Intermittent Fasting Improves Functional Recovery after Rat Thoracic Contusion Spinal Cord Injury

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Abstract

Spinal cord injury (SCI) often results in a loss of motor and sensory function. Currently there are no validated effective clinical treatments. Previously we found in rats that dietary restriction, in the form of every-other-day fasting (EODF), started prior to (pre-EODF), or after (post-EODF) an incomplete cervical SCI was neuroprotective, increased plasticity, and promoted motor recovery. Here we examined if EODF initiated prior to, or after, a T10 thoracic contusion injury would similarly lead to enhanced functional recovery compared to *ad libitum* feeding. Additionally, we tested if a group fed every day (pair-fed), but with the same degree of restriction as the EODF animals (~25% calorie restricted), would also promote functional recovery, to examine if EODF's effect is due to overall calorie restriction, or is specific to alternating sequences of 24-h fasts and *ad libitum* eating periods. Behaviorally, both pre- and post-EODF groups exhibited better functional recovery in the regularity indexed BBB ambulatory assessment, along with several parameters of their walking pattern measured with the CatWalk device, compared to both the *ad-libitum*-fed group as well as the pair-fed group. Several histological parameters (intensity and symmetry of serotonin immunostaining caudal to the injury and gray matter sparing) correlated with functional outcome; however, no group differences were observed. Thus besides the beneficial effects of EODF after a partial cervical SCI, we now report that alternating periods of fasting (but not pair-fed) also promotes improved hindlimb locomotion after thoracic spinal cord contusion, demonstrating its robust effect in two different injury models.

Key words: calorie restriction; every-other-day fasting; functional recovery; intermittent fasting; spinal cord injury

Introduction

THE MAJORITY OF HUMAN SPINAL CORD INJURIES are anatomically incomplete, yet aside from physical rehabilitation, there are no clinically validated treatments available to target the injured spinal cord and improve functional recovery. Pre-clinical research focuses on the prevention of secondary degeneration, and the enhancement of endogenous neural plasticity in the spared spinal cord, as well as axonal regeneration (Bareyre et al., 2004; Park et al., 2004; Ramer et al., 2005).

Ideally, therapeutic interventions in acute SCI should provide neuroprotection as well as increased plasticity and axonal regeneration (Ramer et al., 2005). Dietary restriction (DR), with adequate nutrition, which is well established to increase lifespan in a diverse set of species, could potentially represent such a versatile broad-spectrum approach. There are two main forms of DR: calorie restriction (CR), in which daily calorie intake is reduced 20–40%, and intermittent fasting,

which is usually carried out by fasting the animals for 24 h, followed by 24 h of *ad libitum* access to food. Prophylactic intermittent fasting (e.g., every-other-day fasting [EODF]) can protect neurons against injury by enhancing endogenous cellular stress responses and energy metabolism, by dampening oxygen free radical formation and inflammation, and activating cell survival pathways, as well as by increasing growth factor expression and axonal plasticity (Fontan-Lozano et al., 2007; Mattson et al., 2002, 2005; Plunet et al., 2008).

DR has biochemical and functional benefits in a wide range of animal injury/disease models, including seizures (Bough et al., 1999, 2003), excitotoxicity (Anson et al., 2003; Bruce-Keller et al., 1999; Sharma and Kaur, 2005), stroke (Yu and Mattson, 1999), Parkinson's disease (Maswood et al., 2004), Huntington's disease (Bruce-Keller et al., 1999; Duan et al., 2003), and Alzheimer's disease (Halagappa et al., 2007; Mouton et al., 2009; Patel et al., 2005; Qin et al., 2006; Wang et al., 2005; Wu et al., 2008; Zhu et al., 1999).

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In most studies the benefits of DR were seen in chronic disease models, or in cases of acute insults, with DR pre-treatment of several weeks to multiple months prior to insult. More recently and in contrast to this previous work, a 24-h fast initiated after a moderate traumatic brain injury (TBI) also resulted in improved functional outcome in the Morris water maze, and increased neuroprotection (Davis et al., 2008). Similarly, we reported the effectiveness of EODF, the most widely used form of intermittent fasting, when started after a cervical SCI (Plunet et al., 2008). After a unilateral crush of the dorsolateral funiculus of the cervical spinal cord, the EODF-treated rats performed significantly better than the *ad-libitum*-fed group in a forelimb usage (cylinder) test, as well as in an irregular ladder crossing test. The loss of neurons and gray matter tissue in the vicinity of the lesion was significantly reduced by treatment with EODF, and this correlated with behavioral outcomes. Additionally, we observed an increase of sprouting in the intact corticospinal cord tract, which also correlated with functional outcome. These observations suggest that intermittent fasting might be a potential treatment strategy after acute SCI, that could improve the quality of life in individuals with SCI.

In light of a recently published survey, which revealed that contusion injuries are deemed to be the most clinically relevant model (Kwon et al., 2010), we decided to test the efficacy of EODF after a thoracic spinal cord contusion injury. Data from this widely used model would allow us to compare the efficacy of EODF to other pre-clinical treatments. Furthermore, we included a pair-fed control group to determine whether daily calorie restriction diets exert similar beneficial effects as alternate days of fasting (EODF). This addresses the question of whether the positive results of EODF could be due to the general 20–30% reduction in calories typically observed in rats on EODF (Martin et al., 2007; Wan et al., 2003), or is something more specific to the 24-h fasting periods followed by 24 h of overfeeding. Some patients with SCI may prefer daily caloric restriction over alternate-day fasting regimens.

Methods

All procedures involving animals were approved by the Animal Care Committee of the University of British Columbia (UBC), in accordance with the guidelines of the Canadian Council for Animal Care and the National Institutes of Health. All efforts possible were made to reduce the number of animals used and to minimize pain.

Animals and general housing conditions

Sprague-Dawley rats (UBC breeding facility/Charles River Breeding Laboratories) were housed in the animal facility at the UBC with room temperature controlled at 21–22°C, and an artificial 12-h:12-h light:dark cycle (lights off at 8:00 PM). Upon arrival, the animals were group housed ($n=3-4$ per cage), and given food (LabDiet-5001; Purina Mills, Brentwood, MO) and water *ad libitum* until the start of the experiment. Each cage was supplied with housing tubes.

Feeding regimen at the start of the experiment

Before injury the animals were randomized into four different groups: (1) *ad libitum* (AL) group ($n=12$), which continued to have access to food *ad libitum* (but did not initiate

eating until 4–8 h after surgery); (2) post-EODF group ($n=12$), starting a 24-h fast immediately after injury, then alternating between 24 h of feeding with free access to food and water, and 24 h of fasting with only access to water, and this alternating sequence of feeding/fasting continued throughout the rest of the experiment; (3) pre-EODF group ($n=13$), which started EODF 3 weeks prior to injury and continued this feeding regimen for the remainder of the experiment; or (4) pair-fed group ($n=12$), which were fed daily, but were matched for total food consumption with the EODF groups, starting 3 weeks prior to injury and continuing throughout the rest of the experiment (see Fig. 1 for a timeline of the experiment).

Feeding and body weight monitoring

Daily food intake and the animals' weights were measured for all groups for the first 14 days after injury, and every 8 days after that. Pre-weighed food was provided in the food hopper of their home cage at 8:00 AM (unless a fasting day for the EODF groups), and leftover food was weighed 24 h later. Daily food intake was estimated by cumulative food intake in a given cage divided by the number of animals in that cage. UBC animal care regulations do not allow single housing of rats simply for the estimation of food intake.

Thoracic spinal cord contusion injury

The rats were anesthetized with a ketamine/rumpon mixture (70 mg/10 mg per kg IP, respectively). After inducing a surgical plane of anesthesia, the incision site was shaved and disinfected with alternating povidone-iodine scrub and 70% alcohol. To prevent dehydration, 10 mL lactated Ringer's was injected (SC), and an injection of buprenorphine (0.03 mg/kg SC) was given to reduce pain. Lubricant eye ointment was applied to the eyes to avoid desiccation. Lidocaine containing epinephrine (0.1 mL) was injected at the incision site (IM) as a local anesthetic and to reduce bleeding.

The T10 lamina was removed with a pair of fine rongeurs to visualize the spinal cord. Following the laminectomy, the spine was stabilized by clamping the dorsal processes one segment above and below the lesion site. The rats received a spinal contusion injury utilizing the Ohio State impactor device (The Ohio State University, Columbus, OH), with a set displacement of 1.5 mm. Animals that received a contusion force above 250 kilodynes were excluded from the experiment. Following injury the rats were placed in a temperature-controlled incubator (37°C) until the animals were fully awake and moving. To prevent dehydration and post-operative pain, animals received 10 mL of Ringer's lactate solution and buprenorphine (0.05 mg/kg, 2 times/day, for a total of 2 days post-injury). The surgeon (J.L.) was blinded to the randomization of the rats.

Behavioral testing

Baseline (pre-operative) behavior was tested using three different behavioral tests: (1) the irregularly-spaced horizontal ladder, (2) the CatWalk, and (3) the Basso-Beattie-Bresnahan (BBB) open field assessment. All behavioral testing took place on a feeding day for the EODF animals to control for potential differences in motivation, and no food rewards were used in any of the behavioral testing (Plunet et al., 2008, 2010).

Behavioral testing of the animals did not start until at least 4 h after the animals had access to food for the day. It was noted that after a 24-h fast the EODF animals gorged themselves during the first couple of hours, and had an extended abdomen, and gained the majority of their feeding day weight gains in those first few hours (approximately 75% of their typical feeding day weight gain of 30–40 g). Additionally, by performing behavioral testing on feeding days, it allowed the behavioral investigators to remain blinded to the treatment groups (the video analysis of the behavioral data on the ladder and the CatWalk was also done blinded to treatment group).

Irregularly-spaced horizontal ladder

The rats were trained to walk across a 110-cm-long horizontal ladder motivated by their home cage at the end. The ladder was 15 cm wide and consisted of 2-mm-diameter metal rungs. The scoring was limited to the middle 60-cm portion of the ladder, where the spacing between the rungs was varied, ranging from 1.75 to 4.0 cm. Each week the pattern of rungs was changed so the animal could not learn the sequence of rungs (Metz and Whishaw, 2002). Each animal crossed 7–8 times, and the runs were recorded by a high-definition camcorder. The hindlimb error rate was generated from frame-by-frame video replay, and was expressed as the percent error of the steps taken. A hindlimb foot-slip error/miss was designated when the animals completely missed the rungs and the hindlimb slipped noticeably below the rung's level; the error rate was expressed as a percentage of errors. The horizontal ladder was tested pre-injury, and at 50, 66, and 72 days post-injury (i.e., well after the rats had regained weight-supported stepping).

CatWalk gait analysis

Footprints of animals crossing an acrylic glass runway were marked using frame-by frame video replay of 4–6 uninterrupted crossings per animal (CatWalk; Noldus Information Technology, Wageningen, The Netherlands). Animals were tested on the CatWalk before surgery, and at 22, 36, 50, 64, and 70 days after injury.

The regularity index (RI) is a measurement of interlimb coordination. It is calculated from the number of normal step sequences (based on all patterns of all four limb placements), and the total number of steps. An RI of 100% represents perfect coordination (Hamers et al., 2001). In pre-operative testing 20.4% of our animals did not average a perfect 100% regularity index, with the lowest one averaging 96.01%. Our pre-operative RI percent averages were consistent with previous research using the CatWalk device (Hamers et al., 2001; Van Meeteren et al., 2003).

Typically, during coordinated walking as seen in uninjured animals the swing duration of forelimbs and hindlimbs are similar. Hence, subtracting the hindlimb swing duration from the forelimb swing duration yields a number close to zero (see Fig. 5A), and indeed this value was never smaller than -0.00338 sec, and usually was slightly positive. After injury, swing duration timing becomes disrupted, with longer duration of the hindlimb swing phase compared to the forelimb, typically resulting in a negative value. Similarly, in uninjured animals both the forelimb and hindlimb stride lengths were nearly identical, and hence subtracting the hindlimb stride

length from the forelimb stride length yields a value near zero, but averages ~ 2.0 mm (see Fig. 5B). The largest difference we observed for any individual animal prior to injury was -3.42328 mm. However, after thoracic injury rats take smaller forelimb strides compared to their hindlimb stride, which results in large negative differences for most animals.

Modified BBB open field assessment

Hindlimb locomotion was scored by the open-field locomotor scale described by Basso, Beattie, and Bresnahan (BBB; Basso et al., 1995, 1996) with some modifications. Briefly, the BBB is a 21-point scale (0 = complete hindlimb paralysis and 21 = normal locomotion), in which rats are scored based on hindlimb movements made in an open field during a 4-min interval while freely exploring the surroundings. The animals were tested prior to injury, and at 2, 8, 14, 22, 36, 50, 64, and 70 days post-injury.

The RI, obtained from the CatWalk device (Hamers et al., 2001, 2006), an objective measurement of coordination, was used to determine the coordination criteria of the open-field score from 22 days post-injury onward (after the animals were performing weight-bearing walking and were tested on the CatWalk device; Deumens et al., 2006; Koopmans et al., 2005, 2009). For an animal to be designated as occasional coordination, the animal had to perform at least one CatWalk run at 100% on the RI; for frequent coordination the animal had to average 95% RI or more, and have more than one run of 100%; to be considered as consistent coordination, individual animals had to have an average RI of 95% or above, and with at least half of the runs being at 100% RI. (Note: The RI percentage is not the same as the percentage of coordinated runs as traditionally assessed in the BBB.)

Histology

Animals were anesthetized with an overdose of sodium pentobarbital (107 mg/kg IP; Bimeda-MTC Animal Health Inc., Cambridge, Ontario, Canada), and perfused transcardially with 0.12 M PBS (0.43% NaCl, pH 7.4), followed by a solution of 4% paraformaldehyde (in 0.12 M PBS buffer, pH 7.4). The spinal cord was removed and cryoprotected overnight in 24% sucrose solution (in 0.12 M PBS). A 1.5-cm-long segment of the thoracic spinal cord containing the injury site was frozen for serial cross sectioning, as well as the lumbar enlargement, which was cross-sectioned for subsequent serotonin transporter (SERT) immunostaining caudal to the injury site. These regions of the spinal cord were cut on a cryostat at 20 μ m thickness, serially collected, and frozen at -80°C until further use.

Lesion size and sparing analysis

The amount of spared white matter was measured on cross-sections 400 μ m apart stained with eriochrome cyanine. A Zeiss Axioskop microscope with a 5 $\times$ objective was used to capture images of the stained cross-sections ranging from 2800 μ m rostral to 2800 μ m caudal to the epicenter (as determined by the section with the least amount of spared white matter) of the injury for each animal. The rim of spared white matter was manually traced along with the amount of spared gray matter and quantified with SigmaScan Pro version 5.0 (Systat Software, Inc., Chicago, IL).

Immunofluorescence

For immunohistochemistry, sections were rehydrated in 0.01 M PBS (0.72% NaCl, pH 7.4) for 10 min and blocked with 10% NDS (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) in 0.01 M PBS containing 0.1% Triton X-100 (PBS-T) for 30 min. Thereafter sections were incubated with primary antibodies against rabbit anti-SERT (1:500; ImmunoStar, Inc., Hudson, WI) diluted in PBS-T overnight at room temperature. After washing the slides three times for 5 min each (PBS), the sections were incubated with Cy3-conjugated donkey anti-rabbit IgGs (1:500; Jackson ImmunoResearch Laboratories) in PBS-T for 2 h. After an additional washing step, the slides were cover-slipped with Fluoromount G (Southern Biotechnology, Birmingham, AL).

Quantification of raphe-spinal serotonergic fibers

From each animal, serial cross-sections spaced 400 μm apart starting at the rostral end of the lumbar enlargement (distal to the injury) were stained with SERT antibody. Digitized images of anti-SERT-stained sections (200 $\times$ magnification) were captured and analyzed using Sigma Scan Pro 5. The total intensity per area (μm^2) was analyzed, providing a density measurement (a.u.). We analyzed the gray matter of the ventral horns (Rexed laminae VII, VIII, and IX), along with a more discrete measurement of Rexed lamina IX of the ventral gray matter. We analyzed each side of the ventral gray matter lamina IX separately to obtain a symmetry score by dividing the lowest scoring side by the highest (perfect symmetry would result in a symmetry score of 1.0).

Statistical analysis

For behavioral outcomes a two-way repeated-measures analysis of variance (ANOVA) (time and behavior) was used to compare the four groups, followed by a one-way ANOVA at each time point, and if significance was found, it was followed up by a Bonferroni *post-hoc* test ($p < 0.05$, two-tailed). For histological data a one-way ANOVA was used and followed-up with a Bonferroni *post-hoc* if warranted. Pearson's correlations were calculated to examine relationships between functional

behavioral outcomes (the average of each behavior over the last three testing times, on days 50, 64, and 70), along with the histological outcomes and behavioral results, also using the last three testing time points (r^2 and p values are reported). All data are expressed as average $\pm$ standard error of the mean (SEM). All tests of significance performed were two-tailed.

Results

An overview of the timeline of the experiment is presented in Figure 1.

Animal weight and food consumption

As expected the calorie-restricted groups consumed a lower total amount of food than the *ad libitum* eating (AL) control group, indicating that the restriction on the fasting day was not fully compensated on the eating days when food was offered *ad libitum*: post-EODF group ($78.04 \pm 0.77\%$), and pre-EODF group ($70.73 \pm 2.46\%$; $p < 0.05$ by one-way ANOVA; Fig. 2A). This equates to approximately 22% and 29% calorie restriction for the two EODF groups. In an effort to match the food intake in the pair-fed group, we compromised and restricted it by 25%, at a level in between the two EODF groups.

At the time of the injury the animal body weights did not differ between the groups (Fig. 2B). Both the pre-EODF and the pair-fed calorie-restricted group started their restricted diet 3 weeks prior to injury, and we arranged the age of these animals in such a manner that all groups weighed the same at the time of injury. After injury all groups returned to pre-operative weight within 2 weeks, and continued to gain weight thereafter. However, both the pre-EODF and pair-fed restricted group gained less weight over time compared to the AL and post-EODF group. At the end of the experiment the three dietary-restricted groups weighed less than the AL group (post-EODF = -7% , pair-fed = -12.5% , pre-EODF = -16% ; $p < 0.05$).

Functional recovery after spinal cord injury

Horizontal ladder. On the horizontal ladder with irregular spacing there was a substantial increase in the percentage

FIG. 1. Timeline of the experiment. Both pair-fed and pre-EODF animals started their restricted diet 3 weeks prior to the scheduled spinal cord injury (SCI). The post-EODF group started its diet after the SCI. Baseline testing was performed prior to the injury. Post-injury behavioral testing was done over the next 10 weeks (not all the behaviors were run every week; EODF, every-other-day fasting).

FIG. 2. Animal food intake and weight. Animals in the restricted diet groups consumed fewer calories than the *ad libitum* (AL) group. **(A)** Food intake of the four groups over the experimental period. The post-EODF group consumed ~78% as much as the *ad libitum* control group, the pre-EODF group ~71%, and the pair-fed group ~75%. In an effort to match the food intake in the pair-fed group, we compromised and restricted it by 25%, at a level in between the two EODF groups. **(B)** Both the pre-EODF and pair-fed restricted groups gained less weight over time compared to the AL and post-EODF groups. At the end-point of the experiment the three dietary-restricted groups weighed less than the AL group (post-EODF group=7%, the pair-fed group=12.5%, the pre-EODF group=16%; $p < 0.05$; EODF, every-other-day fasting).

of hindlimb foot errors post-injury in all groups (Fig. 3; Metz and Whishaw, 2002). There were no statistically significant differences between groups for post-injury percentages of hindlimb footslips below the ladder rungs (Fig. 2; $p < 0.05$). The rank order of the lowest to highest percentage of errors was consistent with the subsequent behavioral data, showing the two EODF groups at the top of the behavioral rankings; however, a Kruskal-Wallis rank order test did not reach statistically significant differences between groups.

CatWalk. One of the most-frequently used coordination parameter in the CatWalk is the RI, which indicates the regularity, or degree, to which the animal uses normal step sequence patterns (Deumens et al., 2006; Koopmans et al., 2005, 2009). During the post-injury period a two-way repeated-measures ANOVA revealed group differences ($p < 0.05$; Fig. 4). Follow-up statistical analysis showed that the pre-EODF group had a higher RI than the *ad libitum* control group at 22 and 64 days post-injury, and both the *ad libitum* and pair-fed groups at 70 days post-injury ($p < 0.05$ by one-way ANOVA; $p < 0.05$ by Bonferroni *post-hoc* testing).

Other gait parameters altered as a result of compromised locomotion after injury include swing duration and stride length for both hindlimbs (HL) and forelimbs (FL). Typically, during coordinated walking, as observed in uninjured animals, the swing duration of the FL and HL are similar. After injury, swing duration timing becomes disrupted, with longer duration of the swing phase in the HL compared to the FL, resulting in negative values when you subtract the FL swing duration from the HL measurement (Fig. 5A). A two-way repeated-measures ANOVA revealed differences among the groups in the post-injury period ($p < 0.05$). Both pre-EODF and post-EODF treatment resulted in a smaller difference between FL and HL swing duration scores compared to the *ad libitum* group at 50 and 64 days post-injury ($p < 0.05$ by one-way ANOVA; $p < 0.05$ by Bonferroni *post-hoc* testing). Both of the EODF groups' swing duration differences were not statistically significantly different than their pre-operative baseline levels at 50–64 days post-injury ($p < 0.05$). The *ad libitum* and pair-fed groups retained a deficit throughout the testing period compared to their pre-operative baseline measurements ($p < 0.05$).

A similar effect was observed for the difference between FL and HL stride lengths (Fig. 5B) during stepping. This difference is typically near zero before injury. After injury all groups showed large negative values, indicating relatively smaller FL strides compared to HL strides. A two-way repeated-measures ANOVA indicated group differences ($p < 0.05$; Fig. 5B). At 64 and 70 days after injury the pre-EODF group showed improvement in recovery as evidenced by a smaller difference between FL – HL stride lengths compared to the *ad libitum* control group ($p < 0.05$ by one-way ANOVA; $p < 0.05$ by Bonferroni *post-hoc* testing; Fig. 5B).

Modified Basso-Beattie-Bresnahan open-field analysis

The BBB is the most widely used rating scale to evaluate locomotor function after a thoracic SCI in animal models (Basso et al., 1995, 1996). However, the most difficult and contentious component of the BBB is the accurate assessment of frequent (<95%) versus consistent (100%) coordination (Basso, 2004). Several researchers decided to use footprint analysis to assist in the measurement of coordination (Deumens et al., 2006;

FIG. 3. Ladder crossing hindlimb footslip errors. There were no differences between the groups in the percentage of hindlimb footslip errors (footslips below the level of the rungs) in the post-injury period. All data are expressed as average $\pm$ standard error of the mean (EODF, every-other-day fasting; Pre-op, preoperative).

Koopmans et al., 2005, 2009). Therefore, we used the catwalk RI as an objective measurement of coordination and incorporated it into our weekly BBB measurements (Fig. 6). We designated this measurement as a regularity indexed BBB score (RI-BBB). There was a drop in RI-BBB after injury, which improved over time ($p < 0.05$ by two-way repeated-measures ANOVA; Fig. 6). Analysis over this same time period also indicated a significant effect of group assignment ($p < 0.05$ by two-way repeated-measures ANOVA). The pre-EODF group (at 50, 64, and 70 days post-injury), and the post-EODF group (at 64 and 70 days post-injury) achieved significantly higher RI-BBB scores than both the *ad libitum* control and the pair-fed groups ($p < 0.05$ by one-way ANOVA; $p < 0.05$ by Bonferroni *post-hoc* testing). The *ad libitum* control and pair-fed groups reached an average score of 12.42 ± 0.69 and 12.17 ± 0.31 , respectively, indicating plantar stepping with frequent to consistent weight bearing and occasional FL-HL coordination. In contrast, the pre-EODF and post-EODF animals showed plantar stepping with consistent weight support, and consistent FL-HL coordination (14.17 ± 0.75 and 14.77 ± 0.58 , respectively).

Lesion size and tissue sparing

With our previous finding of a reduced lesion size after a cervical spinal cord injury in the EODF group, we wanted to

ascertain if we would see similar results in our thoracic injury model (Plunet et al., 2008, 2010). The spared white matter rim was quantified in cross-sections at $400\text{-}\mu\text{m}$ intervals spanning from $2800\text{-}\mu\text{m}$ rostral to $2800\text{-}\mu\text{m}$ caudal to the epicenter. As expected, the contusion injury to the thoracic spinal cord resulted in a dramatic loss of gray and white matter at the epicenter, with only a thin rim of residual white matter (Fig. 7A). In all four groups the lesion area extended both rostrally and caudally for $1600\text{--}2000\text{-}\mu\text{m}$. No group differences in the amount of spared white matter were observed ($p < 0.05$ by two-way repeated-measures ANOVA).

Gray matter was virtually eliminated at the epicenter of the injury, but further rostral and caudal to the injury there was some spared gray matter in all groups (Fig. 7B). No group differences were found between the groups in the amount of spared gray matter ($p > 0.05$ by two-way repeated-measures ANOVA), although the amount of sparing $2000\text{-}\mu\text{m}$ rostral to the lesion site correlated with behavioral outcomes (see correlation section below).

Serotonergic fibers

With EODF reported to increase plasticity (Fontan-Lozano et al., 2007; Plunet et al., 2008), we wanted to examine if increased sprouting could account for the functional differences

FIG. 4. EODF improved CatWalk regularity index ratings. The regularity index from the CatWalk device gives a coordination score for each animal. While this percentage cannot be directly compared to the 95% coordination level used in the Basso-Beattie-Bresnahan scale, it can be used as an overall coordination score. Group differences were found between groups ($p < 0.05$ by two-way repeated-measures ANOVA). The pre-EODF group had significantly higher regularity index scores compared to the *ad libitum* group at 22 and 64 days post-injury (*), and both the *ad libitum* and pair-fed groups at 70 days post-injury (** $p < 0.05$ by one-way ANOVA; $p < 0.05$ by Bonferroni *post-hoc* testing). All data are expressed as average $\pm$ standard error of the mean (EODF, every-other-day fasting; ANOVA, analysis of variance; Pre-op, preoperative).

between the groups we observed after SCI. The delayed functional recovery would also be more consistent with enhanced plasticity rather than neuroprotection. Detection of descending raphe-spinal serotonergic tracts was visualized by serotonin transporter (SERT) immunofluorescence. 5-HT transporter (SERT) immunostaining has a better correlation with return of function than 5-HT, and hence we used SERT immunostaining to assess serotonin's influence on functional recovery in our experiment (Saruhashi et al., 2009). 5-HT fibers at the L1–L2 level (below the level of injury) are known to be important for interlimb coordination (Jordan et al., 2008; Schmidt and Jordan, 2000).

Caudal to the injury we found no statistically significant differences among the groups in SERT immunostaining density in the ventral gray matter horns ($p = 0.14$ by one-way repeated-measures ANOVA; Fig. 8), although there was a trend toward higher SERT staining densities in both EODF groups. We additionally examined the more specific location of motor neurons in lamina IX (Fig. 9A), but again the trend toward higher values in the post-EODF group did not reach statistical significance ($p > 0.05$ by one-way ANOVA). Since

some of our behavioral differences among groups centered around interlimb coordination, and 5-HT fibers at the L1–L2 level (below the level of injury) are known to be important for interlimb coordination, we made a more detailed examination (Jordan et al., 2008; Schmidt and Jordan, 2000). The physiological functioning of 5-HT requires some threshold amount on each side of the spinal cord (Saruhashi et al., 1996, 2009). Hence, we obtained a symmetry score by taking the ratio of SERT immunostaining in lamina IX on one side compared to the other side. While a conservative one-way ANOVA did not show a difference among the groups (Fig. 9B), a Pearson correlation analysis revealed interesting relationships between histology and behavior, as did a number of the SERT parameters we measured.

Correlation of behavioral outcomes and histology with behavior

While we did not observe significant group differences on any of the histological parameters, we explored if any of these parameters correlated with the various functional outcomes

FIG. 5. EODF improved swing duration differences between forelimbs and hindlimbs, as well as stride length differences. **(A)** After injury, swing duration timing is altered, resulting in a longer duration of the swing phase in the HL compared to the FL (FL - HL). Both pre-EODF and post-EODF treatment resulted in a smaller difference between FL and HL swing duration compared to the *ad libitum* group at 50 and 64 days post-injury (** $p < 0.05$ by one-way ANOVA; $p < 0.05$ by Bonferroni *post-hoc* testing). **(B)** The difference between FL and HL stride lengths prior to injury is near 0. After injury, all groups showed large negative values, indicating a deficit with small forelimb steps. At 64 and 70 days after injury, the pre-EODF group had better recovery, as evidenced by a smaller difference between FL - HL stride lengths compared to the *ad libitum* control group (* $p < 0.05$ by one-way ANOVA; $p < 0.05$ by Bonferroni *post-hoc* testing). All data are expressed as average $\pm$ standard error of the mean (EODF, every-other-day fasting; ANOVA, analysis of variance; Pre-op, preoperative).

using Pearson correlation analyses, and by examining correlations between the various behaviors (Tables 1 and 2).

First we analyzed the relationships between various behavioral outcomes (i.e., between each other; Table 1). We found a high degree of significant correlations between all the behaviors, with the exception of percentage of ladder errors, which did not correlate with stride length differences (Table 1). Interestingly, the percentage of ladder errors (which did not differ between groups) correlated with all the other behaviors, other than the one mentioned exception (stride length).

We then examined if any of our histological measurements correlated with the various behavioral outcomes. Overall white matter sparing did not correlate with behavior, or any of the individual sparing measurements at the epicenter, or those made at various distances rostral or caudal to the injury site ($p < 0.05$ by Pearson correlation). Overall gray matter sparing did not significantly correlate with any of the behaviors, but the amount of gray matter spared 2000 μ m rostral to the lesion did significantly correlate with two of the five tested behaviors ($p < 0.05$; Table 2).

Despite the absence of significant group differences among the various SERT immunostaining measurements, we observed correlations with several behavioral outcomes. Both the ventral gray matter (Fig. 8) and the lamina IX (Fig. 9A) caudal to the injury site significantly correlated with several behavioral measurements ($p < 0.05$; Table 2). Lamina IX SERT immunostaining levels correlated with swing duration differences and percentage of ladder errors, while the broader ventral horn gray matter SERT staining correlated with four out of the five behaviors (swing duration differences, percentage of ladder errors, RI-BBB, and the RI). Finally, the symmetrical level of SERT immunostaining in lamina IX (Fig. 9B) significantly correlated with RI, swing duration differences, and percentage of ladder errors.

In addition, we examined if correlations of the histology/behavior relationships existed independently in each of the four different animal groups, and for each possible histology/behavior interaction. Of the 80 possible correlations, only 5 reached significance. In the control group there was a correlation between gray matter sparing 2000 μ m caudal to the injury site and swing duration differences, as well as with the RI-BBB; and the RI correlated with the caudal lamina IX SERT symmetry score. The only other two significant correlations were found in the post-EODF group between the caudal lamina IX SERT levels and ladder errors, and between the caudal ventral gray matter SERT levels and swing duration differences. There were no significant relationships between behavior and histology for the other two groups.

Discussion

Here we report that EODF improves functional recovery after a thoracic spinal cord contusion injury of moderate severity in rats. Both EODF starting 3 weeks before the injury, or more importantly, starting it after the injury, were effective. The pre-EODF and post-EODF groups reached significantly higher scores on the RI-BBB for open-field locomotion when compared to the *ad libitum* control group, but also the pair-fed group. The pair-fed group consumed the same total amount of calories offered daily. In addition, on several parameters measured with the CatWalk gait analysis device, both EODF

FIG. 6. EODF improved the regularity indexed BBB (RI-BBB). Using the RI to measure the degree of coordination, we observed a significant effect of group assignment ($p < 0.05$ by two-way repeated-measures ANOVA). The pre-EODF group (at 50, 64, and 70 days post-injury), and post-EODF group (at 64 and 70 days post-injury) achieved significantly higher RI-BBB scores than both the *ad libitum* control and the pair-fed groups ($p < 0.05$; *the pre-EODF group significantly better than the *ad libitum* and pair-fed control groups; **the pre-EODF and post-EODF groups significantly better than the *ad libitum* and pair-fed control groups). All data are expressed as average $\pm$ standard error of mean (EODF, every-other-day fasting; ANOVA, analysis of variance; Pre-op, preoperative; BBB, Basso-Beattie-Bresnahan).

feeding regimens elicited improved functional recovery. Hence, EODF has prophylactic and therapeutic potential after thoracic contusion SCI, and these findings are consistent with our previous findings with pre-EODF and post-EODF in a cervical SCI model, the dorsolateral funiculus crush (Plunet et al., 2008, 2010).

While both EODF groups had better functional recovery than the two control groups (*ad libitum* and pair-fed), the pre-EODF group (starting the diet 3 weeks prior to injury) improved functional recovery to a greater extent than the post-EODF group (Table 3). This observation is consistent with our two separate EODF studies after cervical SCI, although they were not performed at the same time and therefore cannot be directly compared (Plunet et al., 2008, 2010). One can speculate that cellular pathways for neuroprotection have been already activated by 3 weeks of pre-EODF, and that this provides benefits during the early secondary injury phase. In contrast, this activation of EODF-induced effects may occur too slowly to influence the early cascades of secondary death and inflammation if the diet is

started after injury. The clinical significance of our pre-EODF data is that such a regimen could conceivably be implemented prior to elective interventions that put the spinal cord at risk, such as surgery for deformities or tumors. Additionally, EODF started after injury, which would have wider clinical applications, also improved functional recovery after SCI.

Daily caloric restriction (pair-fed group), in contrast to EODF, did not improve functional outcomes after thoracic contusion injury, and the pair-fed rats showed similar behavioral performance as the *ad libitum* group. This differential outcome between the CR and EODF groups is consistent with previous research in mice showing increased neuroprotective efficacy of pre-EODF compared to caloric restriction after kainate-induced brain injury (Anson et al., 2003). This suggests that similar levels of calorie restriction do not produce the same behavioral outcome, and the specific timing of food/fasting can make a substantial difference in the effects. Anson and coworkers suggested that differences in trophic factors may play a role. It is unknown how a change in metabolism and gene expression elicited by 24 h of fasting, followed by a

FIG. 7. Spared white and gray matter. (A) The rostral and caudal extent of spared white matter was quantified at 400-μm intervals spanning from 2800 μm rostral to 2800 μm caudal to the epicenter. No group differences in the amount of spared white matter were observed ($p > 0.05$ by two-way repeated-measures ANOVA). (B) Gray matter was basically eliminated at the epicenter of the injury, but there was some spared gray matter in all groups further rostral and caudal to the injury. No group differences were found between the groups in the amount of spared gray matter ($p > 0.05$ by two-way repeated-measures ANOVA). All data are expressed as average $\pm$ standard error of the mean (ANOVA, analysis of variance).

feasting and free access to food for 24 h, contributes to the increased functional recovery seen after SCI. We propose that the 24-h fasting period increases blood ketone levels, which could exert neuroprotective effects. Such an increase in ke-

tones is not seen on a caloric-restriction regimen (Anson et al., 2003), such as the pair-fed control feeding regimen. This hypothesis is supported by recent data from our laboratory revealing that a ketogenic diet, which also increases ketone

FIG. 8. Serotonin transporter (SERT) sparing/sprouting caudal to the injury in the ventral gray matter. Caudal to the injury we found no statistically significant differences among the groups for SERT immunostaining density in the ventral gray matter ($p=0.14$ by one-way repeated-measures ANOVA). All data are expressed as average $\pm$ standard error of the mean (ANOVA, analysis of variance; a.u., arbitrary units).

levels, after cervical hemi-contusion injuries in rats elicits robust improvements in behavioral outcomes (Streijger et al., in submission). In addition, it is conceivable that 24 h of feasting (eating ~ 140 – 160% of normal intake) contributes to the functional recovery by altering fundamental metabolic pathways, such as stimulation of the mTOR pathway, as has recently been reported in the brains of EODF animals, but not in similarly caloric-restricted animals that were fed every day (Martin et al., 2008). Increased levels of mTOR have recently been shown to enhance the regenerative capacity of corticospinal axons (Liu et al., 2010). Further research is necessary to clarify the relative contributions of these possible fasting and/or feasting effects.

In light of our previous findings of a reduced lesion size after a cervical SCI in both pre-EODF and post-EODF groups (Plunet et al., 2008, 2010), we examined here if similar neuroprotection is seen in our thoracic injury model. Despite functional improvements in both EODF groups after thoracic SCI, we did not observe any differences in the amount of spared white or gray matter between the groups. Of note, in our cervical model EODF rescued mainly gray matter, and gray matter is typically entirely destroyed after thoracic contusions. However, there was a trend toward improved gray matter rescue at a distance of 2000–1600 μm from the epicenter. It is conceivable that the more complete and larger injury seen after thoracic contusion, compared to the unilateral cervical crush injury, masks any of the differences in neuroprotection; in other words, an equally small rim of rescued tissue would be more evident and easier to detect in a small lesion. Hence a small neuroprotective effect of EODF might have gone undetected in this more severe thoracic contusion model.

EODF is known to alter the expression of a large number of proteins that could account for increased plasticity (Mattson et al., 2003), and indeed EODF increased axonal plasticity as

FIG. 9. Serotonin transporter (SERT) expression in Rexed lamina IX caudal to injury and symmetry scores. (A) A one-way ANOVA found no differences between groups in the amount of SERT immunostaining caudal to the injury in lamina IX ($p>0.05$). (B) The level of SERT symmetry in lamina IX is shown. No group differences were observed ($p>0.05$ by one-way ANOVA). All data are expressed as average $\pm$ standard error of the mean (ANOVA, analysis of variance; a.u., arbitrary units).

measured by long-term changes in synaptic efficiency, coinciding with increases in NMDA receptor subunit NR2B, and sprouting of spared corticospinal axons (Fontan-Lozano et al., 2007; Plunet et al., 2008). The delayed functional recovery (50 days) in the both EODF groups would be consistent with enhanced neural plasticity being the major mechanistic player. In the cervical injury model we observed increased sprouting of the intact corticospinal tract (Plunet et al., 2008). In the present study we quantified serotonergic axons, as this system plays an important role in limb coordination (Jordan et al., 2008; Schmidt and Jordan, 2000), and hence is often studied in thoracic SCI models. While the several parameters of SERT sprouting data correlated with functional recovery, we did not detect group differences among our multiple groups using conservative ANOVA statistics. These results suggest that additional fiber systems might be playing a role,

TABLE 1. STRONG CORRELATIONS BETWEEN DIFFERENT FUNCTIONAL RECOVERY BEHAVIORS

Behavior	RI-BBB	Ladder errors	RI	Swing duration differences	Stride length differences
RI-BBB		R = 0.5424 $p < 0.0001$	R = 0.7801 $p < 0.0001$	R = 0.6826 $p < 0.0001$	R = 0.7128 $p < 0.0001$
Ladder errors	R = 0.5424 $p < 0.0001$		R = 0.6851 $p < 0.0001$	R = 0.7736 $p < 0.0001$	R = 0.2471 $p > 0.05$
RI	R = 0.7801 $p < 0.0001$	R = 0.6851 $p < 0.0001$		R = 0.8386 $p < 0.0001$	R = 0.6440 $p < 0.0001$
Swing duration differences	R = 0.6826 $p < 0.0001$	R = 0.7736 $p < 0.0001$	R = 0.8386 $p < 0.0001$		R = 0.5227 $p < 0.0001$
Stride length differences	R = 0.7128 $p < 0.0001$	R = 0.2471 $p > 0.05$	R = 0.6440 $p < 0.0001$	R = 0.5227 $p < 0.0001$	

The table shows the correlations between the various behaviors we tested in this experiment, along with the p values. All but one pairing of behaviors were significantly related to each other ($p < 0.05$). It is interesting to note that even the percentage of ladder errors, which did not reveal group differences, was correlated with the other behaviors for which group differences were observed.

RI-BBB, regularity indexed BBB score; RI, regularity index.

and that individual fiber systems may only show subtle changes.

These results also raise the possibility that changes at the cortical level could have occurred that contributed to the change in functional recovery observed in the EODF groups. The potential cortical level changes could have occurred at a structural level and/or at a molecular level. Since EODF can have systemic affects, including in the brain, this could provide additional mechanisms for beneficial outcomes. Both calorie restriction and EODF have been reported to improve learning/memory in older animals, though there are other researchers that found no improvement (Bond et al., 1989; Beatty et al., 1987; Idrobo et al., 1987; Pitsikas and Algeri, 1992). Improved learning/memory in younger animals, such as those used in our experiment, is not established. In our study we did not specifically examine any cortical structural plasticity changes induced by EODF that could account for our behavioral results. An additional possibility is that a cortical learning effect may account for some of these improvements. However, we think that this is unlikely for several reasons. The behavior with the highest potential for learning, the skilled ladder crossing test, revealed no differences among the groups. The other two tests, open-field, and Cat-Walk, involve arguably less skilled locomotor movements (i.e., walking/running). While there is likely a component of re-learning after SCI for these motor patterns, these are more likely

“hard wired” in comparison to a more complex task such as crossing an irregular ladder with specific foot placements on the slim rungs. Additionally, the equally calorie-restricted pair-fed group did not show any improvement in motor recovery after SCI, and if memory/learning was playing a role we would have expected similar improvements in motor recovery in this group, since the level of calorie restriction these animals were on is similar to what is reported to improve memory/learning, at least in older animals. Overall, this suggests that improved memory/learning did not play a major role in the motor recovery seen after SCI in this experiment.

Conclusion

Here we provide further evidence that rats maintained on an EODF diet after SCI show improved motor function. Both the prophylactic pre-EODF and therapeutic post-injury-initiated EODF resulted in improved functional recovery in a thoracic injury contusion model compared to the control *ad libitum* and pair-fed calorie-restricted groups. Additionally, the pre-EODF performed slightly better than the post-EODF group. Finally, daily calorie restriction at the same level as the EODF groups did not appear to produce the same positive effects as EODF on behavioral recovery after SCI. However, we could not detect significant neuroprotective effects of

TABLE 2. HISTOLOGICAL OUTCOMES WERE SIGNIFICANTLY CORRELATED WITH BEHAVIORAL MEASUREMENTS

Behavior Histology	RI-BBB	Ladder errors	RI	Swing duration differences	Stride length differences	Average R value
Gray matter sparing 2000 μ m caudal	R = 0.2788 $p = 0.055$	R = 0.2590 $p = 0.0755$	R = 0.3333 $p < 0.05$	R = 0.2934 $p < 0.05$	R = 0.2217 $p = 0.1298$	R = 0.2773
Caudal ventral gray matter SERT	R = 0.3558 $p < 0.05$	R = 0.3732 $p < 0.01$	R = 0.4301 $p < 0.01$	R = 0.4805 $p < 0.001$	R = 0.2167 $p = 0.1462$	R = 0.3701
Caudal lamina IX SERT	R = 0.1577 $p = 0.2795$	R = 0.3966 $p < 0.01$	R = 0.2653 $p = 0.0654$	R = 0.3922 $p < 0.01$	R = 0.0523 $p = 0.7211$	R = 0.2528
Caudal lamina IX SERT symmetry	R = 0.2662 $p = 0.0645$	R = 0.2998 $p < 0.01$	R = 0.3366 $p < 0.05$	R = 0.3089 $p < 0.05$	R = 0.1293 $p = 0.3760$	R = 0.2682

The five histological outcomes were statistically related with various behavioral results ($p < 0.05$), with the exception of forelimb – hindlimb stride length differences. The caudal intermediate levels of SERT had the highest average correlations with the behavioral outcomes.

RI-BBB, regularity indexed BBB score; RI, regularity index; SERT, serotonin transporter.

TABLE 3. SUMMARY OF BEHAVIORAL EFFECTS OBSERVED WITH DIFFERENT DIETARY STRATEGIES AFTER THORACIC CONTUSION INJURY COMPARED TO THE *AD LIBITUM* CONTROL GROUP

Parameters	Pre-EODF	Post-EODF	Pair-fed
Horizontal ladder errors (%)	—	—	—
RI-BBB open field score	↑	↑	—
Catwalk			
RI	↑	—	—
Stride length (forelimb – hindlimb)	↑	—	—
Swing duration (forelimb – hindlimb)	↑	↑	—

Both pre-EODF and the clinically relevant post-EODF groups improved functional recovery on multiple behaviors compared to both the *ad libitum* control, but also the pair-fed (equally calorie-restricted) groups. Additionally, the pre-EODF group had better functional recovery on some of the behavioral outcomes on certain parameters, while the post-EODF group did not.

RI-BBB, regularity indexed BBB score; RI, regularity index; BBB, Basso-Beattie-Bresnahan; EODF, every-other-day fasting.

EODF in this injury model, nor were there any pronounced group differences in SERT sparing/sprouting, despite a significant correlation of SERT staining with behavioral outcomes. It is conceivable that multiple mechanisms are mediating the improvements seen in functional recovery, which when measured in isolation may not reach significance. The main result of improved functional recovery elicited by this safe, simple, and inexpensive EODF paradigm in two different experimental groups of thoracic contusion adds to our previous findings of improved recovery after cervical SCI. Taken together, this body of data supports the notion that specific dietary strategies might eventually benefit acutely injured patients with SCI; however, more pre-clinical data about the possible mechanisms and side effects of this approach are needed.

Acknowledgments

This work was supported by a grant from the Canadian Institute for Health Research (CIHR) to W.T.

Author Disclosure Statement

No competing financial interests exist.

References

Anson, R.M., Guo, Z., de Cabo, R., Iyun, T., Rios, M., Hagepanos, A., Ingram, D.K., Lane, M.A., and Mattson, M.P. (2003). Intermittent fasting dissociates beneficial effects of dietary restriction on glucose metabolism and neuronal resistance to injury from calorie intake. *Proc. Natl. Acad. Sci. USA* 100, 6216–6220.

Bareyre, F.M., Kerschensteiner, M., Raineteau, O., Mettenleiter, T.C., Weinmann, O., and Schwab, M.E. (2004). The injured spinal cord spontaneously forms a new intraspinal circuit in adult rats. *Nat. Neurosci.* 7, 269–277.

Basso, D.M. (2004). Behavioral testing after spinal cord injury: congruities, complexities, and controversies. *J. Neurotrauma* 21, 395–404.

Basso, D.M., Beattie, M.S., and Bresnahan, J.C. (1995). A sensitive and reliable locomotor rating scale for open field testing in rats. *J. Neurotrauma* 12, 1–21.

Basso, D.M., Beattie, M.S., and Bresnahan, J.C. (1996). Graded histological and locomotor outcomes after spinal cord contusion using the NYU weight-drop device versus transection. *Exp. Neurol.* 139, 244–256.

Beatty, W.W., Clouse, B.A., and Bierley, R.A. (1987). Effects of long-term restricted feeding on radial maze performance by aged rats. *Neurobiol. Aging* 8, 325–327.

Bond, N.W., Everitt, A.V., and Walton, J. (1989). Effects of dietary restriction on radial-arm maze performance and flavor memory in aged rats. *Neurobiol. Aging* 10, 27–30.

Bough, K.J., Schwartzkroin, P.A., and Rho, J.M. (2003). Calorie restriction and ketogenic diet diminish neuronal excitability in rat dentate gyrus in vivo. *Epilepsia* 44, 752–760.

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

Bruce-Keller, A.J., Umberger, G., McFall, R., and Mattson, M.P. (1999). Food restriction reduces brain damage and improves behavioral outcome following excitotoxic and metabolic insults. *Ann. Neurol.* 45, 8–15.

Davis, L.M., Pauly, J.R., Readnower, R.D., Rho, J.M., and Sullivan, P.G. (2008). Fasting is neuroprotective following traumatic brain injury. *J. Neurosci. Res.* 86, 1812–1822.

Deumens, R., Koopmans, G.C., Honig, W.M., Hamers, F.P., Maquet, V., Jerome, R., Steinbusch, H.W., and Joosten, E.A. (2006). Olfactory ensheathing cells, olfactory nerve fibroblasts and biomatrices to promote long-distance axon regrowth and functional recovery in the dorsally hemisected adult rat spinal cord. *Exp. Neurol.* 200, 89–103.

Duan, W., Guo, Z., Jiang, H., Ware, M., Li, X.J., and Mattson, M.P. (2003). Dietary restriction normalizes glucose metabolism and BDNF levels, slows disease progression, and increases survival in huntingtin mutant mice. *Proc. Natl. Acad. Sci. USA* 100, 2911–2916.

Fontan-Lozano, A., Saez-Cassanelli, J.L., Inda, M.C., de los Santos-Arteaga, M., Sierra-Dominguez, S.A., Lopez-Lluch, G., Delgado-Garcia, J.M., and Carrion, A.M. (2007). Caloric restriction increases learning consolidation and facilitates synaptic plasticity through mechanisms dependent on NR2B subunits of the NMDA receptor. *J. Neurosci.* 27, 10185–10195.

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

Hamers, F.P., Koopmans, G.C., and Joosten, E.A. (2006). Cat-Walk-assisted gait analysis in the assessment of spinal cord injury. *J. Neurotrauma* 23, 537–548.

Hamers, F.P., Lankhorst, A.J., van Laar, T.J., Veldhuis, W.B., and Gispen, W.H. (2001). Automated quantitative gait analysis during overground locomotion in the rat: its application to spinal cord contusion and transection injuries. *J. Neurotrauma* 18, 187–201.

Idrobo, F., Nandy, K., Mostofsky, D.I., Blatt, L., and Nandy, L. (1987). Dietary restriction: effects on radial maze learning and lipofuscin pigment deposition in the hippocampus and frontal cortex. *Arch. Gerontol. Geriatr.* 6, 355–362.

Jordan, L.M., Liu, J., Hedlund, P.B., Akay, T., and Pearson, K.G. (2008). Descending command systems for the initiation of locomotion in mammals. *Brain Res. Rev.* 57, 183–191.

Koopmans, G.C., Deumens, R., Buss, A., Geoghegan, L., Myint, A.M., Honig, W.H., Kern, N., Joosten, E.A., Noth, J., and Brook, G.A. (2009). Acute rolipram/thalidomide treatment improves tissue sparing and locomotion after experimental spinal cord injury. *Exp. Neurol.* 216, 490–498.

- Koopmans, G.C., Deumens, R., Honig, W.M., Hamers, F.P., Steinbusch, H.W., and Joosten, E.A. (2005). The assessment of locomotor function in spinal cord injured rats: the importance of objective analysis of coordination. *J. Neurotrauma* 22, 214–225.
- Kwon, B.K., Okon, E.B., Tsai, E., Beattie, M.S., Bresnahan, I.C., Magnuson, D.K., Reier, P.J., McTigue, D.M., Popovich, P.G., Blight, A.R., Oudega, M., Guest, I.D., Weaver, L.C., Fehlings, M.G., Tetzlaff, W. (2010). A grading system to evaluate objectively the strength of pre-clinical data of acute neuroprotective therapies for clinical translation in spinal cord injury. *J. Neurotrauma* (E-pub ahead of print.)
- Liu, K., Lu, Y., Lee, J.K., Samara, R., Willenberg, R., Sears-Kraxberger, I., Tedeschi, A., Park, K.K., Jin, D., Cai, B., Xu, B., Connolly, L., Steward, O., Zheng, B., and He, Z. (2010). PTEN deletion enhances the regenerative ability of adult corticospinal neurons. *Nat. Neurosci.* 13, 1075–1081.
- Martin, B., Pearson, M., Brenneman, R., Golden, E., Keselman, A., Iyuan, T., Carlson, O.D., Egan, J.M., Becker, K.G., Wood, W. 3rd., Prabhu, V., de Cabo, R., Maudsley, S., and Mattson, M.P. (2008). Conserved and differential effects of dietary energy intake on the hippocampal transcriptomes of females and males. *PLoS One* 3, e2398.
- Martin, B., Pearson, M., Kebejian, L., Golden, E., Keselman, A., Bender, M., Carlson, O., Egan, J., Ladenheim, B., Cadet, J.L., Becker, K.G., Wood, W., Duffy, K., Vinayakumar, P., Maudsley, S., and Mattson, M.P. (2007). Sex-dependent metabolic, neuroendocrine, and cognitive responses to dietary energy restriction and excess. *Endocrinology* 148, 4318–4333.
- Maswood, N., Young, J., Tilmont, E., Zhang, Z., Gash, D.M., Gerhardt, G.A., Grondin, R., Roth, G.S., Mattison, J., Lane, M.A., Carson, R.E., Cohen, R.M., Mouton, P.R., Quigley, C., Mattson, M.P., and Ingram, D.K. (2004). Caloric restriction increases neurotrophic factor levels and attenuates neurochemical and behavioral deficits in a primate model of Parkinson's disease. *Proc. Natl. Acad. Sci. USA* 101, 18171–18176.
- Mattson, M.P., Chan, S.L., and Duan, W. (2002). Modification of brain aging and neurodegenerative disorders by genes, diet, and behavior. *Physiol. Rev.* 82, 637–672.
- Mattson, M.P., Duan, W., and Guo, Z. (2003). Meal size and frequency affect neuronal plasticity and vulnerability to disease: cellular and molecular mechanisms. *J. Neurochem.* 84, 417–431.
- Mattson, M.P. (2005). Energy intake, meal frequency, and health: a neurobiological perspective. *Annu. Rev. Nutr.* 25, 237–260.
- Metz, G.A., and Whishaw, I.Q. (2002). Cortical and subcortical lesions impair skilled walking in the ladder rung walking test: a new task to evaluate fore- and hindlimb stepping, placing, and co-ordination. *J. Neurosci. Methods* 115, 169–179.
- Mouton, P.R., Chachich, M.E., Quigley, C., Spangler, E., and Ingram, D.K. (2009). Caloric restriction attenuates amyloid deposition in middle-aged dtg APP/PS1 mice. *Neurosci. Lett.* 464, 184–187.
- Park, E., Velumian, A.A., and Fehlings, M.G. (2004). The role of excitotoxicity in secondary mechanisms of spinal cord injury: a review with an emphasis on the implications for white matter degeneration. *J. Neurotrauma* 21, 754–774.
- Patel, N.V., Gordon, M.N., Connor, K.E., Good, R.A., Engelman, R.W., Mason, J., Morgan, D.G., Morgan, T.E., and Finch, C.E. (2005). Caloric restriction attenuates Abeta-deposition in Alzheimer transgenic models. *Neurobiol. Aging* 26, 995–1000.
- Pitsikas, N., and Algeri, S. (1992). Deterioration of spatial and nonspatial reference and working memory in aged rats: protective effect of life-long calorie restriction. *Neurobiol. Aging* 13, 369–373.
- Plunet, W.T., Lam, C.K., Lee, J.H., Liu, J., and Tetzlaff, W. (2010). Prophylactic dietary restriction may promote functional recovery and increase lifespan after spinal cord injury. *Ann. NY Acad. Sci.* 1198 (Suppl. 1), E1–E11.
- Plunet, W.T., Streijger, F., Lam, C.K., Lee, J.H., Liu, J., and Tetzlaff, W. (2008). Dietary restriction started after spinal cord injury improves functional recovery. *Exp. Neurol.* 213, 28–35.
- Qin, W., Yang, T., Ho, L., Zhao, Z., Wang, J., Chen, L., Zhao, W., Thiagarajan, M., MacGrogan, D., Rodgers, J.T., Puigserver, P., Sadoshima, J., Deng, H., Pedrini, S., Gandy, S., Sauve, A.A., and Pasinetti, G.M. (2006). Neuronal SIRT1 activation as a novel mechanism underlying the prevention of Alzheimer disease amyloid neuropathology by calorie restriction. *J. Biol. Chem.* 281, 21745–21754.
- Ramer, L.M., Ramer, M.S., and Steeves, J.D. (2005). Setting the stage for functional repair of spinal cord injuries: a cast of thousands. *Spinal Cord* 43, 134–161.
- Saruhashi, Y., Matsusue, Y., and Fujimiya, M. (2009). The recovery of 5-HT transporter and 5-HT immunoreactivity in injured rat spinal cord. *Arch. Orthop. Trauma Surg.* 129, 1279–1285.
- Saruhashi, Y., Young, W., and Perkins, R. (1996). The recovery of 5-HT immunoreactivity in lumbosacral spinal cord and locomotor function after thoracic hemisection. *Exp. Neurol.* 139, 203–213.
- Schmidt, B.J., and Jordan, L.M. (2000). The role of serotonin in reflex modulation and locomotor rhythm production in the mammalian spinal cord. *Brain Res. Bull.* 53, 689–710.
- Sharma, S., and Kaur, G. (2005). Neuroprotective potential of dietary restriction against kainate-induced excitotoxicity in adult male Wistar rats. *Brain Res. Bull.* 67, 482–491.
- Streijger, E., Plunet, W.T., Lee, J.H.T., Liu, J., Lam, C.K., Park, S., Hilton, B.J., Fransen, B.J., Matheson, K.A.I., Assinck, P., Kwon, B.K., Tetzlaff, W. (2011). Post-injury ketogenic diet improves gross and skilled forelimb motor function after cervical cord contusion in rats (In submission).
- Van Meeteren, N.L., Eggers, R., Lankhorst, A.J., Gispen, W.H., and Hamers, F.P. (2003). Locomotor recovery after spinal cord contusion injury in rats is improved by spontaneous exercise. *J. Neurotrauma* 20, 1029–1037.
- Wang, J., Ho, L., Qin, W., Rocher, A.B., Seror, I., Humala, N., Maniar, K., Dolios, G., Wang, R., Hof, P.R., and Pasinetti, G.M. (2005). Caloric restriction attenuates beta-amyloid neuropathology in a mouse model of Alzheimer's disease. *FASEB J.* 19, 659–661.
- Wan, R., Camandola, S., and Mattson, M.P. (2003). Intermittent fasting and dietary supplementation with 2-deoxy-D-glucose improve functional and metabolic cardiovascular risk factors in rats. *FASEB J.* 17, 1133–1134.
- Wu, P., Shen, Q., Dong, S., Xu, Z., Tsien, J.Z., and Hu, Y. (2008). Calorie restriction ameliorates neurodegenerative phenotypes in forebrain-specific presenilin-1 and presenilin-2 double knockout mice. *Neurobiol. Aging* 29, 1502–1511.
- Yu, Z.F., and Mattson, M.P. (1999). Dietary restriction and 2-deoxyglucose administration reduce focal ischemic brain damage and improve behavioral outcome: evidence for a preconditioning mechanism. *J. Neurosci. Res.* 57, 830–839.
- Zhu, H., Guo, Q., and Mattson, M.P. (1999). Dietary restriction protects hippocampal neurons against the death-promoting action of a presenilin-1 mutation. *Brain Res.* 842, 224–229.

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