – B. Pre-surgical Data Collection and Surgical Procedure

DigiGait Behavioral Testing:

Rats were placed on the DigiGait automated treadmill at a decline of 13 degrees and a treadmill speed of 18cm/s. Rats from each study group were selected for behavioral testing based on their initial willingness to walk. Representatives from each study group were established and tested once every month starting at 3 months old. During testing, rats that were reluctant to walk were placed in a ‘redo’ category and tested again at the end of the session.

Blood Testing for β HB and Glucose:

Blood tests were conducted three times, once during each of the last three months before sacrifice, by the tail prick method. Ketone and glucose levels were measured using β HB and glucose test strips and digital meters.

Surgical Procedure:

Scrubs, lab coats, facemasks, protective eyewear, and gloves were worn at all times during surgeries. Approximately 4-5mL of isoflurane liquid anesthetic was added to an isolation chamber located in a fume hood. Animals were placed in the chamber until signs of consciousness were no longer observed. To ensure that the animal was deeply anesthetized a sharp foot pinch was administered. A nose cap containing a cotton ball and approximately 1mL of isoflurane was used intermittently during surgery to ensure that animals remained fully anesthetized. An incision below the ribcage was made so that the heart was exposed. A

sharp 18-gauge needle was placed in the left ventricle of the heart and valve of the gravity-driven 1x PBS perfusion apparatus was opened. A small incision in the right atrium was made to allow efflux of blood. Perfusions were allowed to continue until fixation tremors were observed. At this point, the sharp-tipped needle was replaced with a dull-tipped needle which was reinserted into the left ventricle (Note: rats A-D were not perfused with the dull-tipped needle, nor was the sharp-tipped needle position adjusted during perfusion). To ensure a complete perfusion of the cerebral vasculature the dull-tipped needle was positioned in the ascending aorta. The perfusion was continued until all of the following signs were observed: fluid exiting the incision in the right atrium was clear (no blood), heart and liver were observed to show significantly reduced blood content (less red, more pale/pink in color), and fixation tremors ceased. Animals were then decapitated and brains were rapidly dissected and placed in a solution of ethanol and dry ice.

APPENDIX – C. Post-surgical Tissue Processing and Data Collection Procedures

Brain Slicing, Tissue Punching, and Brain Sample Preparation:

1. Microtome was cooled to -20 C using Physitemp regulator. The blade was cooled with dry ice
2. Brains were mounted using freezing medium and oriented with frontal regions facing upward.
3. Five 1.5mm slices were taken in order to take tissue punches from the following regions:
 - Slice 1: Motor cortex
 - Slice 2: Nucleus accumbens, somatosensory cortex, and anterior caudate
 - Slice 3: Posterior caudate
 - Slice 4: Hippocampus
 - Slice 5: Substantia nigra
4. Bilateral punches were taken from the regions described above, placed in blue 1.5mL centrifuge tubes containing 400uL HPLC-grade ethanol, and sonicated using the Tekmar pestle sonicator until tissue was thoroughly broken up.
5. Samples were then spun down at 2400rpm for 30 min in a microcentrifuge stored in -4 C.
6. After 30 min, the supernatant was carefully removed and transferred to yellow 1.5mL microcentrifuge tubes. (Note: blue microcentrifuge tubes were stored in a -80 C freezer for protein assays).
7. The ethanol in the supernatant was evaporated in the rotovap at 45 C for 30 min.

For Rats A-H:

8. The neurotransmitter pellet remaining in the yellow tubes were resuspended in 1mL of a 0.1 μ g/mL solution of DHBA in DiH₂O.

For Rats I-L:

8. 7 μ L of an 8 μ g/mL DHBA solution was added to 500 μ L of DiH₂O and neurotransmitter pellet.

For Rats M-X:

8. 7 μ L of a 16.6 μ g/mL DHBA solution was added to 500 μ L of DiH₂O and neurotransmitter pellet.
9. All brain samples were stored in a -80 C freezer until analyzed by HPLC.

HPLC Analysis:

A 100 μ L sample loop attached to a manual injector was used for sample injections. Prior to running calibration standards or brain samples, an injection of DiH₂O was run with the dual MP gradient protocol to establish an acceptable baseline. In a random order, each of the five calibration standards was injected and a calibration curve was generated for each of the 7 purine analytes (Note: detection of guanine and uric acid was not consistent and, as such, analysis of these two compounds could not be included in the study). Calibration standards were prepared as follows:

Preparation of Purine Calibration Standards:

Standard Concentration	Volume of DISS	Volume of PurM	Volume of DiH ₂ O
0.01 μ g	25 μ L	2 μ L	1973 μ L
0.05 μ g	25 μ L	10 μ L	1965 μ L
0.1 μ g	25 μ L	20 μ L	1955 μ L
0.5 μ g	25 μ L	100 μ L	1875 μ L
1.0 μ g	25 μ L	200 μ L	1775 μ L

Once a full set of calibration standards had been run and calibration curves generated, brain samples were run. On days following the injection of all five calibration standards the typical injection order was as follows:

DiH₂O

Calibration Std

3-4 Brain Samples

Calibration Std

3-4 Brain Samples

Calibration Std

Between each injection, the injection syringe and sample loop were rinsed first with a 50/50 Methanol/DiH₂O solution, then with 100% DiH₂O.

Protein Assays:

Standards:

- 1) Created 1N Folin Reagent by diluting 2N Folin & Ciocalteu Reagent by half using DiH₂O
- 2) Protein standards were created in 2mL microcentrifuge tubes as follows:

Std #	$\mu\text{L DiH}_2\text{O}$	$\mu\text{L Albumin Protein}$	Protein (mg)
0	500	0	0
1	480	20	0.04
2	470	30	0.06
3	460	40	0.08
4	450	50	0.10
5	430	70	0.14

- 3) 500 μL of Modified Lowry Protein Assay Reagent (MLPAR) was added to each standard, mixed, and allowed to react for 10 min.
- 4) After 10 min 100 μL of 1N Folin reagent was added to each standard, mixed, and allowed to react for 30 min.
- 5) Absorbance of each standard was measured at 750nm

For Brain Samples:

- 1) 500 μL of DiH_2O was added to each protein sample, then sonicated to mix
- 2) 500 μL of MLPAR was added to each sample, mixed, and allowed to react for 10 min.
- 3) After 10 min 100 μL 1N Folin reagent was added to each sample, mixed, and allowed to react for 30 min.
- 4) Absorbance at 750nm was measured for each sample.

TH-Staining Procedure:

Four rats (one from each study group) were perfused using 4% paraformaldehyde and post-fixed in 4% paraformaldehyde until they could be sliced. Full brains were placed in a sucrose gradient solution 30% sucrose and PBS for 24 hours, then transferred to 20% sucrose for 24 hours, and finally to a 10% solution for 24 hours. Brains were then frozen in a slurry of dry ice and 2-methyl butane for 4-8 min. Stored at -80 C for 24 hours. Defrosted in a -20 C freezer prior to slicing. Slices 20 microns thick of the substantia nigra were taken using a Cryocut 1800

set to 22 C and placed on Superfrost (ThermoFisher brand) slides. Slides were stored at -20 C until staining procedure was carried out. Slices were then fixed with acetone pre-cooled to -20 C. Once acetone had evaporated sections were washed twice in 1x PBS for 5 minutes. Slides were then incubated in 0.3% H₂O₂ in a 5% solution of goat serum in 1x PBS for 10 min to quench peroxidase enzyme activity. After 10 min, slides were rinsed with DiH₂O for 3 min. Slides were then washed again using 1x PBS for 5 min. Slides were then incubated for 30 min with diluted normal blocking serum 1x PBS and goat serum. After 30 min, excess serum was blotted from slides. Slides were then incubated overnight in 1:500 ratio of anti-TH to 1x PBS and goat serum solution of primary anti-TH antibody diluted using 1x PBS and 5% serum. Slides were then placed in a makeshift humidity chamber with a wet paper towel to keep slides moist. Paraffin covers were then placed on each slide and left for overnight (ideally left for 10 hours). The following day paraffin covers were removed slides were washed 3 times for 10 min with 1x PBS. Sections were then incubated for 30 min with 1:200 solution of biotinylated secondary antibody diluted using PBS serum. After 30 min slides were washed with 1x PBS for 5 min, then incubated for 30 min with Vectastain ABC reagent. After 30 min slides were washed in 1x PBS for 5 min, then 100 μ L of 3,3'-diaminobenzidine (DAB) substrate working solution was applied for 2-10 min (until color of staining was revealed). Once color of stains appeared, slides were placed in 1x PBS to stop the reaction. 1x PBS was then blotted from slides and 100 μ L of glycerol was carefully applied to the coverslips before placing the coverslips on the slides.

APPENDIX – D. Supplementary Data

Table 1. Summary of purine neurochemistry by brain region¹ in ketogenic diet (KD)- and normal diet (ND)-fed PINK1-KO (KO) and wild-type (WT) animals²

	MC	SC	NA	AC	PC	HC	SN
Adenosine							
WT/ND	2.17 ±0.59	1.29 ±0.11	2.18 ±0.29	1.35 ±0.38	2.45 ±0.42	1.45 ±0.13	0.80 ±0.06
WT/KD	0.78 ±0.28	0.89 ±0.45	0.67 ±0.36	0.90 ±0.44	0.78 ±0.45	0.85 ±0.43	0.41 ±0.37
KO/ND	2.21 ±0.99	1.38 ±0.47	1.43 ± 0.28	1.47 ±0.17	1.58 ±0.25	1.31 ±0.32	2.88 ±1.36
KO/KD	1.20 ±0.15	1.06 ±0.11	0.94 ±0.17	1.29 ±0.03	1.04 ±0.10	1.02 ±0.04	0.50 ±0.05
Guanosine							
WT/ND	0.07 ±0.02	0.13 ±0.04	0.11 ±0.03	0.07 ±0.02	0.12 ±0.05	0.07 ±0.03	0.05 ±0.01
WT/KD	0.31 ±0.22	0.21 ±0.14	0.17 ±0.10	0.18 ±0.08	0.19 ±0.10	0.28 ±0.12	0.35 ±0.22
KO/ND	1.10 ±0.73	1.05 ±0.49	1.10 ±0.48	1.04 ±0.49	1.17 ±0.59	1.29 ±0.71	0.50 ±0.29
KO/KD	0.08 ±0.02	0.08 ±0.03	0.06 ±0.02	0.09 ±0.03	0.04 ±0.02	0.08 ±0.04	0.07 ±0.00
Inosine							
WT/ND	0.66 ±0.08	0.60 ±0.03	0.81 ±0.04	0.63 ±0.10	0.59 ±0.15	0.83 ±0.21	1.16 ±0.15
WT/KD	0.38 ±0.08	0.38 ±0.16	0.35 ±0.12	0.30 ±0.13	0.34 ±0.13	0.47 ±0.20	0.47 ±0.44
KO/ND	0.23 ±0.13	0.28 ±0.23	0.31 ±0.26	0.13 ±0.11	0.26 ±0.15	0.54 ±0.47	0.46 ±0.36
KO/KD	0.36 ±0.06	0.42 ±0.07	0.42 ±0.02	0.39 ±0.04	0.34 ±0.06	0.59 ±0.11	0.74 ±0.15
Xanthine							
WT/ND	0.09 ±0.02	0.08 ±0.03	0.11 ±0.01	0.11 ±0.02	0.12 ±0.01	0.08 ±0.01	0.07 ±0.01
WT/KD	1.11 ±1.04	0.43 ±0.37	0.77 ±0.67	0.43 ±0.35	0.51 ±0.42	0.63 ±0.53	0.68 ±0.60
KO/ND	0.46 ±0.17	0.35 ±0.12	0.34 ±0.11	0.34 ±0.12	0.32 ±0.14	0.56 ±0.26	0.61 ±0.29
KO/KD	0.16 ±0.07	0.10 ±0.05	0.08 ±0.03	0.08 ±0.02	0.06 ±0.01	0.07 ±0.02	0.05 ±0.01

Hypoxanthine							
WT/ND	2.39 ±0.45	1.97 ±0.58	2.06 ±0.33	1.89 ±0.21	2.16 ±0.25	1.64 ±0.24	2.28 ±0.44
WT/KD	2.01 ±0.60	1.38 ±0.14	1.35 ±0.10	1.27 ±0.12	1.36 ±0.09	1.60 ±0.12	1.41 ±0.14
KO/ND	0.86 ±0.77	0.73 ±0.66	0.74 ±0.66	0.72 ±0.67	0.81 ±0.69	0.53 ±0.45	0.65 ±0.58
KO/KD	1.52 ±0.07	1.52 ±0.16	1.30 ±0.06	1.31 ±0.18	1.27 ±0.04	1.56 ±0.19	1.49 ±0.09

¹ MC – motor cortex; SC – somatosensory cortex; NA – nucleus accumbens; AC – anterior

caudate; PC – posterior caudate; HC – hippocampus; SN – substantia nigra

² Values given in $\mu\text{g}/\text{mg}$ protein \pm SEM

Images of Various Lesions and Ventricular Abnormalities



Image 1 Rat D incomplete perfusion of cerebral vasculature and third ventricle asymmetry in posterior caudate (PC) slice.



Image 2 Rat D incomplete perfusion of cerebral vasculature and lateral lesion of caudate observed in hippocampal (HC) slice.



Image 3 Rat F lesion and perfusion-induced ventricular asymmetry.



Image 4 Rat H perfusion-induced asymmetrical third ventricle in posterior caudate (PC) slice.



Image 5 Rat H perfusion-induced exploded left third ventricle in hippocampal (HC) slice.



Image 6 Rat L lesion first observed in motor cortex (MC) slice.

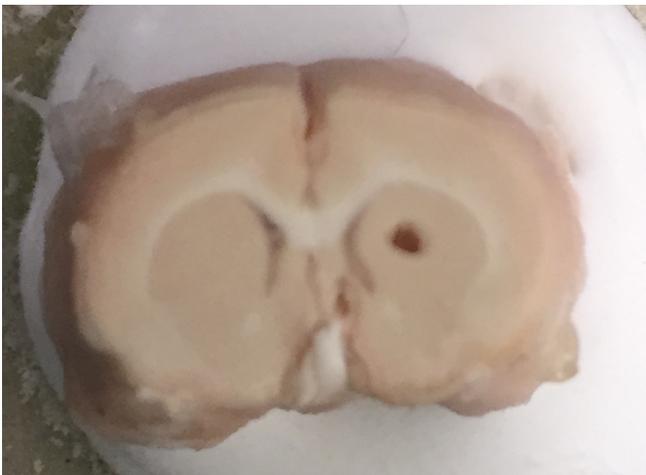


Image 7 Rat L lesion continued into posterior caudate (PC) slice.



Image 8 Rat L lesion started to close up after posterior caudate (PC) slice.



Image 9 Rat K lesion in right dorsal accumbal tissue.

APPENDIX – E. Manuscript from related undergraduate work

The following is a manuscript submitted for publication. It represents my undergraduate work which used the same HPLC method for quantifying purines as the current thesis to examine how the KD affects this neurochemical system.

The ketogenic diet does not alter tissue purine levels in mouse brain

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Highlights

- 3-week ketogenic diet did not alter tissue purine levels in wild-type mice
- Control-fed mice showed negative correlation between dopamine activity and adenosine levels in the cortex
- Ketogenic-fed mice did not exhibit negative correlation between dopamine activity and adenosine levels in the cortex

Abstract

Adenosine has been implicated in the therapeutic effect of the ketogenic diet. It is also known to modulate dopaminergic activity. We previously showed that the ketogenic diet increased cortical dopamine activity. This study evaluated whether the ketogenic diet produced changes in brain adenosine levels and purine activity. Samples from the previous study were analyzed for purines using a high performance liquid chromatography method for the quantification of

adenosine, hypoxanthine, xanthine, and inosine. No alteration in tissue levels of purinergic compounds was found in the ketogenic diet treatment group when compared to the control diet group. A negative correlation between dopaminergic activity and adenosine tissue levels was found in the cortex of the control diet group but was absent in samples from the ketogenic diet group. These findings support previous literature regarding interaction between the dopaminergic and purinergic neuronal systems and suggest a possible ketogenic diet-induced change in the purinergic modulation of cortical dopaminergic activity in mice.

Keywords: Adenosine; Ketogenic Diet; Dopamine; Purines; Epilepsy

1. Introduction

Epilepsy is a neurological disorder associated with episodic seizures, sensory disturbances, loss of consciousness, and unusual behavior. The ketogenic diet (KD) has successfully been used to treat medically refractory epilepsy, especially in children (Sirven et al., 1999; Hemingway et al., 2001). The diet consists of a high fat, low protein, and low carbohydrate regimen that produces a change in metabolism such that blood glucose levels decrease and β -hydroxybutyrate levels increase (Hartman et al., 2007). Currently the mechanisms through which the KD ameliorates epileptic seizures is poorly understood.

Studies examining the neurochemical changes associated with the KD have been limited to date. The KD has previously been reported to affect glutamatergic systems (Yudkoff et al., 2004; Dahlin et al., 2005), adenosine (Masino et al., 2011; Ruskin and Masino, 2012) and catecholamine transmitters, including dopamine (DA) (Szot et al., 2001; Weinshenker, 2008; Dahlin et al., 2012). Increased levels of the tryptophan metabolite kynurenic acid in the striatum

and hippocampus, but not the cortex, have also been observed in rats fed a KD (Żarnowski et al., 2011). Previous work in our lab found that mice fed a chronic (three-week) KD had increased DAergic activity in the motor and somatosensory cortices (Church et al., 2014).

The KD has been found to improve mitochondrial function and thus energy metabolism (Stafstrom and Rho, 2012). It has been suggested that metabolic changes induced by the KD impact purine neurochemistry. Kawamura et al. (Kawamura et al., 2010) showed that reducing extracellular glucose causes the neuronal release of ATP. Zhang et al. (Zhang et al., 1995) found that stimulation of P_{2Y} receptors, by ATP, resulted in increased extracellular DA in the rat striatum. Adenosine, the breakdown product of extracellular ATP, also has the ability to modulate DAergic activity (Krügel et al., 2003; Fuxe et al., 2007). Alteration of adenosine levels has been suggested to be associated with the anti-seizure effects of the KD (Masino and Geiger, 2008; Greene, 2011). Taken together, these findings suggest a potential change in the interaction between adenosine and DAergic activity under the metabolic state induced by the KD.

The purpose of the present work was to determine if the KD (1) altered adenosine levels in brain tissue and (2) altered the activity of the purinergic system. We analyzed samples from six brain regions in mice fed a KD for three weeks for adenosine and its metabolites. While no change in tissue levels of purines was found, we observed that a negative correlation between cortical DA activity and adenosine levels in control diet mice was absent in KD mice.

2. Materials & Methods

2.1. General overview, and animals

The brain samples were obtained from the study by Church et al. (Church et al., 2014). The current study evaluated the neurochemical concentrations of adenosine (Ado), inosine (Ino),

xanthine (Xanth), and hypoxanthine (Hypo). Purine content was measured in the motor cortex (MC), somatosensory cortex (SC), nucleus accumbens (NA), anterior and posterior caudate-putamen (ACP and PCP), and the midbrain (MB) using high performance liquid chromatography (HPLC) with UV and electrochemical detection (Burdett et al., 2013). All animal care, use, and surgical procedures were approved by the Institutional Animal Care, the Use Committee of Trinity College, and are in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. Chemicals

Two mobile phases were used for HPLC analysis. Mobile phase A (MP-A) contained 0.52 mM sodium 1-pentane sulfonate, 0.20 M potassium phosphate monobasic and was pH adjusted to 3.5 using 85% phosphoric acid (Baker Analyzed; Phillipsburg, NJ, USA). Mobile phase B (MP-B) contained identical concentrations as MP-A plus an addition of 10% acetonitrile (HPLC-Grade, Fisher Scientific, Pittsburgh, PA, USA). All purine chemicals used as standards were acquired from Sigma Aldrich (St. Louis, MO, USA).

2.3. HPLC Parameters

The neuroactive compounds were separated using a dual-gradient reverse-phase HPLC system with electrochemical (EC) and UV detection. The mobile phases were run at a flow rate of 0.5mL/min. Separation was carried out at 22°C on a 150 x 2.00mm LUNA 5 μ m C18 analytical column (Phenomenex, Torrence, CA, USA) using a dual mobile phase gradient to achieve proper separation of the analytes. The gradient was 100% MP-A for 6 minutes increasing to 55% MP-B at 14 minutes. The 45% MP-A and 55% MP-B gradient was maintained from 14 – 18 minutes then returned to 100% MP-A. Total separation time was 20 minutes and the system was allowed to equilibrate for 15 min between each sample injection.

The sample injection volume was 100 μ L. Dual electrochemical detection (ESA Coulochem III; $E_1 = -150\text{mV}$; $E_2 = +500\text{mV}$; Thermo Scientific, Sunnyvale, CA, USA) and UV detection ($\lambda_1 = 254\text{nm}$; BioAnalytical Systems, West Lafayette, IN, USA) were used to quantify the purines. Chromatographic data was collected, stored, and analyzed using EZ Chrom chromatography software (Thermo Scientific).

2.4. Statistical Analysis

Differences in the levels of purines in the brain tissue were evaluated using two-way ANOVA with post hoc comparisons (Bonferroni; $n = 6-7$ brains per diet group; GraphPad Prism 6.0, GraphPad Software, Inc., San Diego, CA, USA).

3. Results & Discussion

Table 1. Purine tissue content from selected brain regions of mice fed either a normal chow (Control) or a ketogenic diet (KD) for three weeks.^a

Brain Region	Hypoxanthine		Xanthine		Inosine		Adenosine	
	Control	KD	Control	KD	Control	KD	Control	KD
	132.9 \pm							
MC	40.7	79.6 \pm 7.7	2.5 \pm 0.4	3.4 \pm 1.1	29.9 \pm 10.3	24.7 \pm 5.4	71.8 \pm 25.5	50.4 \pm 6.6
	102.8 \pm							
SC	23.3	90.1 \pm 6.5	1.7 \pm 0.3	3.6 \pm 1.0	19.5 \pm 6.4	26.7 \pm 4.7	54.4 \pm 19.8	49.2 \pm 7.2
	78.3 \pm							
NA	18.9	61.1 \pm 13.1	8.5 \pm 4.9	4.0 \pm 2.3	29.2 \pm 9.9	30.2 \pm 7.1	40.2 \pm 12.3	42.9 \pm 11.6
	155.1 \pm							
ACP	88.8	48.8 \pm 9.5	6.5 \pm 2.9	1.7 \pm 0.7	48.4 \pm 19.5	15.8 \pm 2.6	71.2 \pm 32.4	34.6 \pm 7.3
	42.2 \pm							
PCP	18.9	51.6 \pm 7.4	6.2 \pm 2.4	2.6 \pm 1.3	16.7 \pm 5.2	20.9 \pm 3.5	40.8 \pm 13.0	31.1 \pm 3.2
	111.9 \pm							
MB	37.4	44.8 \pm 15.4	4.2 \pm 2.4	1.8 \pm 0.5	42.0 \pm 9.7	34.5 \pm 13.1	23.1 \pm 7.2	13.9 \pm 4.0

^aData are reported as ug/mg protein \pm S.E.M. for $n = 6$ and 7 KD and control, respectively

3.1. A ketogenic diet does not alter tissue purine levels in mice

A three-week KD regimen did not alter endogenous purine levels in any of the brain regions analyzed (Table 1). Figure 1 shows that a KD had no effect on adenosine activity in the regions analyzed. The mechanism by which the KD ameliorates seizures in animals and humans is not known. Numerous neurochemical systems have been implicated with recent studies focusing on energy metabolism and adenosine (Greene, 2011; Ruskin and Masino, 2012). Previously it has been reported that transgenic mice with spontaneous hippocampal seizures experienced decreased seizure frequency following a KD. After being fed a three-week KD these transgenic mice were reported to have low endogenous adenosine levels due to an overexpression of adenosine kinase (Fedele et al., 2005). After being fed a three-week KD, seizure frequency decreased by almost 90% but could be restored by injecting exogenous glucose or adenosine A₁ receptor antagonists (Masino et al., 2011). The current findings indicate that the KD did not alter endogenous purine levels in wild-type (WT) mice (Table 1). Although not statistically significant, KD-fed mice were observed to have lower adenosine levels than control-fed mice across almost all the brain regions analyzed. This finding is consistent with a recent paper which reported that non-disease-state mice fed a KD show lower levels of adenosine in the hippocampus than controls (Lusardi et al., 2015). However, this may not be reflective of diet-induced changes in neuronal purinergic activity. One measure frequently used to evaluate neuronal activity is to compare the levels of metabolites to the parent compound (Heffner et al., 1980; Kato et al., 1984; Church et al., 1986; Desole et al., 1996). We subsequently evaluated adenosine activity (Fig 1) and saw no alteration as a result of the KD. These findings do not support an enhanced augmentation of adenosine as a possible anti-seizure mechanism for the

KD. However, the methods used in this study measure both intra- and extracellular purine levels. It is possible that the KD alters mechanisms exclusively impacting extracellular levels of adenosine that would not be observable under the present experimental conditions. Additionally, the contribution of purines from residual blood found in post-mortem brain tissue could be a confounding factor. This is currently under investigation.

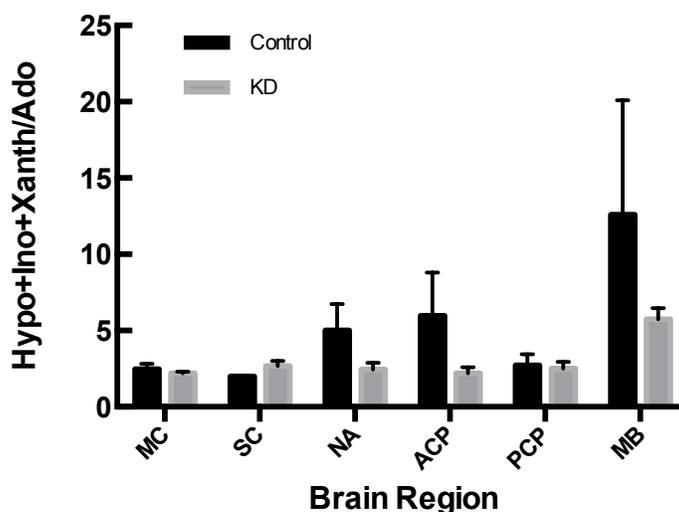


Figure 1. Effect of the KD on adenosine activity. Adenosine activity was defined as the sum of adenosine metabolite levels (hypoxanthine, xanthine, and inosine) divided by adenosine levels. No significant differences in the ratio of adenosine metabolites to adenosine were detected; ($p > 0.05$), Control $n = 7$, KD $n = 6$.

3.2. The KD alters the relationship between purinergic and dopaminergic systems

Since adenosine has a main role in the CNS as a modulator of DA activity, we were interested to see if the KD altered the relationship between DA activity and adenosine levels. Previous research from this lab has shown that the KD increases DA activity exclusively in the motor and somatosensory cortices (Church et al., 2014). Figure 2 reports the correlation between DAergic activity and adenosine in brain samples of both cortical regions. A negative correlation was observed in animals fed the control diet ($r = -0.88$, $p < 0.05$, $n = 6$). This negative correlation

was not observed in animals fed the KD ($r=+0.44$, $p=n.s.$, $n=10$). Midbrain samples from control and KD animals showed similar results (CD: $r=-0.81$, $p=0.09$; KD: $r=+0.41$, $p=n.s.$). No other brain regions showed this type of relationship. Interactions between adenosine and the DAergic system are well documented (Ferre et al., 1991; Ferré et al., 1991; Pinna et al., 1996; Ginés et al., 2000; Salim et al., 2000; Short et al., 2006). Krügel et al. (Krügel et al., 2003) reported that perfusion of adenosine into the nucleus accumbens significantly reduced extracellular DA concentration and suggests that stimulation of A_1 receptors facilitates this decrease. Both the DAergic and adenosinergic systems have been implicated in the modulation of seizures (Fedele et al., 2006; Bozzi and Borrelli, 2013). The negative correlation between adenosine and DA activity is consistent with an inhibitory modulation by adenosine. Inhibitory A_1 receptors (A_1R) are heavily expressed in the cortex (Rivkees et al., 1995). While it is not clear if the present findings represent a loss of inhibitory modulation, a putative mechanism could be as follows: (a) the KD produced increased release of ATP with resultant increased extracellular adenosine levels; (b) this increase in extracellular adenosine could alter the modulatory effect on DA cells from one of A_1R -dependent inhibition to one of excitation by presynaptic A_{2A} receptors; (c) increased A_{2A} receptor stimulation would result in increased DAergic activity (Okada et al., 1996; Quarta et al., 2004); (d) the increased DAergic activity may then act on cortical glutamatergic neurons impacting seizure activity. De Sarro et al. (De Sarro et al., 1999) reported seizure suppression in an animal model of reflex epilepsy by A_{2A} agonists. Furthermore, the results of a recently published study indicate that selective antagonism of A_1Rs and $A_{2A}Rs$ reverses the anticonvulsant effect of caprylic acid in mice (Socąła et al., 2015).

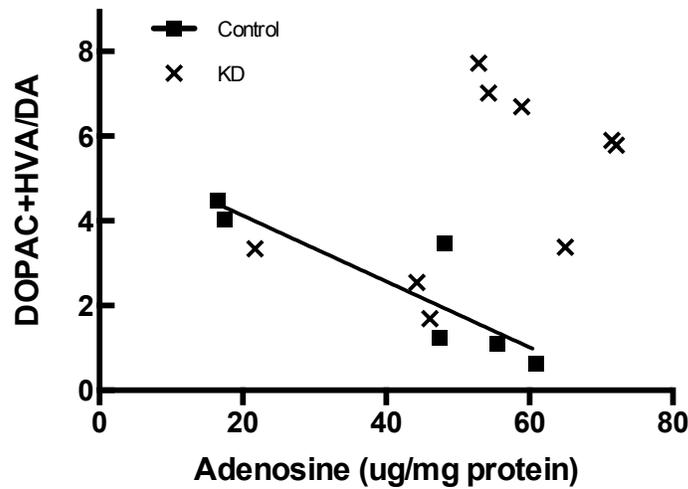


Figure 2. The correlation between cortical tissue adenosine levels and DA activity^a as measured by the sum of DA metabolites (DOPAC and HVA) divided by DA levels. Correlation coefficients (r) were -0.88 (p<0.05, n=6) and +0.44 (p=n.s., n=10) for control and KD, respectively. Values from motor and somatosensory cortices were combined for each diet group. ^a DA activity values taken from ref. 3

To our knowledge, the current paper is the first to evaluate the effect of a chronic KD on tissue levels of purines in multiple brain regions of experimental animals. Figure 3 is a graphical representation of adenosine tissue content in the brain structures analyzed. Interestingly, adenosine levels varied in a descending manner in both diet groups from anterior to posterior ranging from 61.1 $\mu\text{g}/\text{mg}$ protein in the motor cortex to 18.5 $\mu\text{g}/\text{mg}$ protein in the midbrain. A recent paper measuring cerebral adenosine levels in five strains of mice using HPLC reported wide variability across five inbred mouse strains (Pani et al., 2014). For example, in the cerebral cortex the strain with the highest adenosine levels was 415% greater than the strain with the lowest adenosine levels. The present paper reports cortical adenosine levels at 71.8 $\mu\text{g}/\text{mg}$ protein compared to 0.013 $\mu\text{g}/\text{mg}$ wet weight reported by Pani and his colleagues. One possible explanation for could be that the samples used in the current study had previously been used in

another and may have been thawed and frozen multiple times allowing for possible breakdown of upstream adenosine metabolites.

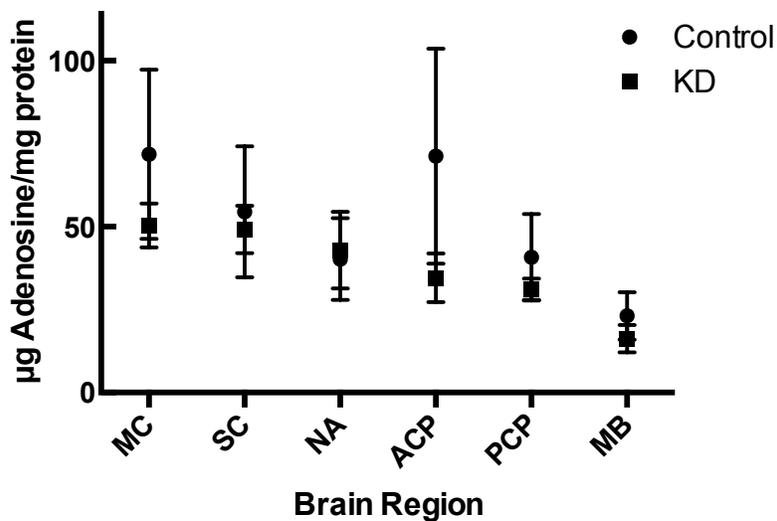


Figure 3. Adenosine levels in various structures of mouse brain were evaluated in eight week-old mice fed either a ketogenic diet or control diet for three weeks. No significant differences in adenosine levels were detected; ($p>0.05$). Brain structures: MC = motor cortex, SC = somatosensory cortex, NA = nucleus accumbens, ACP = anterior caudate putamen, PCP = posterior caudate putamen, MB = midbrain.

4. Conclusion

While the current results suggest that the KD does not chronically alter the endogenous levels of adenosine in brain tissue, they provide valuable insight as to the potential interactions between the DAergic and purinergic systems and their role in the anti-epileptic effects of the KD. Further study aims to determine whether extracellular purine levels specifically are affected by the KD in order to more directly elucidate the role of adenosine in the diet's efficacy.

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Author Contributions

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Notes

The authors declare no competing financial interest.

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