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The effects of a ketogenic diet on sensorimotor function in a thoracolumbar mouse spinal cord injury model

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Abstract

Spinal cord injury (SCI) and peripheral nerve injuries are traumatic events that greatly impact quality of life. One factor that is being explored throughout patient care is the idea of diet and the role it has on patient outcomes. But the effects of diet following neurotrauma need to be carefully explored in animal models to ensure that they have beneficial effects. The ketogenic diet provides sufficient daily caloric requirements while being potentially neuroprotective and analgesic. In this study, animals were fed a high fat, low carbohydrate diet that led to a high concentration of blood ketone levels that was sustained for as long as the animals were on the diet. Mice fed a ketogenic diet had significantly lower levels of tyrosine and tryptophan but the levels of other monoamines within the spinal cord remained similar to control mice. Mice were fed a standard or ketogenic diet for 7 days before, and 28 days following the injury. Our results show that mice hemisected over the T10-11 vertebrae showed no beneficial effects of being on a ketogenic diet over a 28 day recovery period. Similarly, ligation of the common peroneal and tibial nerve showed no differences between mice fed normal or ketogenic diets. Tests included von Frey, open field, and ladder-rung crossing. We add to existing literature showing protective effects of the ketogenic diet in forelimb injuries by focusing on neurotrauma in the hindlimbs. The results suggest that ketogenic diets need to be assessed based on the type and location of neurotrauma.

Significance Statement

There is an urgent need for therapeutics to improve outcomes for patients with neurotrauma. Here we test the effects of a non-invasive diet-based therapy. Ketogenic diets, which are high fat and low carbohydrate-based, have been shown to be effective in treating epilepsy, Parkinson's Disease, and show promise for treating other neurotrauma and neurodegenerative conditions. Here we show that while we were successful in producing high ketone concentrations in mice, the effects on recovery of function and pain following a thoracic spinal cord hemisection or spared nerve injury were minimal. Therefore, ketogenic diets, while effective in certain cases, should be evaluated depending on the injury type.

Introduction

Nerve injuries to the central and peripheral nervous systems are a heterogeneous group of conditions that cause significant disability, which also share common characteristics. There are multiple underlying causes contributing to peripheral nerve injury and spinal cord injury (SCI) such as neurotrauma, demyelination, and autoimmune disorders (Ahuja and Fehlings, 2016). In addition to movement disorders, patients with neural injuries have high incidences of persistent pain. Epidemiological data suggests 53% of SCI patients experience pain (Burke et al., 2017), with the global prevalence of neuropathic pain (pain as a result of nerve injuries) estimated at 7% (van Hecke et al., 2014). More importantly, persistent pain inhibits patients from adhering to rehabilitative therapies (Turk and Rudy, 1991), thus pain and loss in motor functions form a negative feedback loop that further perpetuates disabilities (Thompson et al., 2016).

For patients with nerve injuries, improvement in motor functions and reduction in chronic pain are important for the quality of life (Adams and Hicks, 2005; Jensen et al., 2007). However, few therapies exist to target both pain and motor functions simultaneously, in part due to the diversity in etiologies and disease progress. Most therapies targeting neurodegeneration and pain are symptomatic and do little to halt further loss in function (Scholz et al., 2009). Existing pain therapies including gabapentin and opioids all have side effects (Teasell et al., 2010), with the development of tolerance and dependence hindering treatment success. Overall, patients rely on combinatorial therapies, requiring multiple types of medications and management, thus resulting in suboptimal rates of adherence to their prescriptions. The identification of a more comprehensive therapy that targets pain and movement disorders is urgently needed for improving care in nerve injuries (Simpson et al., 2012; Teasell et al., 2010).

A key feature of nerve injury is neuroplasticity, which broadly manifests as changes in neuronal excitability and may aid spontaneous recovery by allowing sprouting of neural fibres reinforcing reflex and propriospinal pathways (Bareyre et al., 2004; Finnerup and Baastrup, 2012). However, mechanisms underlying neuroplasticity may also negatively impact upon pain and spasticity (Brown and Weaver, 2012) when maladaptive synaptogenesis occurs. Changes in levels of neuromodulators including serotonin, dopamine and noradrenaline are crucial facilitators of neuroplasticity (Azmitia, 1999; Marzo et al., 2009; Nitsche et al., 2006). Importantly, each of these neuromodulators engages in diverse physiological functions including cognition, mood regulation, movement and somatosensation; the therapeutic potential in targeting these neuromodulatory systems is hampered by their diverse physiological roles.

Different diet regimes hold promise as a non-pharmacological alternative to treat neural injury. Metabolism influences brain activity (Ruskin and Masino, 2012) and metabolic functions, in turn, depend on the diet (Flint et al., 2015). Studies investigating the clinical utility of metabolic therapies, such as the ketogenic diet (KD), indicate effectiveness in epilepsy, brain cancer, type II diabetes and neurodegeneration (Barañano and Hartman, 2008). KD promotes the use of ketone bodies as energy sources by minimizing glucose metabolism and increasing ketolysis. By replacing glucose with ketone bodies, a direct consequence is the enhanced cellular capacity in energy generation, thus increasing the availability of high energy molecules including adenosine triphosphate (ATP) and phosphocreatine (Ruskin and Masino, 2012). Other studies

have also shown an indirect link between KD and the increase in inhibitory neuromodulators such as adenosine and gamma-butyric acid (GABA) (Yudkoff et al., 2007). Hence, KD may be associated with a role in balancing neuronal excitability. In SCI, KD increased mobility, range of motion, and dexterity, in the forepaws of rats following a high cervical SCI (Streijger et al., 2013). Interestingly, a combinatorial therapy that included KD, ibuprofen, ghrelin, and a peptide (C16), did not improve motor function (Streijger et al., 2014). However, there has been little research on the effects of KD on lumbar SCI or peripheral nerve injury. This is important since cervical segments control precision movements of the digits, during grasp for example, compared to more rhythmic movements of the hindlimbs that can be generated by spinal circuits.

The current study explores the therapeutic potential of KD in regulating motor and pain dysfunctions in nerve injuries. In C57/BL6 mice, two of the most common nerve injury models including a thoracic hemisection (SCI) and the spared nerve injury (SNI) were performed to mimic damage to the central and peripheral nervous systems, respectively. A battery of sensory and motor behavioural tests were performed before and after the injuries to test baseline and post-injury effects of KD. The levels of neuromodulators were compared between mice fed standard chow and KD.

Methods

All animals used were C57/BL6 male mice between 8-16 weeks of age during testing. All animal experiments were approved by the University of Calgary animal care committee and are catalogued under the protocol AC15-0026.

Ketogenic Diet administration

Two standard diets were used with the animals in this study and were given *ad libitum*: Pellet Diet (PD) and 6:1 Ketogenic Diet (KD). All animals were initially fed a standard pellet diet ad libitum (Pico-Vac Mouse Diet 20 (Lot: 5062), LabDiet, St. Louis, MO, USA) given in the hanging feeding rack of the cages. KD animals were fasted for one evening (12 hours), a week before testing and given approximately 15 g of frozen KD chow daily (Bio-serv S3666, Bio-Serv, Flemington, NJ, USA) to ensure ketone blood levels were above the threshold for ketosis (Smith et al., 2016). Nutritional composition and macronutrient levels are listed in Table 1.

Ketone blood sampling

Blood ketone concentrations were sampled using blood draws from the tail. Approximately 1.5-2 µL was extracted by making a superficial cut approximately 1 mm from the end of the tail using a scalpel blade (# 10, cat no:10010-00, Fine Science Tools, North Vancouver, BC, Canada). The blood was massaged out of the tail and this was tested using a ketone blood monitor (Freestyle Precision Neo, Abbott Laboratories Inc., Mississauga, ON, Canada) and keto testing strips (Abbott blood beta-ketone test strip, Abbott Laboratories Inc., Mississauga, ON, Canada). All values were recorded in the software for offline analysis.

HPLC tissue collection

In a cohort of 26 Animals, mice were randomized and coded prior to sacrificing after 28 days of being in ketosis. KD animals underwent the standard KD diet paradigm as outlined above. On the day of tissue collection, animals were sacrificed using high doses of isoflurane (5%) in an induction chamber. The spinal column was extracted by cutting the sacrum and cervical vertebral levels using large scissors. The spinal cord was extracted using a 10 ml syringe loaded with aCSF with fluid pressure initiated in the lumbar region. The spinal cord was trimmed to contain only the lumbar enlargement (L1-L6) and the spinal cords were flash-frozen in liquid nitrogen. The spinal cord was analyzed for biogenic amines by modifications of a previously reported method (Parent et al., 2001). Tissue was homogenized in ice-cold 0.1 N perchloric acid containing EDTA (10 mg%) and ascorbic acid (50 µM). The homogenate was centrifuged and 10 µL of supernatant was used in the high performance liquid chromatography (HPLC) assay employing an Atlantis dC18 column (Waters) and an electrochemical detector.

Surgical intervention

All surgical procedures were performed using aseptic techniques under isoflurane anesthesia between 1-2 % delivered by 0.4 L/min of medical grade oxygen (Vitalair 1072, 100 % oxygen). The area of surgical intervention was shaved using animal shears and cleaned with betadine 5% solution and sterilized with 95 % ethanol.

Spared nerve injury

Once anesthetized and prepared, a superficial cut of 1 cm was made in the left hind leg in the skin above the midline of the femur; above the knee joint to below the hip joint. The skin was resected from the fascia by blunt dissection to properly view the musculature of the triceps surae and the biceps femoris. The muscles were separated along where both intersect. The sciatic nerve was isolated between the trifurcation of the common peroneal (CP)/Tibial nerve(Tib) and the sural nerve with surrounding fascia removed. Both the CP and Tib were ligated using one 6-0 suture (Cat No. 18020-60, Fine Science Tools, North Vancouver, BC, Canada). The nerves were then cut on the distal end of the suture to create a full transection of both the Tib and CP while leaving the sural nerve intact. For sham procedures, the nerves were exposed and identified but were left intact. Open wounds and musculature were sutured using 4-0 dissolvable suture in an interrupted fashion with tissue adhesive (Vetbond, 3M, St. Paul, MN, USA) on the skin and sutures to secure the knots. SNI procedures were verified during surgeries and post-hoc.

Spinal cord injury

A superficial incision was made 2 cm in length along the midline of the thoracolumbar vertebral column (T10-T13) where the curvature of the back is most pronounced. The skin and fascia were dissected away from the underlying musculature to expose the underlying vertebrae and surrounding muscles. An incision along both sides of the midline of the T10-11 dorsal spinous processes was performed and a muscular resection exposed the underlying vertebrae. The underlying vertebrae were blunt dissected using curved forceps (Cat No. 11154-10, Fine

Science Tools, North Vancouver, BC, Canada) to expose the dorsal processes and the intervertebral disc. The intervertebral disc was removed and the underlying spinal cord was exposed. Partial laminectomies (half of the vertebrae) were performed between the T10-11 vertebrae to expose sufficient spinal cord for a hemisection while maintaining structural integrity and preserving the dorsal roots (Rongeurs: Fine Science Tools, Cat no: 16221-14). The spinal cord hemisection was performed using a 30 gauge ½" needle (BD, Cat No. 305106, Franklin Lakes, NJ, USA,) inserted into the midline of the spinal cord until the ventral vertebrae were reached. The cord was then cut in a perpendicular motion toward the lateral edge of the vertebrae, hemisecting the spinal cord on the left side of the animal. For sham procedures, the spinal cord was exposed with a laminectomy but no hemisection was performed. Animals were given buprenorphine at a dose of 0.1 mg/kg delivered intraperitoneally (IP) before removing from isoflurane anesthetic. Animals were assessed daily after injuries to verify proper pain management and if any postoperative complications had occurred. No extra analgesic was required following the day of surgery. SCIs were verified visually at the time of surgery and *post hoc*.

Behavioural and pain testing

Randomization

All experiments were randomized and blinded. Randomization was done using a random number generator (random.org) each day of experimentation to make sure animals were not habituated to specific boxes or positions in the tests. Animals were tested in cohorts of 12, divided into three groups: SHAM, Injury+PD, Injury+KD. All animals in each cohort were assigned a reference number between 1-12. On testing days, the random number generator provided a 2x6 matrix of randomly assigned numbers between 1-12 indicative of either the position of placement in the testing apparatus or the order in which an animal was tested. This method ensured that animals were not habituated to either the specific location in the testing apparatuses or the time of day that the testing was performed.

Von Frey

Mechanical allodynia was assessed by measuring the hind paw withdrawal threshold to von Frey filaments (vFh). Animals were placed individually into plexiglass testing chambers on a raised mesh surface to allow access to the hindpaws, and habituated for 30 minutes before testing. Baseline mechanical sensitivity was determined for each animal prior to the injuries. A range of vFh were used (0.2 to 2 g), the presence or absence of a withdrawal response was recorded, and the withdrawal threshold was calculated using the up-down method of Dixon (Chaplan et al., 1994).

Open Field Test

For open field tests (OFT), animals were habituated to the room for 1 hour before tests were administered. Animals were placed into the open field boxes (Cleversys Systems Inc., Reston, VA, USA) 4 boxes at a time. The open field protocol was set for 30 minutes from the time of insertion into the arena. The center of the box was defined by 25% of the size of the total arena

in the center of the box. The periphery was defined as the total area of the box minus the center area and covers the periphery around the center to the walls of the open field.

Ladder Rung

For ladder rung testing and scoring we adapted previously reported methods (Metz and Whishaw, 2009). The ladder rung system was constructed of plexiglass sides and used a 2 mm diameter round, 100 mm long, steel bar for the rungs. The rungs were spaced randomly and the spacing was not changed throughout the experiments. Animals were habituated and exposed to the test on 3 separate testing days before injuries occurred. During habituation and data acquisition, animals were passed over the ladder rung a total of 3 times and the average step scores and faults were determined daily. Animals were introduced into the right side of the apparatus to allow full visualization of the injured left hindlimb. The order of the animals placed on the ladder rung was randomized on each testing day. Each session was recorded using a high-speed camera (Canon VIXIA HF-R52 HD) recorded at 60 frames per second with a 1080p resolution. Scoring was done post hoc by separate personnel that were blinded to the condition of the mice. Scoring was based on the 6 point scale outlined in (Metz and Whishaw, 2009) (Table 2) with a score of 0 denoting when a hindlimb completely missed the rung leading to a break in the weight-bearing gait. By comparison, a score of 5 would indicate a slight malposition of the hindlimb on a rung of the test with a score of 6 being perfect placement on the ladder rung. Decoding and interpretation of the data were performed by other personnel of the lab to minimize bias.

Statistics

All data were tested for normality using the D'Agostino and Pearson tests. Data that were normally distributed were subsequently analyzed using parametric tests; non-parametric tests were used to analyze data that were not normally distributed. Analysis of behavioural data was performed using repeated measure two-way ANOVAs with Bonferroni multiple comparisons, or a mixed-effects analysis. The factors were treatment (KD vs. PD) and time (before and after injuries). Analysis of baseline behavioural changes, neuromodulator levels (HPLC) and ketone blood concentrations between KD and PD animals were performed with unpaired t-tests or Mann Whitney tests. For all statistical analyses, Prism 8 software (GraphPad, San Diego, CA, USA) was used. A significance level of $p < 0.05$ was used throughout represented by an asterisk in figures.

Results

Mice fed ketogenic diet had increased ketone levels and no changes in body weight.

We first examined the efficacy of the KD in producing a steady ketosis state in uninjured mice. All animals were tested for blood ketone levels before and following diet administration (Figure 1A). Animals fed a ketogenic diet (KD) showed the concentration of blood ketones (unpaired t-test, $t=6.624$, $df=18$, $p<0.0001$), reaching an average of 1.8 mmol/L of ketones indicative of

ketosis (Ruskin et al., 2009). Animals fed a pellet diet (PD) had a blood ketone level at an average of 0.7 mmol/L. When animals in ketosis were fed PD, they showed a decrease in blood ketone levels 60 minutes following ingestion (two-way repeated-measures ANOVA, time x diet: $F(4, 12) = 28.51$, $p<0.0001$, time: $F(1.327, 3.980) = 22.78$, $p=0.0079$, diet: $F(1, 3) = 51.35$, $p=0.0056$, Figure 1B), suggesting that maintenance of high blood ketone levels reliably indicate the state of ketosis. No significant difference in weight between animals fed either KD or PD was detected during the course of the study (mixed-effect analysis, time x diet $p=0.0164$, time: $p=0.005$, diet: $p=0.131$, Figure 1C).

Levels of monoamines in the mouse spinal cord were unaffected by the ketogenic diet

To investigate whether changes in diet influence expression of monoamines, crucial neuromodulators of locomotion and nociception, we next measured levels of monoamines within the lumbar spinal cord of mice fed with KD or PD using HPLC.

Mice fed KD had significantly lower levels of the essential amino acids tyrosine (unpaired t-tests, $t=3.221$, $df=21$, $p=0.0041$, Figure 2A) and tryptophan (unpaired t-tests, $t=2.489$, $df=22$, $p=0.0209$, Figure 2B). This suggests a lower availability of substrates for the production of monoamines. However, no changes were detected between the diet groups in levels of noradrenaline (unpaired t-test, $t=1.022$, $df=22$, $p=0.3177$, Figure 2C), dopamine (unpaired t-test, $t=0.4043$, $df=22$, $p=0.6899$, Figure 2D) or serotonin (unpaired t-test,$t=0.1737$, $df=22$, $p= 0.8637$, Figure 2E). Furthermore, levels of the main metabolite of serotonin, 5-hydroxyindoleacetic acid remained unaltered (unpaired t-test, $t=0.6498$, $df=21$, $p=0.5228$, Figure 2F).

The ketogenic diet had no effect on sensorimotor recovery in the context of a peripheral nerve injury

We sought to understand the effects of KD in sensorimotor recovery following peripheral nerve injury. Spared nerve injury (SNI) was performed as animals exhibit motor deficits and pro-nociceptive phenotypes.

We first examined general locomotion before and after SNI surgeries using open field tests. Mice were allowed to move freely in the open field boxes and activity recorded for 30 minutes. Before SNI surgeries (baseline), mice showed no difference in total distance travelled (Figure 3A), crossing from the periphery of the box to the centre (Figure 3C) and duration of in place activity in the OFT (Figure 3E). Interestingly, the duration of in place activity in the centre, when the animal is active, but not moving outside of a prescribed area, was increased in KD mice compared to PD (unpaired t-test, $t=2.676$, $df=22$, $p=0.0138$, Figure 3G). No significant time in centre locomoting was observed (Mann-Whitney test, $p=0.054$, Figure 3I). These results indicate that at baseline, mice fed KD may be less opposed to spending time in the centre of the box, suggestive of an anxiolytic effect.

General locomotive behaviours were tracked for 28 days after SNI surgeries. No differences were detected between the diets in total distance travelled (Figure 3B), duration of in place activity (Figure 3D) and crossing over from peripheral to the centre (Figure 3F). No changes in the duration of in place activity (Figure 3H) or locomotion (Figure 3J) in the centre were observed.

To investigate the effect of diet on nociception, von Frey hair tests were performed to measure mechanical withdrawal thresholds at baseline and after SNI surgeries. There were no differences at baseline (Figure 4A), but mechanical withdrawal threshold was significantly lower after SNI (two-way repeated-measures ANOVA, time x treatment: $F(8, 80) = 1.343$, $p=0.2347$, time: $F(2.98, 59.6) = 2.29$, $p=0.0879$, treatment: $D(2, 20) = 7.185$, $p=0.0045$, Figure 4B). Post-hoc analysis revealed that mice fed KD had significantly lower mechanical withdrawal thresholds 3 days after SNI compared to PD (KD vs. PD, 0.9 g vs. 0.5575 g, $p=0.0231$), but not in the latter timepoints. This indicates an initial worsening of allodynia (pain evoked by normally non-noxious stimulations) after SNI in KD animals.

To quantify the effects of diet on the recovery of fine motor skills, the ladder rung test was used to qualitatively score the animals' motor ability. Animals showed no statistical difference before SNI between groups fed either a PD or KD at baseline (unpaired t-test, $t=0.7329$, $df=21$, $p=0.4717$; Figure 4C). Following injury, animals initially had a reduced ability to cross the ladder rung platform, which recovered over time. However, no difference in recovery was observed between animals fed KD or PD (Figure 4D) (2-way ANOVA, time x treatment: $F(6, 60) = 1.796$, $p=0.1152$, treatment: $F(2, 20) = 1.882$, $p=0.1783$).

Before the commencement of sensorimotor behaviour tests, animals were in ketosis with ketone levels of 2.0 +/- 0.580 compared to the PD fed animals with ketone levels of 0.653 +/- 0.195 SD (unpaired t-test, $t=9.228$, $df=22$, $p<0.0001$). Before euthanasia of the animals, we took a blood sample and determined the ketone concentrations. The PD average was 0.725 +/- 0.084 SD while the KD concentration was 2.238 +/- 1.509 (unpaired t-test, $t=3.954$, $df=21$, $p<0.0007$).

The ketogenic diet had no effect on sensorimotor recovery in the context of a spinal cord injury

We next sought to examine the effects of a KD diet on neurotrauma to the CNS, in particular the thoracolumbar spinal cord. We used the spinal cord hemisection model to produce a repeatable injury. We first examined the motor behaviour of the mice in open field boxes. Before SCI (baseline), mice showed no significant differences in motor behaviours when fed KD or PD. Specifically, we found no difference in the distance in the open field (Figure 5A), time spent in place in the open field (Figure 5C), total crosses of the outside periphery to the inside area (Figure 5E), the duration of in place activity in the center of the open field (Figure 5G), and time spent locomoting in the center of the box (Figure 5I).

General locomotor behaviors were tracked for 28 days following SCI. No differences were detected between the diets in total distance travelled in the open field (2-way ANOVA, time x treatment: $F(8,84)=2.617$, $p=0.0131$, treatment: $F(2,21)=0.5886$, $p=0.5640$, Figure 5B), duration of in-place activity (Figure 5D), crossing from the periphery to the centre of the box (2-way ANOVA, time x treatment: $F(8,84)=2.907$, $p=0.0065$, treatment: $F(2,21)=0.007431$, $p=0.9926$, Figure 5F). No differences were present between treatment groups in the duration of in-place activity in the centre of the box (Figure 5H), along with no difference in the time spent locomoting in the center of the open field (Figure 5J).

vFh tests were performed to investigate nociception following SCI. At baseline, animals fed PD chow did not show any marked differences from animals fed KD diet (unpaired t-test, $t=0.385$, $df=22$, $p=0.7038$). Following SCI, no significant changes to the withdrawal threshold over the course of recovery were found (2-way ANOVA, time x treatment: $F(6,84) = 0.9613$, $p=0.457$, treatment: $F(1,14) = 2.02$, $p=0.178$).

To quantify the effects of diet on the recovery of fine motor skills, the ladder rung test was used to qualitatively score the animals' motor ability. Animals showed no statistical difference between groups fed either PD or KD diets at baseline (unpaired t-test, $t=0.1162$, $df=22$, $p=0.9086$). Following injury, both injury groups had a reduced ladder rung score, compared to the sham mice (2-way ANOVA, time x treatment, $F(8,84) = 5.024$, $p<0.0001$, treatment; $F(2, 21) = 23.87$, $p<0.0001$). However, no difference in recovery was observed between animals fed PD or KD in SCI animals.

Preinjury, animals were in ketosis with ketone levels of 1.97 +/- 1.25 SD compared to the PD fed animals with ketone levels of 0.75 +/- 0.241 SD (unpaired t-test, $t=3.651$, $df=21$, $p<0.0015$). Before euthanasia of the animals, we obtained a blood sample and determined the ketone concentrations. The PD average was 0.750 +/- 0.259 SD while the KD concentration was 2.063 +/- 0.830 SD (unpaired t-test, $t=4.752$, $df=16$, $p<0.0002$).

Discussion

There is an urgent need to assess the potential of new therapeutic solutions, including diet-based interventions, to improve recovery following neurotrauma. Our study concluded minimal effects of the ketogenic diet (KD), a common form of metabolic therapy on recovery from neural injuries (Li et al., 2020; Streijger et al., 2013). At baseline, before the injuries, we observed no significant differences in locomotor behaviours using the open field and ladder rung paradigms, or mechanosensory behaviours tested with von Frey hairs. Animals who were on the KD diet also had no difference in levels of neuromodulators (dopamine, serotonin and noradrenaline) in the spinal cord, although we did observe a decrease in levels of tyrosine and tryptophan. Similar reductions in tryptophan have been found in rats fed a KD (Heischmann et al., 2018). Following spared nerve injury (SNI) mimicking injury to the peripheral nervous system, an increase in mechanical allodynia (pain evoked by normally non-noxious stimulus) was observed in KD animals compared to those fed normal pellet diet (PD) during the early phase of pain development. No other changes were detected in locomotor behaviours. In our SCI model, KD

did not mitigate nociception or locomotor disability following thoracic spinal cord hemisection. Altogether our data suggest that KD is not a suitable therapy for improving disability after peripheral or spinal cord injuries affecting the hindlimb.

This study was conceived on the premise that KD contributes to the reprogramming of cellular metabolism, thus modulating processes related to disorders of the central nervous system including aberrant neuronal activity, neuroinflammation and neurodegeneration (Cullingford, 2004). The KD replaces glucose with ketone bodies as the source for generating high-energy molecules, including adenosine triphosphate (ATP). It has been reported that by switching to ketone bodies, cellular energy production becomes more efficient (Ruskin and Masino, 2012). We hypothesized this change in cellular metabolism would regulate cellular processes consequential to nerve injuries. Importantly, pain and motor deficits resulting from nerve injuries share similar cellular mechanisms. Immediately after a nerve is injured, neurodegeneration occurs followed by robust inflammatory processes, which aid removal of cellular debris and subsequent sprouting of neural fibres (Benowitz and Popovich, 2011). These processes require precise temporal and spatial signalling of a plethora of trophic factors, relying on receptor binding on efferent neuronal targets for successful circuit remodelling (Hermann and Chopp, 2012). Importantly, errors in these intricate processes would result in aberrant neuronal function, thereby perpetuating motor deficits and pain following nerve injuries (Hains et al., 2005; Kohama et al., 2000). Often these changes can occur quickly, for example leading to hyperexcitability of neurons within 24 hours of peripheral nerve injury (Iwata et al., 1999).

There are other reports which elucidated the molecular underpinnings in the benefits of KD. In a murine model of kainic-acid induced seizures, KD activates peroxisome proliferator-activated receptor-gamma (PPARγ), which suppressed the expression of pro-inflammatory tumour-necrosis factor-alpha (TNFα) and cyclooxygenase-2 (Cox-2) (Jeong et al., 2011). Using the progressive genetic model of Alzheimer's disease (APP/V7171 mutation), mice on the KD had overall lower levels of amyloid-β (Aβ) (Auwera et al., 2005), a neurotoxin which upon accumulation forms plaques and leads to cellular death (Mark et al., 1995). However, the same study also reported that KD did not alter behavioural outcomes, object recognition, as a measure of memory function was not improved by KD in the APP/V7171 mutation model (Auwera et al., 2005). An older study, using a different epileptic model (bicuculline-induced seizures) also found the effect of KD to be age-dependent: KD initiated at postnatal day 16 protected animals against the tonic-extensor phase of bicuculline-induced seizures, whereas these effects were diminished if KD was initiated at postnatal day 26 (Uhlemann and Neims, 1972).

Our current study showed no overall improvements in nerve injury-induced sensorimotor deficits after KD, which is in contrast to other published observations. Rats fed KD for 3 to 4 weeks showed reduced thermal hyperalgesia in a model of complete Freund's adjuvant-induced chronic inflammation (Ruskin et al., 2009). In a murine model of obesity and prediabetes, the development of mechanical allodynia and loss of peripheral axonal termination was not observed in KD animals, suggesting a neuroprotective mechanism (Cooper et al., 2018). Despite differences in an animal model of choice and testing modality, one experimental

concern could be the duration of testing, as pathogenesis of pain and motor deficits is dynamic and could differ between early and late phases of the injury (Kawasaki et al., 2008).

While no studies that we are aware of have addressed the effects of KD on thoracic SCI or peripheral nerve injury, there have been reports of KD beneficial effects on recovery of reaching behaviour in high cervical injuries in both mice (Streijger et al., 2013) and in human studies (Yarar-Fisher et al., 2018). These effects on reaching behaviour in mice were confined to supination and grasping, suggesting regional effects on the cervical motor circuit. Other metrics showed a rapid increase in use behaviour of the affected limb in KD cohorts. Importantly, our study concentrated on hindlimb, and connectivity onto the CPG is different with large projections from the reticulospinal tract contributing to locomotor activity along with inherent plasticity of the CPG itself (Girgis et al., 2007). Also, we used mice, whereas rats were used in the forelimb study, which score lower on the BBB scale for motor recovery following SCI (Byrnes et al., 2010). To our knowledge, the time course of testing employed in this study was comprehensive. The testiing evaluated the most significant time points for motor recovery following injury, characterizing changes both before the onset of injury, and up to 4 weeks after. Most of the behavioural observations steadily plateaued before the end of the study. However, we do note some of the metrics observed following SCI forelimb recovery occurred following six weeks of KD treatment. Surprisingly, none of these beneficial effects were observed if KD was combined with other treatments (ibuprofen, ghrelin, and C16) (Streijger et al., 2014). Future studies could extend the testing period beyond four weeks, and include measures of thermal sensitivity and neurite outgrowth to fully elucidate the effects of the KD. Our study used a hemisection model for SCI, producing a relatively mild injury, and hindlimb specific effects on ascending afferent projections. It is, therefore, possible that KD could be effective in a severe contusion model.

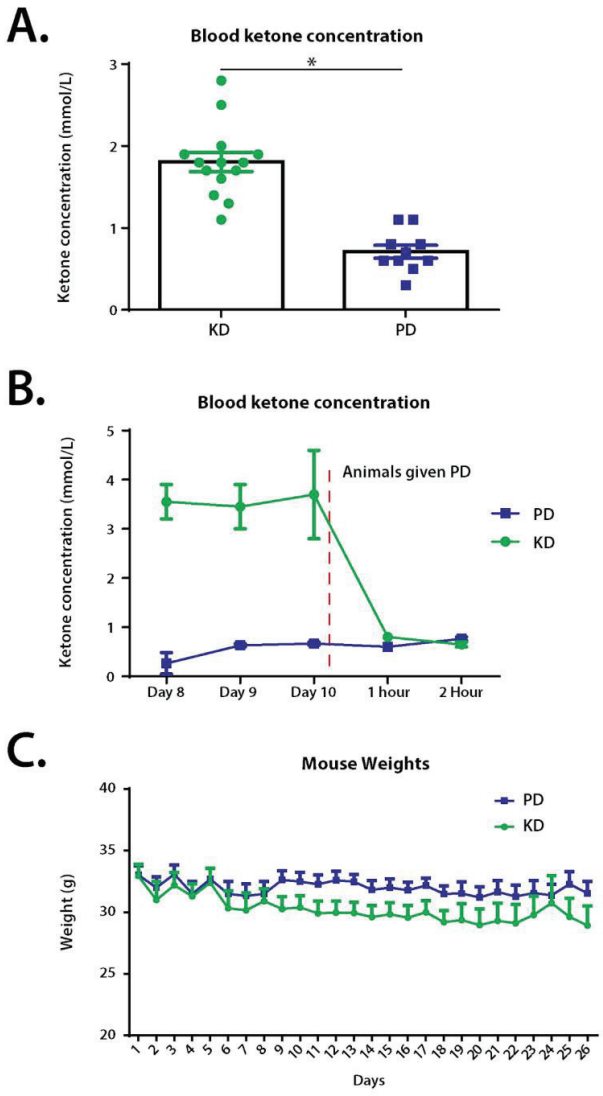
Another experimental consideration relates to the time required for animals to calibrate to the state of ketosis. Varying paradigms have been reported, ranging from introducing KD for ten days to four weeks before the commencement of experiments (Jeong et al., 2011; Uhlemann and Neims, 1972). In our study, animals fasted for 12 hours and behavioural testing commenced after three weeks of KD. We also reported that animals were no longer in a state of established ketosis 1 hour after the introduction of the normal pellet diet, indicating the sustainability of ketosis is dependent on dedicated, timely, feed of KD. In this work, we ensured that all animals were in states of ketosis before neural injury. However, when animals first enter ketosis, there are spikes in adenosine levels, which could have beneficial short-term effects (Yang et al., 2017). Future studies, with careful monitoring of the state of ketosis, could introduce the diet after nerve injuries, to test the therapeutic potential of KD in alleviating the maintenance of neuropathic pain and motor disability.

Mirroring this behavioural observation are our molecular findings showing no changes in levels of neuromodulators after the commencement of KD. We chose to study the levels of dopamine, serotonin and noradrenaline as they share common mechanisms underlying pain and motor functions (Hains et al., 2001; Rommelfanger et al., 2007; Scott et al., 2006). Their levels were determined in the spinal cord, thereby a proxy measure of monoamine release at the terminals rather than cell bodies originating from the brain. Nonetheless, the pathophysiology of pain and

motor disabilities is complex, and systemic administration of KD is likely to affect multiple downstream signalling pathways despite the monoamine systems. Of particular interest is the expression of brain-derived neurotrophic factors (BDNF) (Keefe et al., 2017), and other inflammatory mediators which may be modulated by KD (Vizuete et al., 2013) and has an established role in nerve repair (Zhang et al., 2000), plasticity (Seebach et al., 1999), and pain (Coull et al., 2005). Moreover, KD can decrease oxidative stress following traumatic brain injury (Greco et al., 2016). It was found by Tetzlaff and colleagues KD upregulated glucose transporter 1 and monocarboxylate transporter-1 (Streijger et al., 2013), and it is possible that this also occurred in our work. In addition, our study detected a decrease in the levels of tyrosine and tryptophan from KD mice, substrates important for the production of monoamines (Wurtman and Fernstrom, 1975). A plausible explanation for the mismatch in levels of precursors and monoamines is the bioavailability of converting enzymes, including tyrosine hydroxylase controlled by changes in diets (DeCastro et al., 2005). Alternatively, the diets used have different concentrations of the amino acids tryptophan and tyrosine with the KD diet (Bio-Serv 3666) having lower amounts of both (Table 1). To complete the findings, further investigation of the expression of other amino acid precursors and converting enzymes would be beneficial.

In summary, our study represents the initial exploration in the therapeutic potential of KD in nerve-injury related disease and showed that KD might not be suitable as therapies for pain and motor recovery for the hindlimb. A translational approach was employed to study the effects of pain and motor recovery from sciatic nerve injury and T10-11 SCI. Our outcome measures showed a minimal effect of the diet and provided a mechanistic insight describing no change in levels of monoamine release at spinal terminals. Other studies could consider a different direction to further elucidate the effect of KD, including a later initiation of the diet, or refocus on a different disease phenotype.

462 **Figure 1**



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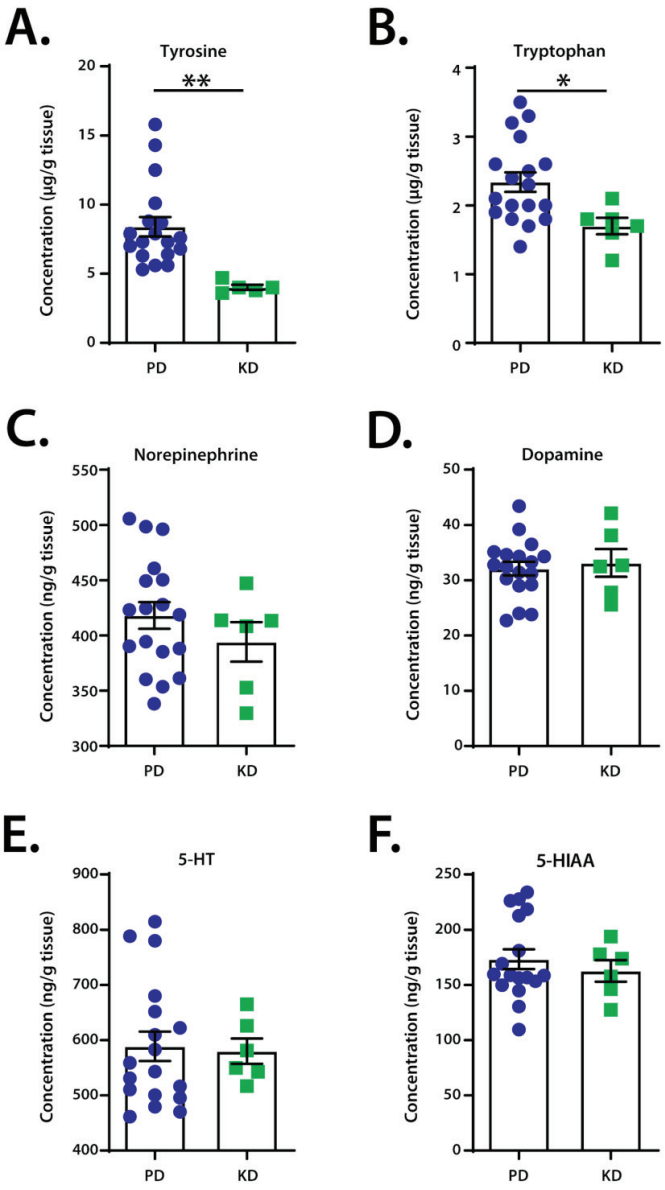
466 **Figure 2**



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468 **Figure 3**

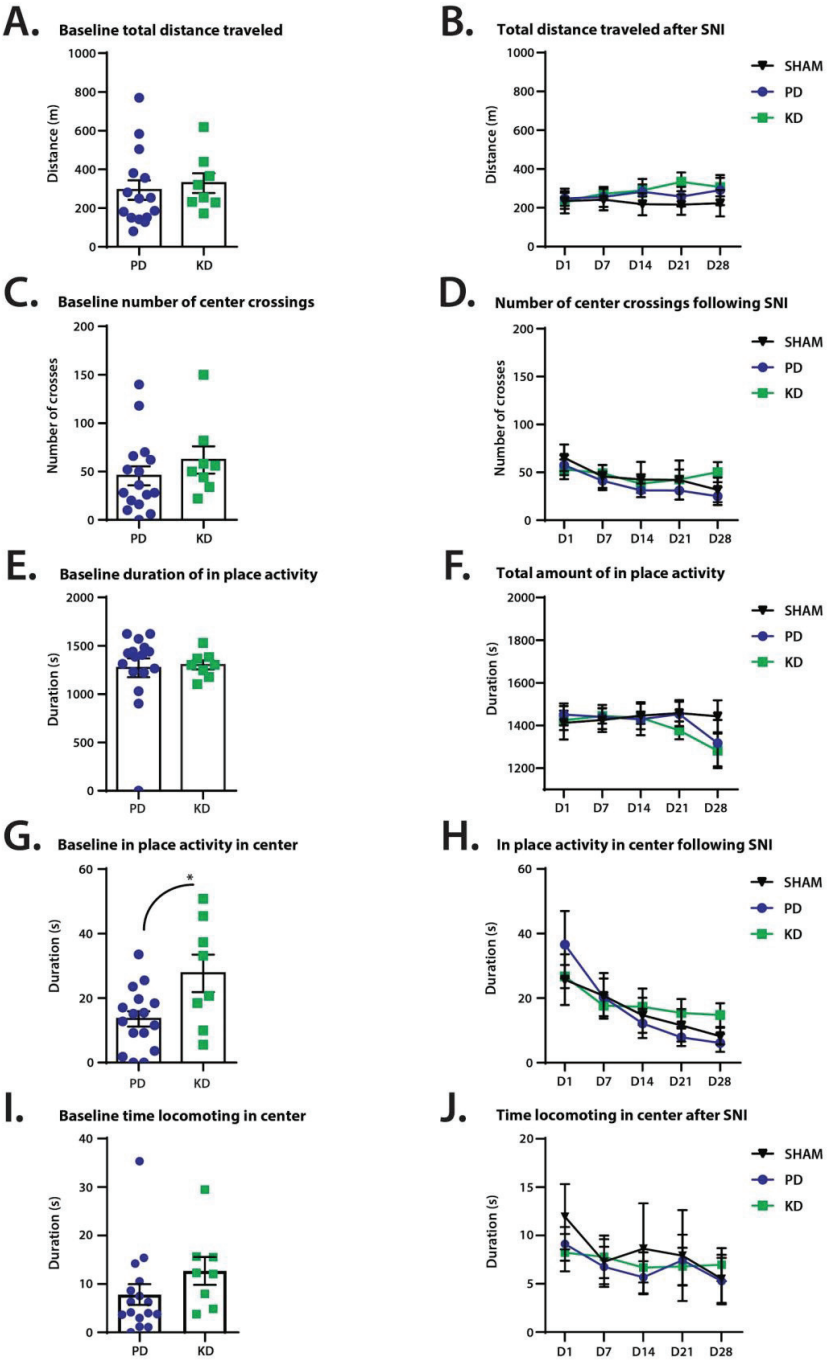

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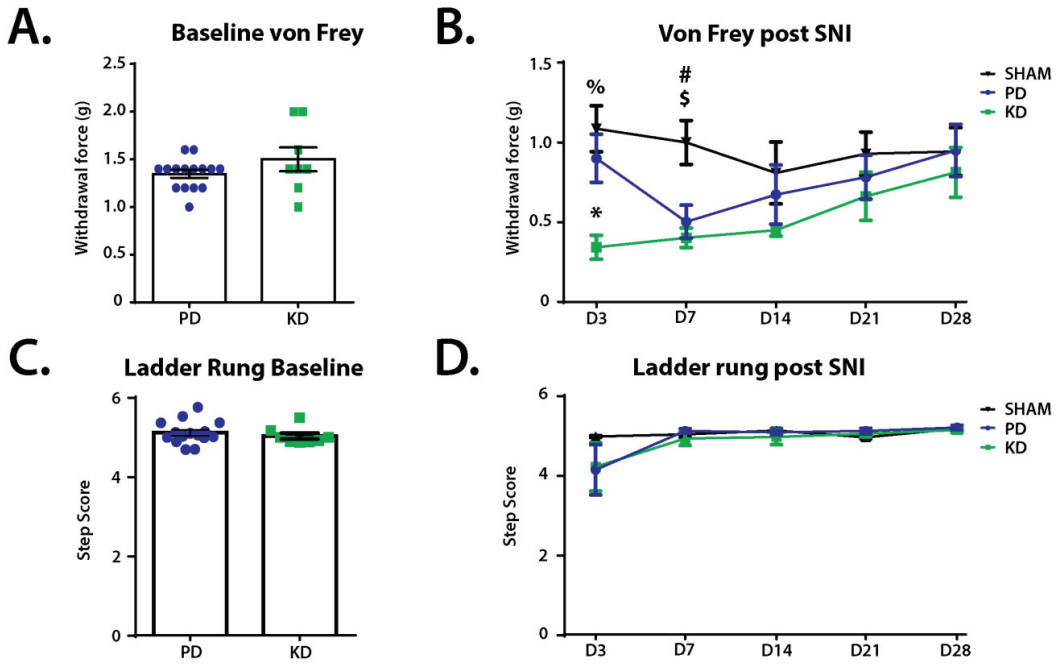
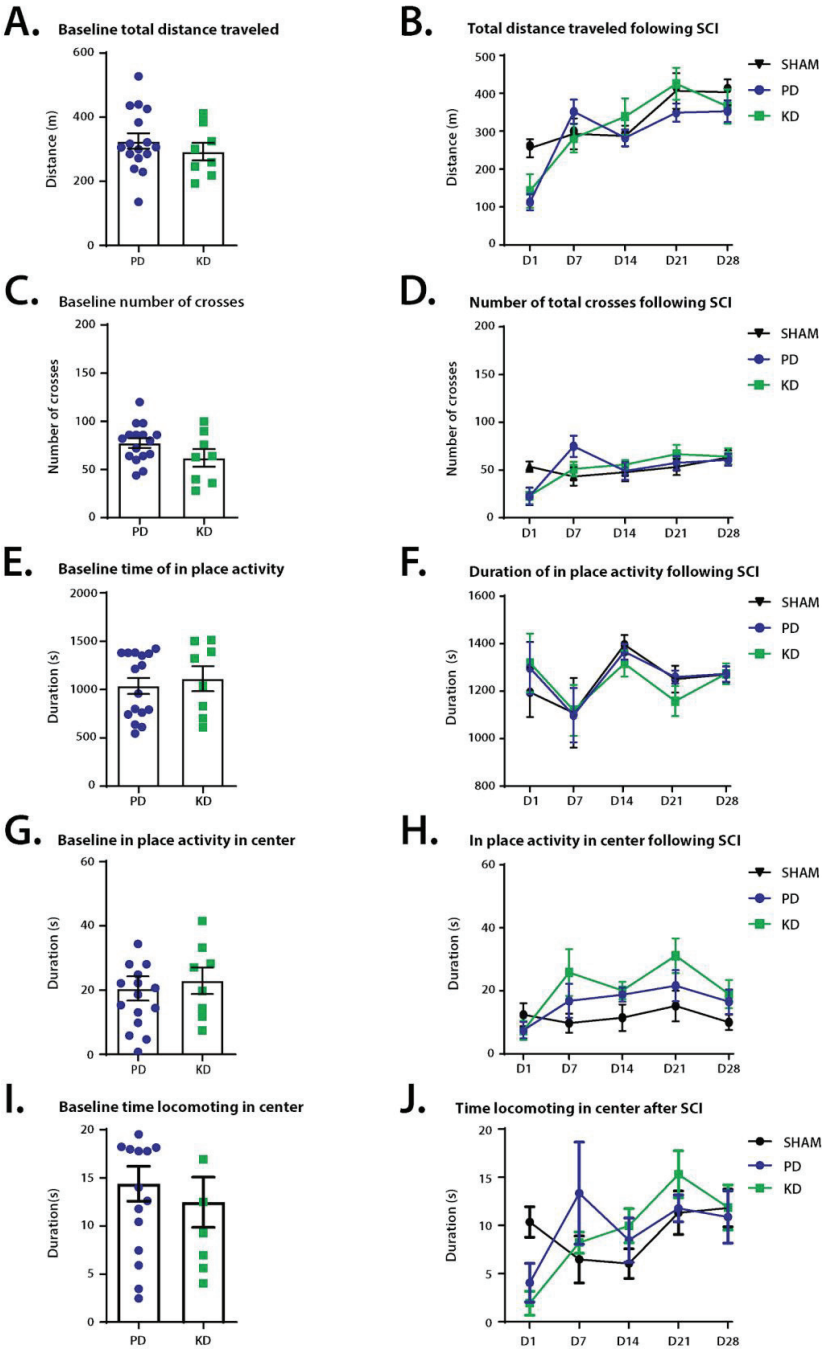
Figure 4



478 **Figure 5**



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480 **Figure 6**

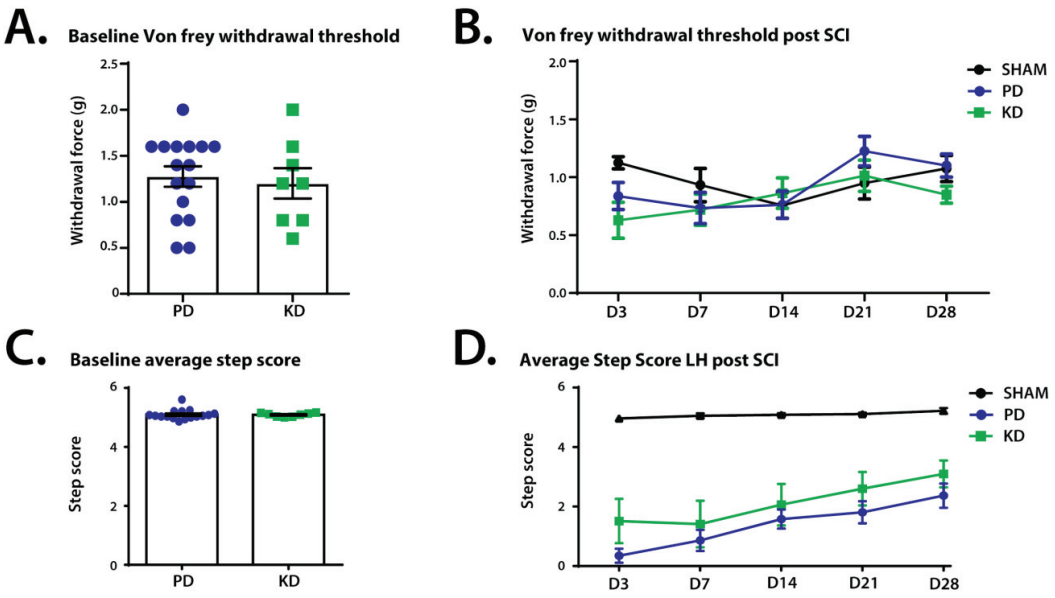

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Table 1

Macronutrients	Pellet Diet (PD) Labdiet mouse diet 20	Ketogenic Diet (KD) Bioserve F3666
Protein	24.65%	8.60%
Fat	13.21%	75.10%
Carbohydrate	62.14%	3.20%
Other		
Fiber	4.00%	4.80%
Ash	6.10%	3.00%
Moisture	12.00%	<10
Amino Acids	%	%
Alanine	1.15	0.23
Arganine	1.22	0.31
aspartic Acid	2.19	0.55
Cystine	0.28	0.03
Glutamic Acid	4.34	1.73
Glycine	0.96	0.21
Histidine	0.5	0.23
Isoleucine	0.97	0.47
Leucine	1.56	0.71
Lysine	1.16	0.63
Methionine	0.7	0.22
Phenylalanine	0.9	0.38
Proline	1.47	0.87
Serine	1.03	0.48
Threonine	0.77	0.37
Tryptophan	0.26	0.1
Tyrosine	0.59	0.48
Valine	1	0.55
Minerals		
Calcium	0.81	0.57
Chloride	0.42	0.17
Copper	13 ppm	6.6 ppm
Chromium	0.81 ppm	2.2 ppm
Flouride/Flourine	10 ppm	0.0 ppm
Iodine	1.5 ppm	0.2 ppm
Iron	220 ppm	38.7 ppm
Magnesium	0.16	0.56
Manganeese	85 ppm	63.6 ppm
Phosphorus	0.6	0.49
Potassium	0.7	0.39
Selenium	0.3 ppm	0.19 ppm
Sodium	3000 ppm	1128 ppm

** All values are % unless otherwise indicated

487 Table 2

Score	Metz Scale 2009	Explanation
0	**Total miss**	0 points were given when the limb completely missed a rung, i.e. did not touch it, and a fall occurred. A fall was defined as a limb deeply falling in-between rungs and body posture and balance were disturbed.
1	**Deep slip**	The limb was initially placed on a rung, then slipped off when weight-bearing and caused a fall.
2	**Slight slip**	The limb was placed on a rung, slipped off when weight bearing, but did not result in a fall nor interrupt the gait cycle. In this case, the animal was able to maintain balance and continue a coordinated gait.
3	**Replacement**	The limb was placed on a rung, but before it was weight bearing it was quickly lifted and placed on another rung.
4	**Correction**	The limb aimed for one rung, but was then placed on another rung without touching the first one. Alternatively, a score of 4 was recorded if a limb was placed on a rung and was quickly repositioned while remaining on the same rung.
5	**Partial placement**	The limb was placed on a rung with either wrist or digits of the forelimb or heel or toes of the hindlimb.
6	**Correct placement**	The midportion of the palm of a limb was placed on the rung with full weight support.

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Figure Captions

Figure 1. Blood ketone levels and stability one week after administration of ketogenic diet in uninjured mice

A) A schematic showing a timeline of diet intervention relative to surgery, and behavioural testing. **B)** Blood samples were collected from the tail vein and determined by ketone testing strips and monitoring. Significantly higher ketone blood concentrations were found in mice fed the ketogenic diet compared to a conventional pellet diet. *N=10 each for KD and PD, p<0.0001*****. **C)** Mice fed a ketogenic diet reliably remained in ketosis with KD being introduced 7 days prior. In addition, within one hour of being given a pellet diet mice were no longer in a state of ketosis. *N=2 for KD, 3 for PD.* **D)** Comparison of body weights between mice who were fed the ketogenic and pellet diet without surgical intervention, no significant difference was detected at each time point between the diets. *N=6-10 each for PD and KD. PD=pellet diet, KD=ketogenic diet. Error bars indicate mean +/- SEM.*

Figure 2. The effect of diets on neuromodulator content in the lumbar spinal cord of mice

Mice fed the ketogenic diet had significantly lower content of the monoamine precursors tyrosine (**A**) and tryptophan (**B**), *p=0.0041 ** and p=0.0209 * respectively*. No changes were detected in the levels of monoamines noradrenaline (**C**), dopamine (**D**), and serotonin (5-HT, **E**). There were also no changes in the content of the main metabolite of serotonin, 5-hydroxyindoleacetic acid (5-HIAA, **F**). *N=18 for PD, 6 for KD. PD=pellet diet, KD=ketogenic diet. Error bars indicate mean +/- SEM.*

Figure 3. The effect of diet on open-field locomotor behaviours in a model of peripheral nerve injury

Peripheral nerve injury was modelled by spared nerve injury (SNI), comparison of general locomotor behaviours were made at before (baseline) and after SNI. No significant differences were observed between the diets in distance travelled (**A-B**), duration of in place activity (**C-D**), and number of crossing from the periphery of the open field box to centre (**E-F**). At baseline, mice fed the ketogenic diet displayed more in place activity in the centre of the box, (**G**) *p=0.0138 **. However, no effects of diet were observed after SNI between mice fed pellet or ketogenic diet (**H**)**.** Duration of locomotion in the center of the open field box is a proxy measure of anxiety-like behaviours. The more time spent in the center of the box suggests less anxiety as mice innately avoid bright open spaces. **I**) At baseline, no significant movement to the centre was observed, p=0.054. **J**) No effects of the diet were observed after SNI. *N=7-8 for SHAM, 8 for SNI+PD, 8 for SNI + KD. PD=pellet diet, KD=ketogenic diet. Error bars indicate mean +/- SEM.*

Figure 4. The effect of diet on other sensorimotor behaviours before and after peripheral nerve injury

Comparison of mechanical withdrawal threshold (von Frey hair test) and fine motor behaviours (ladder rung) made before (baseline) and after SNI. **A**) No difference in the mechanical withdrawal threshold between the diets at baseline. **B**) Mechanical withdrawal threshold was significantly lower in SNI mice compared to sham, indicating the development of mechanical allodynia (pain evoked by normally innocuous stimulations). *Post-hoc* Bonferroni analysis revealed a significant difference between SHAM+PD and SNI+KD groups on day 3 and 7 after SNI, *p=0.0038 % and p=0.0117 # respectively*, SNI+PD also had lower mechanical withdrawal threshold compared to SHAM+PD on day 7 after SNI, *p=0.0428 $*. 3 days after SNI, SNI+KD also had significantly lower mechanical withdrawal threshold compared to SNI+PD, *p=0.0231 **. N=7 for SHAM, 8 for SNI+KD and SNI+PD. PD=pellet diet, KD=ketogenic diet. *Error bars indicate mean +/- SEM.*

Figure 5. The effect of diet on open-field locomotor behaviours in a model of spinal cord injury

Spinal cord injury (SCI) was modelled by a hemisection T12-13, left side of the spinal cord. A comparison of general locomotor behaviours was made before (baseline) and after SCI. Distance travelled after 30 minutes in the OFT; there was no significant difference at baseline PD vs KD fed animals **(A)**, nor was there difference following injury **(B)**. No difference in the number of crosses from the periphery of the open field box to centre (**C-D**). At baseline, mice fed the ketogenic diet displayed no differences in activity in place in the entirety of the arena **(E)**, nor in the centre of the box (**G**). No significant effects of diet were observed for in place activity after SCI between PD or KD fed mice in either the arena **(F)** or the center **(H).** The duration of locomotion in the center of the open field box is a proxy measure of anxiety-like behaviours. The more time spent in the center of the box indicates less anxiety as mice innately avoid bright open spaces. **I**) non-significant difference at baseline in mice fed KD vs mice fed PD during locomotion in the centre of the open field (*student's t-test, p=0.548*.) **J)** No effects of the diet were observed after SCI except for in the time domain, 2-way ANOVA, p=0.008. There were no significant differences between animals in the KD group and the PD group following injury (2-way ANOVA, p=0.8994). *N=8 for SHAM, 8 for SCI+PD, 8 for SCI + KD. PD = pellet diet, KD = ketogenic diet. Error bars indicate mean +/- SEM.*

Figure 6. The effect of diet on other sensorimotor behaviours before and after spinal cord injury

Spinal cord injury (SCI) was modelled by a hemisection T12-13, left side of the spinal cord. Comparison of mechanical withdrawal threshold (von Frey hair test) and fine motor behaviours (ladder rung) was made at before (baseline) and after SNI. **A**) No difference in mechanical withdrawal threshold between the diets at baseline, or after SCI (**B**). There was no difference in the step score of animals at baseline between animals fed the KD vs PD **(C)**. Diet produced no significant changes over the course of 28 days on the rehabilitation of motor recovery **(D)**. *N=8 each for SHAM, SNI+KD and SNI+PD*. PD=pellet diet, KD=ketogenic diet. *Error bars indicate mean +/- SEM.*

Table 1. Nutritional composition of administered diets

Comparison table of the two diets used throughout this study. All values have been converted to percent based on weight from company data sheets of the diets. Pellet diet (PD); Pico-Vac Mouse Diet 20 from LabDiet compared to Ketogenic diet (KD) F3666 6:1 from Bio-Serv. Diets have been sorted into four categories for readability: Macronutrients, Other, Amino Acids, Minerals.

Table 2. Ladder rung foot scoring scale

This 6 point scale was used to score the mouse hindlimb positioning when crossing over the ladder rung test. 0 being the complete miss of the hindpaw placement on the ladder rung, up to a maximum score of six with correct paw placement achieved. This scale was used throughout the experiments and was adapted from (Metz and Whishaw, 2009).

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