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Effects of passive immobilization on locomotor recovery after spinal cord injury in adult rats.

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EFFECTS OF PASSIVE IMMOBILIZATION ON LOCOMOTOR RECOVERY
AFTER SPINAL CORD INJURY IN ADULT RATS

By

Kelsey Lee Stipp

B.S., University of Louisville, 2011

A Thesis

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A Thesis Approved on
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ABSTRACT

EFFECTS OF PASSIVE 'IMMOBILIZATION' ON LOCOMOTOR RECOVERY IN THORACIC SPINAL CONTUSED RATS

Kelsey Lee Stipp

August 6, 2014

Background: Spontaneous locomotor recovery in spinal rats has been attributed to animals moving freely in-cage. Environmental enrichment has been shown to increase in-cage movement and functional recovery subsequently. Anxiety has been shown to decrease overnight activity in rats.

Methods: Rats were double-housed in medium cages (MC) or single-housed in tiny sized cages (TC). Slotted dividers allowed for partial isolation in TC. Overnight activity was monitored bi-weekly. The open field test and BBB's were taken weekly. Gait analysis was performed at weeks six and eight.

Results: MC showed higher overnight activity and improved gait overtime. No differences were found in BBB scores. Differences in anxiety began to show in the last few weeks of the study.

Discussion: The opportunity for movement in MC led to these animals having higher in-cage activity and an improvement in gait. A more severe injury than anticipated perhaps caused low BBB scores. MC animals may have been anxious due to unwanted stressors.

TABLE OF CONTENTS

	PAGE
ACKNOWLEDGMENTS.....	iii
ABSTRACT.....	iv
LIST OF FIGURES.....	vii
INTRODUCTION.....	1
METHODS.....	7
Study design.....	7
Cage design.....	8
Spinal cord injury.....	12
Recovery.....	13
Functional locomotor recovery.....	13
Overnight activity.....	14
Open field test.....	16
Analysis of chromodacryorrhea.....	19
Light cycle recordings.....	20
Overground gait assessment.....	20
Nociception assessment.....	21
Perfusion.....	22
Histology.....	22

Statistics.....	23
RESULTS.....	24
Functional locomotor recovery.....	24
Overnight activity.....	25
Open field test.....	27
Analysis of chromodacryorrhea.....	31
Overground gait assessment.....	32
Nociception assessment.....	34
Histology.....	35
DISCUSSION.....	37
REFERENCES.....	44
CURRICULUM VITAE.....	49

LIST OF FIGURES

FIGURE	PAGE
Figure 1: Timeline of study.....	8
Figure 2: Image of medium cages.....	9
Figure 3: Image of tiny cages.....	10
Figure 4: Tiny cage showing dividers.....	11
Figure 5: Single frame image of overnight recording.....	12
Figure 6: Overnight setup.....	15
Figure 7: Single frame image of digitized overnight activity.....	16
Figure 8: Open field test arena.....	17
Figure 9: Single frame of digitized open field test.....	18
Table 1: Chromodacryorrhea scoring system.....	19
Figure 10: Example of images used for scoring chromodacryorrhea.....	20
Figure 11: Single frame image of gait analysis.....	21
Figure 12: Average BBB scores between medium and tiny cage groups.....	24
Figure 13 Medium and tiny cage BBB scores.....	25
Figure 14: Plot of overnight activity averages between groups.....	26
Figure 15: Plot of overnight activity between groups.....	27
Figure 16: Plot of distance traveled between groups.....	28

Figure 17: Plot of distance traveled in the center of the arena between groups.....	29
Figure 18: Plot of distance traveled in the periphery between groups.....	29
Figure 19: Plot of time spent stationary between groups.....	30
Figure 20: Plot of number of entries into the center of the arena between groups.....	30
Figure 21: Plot of number of boxes crossed in arena between groups.....	31
Figure 22: Plot of chromodacryorrhea between groups.....	32
Figure 23: Plot of CPI, RI and PSI in tiny cage group.....	33
Figure 24: Plot of CPI, RI and PSI in medium cage group.....	33
Figure 25: Tail flick latency by group.....	34
Figure 26: Representative images of stained epicenter showing spared white matter.....	35
Figure 27: Percent spared white matter between groups.....	35
Figure 28: Scatterplot showing correlation between spared white matter and BBB subscore in the medium cage group.....	36

INTRODUCTION

Successful treatment of spinal cord injury (SCI) in humans is dependent upon animal models. The contusion model of SCI in rats has proven to be a successful translational model of injury [Gruner, 1992] as most human injuries are the result of contusion or compression of the spinal cord [Young, 2002]. While loss of locomotion is undesirable for many reasons, recovery of locomotion is not the most crucial function desired by patients with SCI [Fouad & Pearson, 2004; Anderson et al., 2007]. However, functional recovery is imperative and is widely assessed in animal models. The goal of treatments for functional recovery is to help the patient regain their locomotion which ultimately helps them return to their usual activities. The current treatment plan for an individual with SCI is immediate and sustained bed rest, with the exception of scheduled physical therapy and rehabilitation, resulting in very limited activity. When patients are mobile during the acute phase after injury they rely on wheelchairs, continuing an immobility trend. In the rat model, immobilization of paralyzed limbs via the utilization of wheelchairs was recently found to prevent and/or alter locomotor recovery [Caudle et al., 2011].

Rehabilitation programs aimed at functional recovery in humans usually begin no sooner than few months after injury, which differs from rodent models of SCI in which

animals begin moving in their cages almost immediately following injury. Rehabilitation and physical therapy in humans can include treatments such as treadmill training, swimming, stretching and bicycle training. Rehabilitation often involves a widely accepted model of functional training, body weight supported step training. Developed by Wernig and Muller, step training incorporates manual movement of patients' lower extremities through a step cycle by a therapist while a harness supports their body weight over a treadmill [Wernig & Muller, 1992]. Over the course of training the body weight support and manual assistance is reduced as the patient regains function [Fouad & Pearson, 2004]. This form of locomotor training has shown to be effective in improving walking in some SCI patients [Behrman et al., 2005; Harkema, 2001; Wernig & Muller, 1992; Wernig, Muller, Nanassy & Cagol, 1995; Wirz et al., 2005], but has been found to be ineffective in some recent studies [Dobkin et al., 2006].

A few studies have also found that step training can improve locomotor recovery following SCI in rats [Heng & de Leon, 2009; Multon, Franzen, Poirrier, Scholtes & Schoenen, 2003]. However, many studies have noted substantial functional recovery in control groups that received no treatment following SCI [Basso et al., 1996; Fouad, Metz, Merkler, Dietz & Schwab, 2000; Heng & de Leon, 2009; Miranda et al., 2012;]. This spontaneous recovery suggests the rats are training themselves, most likely by walking while in their cages [Fouad et al., 2000]. This particular spontaneous recovery will not be seen in patients, as their exposure to locomotion or locomotor like movements is strictly limited to their rehabilitation program.

In summary, step training remains one of the most promising treatments for SCI in patients. However, current clinical approaches result in limited to no use of paralyzed

limbs for several months, post-injury, outside of scheduled physical therapy. In rat models of SCI in-cage activity is rarely monitored to ensure disuse of affected limbs outside of treatment; when in-cage activity is monitored current techniques require that rats be housed individually, which is known to increase an animals stress and/or anxiety levels [Sharp, Azar & Lawson, 2003]. This in-cage, self-training may be affording the spontaneous recovery not seen in human SCI.

Our lab believes locomotor pattern recovery in SCI rats occurs because of, or is improved, by in-cage activity. Rats' nocturnality causes this activity to be 4 times greater at night [Tsvirkun et al., 2012]. Lankhorst et al. (2001) used environmental enrichment to measure the effects of increased locomotor activity on functional recovery in rats. Various stimuli such as tubes and running wheels were provided to animals in environmentally enriched housing in order to encourage locomotor activity. To ensure a minimum amount of movement throughout the cage, food and water were placed at opposite ends of the cage. While both the control and enriched housing groups showed an initial increase in locomotor function, the enriched housing group continued to increase function as the control group plateaued [Lankhorst et al., 2001].

Fischer and Peduzzi (2007) saw similar results; functional recovery was greater in animals placed in environmentally enriched housing than in controls [Fischer & Peduzzi, 2007]. In contrast to Lankhorst et al. (2001), the environmental enrichment in this study included an increased social component by housing rats 5 per cage rather than 2 per cage [Fischer & Peduzzi, 2007]. As rats are naturally social, the increased opportunity for social interaction may have positively influenced locomotor activity, therefore, positively affecting functional recovery.

Interestingly, van Meeteren et al. (2003) suggested social interaction in rats has little to no effect on functional recovery following SCI. When singly housed and provided a running wheel, isolated rats' functional recovery was comparable to rats housed 12 per cage and given significantly more enrichment [Van Meeteren, Eggers, Lankhorst, Gispen & Hamers, 2003]. This experiment suggests environmental enrichment, with or without the presence of a cage mate, positively influences activity, which enhances locomotor recovery.

While the studies conducted by Lankhorst et al. (2001), Fischer and Peduzzi (2007), and van Meeteren et al. (2003) all indicate environmental enrichment heightens functional recovery presumably by increasing locomotor activity [Lankhorst et al., 2001; Fischer & Peduzzi, 2007; van Meeteren et al., 2003], they all neglect to measure the amount of activity occurring in-cage. Without a proper quantitative measure of in-cage locomotor activity it cannot be determined that environmental enrichment is inducing physical activity and subsequent recovery in these rats. Similarly, studies of various training models that have seen spontaneous recovery in control groups failed to measure in-cage activity as a possible treatment for SCI [Multon et al., 2003; Heng & de Leon, 2009; Miranda et al., 2012; Fouad et al., 2000]. The amount of in-cage, over ground stepping exhibited by SCI rats may have a significant effect on recovery. In our lab, preliminary studies suggest that the presence of a cage mate, without environmental enrichment, increases in-cage activity, therefore potentially improving their overall functional recovery.

As already mentioned, several studies have shown the absence of a cage mate has detrimental effects on rats that can have an impact on their behavior, consequently

affecting their recovery. The chronic mild stress protocol is an animal model of depression and anxiety in which rats are exposed to mild stressors for a period usually lasting 5-9 weeks [Willner, Towell, Sampson, Sophokleous & Muscat, 1987]. Depressive and anxiety-like behaviors develop from these stressors, which can include social isolation, food and water deprivation and confinement [Bessa et al., 2013; Sabban, Schilt, Serova, Masineni, Stier Jr., 2009; Grippo, Beltz & Johnson, 2003]. One study found when rats were immobilized two hours a day for six days their in-cage overnight locomotor activity decreased significantly [Sabban et al., 2009]. This study found exposure to even one session of immobilization led to significant decreases in locomotor activity overnight [Sabban et al., 2009]. Furthermore, another study found chronic mild stress resulted in reduced spontaneous locomotor activity throughout the duration of exposure when presented for four weeks [Grippo et al., 2003]. In a third study sustained inactivity and decreased locomotor activity overnight was produced when rats were subjected to a two week period of chronic mild stress, specifically social isolation [Tsvirkun et al. 2012].

In summary, previous studies strongly suggest the amplified functional recovery seen with environmentally enriched housing is due to an increase in locomotor activity that has not yet been quantified. Activity quantification is necessary in order to examine its affect on recovery. Notably, locomotor activity has been measured in studies of chronic mild stress on otherwise healthy rats. These studies illustrate that stressors can cause depression and anxiety, which result in hampered spontaneous locomotor activity. Thus, we hypothesize that reducing cage size and partially isolating rats should increase

anxiety and lead to a measurable decrease in in-cage activity that should result in reduced functional recovery.

The current study proposed to assess the effects of passive ‘immobilization’ on overnight activity and functional recovery by reducing the opportunity for locomotion. Furthermore, we looked for a relationship between socialization and level of recovery. We achieved ‘immobilization’ by placing rats in cages small enough to dramatically restrict their movement. Socialization was accomplished in varied degrees by either double housing rats or by placing a slotted divider between single housed rats.

METHODS

Study Design

The Institutional Animal Care and Use Committee of the University of Louisville approved all procedures. For the purpose of this study, 21 adult female Sprague-Dawley rats (Harlan Laboratories, Indianapolis, Indiana) with initial body weights between 190 and 210 grams were used.

Throughout the study rats had to be handled daily for various assessments, cage changes, and care after injury. It was essential to expose the rats to human interaction prior to injury in order for them to become acclimated to handling and remove any stress associated with human interaction. This exposure occurred through the process of gentling. Gentling involved removing rats from their cages and holding them. Each rat was gentled for 10 minutes twice daily for three weeks prior to injury. Rats that were persistently uncomfortable during gentling were gentled thrice daily to reduce future unwanted anxiety from handling.

Three rats died immediately after injury and two rats were determined to be outliers based on their week 1 locomotor assessment score and were excluded from the study. After injury, the remaining rats were divided into two groups: medium sized cage, double housed (n=8), and small cage, partial isolation (n=8). Providing each rat with a

clean cage was essential in order to prevent adding undue stress to the animals. Due to their small size, the cages became dirty quickly; therefore, cages were cleaned daily.

Each rat was given 60 grams of food per day and monitored for necessary adjustments.

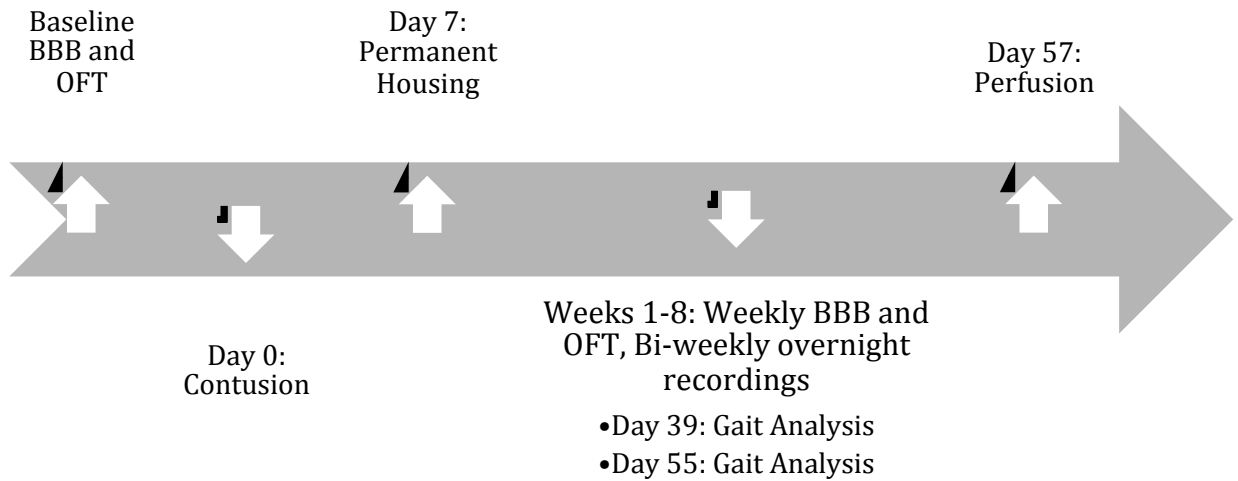


Figure 1: Timeline of study

Cage design

As mentioned previously, socialization and ‘immobilization’ between rats was varied. In order to achieve maximum socialization and mobilization a large opaque cage was subdivided into two medium sized cages measuring 34 cm x 22 cm that each held two rats. In order to further facilitate locomotor activity in the cage, the water bottle was placed at one end of the cage and food was placed at the opposite end of the cage.



Figure 2: Large cage subdivided into two medium sized cages

To achieve ‘immobilization’, rats were placed in a large cage subdivided into four small cages measuring 17 cm x 22 cm that each held one rat. To accomplish partial isolation, a slotted divider separated these singly housed rats from each other. On the shortest width divider there were four columns of holes, each one cm apart, measuring one cm in diameter. These holes began one inch from the bottom of the cage and one centimeter from the side of the cage. The slotted divider allowed minimal interaction between two rats. To further impede locomotor activity, water bottles and food were placed directly beside the slotted divider.



Figure 3: Large cage subdivided into four tiny sized cages



Figure 4: Tiny cage, from side showing dividers

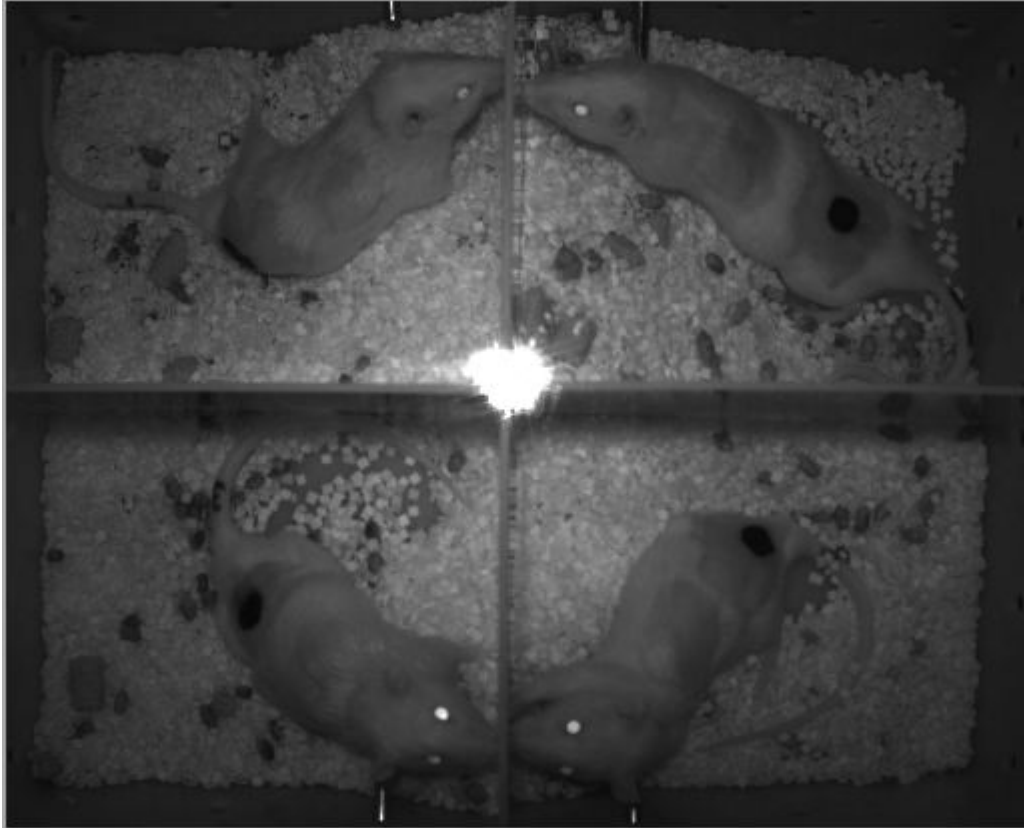


Figure 5: Single frame image of rats interacting during overnight recording

Spinal cord injury

Prior to surgery, all rats were housed under normal housing conditions. For contusions, all rats were anesthetized using a ketamine (80 mg/kg)/xylazine (4mg/kg) combination injected intraperitoneally, and brought to a surgical plane. Rats were given isoflurane gas via a nose-cone as a supplement anesthetic as needed. Each rat received a laminectomy at thoracic level 9 of the vertebral column in order to expose the spinal cord and a contusion injury at thoracic level 10 of the spinal cord.

The contusion injury was performed using the NYU weight drop device (W. M. Keck Center for Collaborative Neuroscience, Piscataway, New Jersey). The NYU weight drop device administers varying severities of spinal contusion injuries by dropping a 10-

gram rod onto an exposed spinal cord from 6.25, 12.5, 25 or 50 millimeters above the spinal cord [Basso, Beattie & Bresnahan, 1996]. Each rat was given a moderate 12.5 g/cm contusion. After injury the wound was closed using surgical sutures and the incision was closed using surgical stainless steel clips. A topical antibiotic was applied to each incision.

Recovery

Beginning the day of injury rats were given 0.1 ml Gentamicin daily as a prophylactic and 5 ml 0.9% saline solution for hydration twice daily for seven days. For pain, rats were given Buprenorphine twice daily, 0.15 ml for the first three days and 0.08 ml for two days after in order to wean them off the medication. Bladders were expressed manually twice per day and Baytril (0.1 ml) was given daily for bladder infections as needed. Surgical clips were removed 10 days after surgery. Animals were placed into their permanent housing conditions seven days after surgery.

Functional locomotor recovery

The BBB open field locomotor scale is a measure of recovery of hindlimb function in rats developed by Basso Beattie and Bresnahan [1995]. Rats are placed in an open field and monitored for four minutes. Rats are given a score between 0 and 21 based on walking characteristics. A score of 0-8 is given if the rat cannot weight support, 9-14 if there is weight support without coordination and 15-21 if there is weight support, coordination and refined stepping. BBB's were performed weekly beginning seven days after injury.

Overnight activity

In order to quantify overnight locomotor activity, the rats were filmed in their permanent cages twice per week during their 12-hour dark cycle. Two infrared LED lights and one Basler ACA 645-100GM (Basler, Exton, PA) digital video camera per cage were mounted to a rack designed for recordings. Using a program written in LabVIEW (National Instruments, Austin, TX), the rats were recorded for one minute out of every ten minutes at 4 Hz for the entire 12-hour dark cycle. Prior testing concluded the resulting 72 recorded loops were sufficient to accurately capture the rats' overall nightly activity. Overnight recordings were made twice weekly beginning seven days after injury.

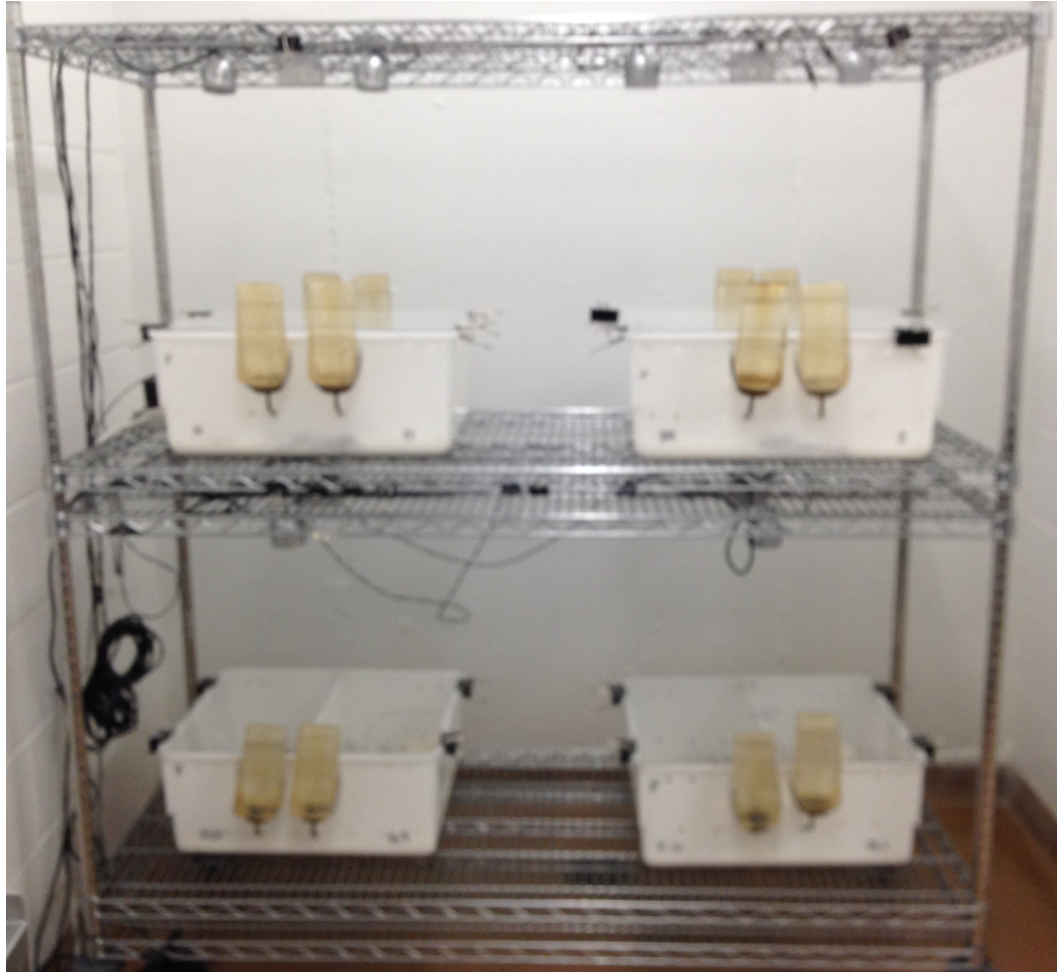


Figure 6: Overnight setup

MaxTRAQ (Innovision Systems, Columbiaville, MI) software allows a point to be tracked digitally. Permanent marker was used to draw a two cm circle on the rats' backs to track them during their overnight recording. Each circle was placed over the iliac crest to accurately measure over ground movement in their cages. MaxTRAQ software was used to track the center of each circle and digitize all the overnight video. The data was then exported to Microsoft Excel to quantify activity. A macro calculated the distance each digitized point traveled in each video. The resulting calculation was

multiplied by ten to estimate the total distance traveled per rat, per night. Distance is calculated in centimeters and converted to meters.

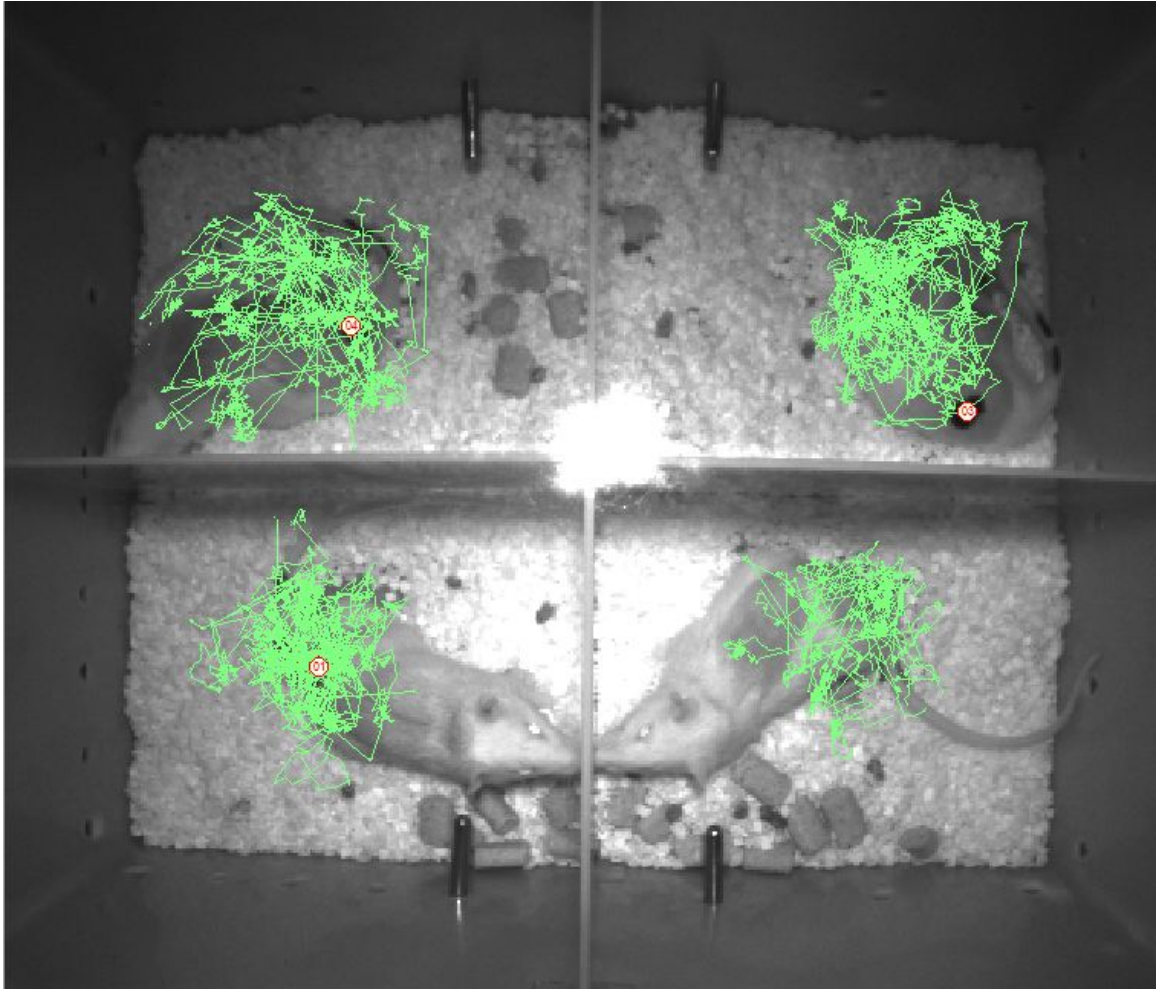


Figure 7: Single frame image of digitized overnight activity

Open field test

The rats' anxiety levels were measured weekly using the open field test. The arena was designed as previously described by Bignami [1996]. The arena was constructed in-house using four black Plexiglas walls that fit together to make a square bottomless box measuring 70 cm by 70 cm. The base of the arena was divided into 16

squares measuring 17.5 cm by 17.5 cm. The test was recorded using the same Basler camera used to record overnight videos. At the start of the assessment one rat was placed directly in the center of the arena and recorded at 8 Hz for five minutes. The arena was thoroughly cleaned between each assessment.

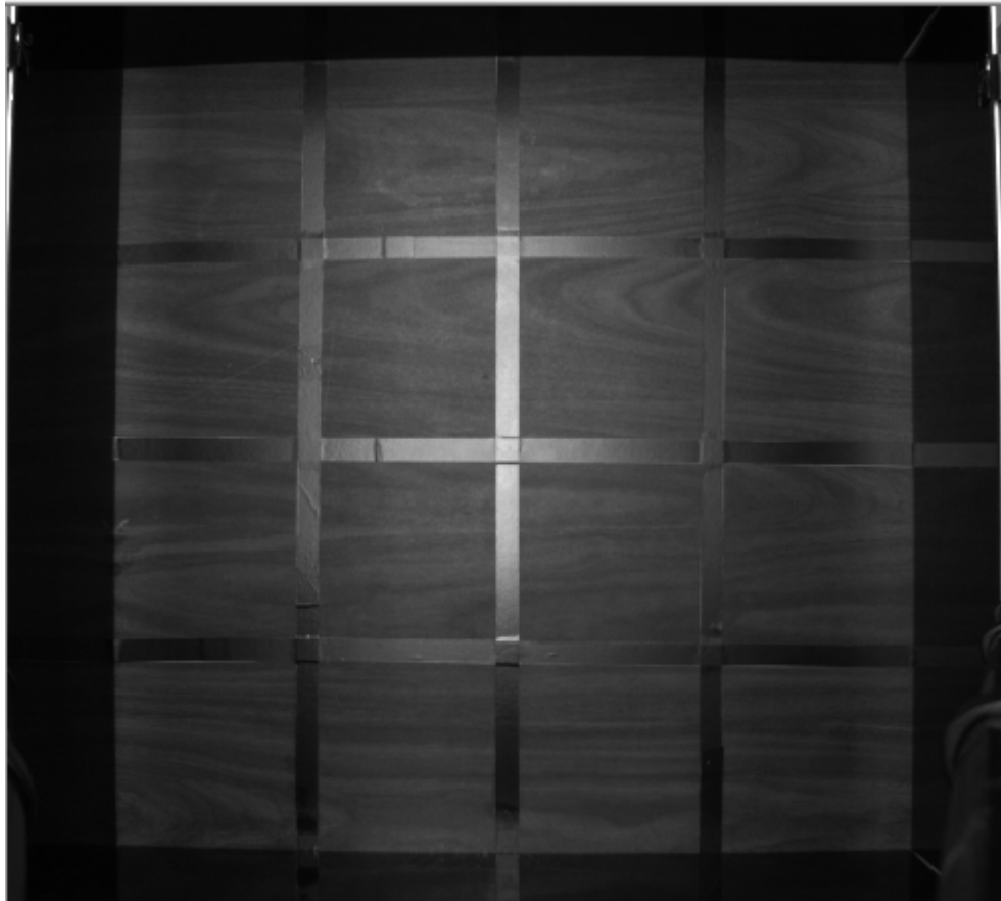


Figure 8: Open field test arena

The rats' movement was digitized using MaxTRAQ software as previously described for overnight recordings. The data was then exported for analysis. A macro in Microsoft Excel was used to calculate total distance traveled, distance traveled in both the center and periphery, the total time travelling, time travelling in both the center and the

periphery, the number of entries into the center and the number of squares crossed. The center of the arena was defined as the innermost four squares and the periphery was defined as the outermost 12 squares.

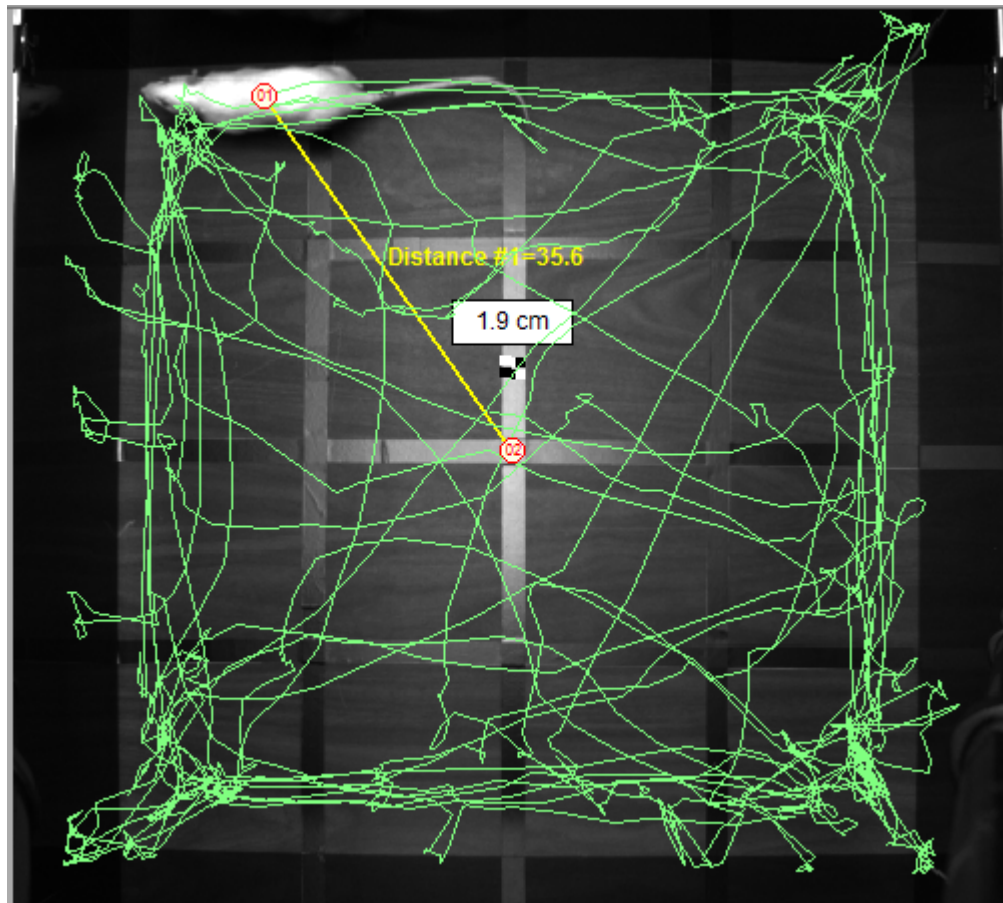


Figure 9: Single frame image of digitized open field test

The open field test is a measure of locomotion, exploration and anxiety [Roth & Katz, 1979]. A high frequency of squares crossed, total time spent travelling and total distance traveled indicates increased locomotion and exploration and low anxiety. A high frequency of entries into the center squares and time and distance traveled in the center also indicates increased exploration and low anxiety levels. Conversely, a high

frequency of time and distance traveled in the periphery of the arena is indicative of decreased exploration and high anxiety levels.

Analysis of chromodacryorrhea

The amount of chromodacryorrhea present on each animal was quantified daily to assess their level of stress throughout the study. An image of the face of each rat was taken daily using a Sony camcorder and chromodacryorrhea was quantified using a modified version of a scaled developed by Mason, Wilson, Hampton and Wurbel [2004]. The nose and eyes of each rat was given a score between 0 and 4 depending on the severity of the chromodacryorrhea.

Score	Description
0	No chromodacryorrhea
1	Slight chromodacryorrhea on one eye/nostril
2	Slight chromodacryorrhea on both eyes/nostrils or Moderate on one eye/nostril
3	Moderate chromodacryorrhea on both eyes/nostrils
4	Severe chromodacryorrhea on both eyes nostrils

Table 1: Chromodacryorrhea scoring system

The Harderian gland sits behind each orbit in rodents; this gland produces and releases a porphyrin via the nasolacrimal ducts [Harper, Kerins, McIntosh, Spears & Bellinger, 2001]. This chromodacryorrhea dries on the fur creating a reddish stain around the eyes and nose. In times of stress this gland releases excessive amounts of porphyrin making chromodacryorrhea a useful qualitative sign of stress.

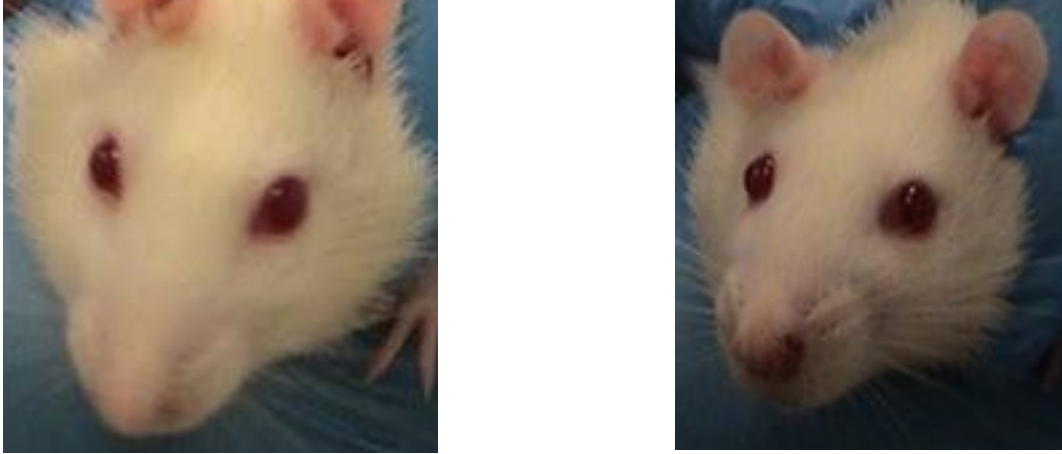


Figure 10: Example of images used for scoring chromodacryorrhea.

Score of 0 eyes, 0 nose and score of 4 eyes, 4 nose respectively.

Light cycle recordings

To observe the rats' behavior during the 12-hour light cycle, the same recording procedures were used during the day once every other week for a total of four times throughout the study.

Overground gait assessment

In order to further assess hindlimb movement during overground walking, gait analysis was performed at week six and week eight of the study. Rats walked the length of a clear Plexiglass walking tank (150 cm x 18 cm x 30 cm) made in-house. A ventral view recording using a Basler camera at 100 Hz was taken of each rat walking a minimum of six passes. A pass was considered complete if the animal walked the entire length of the tank without hesitating, stopping, changing direction or having hindlimb spasms.

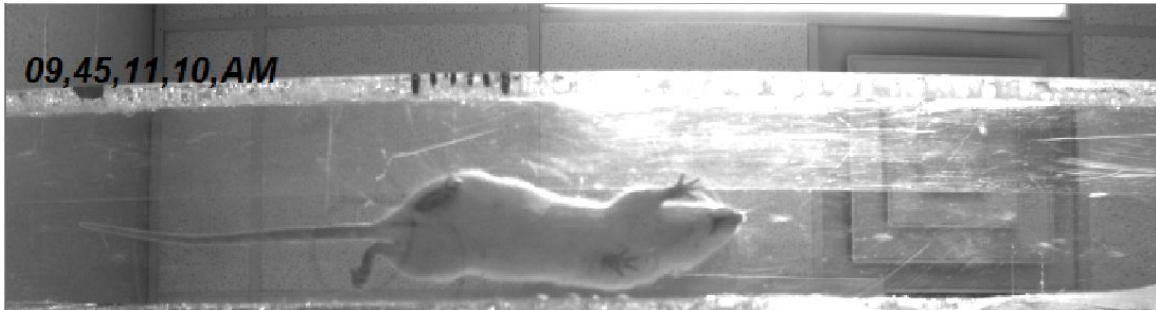


Figure 11: Single frame image from gait analysis video

A total of 12-15 gait cycles were digitized using MaxTRAQ software as previously described [Kuerzi et al., 2010]. The Regularity Index (RI), Plantar Stepping Index (PSI) and Coordinated Pattern Index (CPI) were determined from the ventral view recording. The RI is a measure of forelimb-hindlimb coordination during plantar stepping [Hamers, Lankhorst, van Laar, Veldhuis & Hendrik, 2001]. The PSI is a measure of plantar steps taken by the forelimbs and hindlimbs [Kuerzi et al., 2010]. The CPI is a measure of forelimb-hindlimb coordination regardless of plantar stepping [Caudle et al., 2011].

Nociception assessment

The tail flick test developed by D'Amour and Smith [1941] was used to measure nociception at weeks six and eight following injury. A small towel was used to restrain the rats' upper body while their tails and hindlimbs remained free. The Tail-Flick Analgesia Meter (Columbus Instruments, Columbus, OH) focused a high intensity light on the ventral surface of the tail approximately three cm from the base of the tail. The light emitted from the device created a noxious thermal stimulus resulting in removal of

the tail from the heat source. The time taken to remove the tail is referred to as tail flick latency. If a tail removal response did not occur within ten seconds the trial was terminated. This measure was repeated thrice per animal with a minimum of 30 seconds between each trial.

Perfusion

Eight weeks post injury rats were anesthetized with a weight dependent dose of Nembutal and perfused intracardially with 200 ml 0.1M phosphate buffer.

After perfusion, spinal cords from thoracic level 6 to the cauda equina were dissected from rats, and post-fixed in 4% paraformaldehyde in phosphate buffer overnight. Once fixed, spinal cords were removed from paraformaldehyde and placed in 30% sucrose in phosphate buffered saline for cryoprotection.

Histology

After cryoprotection, the contusion area was cut from the spinal cord for histology. This one cm section of cord was blocked in a tissue-freezing medium and cut at 30 microns on the cryostat. Eriochrome cyanine was used to stain white matter at the epicenters of the injuries. A SPOT digital camera (Medical Diagnostics) was used to photograph the epicenters, which were then analyzed in ImageJ (National Institutes of Health, Bethesda, MD). Using a Wacom Intuos (Vancouver, WA) drawing tablet the total area each section and the areas of spared white matter were traced for quantification. The section found to have the least amount of spared white matter was determined to be the epicenter of the injury. Only the ventral spared tracts were measured as the

reticulospinal tracts in the ventrolateral funiculus are involved in initiating overground [Basso et al., 2002]. The remaining sections rostral and caudal to the epicenter were also traced until the cord no longer showed signs of injury.

Statistics

BBB scores were compared using repeated measures analysis of variance (ANOVA). Non-parametric Mann-Whitney U tests were used to compare overnight activity, the measures of the open field test and the analysis of chromodacryorrhea. Gait was compared using the Wilcoxon signed ranks test. Oneway ANOVA was used to compare the spared white matter and the tail flick assessment. All data are presented as group means and standard deviations.

RESULTS

Functional locomotor recovery

Repeated measures analysis of variance (ANOVA) showed no differences between medium and tiny cage groups in locomotor recovery as seen in BBB scores.

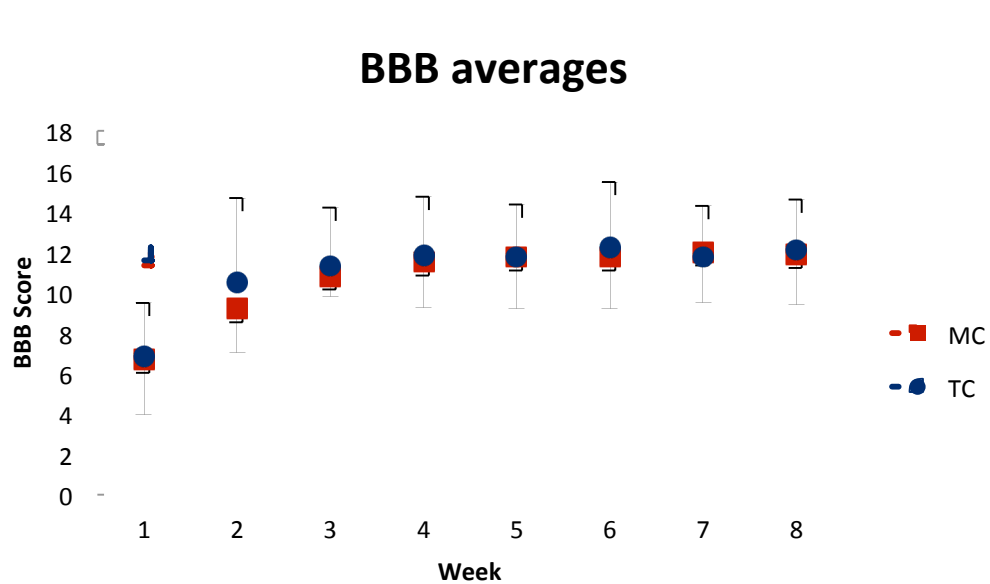


Figure 12: Average BBB scores between medium and tiny cage groups.

There were no differences found in BBB scores between groups. Scores are reported as means \pm standard deviations.

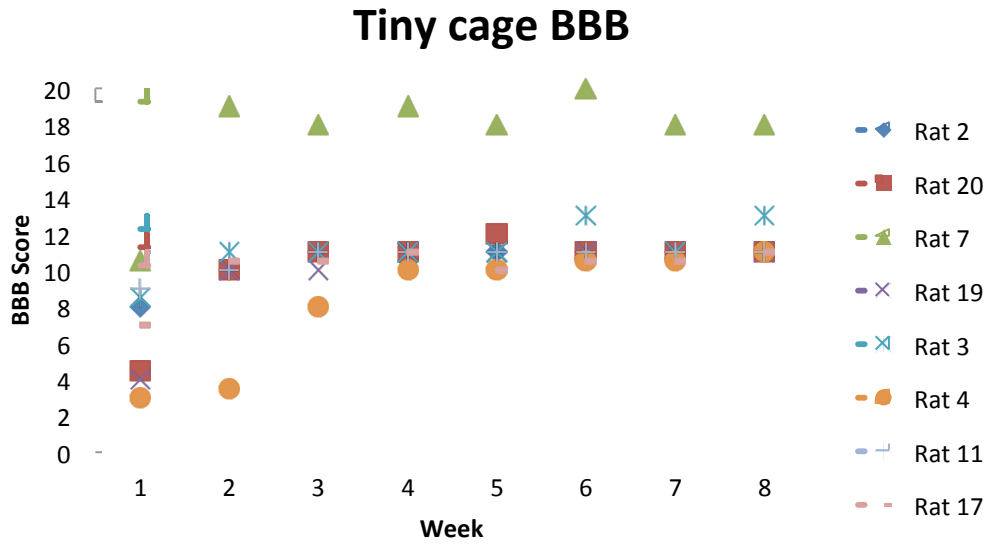
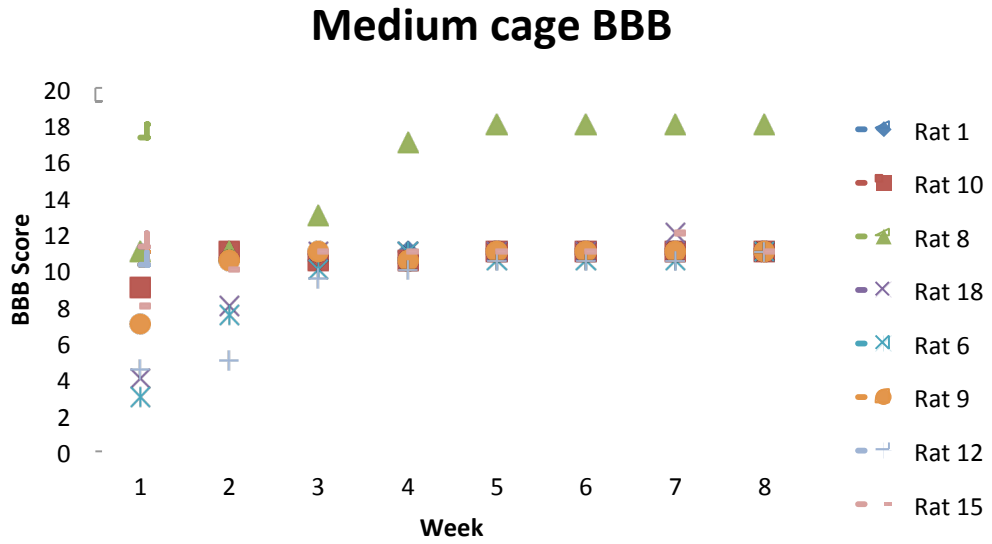


Figure 13: Medium and tiny cage BBB scores graphed separately.

Overnight activity

Non-parametric Mann-Whitney U test found differences between medium and tiny cage groups in in-cage overnight activity at all time points with the exception of day 10. [Day 7 (59.07 ± 16.74m vs. 35.95 ± 12.30m). Day 13 (71.36 ± 16.59m vs. 44.60 ±

18.89m) Day 17 ($86.96 \pm 34.52\text{m}$ vs. $48.81 \pm 14.47\text{m}$) Day 20 ($135.83 \pm 42.04\text{m}$ vs. $70.23 \pm 32.34\text{m}$) Day 24 ($108.47 \pm 33.22\text{m}$ vs. $49.54 \pm 17.90\text{m}$) Day 27 ($101.31 \pm 42.29\text{m}$ vs. $51.23 \pm 16.25\text{m}$) Day 31 ($125.56 \pm 37.68\text{m}$ vs. $52.53 \pm 18.62\text{m}$) Day 34 ($97.43 \pm 43.81\text{m}$ vs. $54.61 \pm 15.95\text{m}$) Day 37 ($121.19 \pm 27.43\text{m}$ vs. $62.51 \pm 20.67\text{m}$) Day 40 ($95.30 \pm 32.51\text{m}$ vs. $55.57 \pm 21.10\text{m}$) Day 45 ($100.86 \pm 19.42\text{m}$ vs. $57.37 \pm 17.20\text{m}$) Day 48 ($119.90 \pm 36.84\text{m}$ vs. $46.56 \pm 17.43\text{m}$) Day 52 ($120.05 \pm 29.28\text{m}$ vs. $53.47 \pm 17.03\text{m}$).

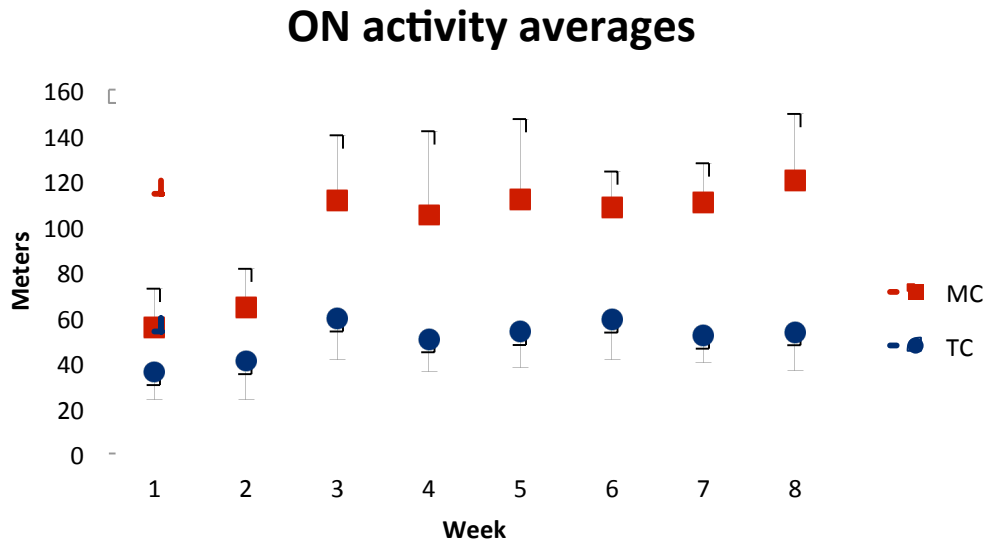


Figure 14: Averages of overnight activity showing differences between medium and tiny cage groups. Scores are reported as means \pm standard deviations.

□

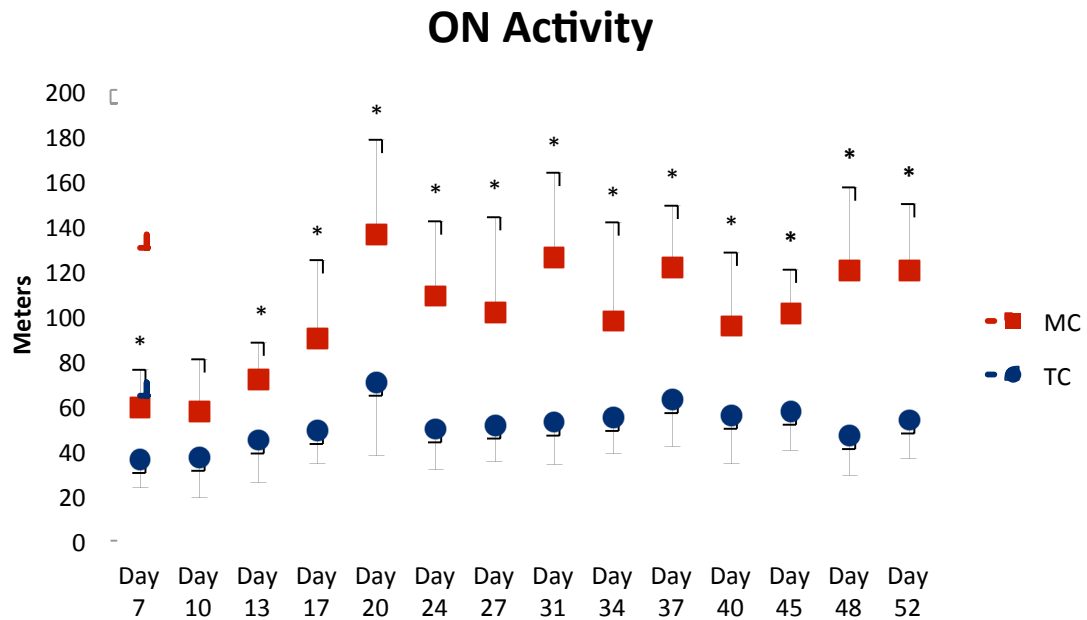


Figure 15: In-cage overnight activity showing difference between groups at all time points except day 10 (* $p < .05$, \pm SD, Mann-Whitney U test).

Open field test

The open field test was used to assess the rats' anxiety levels throughout the study. Non-parametric Mann-Whitney U tests found group differences in each measure of the test at different time points. The total distance traveled was different between medium cage and tiny cage groups at week 8 ($29 \pm 4.50\text{m}$ vs. $22.69 \pm 5.37\text{m}$, $p < .05$). The distance traveled in the center squares differed between groups at weeks 7 and 8 ($2.01 \pm 1.10\text{m}$ vs. $0.75 \pm 0.43\text{m}$, $p < .05$; and $1.74 \pm 1.48\text{m}$ vs. $0.59 \pm 0.42\text{m}$, $p < .05$) and the distance traveled in the peripheral squares differed between groups at week 8 ($27.26 \pm 3.67\text{m}$ vs. $22.10 \pm 5.12\text{m}$, $p < .05$). There were group differences in the time each rat spent stationary at weeks 6, 7 and 8 ($1.63 \pm 0.34\text{min}$ vs. $2.00 \pm 0.41\text{min}$, $p < .05$; $1.64 \pm 0.24\text{min}$ vs. $2.1 \pm 0.57\text{min}$, $p < .05$; and $1.72 \pm 0.30\text{min}$ vs. $2.26 \pm 0.40\text{min}$, $p < .05$). There was a

group difference at week 7 in the number of times a rat entered the center of the arena (7.13 ± 4.97 vs. 2.63 ± 1.51 $p < .05$). Lastly, there were group differences in the number of boxes crossed at weeks 3, 7 and 8 (112.13 ± 17.34 vs. 89.5 ± 21.97 , $p < .05$; 114.13 ± 14.34 vs. 92.38 ± 22.19 , $p < .05$; and 104 ± 12 vs. 76.75 ± 12.21 , $p < .05$).

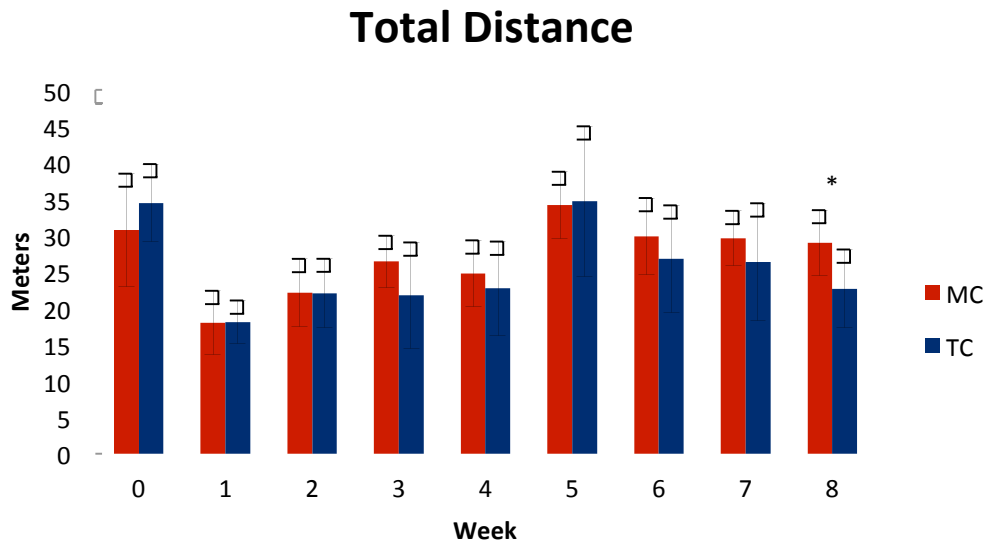


Figure 16: Total distance traveled showing a difference between groups at week 8 (* $p < .05$, \pm SD, Mann-Whitney U test).

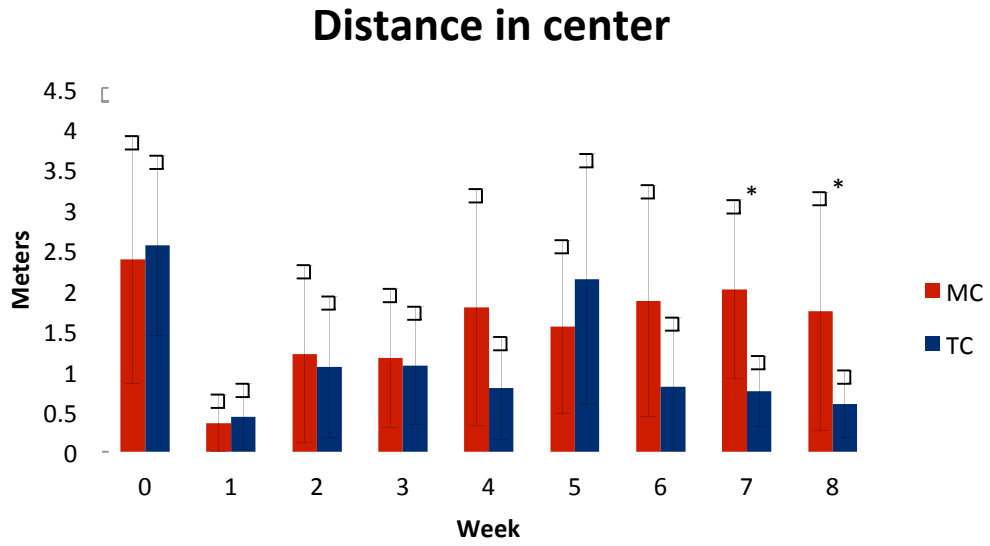


Figure 17: Distance traveled in the center of the arena showing differences between groups at weeks 7 and 8 (* $p < .05$, \pm SD, Mann-Whitney U test).

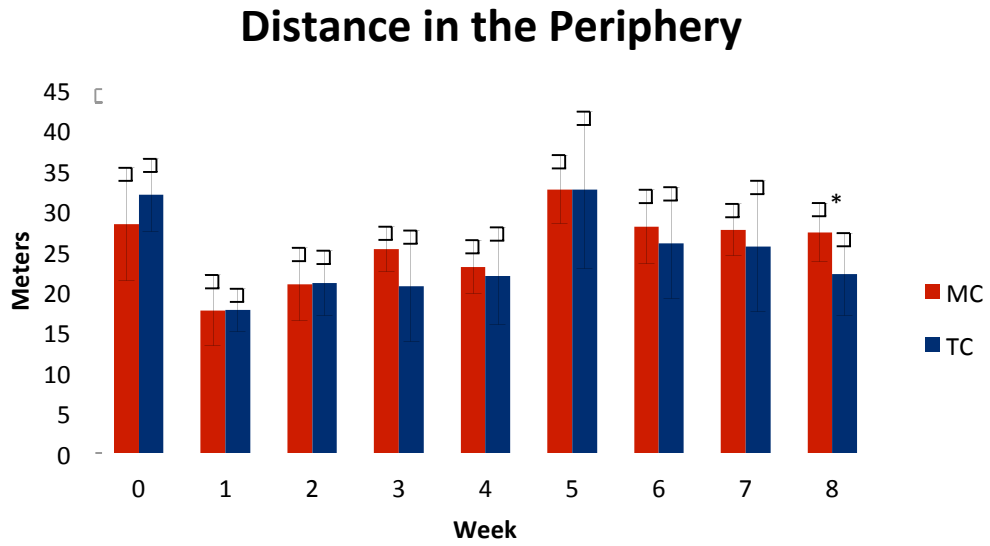


Figure 18: Distance traveled in the periphery of the arena showing a difference between groups at week 8 (* $p < .05$, \pm SD, Mann-Whitney U test).

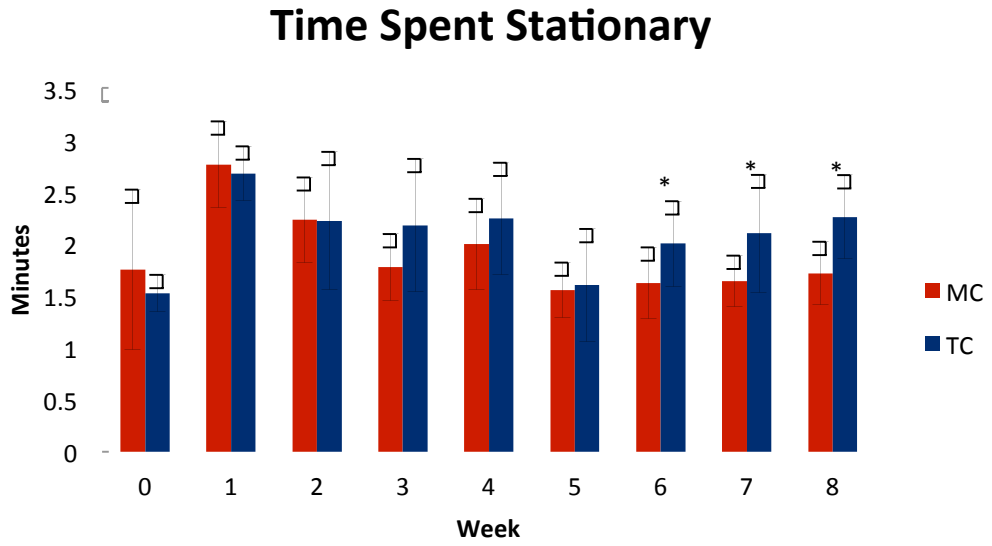


Figure 19: Time spent stationary showing differences between groups at weeks 6, 7 and 8 (* $p < .05$, \pm SD, Mann-Whitney U test).

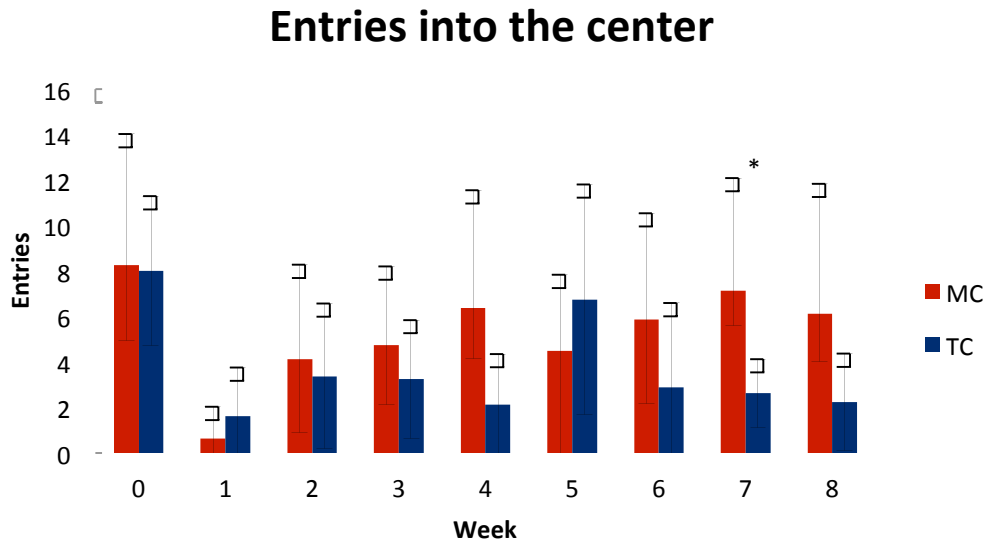


Figure 20: Number of entries into the center of the arena showing a difference between groups at week 7 (* $p < .05$, \pm SD, Mann-Whitney U test).

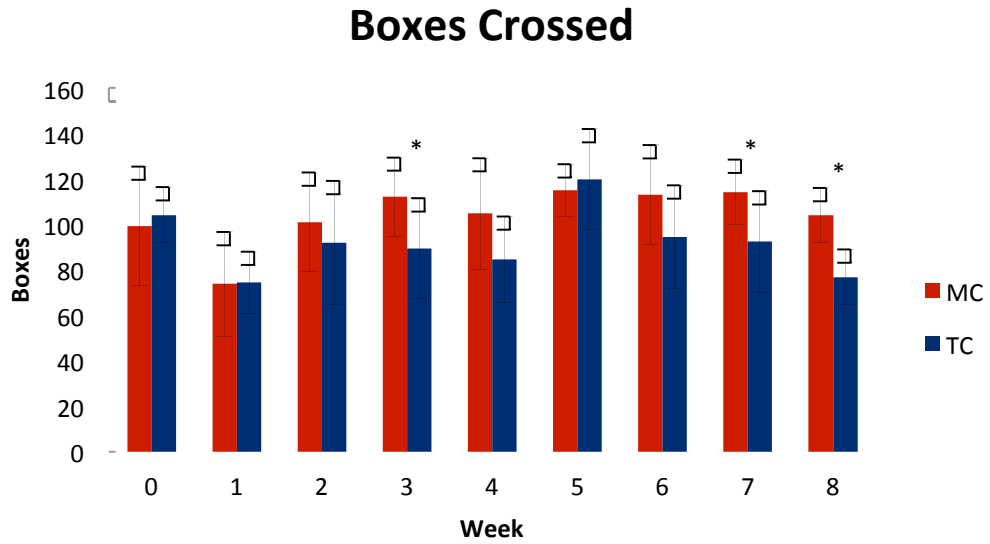


Figure 21: Number of boxes crossed in arena showing a difference between groups at week 3, 6 and 8 (* $p < .05$, \pm SD, Mann-Whitney U test).

Analysis of chromodacryorrhea

Non-parametric Mann-Whitney U test found a difference in chromodacryorrhea between medium and tiny cage groups at week 3 (4.81 ± 0.95 vs. 6.06 ± 0.58 , $p < .05$).

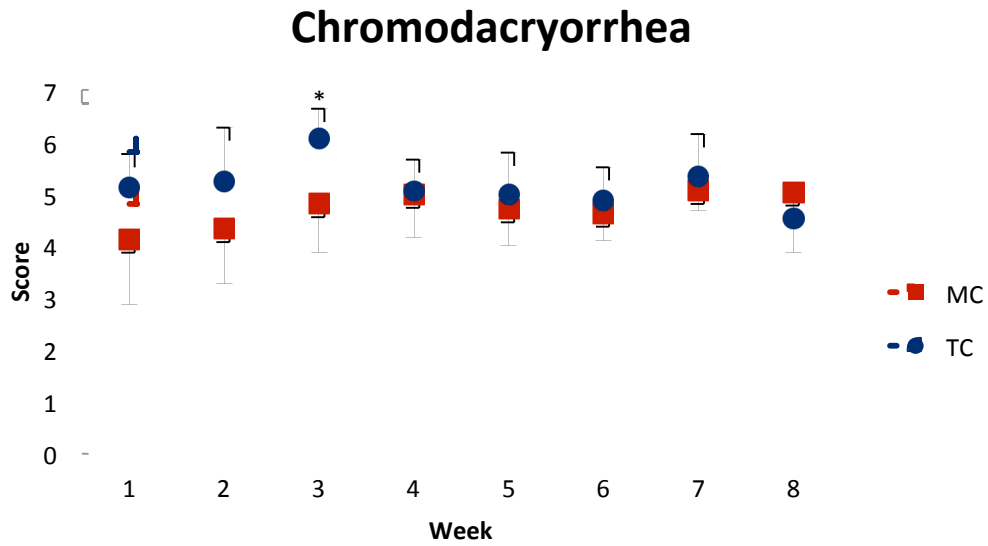


Figure 22: Chromodacryorrhea showing a difference between groups at week 3 (* $p < .05$, \pm SD, Mann-Whitney U test).

Overground gait assessment

Repeated measures ANOVA found no difference between groups in the overground gait assessment. The Wilcoxon signed ranks test found a difference in the medium cage group between week 6 and week 8 on the RI ($0.345 \pm 0.352\%$ vs. $0.531 \pm 0.394\%$), CPI ($0.414 \pm 0.344\%$ vs. $0.595 \pm 0.319\%$) and PSI measures ($0.435 \pm 0.333\%$ vs. $0.605 \pm 0.331\%$).

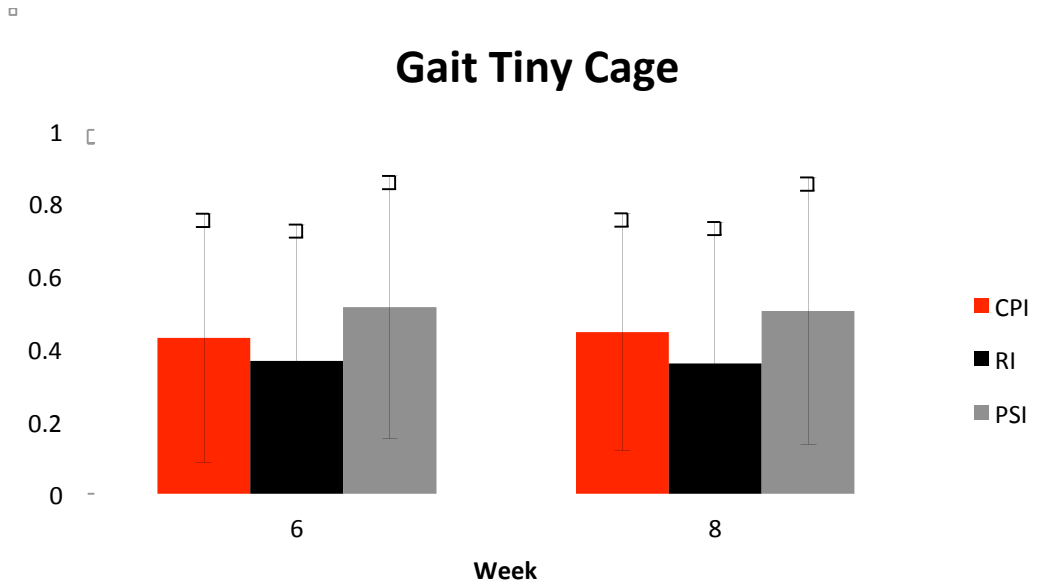


Figure 23: CPI, RI and PSI analysis in the tiny cage group showing no difference overtime. Scores are reported as means \pm standard deviations.

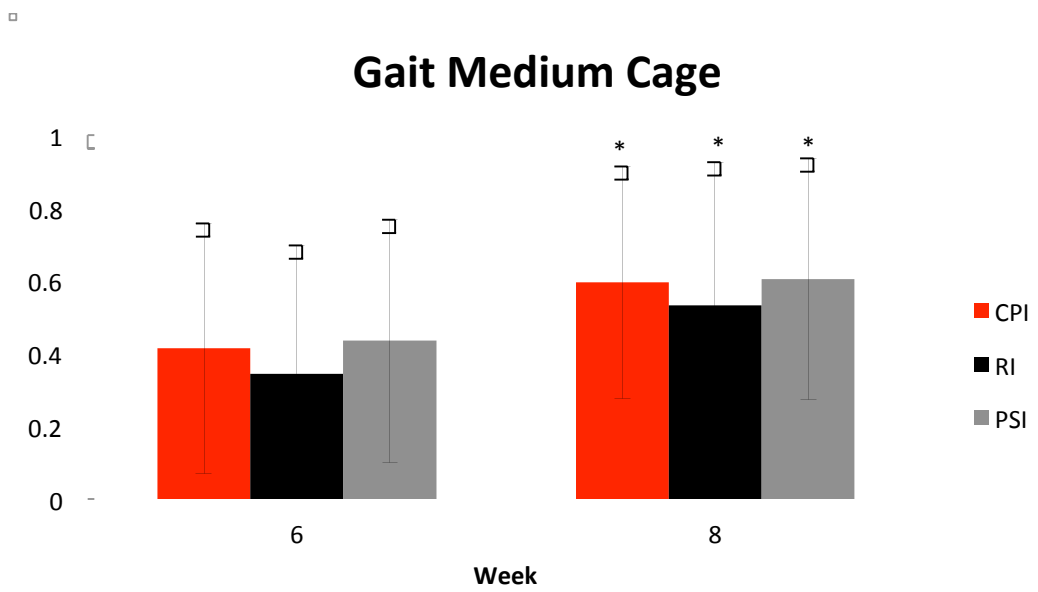


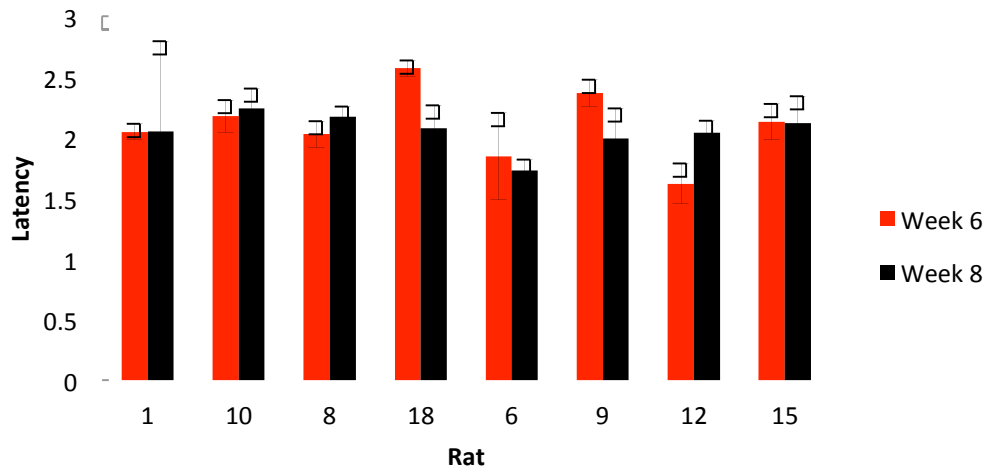
Figure 24: CPI, RI and PSI analysis in the medium cage group showing a difference from week 6 to week 8 (* $p < .05$, \pm SD, Wilcoxon signed ranks test).

Nociception assessment

An oneway ANOVA found no difference in the tail flick assessment between groups at either time point.

□

Tail Flick Medium Cage



□

Tail Flick Tiny Cage

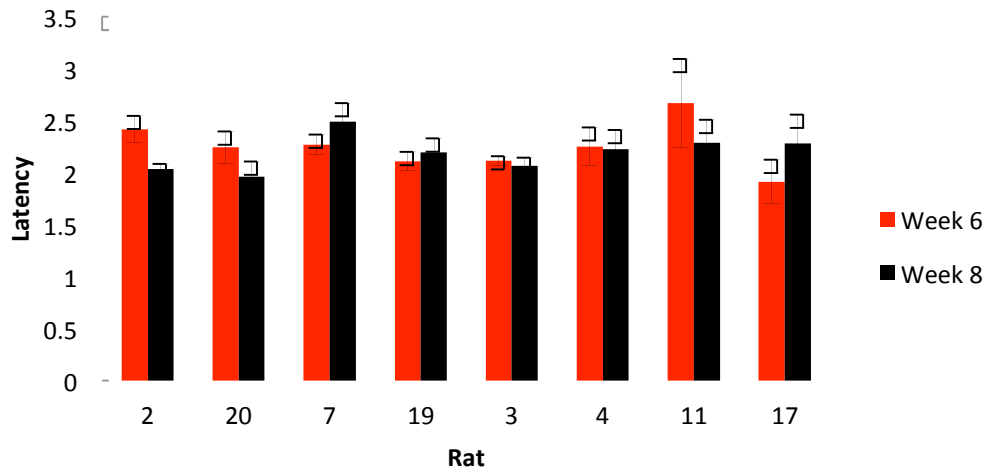


Figure 25: Tail flick latency by cage showing no difference between week 6 and week 8.

Latencies reported as means \pm standard deviations.

Histology

An oneway ANOVA found no difference in percentage of spared white matter between groups.

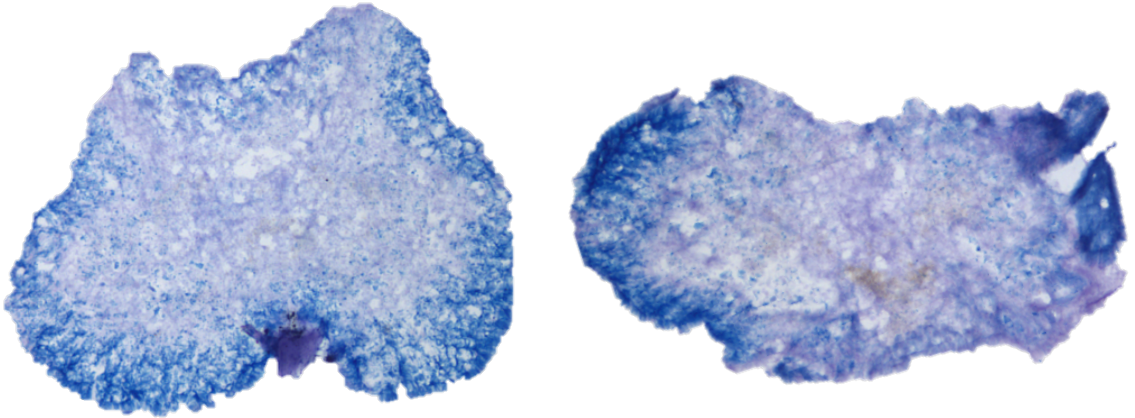


Figure 26: Representative images of stained epicenter showing spared white matter. Stained sections of a medium cage and tiny cage spinal cord respectively.

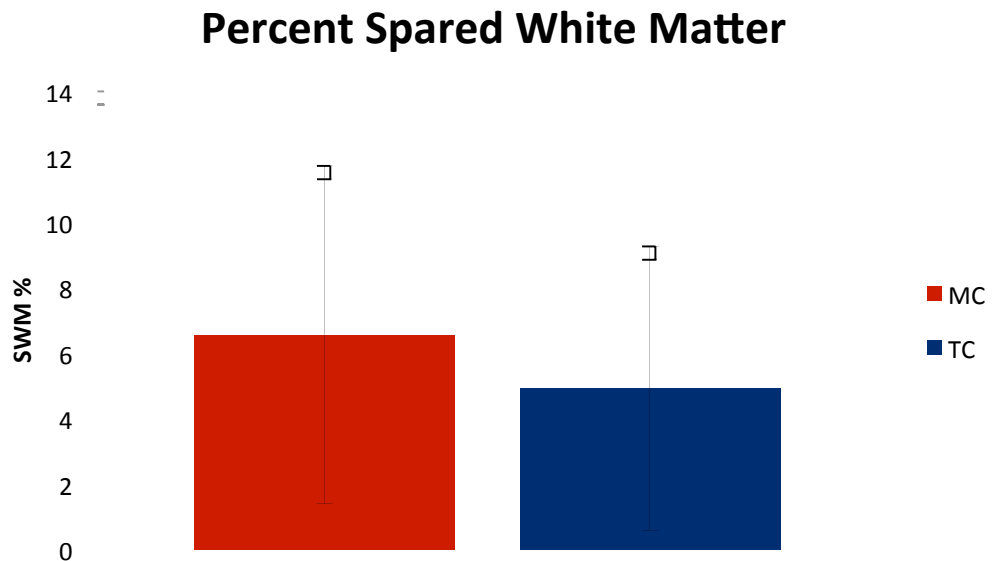


Figure 27: Percent spared white matter showing no difference between medium and tiny cage groups. Percentages reported at means \pm standard deviations.

Non-parametric Spearman's rank test found a correlation between spared white matter and BBB subscores in the medium cage group but not in the tiny cage group ($r_s = .852$, $p < .05$, $n = 7$; $r_s = .152$, $p < .805$, $n = 5$).

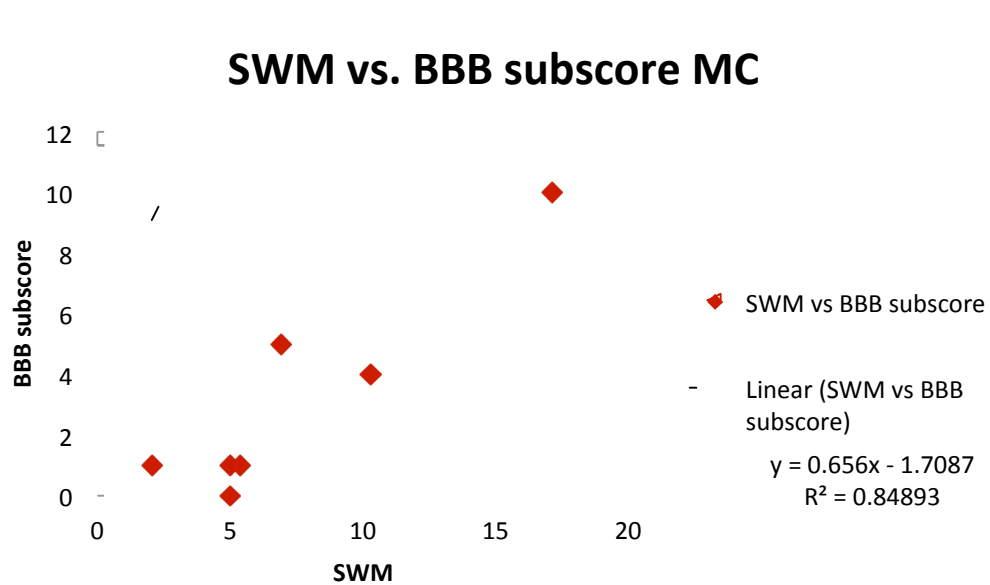


Figure 28: Scatterplot showing a correlation between SWM and BBB subscore in the medium cage group.

DISCUSSION

In partial support of our hypothesis, the amount of overnight in-cage activity differed significantly between medium and tiny cage groups at all time points except day 10. This difference in activity suggests that increasing the opportunity for movement leads to increased in-cage activity while decreasing the opportunity for movement leads to decreased activity. Additionally, the presence of a cage mate in the medium cage group may have had a positive influence on in-cage activity. Interestingly, the amount of in-cage activity seen in the medium cage group began low and increased overtime while the activity seen in the tiny cage group began low and stayed low throughout the duration of the study. This increase overtime in the medium cage group may be attributed to the animals needing to acclimate themselves to a new housing condition, or may be due to a direct or indirect result of the injury. Rats were housed in standard cages during the gentling period leading up to spinal cord injury as well as for a week following injury before being placed in their permanent housing conditions. This sudden change in housing conditions after weeks in standard cages may have initially altered the behavior of the animals. The initial low in-cage activity in the medium cage group may also be attributed to the presence of a new cage mate. After a week of recovery following injury rats were divided into two groups and assigned their permanent housing with their new

cage mates. Becoming acquainted with a new cage mate and no longer interacting with their old cage mate may have led to initial low levels of in-cage activity during the adjustment period.

While in-cage overnight activity did differ between groups it did not lead to a difference in BBB scores between groups. Both groups showed a ceiling effect in BBB scores; with the exception of two animals, all rats achieved recovery to BBB scores of 11 that is described as weight-supported stepping without forelimb-hindlimb coordination. With the 12.5 g/cm contusion injury used in this study we expected animals in medium cages to regain function to BBB scores of 15-16 that is described by coordinated, weight-supported stepping. Perhaps there was no difference in functional recovery as seen in BBB scores between groups because the animals were allowed a seven day recovery period in standard cages during which they experienced a temporary loss of spinal reflex activity known as spinal shock. If animals had been placed in their permanent housing conditions before or immediately following injury the differing cage sizes may have had a greater effect on locomotor recovery.

Due to the plateau of BBB scores, gait analysis was added to the study at week 6 and terminally. This analysis did not show a difference in gait between groups at either time point. However, the medium cage group did show an improvement in all measures of gait between week 6 and week 8. This improvement suggests that the higher levels of activity in the medium cage group, and perhaps the type of activity occurring, had a positive effect on gait. Higher levels of activity may afford the medium cage group the ability to improve their coordination as well as their plantar stepping. Furthermore, the type of movement seen in medium cage groups may have a positive effect on gait. The

size and shape of the medium cage allows the animals to take multiple consecutive steps in a straight line while the size of the tiny cage restricts movement to pivoting and fewer consecutive steps. Repeatedly walking in a straight line could act as training for animals leading to rewiring of reticulospinal pathways thus improving finer aspects of locomotion such as forelimb-hindlimb coordination. Further studies are needed to quantify the amount of straight line passes taken in-cage overnight and look for a correlation with gait analysis.

To further understand the effects of cage size on gait it may be beneficial to conduct a similar study in which animals are housed in either medium or tiny cages for 8 weeks then switched to the opposite group for another 8 weeks. When moved from an environment that restricts in-cage activity to an environment that allows greater in-cage activity it is possible the animals may improve their gait by retraining themselves while walking in-cage. Spinal cord neural networks that have the ability to produce rhythmic motor patterns without supraspinal input are known as central pattern generators (CPGs) [Grillner & Zangger, 1979]. With partial body weight support, previous studies have shown the CPG is able to generate near-normal plantar stepping and forelimb-hindlimb coordination [Heng & de Leon, 2009; Kuerzi et al., 2010]. The CPG is an important contributor to the type of recovery seen in the medium cage group as their plantar stepping and forelimb-hindlimb coordination increased overtime. Interestingly, previous studies have shown forelimb-hindlimb coordination begins to decrease 6 weeks after injury and training is necessary to maintain coordination [Heng & de Leon, 2009; Kuerzi et al., 2010]. These studies suggest that removing the opportunity for in-cage training by moving animals from a medium cage to a tiny cage 8 weeks after injury may lead to a

loss in coordination overtime; while animals moved from a tiny cage to a medium cage, thus given the opportunity to train, may improve their gait.

When comparing spared white matter, no difference was found between groups. However, the amount of spared white matter of these animals was not consistent with 12.5 g/cm contusions seen in previous studies in the Magnuson Lab. The injury severities seen in these animals were more akin to 25 g/m contusions. The severity of these injuries may contribute to plateauing of the BBB scores at 11. When comparing spared white matter and BBB subscores, there was a correlation for the medium cage group that is not seen for the tiny cage group. This correlation suggests that the increased opportunity for movement in the medium cage may have afforded the animals the ability to improve fine motor skills such as toe clearance and paw rotation.

The chromodacryorrhea assessment showed a difference between groups at week 3. This suggests that the groups showed the same level of stress at all time points except at week 3. We anticipated that ‘immobilizing’ animals and placing slotted dividers between them would increase their stress level in comparison to the medium cage group. However, it may be possible that seeing, smelling and having even minimal interaction with another animal mitigated the stress caused by ‘immobilization’ and partial isolation, therefore, making the stress levels between the two groups comparable. It is also possible that by housing two medium cage groups within the same large cage we added an unwanted stressor to this group. During daily cage cleaning the divider between two medium cages was smeared with porphyrin secretions suggesting that the two groups of animals knew there were animals on the other side of the divider and the inability to reach them caused stress. Future studies are needed to assess chromodacryorrhea in

animals in tiny cages with no slotted dividers between animals, complete isolation, as well as with only one double-housed medium cage per large cage. Assessing stress in these housing conditions and comparing them to the stress seen in the current housing conditions would give insight into what is causing and/or alleviating stress.

The results of the open field test began to show differences between groups toward the end of the study with the earliest consecutive differences seen in time spent stationary beginning at week 6. Again this may be due to a mitigation of stress in the tiny cage, partial isolation group and possibly the addition of an unwanted stressor in the medium cage group. To better ascertain anxiety difference between groups similar studies may need to be extended further than eight weeks post-injury. It also may be beneficial to introduce animals to permanent housing conditions prior to injury to further increase stress and anxiety in tiny cage groups. Interestingly, the medium cage group began to show improvements in gait at the same time in which the tiny cage group began to exhibit behavior we attribute to anxiety. The development of anxiety toward the end of the study may have had an effect on the rats' ability to recover coordination overtime in the tiny cage group.

During this study there were a number of unexpected factors that may have affected the outcomes of our assessments. A flood in the building before baseline assessments were taken lead to animals being moved from a room with no other animals to a different building in a room with numerous rats of differing strains. The animals in this study stayed in the room with multiple other rats throughout the gentling period as well as for their week of recovery following injury. Being moved suddenly from a room by themselves to an environment with numerous other rats may have had detrimental

effects on these animals prior to week 1 assessments. Recovering from a spinal cord injury surrounded by so many other animals may have also affected the rats used for this study. As mentioned previously, the injuries sustained by these rats were not consistent with 12.5 g/cm contusions. The NYU weight drop device had just been recalibrated and new vertebral stabilizers were used for this study; these differences led to a much more severe injury than we desired for this study.

Other limitations of this study are the sample sizes and housing conditions used. The computers used for recording activity only allow four cameras to be used at once. This means there is a total capacity for recording four large cages per night that led to two groups of eight rats. The housing conditions used in this study were also limited by our capacity for recording. Four housing conditions were originally designed for this study to further investigate the effects of isolation and immobilization on locomotor recovery; these groups included the two seen in this study (tiny cage, partial isolation and medium cage, double housed) but also involved tiny cages with no slotted dividers between rats in order to achieve complete isolation and a medium cage single housed to assess the effect of a cage mate when there is an opportunity for in-cage activity. Again, our limited capacity for overnight recordings required us to have two housing conditions rather than four.

The current findings partially support our hypothesis; they suggest that limiting the opportunity for movement and removing a cage mate can dramatically affect the amount of activity seen in-cage overnight. We also show that applying the stressors of immobilization and partial isolation for several weeks can cause behaviors we attribute to anxiety. Additionally, we found improvement overtime in gait in the animals with

greater in-cage activity and not experiencing anxiety behaviors. These findings suggest activity may enhance aspects of functional recovery while immobility may hinder this recovery.

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