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Evaluation of the anatomical and functional consequences of repetitive mild cervical contusion using a model of spinal concussion

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Abstract

Spinal cord concussion is characterized by a transient loss of motor and sensory function that generally resolves without permanent deficits. Spinal cord concussions usually occur during vehicular accidents, falls, and sport activity, but unlike brain concussions, have received much less attention despite the potential for repeated injury leading to permanent neurological sequelae. Consequently, there is no consensus regarding decisions related to return to play following an episode of spinal concussion, nor an understanding of the short- and long-term consequences of repeated injury. Importantly, there are no models of spinal concussion to study the anatomical and functional sequelae of single or repeated injury. We have developed a new model of spinal cord concussion focusing on the anatomical and behavioral outcomes of single and repeated injury. Rats received a very mild (50kdyn, IH impactor) spinal contusion at C5 and were separated into two groups three weeks after the initial injury – C1, which received a second, sham surgery, and C2, which received a second contusion at the same site. To track motor function and recovery, animals received weekly behavioral tests - BBB, CatWalk™, cylinder, and Von Frey. Analysis of locomotor activity by BBB demonstrated that rats rapidly recovered, regaining near-normal function by one week after the first and second injury, which was confirmed using the more detailed CatWalk™ analysis. The cylinder test showed that a single contusion did not induce significant deficits of the affected limb, but that repeated injury resulted in significant alteration in paw preference, with animals favoring the unaffected limb. Intriguingly, Von Frey analysis demonstrated an increased sensitivity in the contralateral hindlimb in the C2 group vs. the C1 group. Anatomical analyses revealed that while the lesion volume of both groups was minimal, the area of spared white matter in the C2 group was significantly reduced 1 and 2 mm rostral to the lesion epicenter. Reactive astrocytes were present in both groups, with the majority found at the lesion epicenter in the C1 group, whereas the C2 group demonstrated increased reactive astrocytes extending 1mm caudal to the lesion epicenter. Macrophages accumulated within the injured, dorsal and ipsilateral spinal cord, with significant increases at 2 and 3mm rostral to the epicenter in the C2 group. Our model is designed to represent the clinical presentation of spinal cord concussion, and highlight the susceptibility and functional sequelae of repeated injury. Future experiments will examine the temporal and spatial windows of vulnerability for repeated injuries.

Keywords

Repetitive spinal cord contusion; vulnerability; behavioral consequences; morphological changes; spinal cord concussion model

Introduction

Concussion is a temporary disruption of neuronal function caused by traumatic biomechanical forces, which initially was thought to result in neuronal, chemical, or neuroelectrical changes without significant structural damage (Cantu, 1996). Spinal cord concussion is a variant of mild cord injuries that results in transient neurologic disturbances, associated motor and sensory loss, and complete recovery within 48–72 hours after injury (Del Bigio and Johnson, 1989; Zwimpfer and Bernstein, 1990). Spinal cord concussion has assumed a variety of clinical designations, including transient paraplegia, transient traumatic paraplegia/quadriplegia, transient paraplegia/quadriplegia/paresis, and neurapraxia (Brigham and Capo, 2013; Cantu and Cantu, 2005; Maroon et al., 2007; Torg et al., 1997; Torremans et al., 1996; Winder et al., 2011; Zwimpfer and Bernstein, 1990). Patients who suffer spinal cord concussion exhibit a stereotypic clinical syndrome: transient motor and sensory loss immediately preceded by an acute blow to the spinal cord that gradually resolves without additional intervention. Spinal cord concussion occurs across a wide variety of sports in both professional and amateur adult and pediatric athletes (Rathbone et al., 1992) – wrestling (Bailes, 2005; Rathbone et al., 1992), rugby (Scher, 1991), American football (Cantu and Cantu, 2005; Torg et al., 1986; Winder et al., 2011), hockey (Winder et al., 2011), gymnastics (Rathbone et al., 1992), and diving (Rathbone et al., 1992). Importantly, many individuals have experienced multiple episodes of spinal cord concussion (Brigham and Capo, 2013; Cantu and Cantu, 2005; Gronwall and Wrightson, 1974; Maroon et al., 2007; Torg et al., 1997; Torremans et al., 1996; Winder et al., 2011; Zwimpfer and Bernstein, 1990). In addition, spinal cord concussions have also been reported to occur after a variety of non-sporting activities, such as car accidents and falls (Zwimpfer and Bernstein, 1990).

Unlike brain concussion, or mild traumatic brain injury (mTBI), there have been no appropriate models to study the effects and risks of spinal cord concussion. Studies on brain concussion have revealed that individuals who suffered a single concussion are more susceptible to another, especially if the new injury occurs before symptoms from the previous concussion have completely resolved (Gronwall and Wrightson, 1974). In addition, there is also a negative progressive process in which smaller, subsequent impacts cause the same symptom severity as the initial injury. Moreover, repeated concussions may increase the risk in later life for dementia, Parkinson's disease, and/or depression (Mannix et al., 2013; Plassman et al., 2000). Animal studies have indicated that concussion effects a complex pathology that includes the disruption of neuronal cell membranes accompanied by release of glutamate and a lower metabolic state, which may persist for weeks after injury (Giza and Hovda, 2001). The efforts to increase education about symptoms of mTBI and how to manage them are exemplified by the 2008 Zurich Consensus Statement on Concussion in Sport, which recommends persons to be symptom free before restarting and then, not all at once, but rather through a series of graded steps (McCorry et al., 2009).

In contrast, relatively little was known about the consequences and long-term sequelae of spinal cord concussion at its outset. There are no answers to the questions related to 1) when can athletes return to play after spinal cord concussion and 2) what are the consequences of the repeated spinal cord concussions that athletes suffer. So far, no experimental models have been developed or data on repeated spinal cord concussion reported. Our previous study (Jin et al., 2014) on repetitive mild SCI at the thoracic level indicated that functional deficits worsened with a second injury and resulted in increased tissue damage, inflammation, and cell death. However, the established “mild SCI” produced by the NYU impactor in that study resulted in massive tissue damage at the lesion epicenter and a slow functional recovery that returned to near-normal levels only by three weeks post-injury. This mild SCI model (Jin et al., 2014) therefore does not faithfully reproduce the clinical presentation of spinal cord concussion, which is characterized by minimal tissue damage and rapid functional recovery. In the present study, we developed a new spinal cord concussion model using the Infinite Horizon (IH) impactor to examine the behavioral and anatomical consequences of single and repeated injury. We also compared this new, very mild spinal cord concussion model (referred to as minimal contusion) with the standard mild thoracic contusion we have previously studied (Jin et al., 2014).

Materials and Methods

Animals

Female Sprague-Dawley rats (225–250g) were obtained from Taconic Farms (Germantown, NJ) and were housed 3 per cage with a 12h light/dark cycle. Food and water were available *ad libitum*. All procedures were approved by the Institutional Animal Care and Use Committee of Drexel University College of Medicine and were carried out according to the *NIH Guide for the Care and Use of Laboratory Animals*.

Surgical procedure

Nineteen rats received a minimal contusion using a 50 kilodyne (kdyn) force by the IH impactor and were divided into two groups three weeks post-injury, after complete recovery (defined as an average BBB score around 21): animals that received one contusion (C1; n=9), and animals that received a second contusion (C2; n=10).

Rats received a mixture of XAK containing Xylazine (10 mg/ml), Acepromazine maleate (0.7 mg/kg), and Ketamine (95 mg/kg) injected intraperitoneally (i.p., volume/rat = body weight/rat $\times$ 0.00108 ml). The skin over the upper cervical area was shaved and cleaned with betadine solution and 70% ethanol. The skin and muscles were incised to exposure the fifth cervical vertebral body (C5). A laminectomy was performed at C5, and a contusion was made on the right side of the cord with the IH impactor (Precision Systems and Instrumentation, LLC, Lexington, KY). Dwell time was set to 0 seconds. The IH device was set to deliver a force of 50kydn. Following injury, the muscle and skin were closed in layers. Three weeks after contusion, animals were re-anesthetized with XAK and divided into two groups: C1: control animals where the lesion site was reopened, and C2: experimental animals where the lesion was re-opened and delivered the same minimal contusion (50kydn) as in the first injury at the same location (C5). Rats were placed back in their cages with

heating pads and closely observed until they awoke. Buprenex (0.015–0.02mg/kg, 0.3mg/ml, Reckitt Benckiser, Richmond, VA) was administered subcutaneously post-surgery and once a day after surgery for one day.

Evaluation of motor and sensory function

Baseline values for all behavior tests were acquired prior to the first surgery. All animals were then tested weekly.

Open-field locomotion (BBB)—The rats were placed in an enclosure and scored by two blinded observers according to the Basso, Beattie, and Bresnahan (BBB) rating scale (Basso et al., 1995). The test was performed prior to the initial contusion to establish a baseline, 2–3 days after 1st and 2nd surgery, and once a week after surgery until the end of the experiment.

Paw Placement Test (Cylinder test)—The rats were placed into a Plexiglas cylinder (17.8cm by 35.5cm). A mirror placed behind the cylinder allowed for observation of all movements. The test was video-recorded and the numbers of exploratory paw placements making contact with the cylinder were counted and categorized as left, right, or both paws. The score was expressed as a percentage of use of the affected limb (% of paw placements of the affected limb (right) + half of the % of both paws placements divided by the total number of paw placements) (Liu et al., 1999; Shumsky et al., 2003).

CatWalk™ gait analysis—Animals crossed a Plexiglas floor walkway, which allowed for the visualization of foot contacts, and a high-speed camera recorded each of the animal runs in a dark room (Hamers et al., 2006; Hamers et al., 2001). Locomotion and gait was analyzed using CatWalk™ software NXT 10.5 (Noldus Information Technologies). Animals were pre-trained for 5 days prior to the first surgery and tested once a week until the end of the experiment. Base of support, step sequence, phase dispersion, swing, stance, duty cycle were analyzed for locomotion changes. Mean intensity mean, maximum intensity mean, and maximum contact intensity were analyzed to describe sensory changes in all 4 limbs (Vrinten and Hamers, 2003).

Von Frey—The up-down method (Detloff et al., 2010; Detloff et al., 2012) for Von Frey hair (VFH) monofilaments (VFH, Stoelting Co., Wood Dale, IL) was used to measure the degree of tactile sensory changes after minimal contusion. A total of 10 VFH stimulus applications were collected for each hind paw (Detloff et al., 2013). The response threshold was the lowest force (in grams) that produced a paw withdrawal in at least 50% of the applications. Paw testing order was determined randomly to minimize an order effect.

Tissue preparation

Animals were anesthetized with i.p. injection of sodium pentobarbital (100mg/kg, Abbot Laboratories, Chicago, IL) and transcardially perfused with 100ml saline followed by 500ml of ice-cold 4% paraformaldehyde fixative in phosphate buffer (PB, pH 7.4). A 30mm long segment including the lesion area was dissected. Tissues were post-fixed in the same fixative for 2–3 days and then transferred into a solution of 30% sucrose in 0.1M PB for 3–5 days. Spinal cord segments were embedded in M1 mounting medium (Fisher Scientific,

Pittsburgh, PA), and cut into 20µm coronal sections on a cryostat, and mounted onto gelatin-coated slides in 10 serial sets. After harvesting the first 20µm section, the subsequent four sections were discarded, prior to harvesting another section, making the interval between sections on the same slide 1mm.

Histology and Immunocytochemistry

Spinal cord sections were processed for Nissl/myelin staining. Immunocytochemistry was performed with antibodies against ED1, for macrophages, GFAP, for reactive astrocytes, PKCγ, for corticospinal tract axons, and RECA1, for blood vessels. Spinal cord sections were washed three times in PBS and blocked with 5% goat serum for 1h. Primary antibodies were incubated with 5% goat serum in PBS containing 0.3% Triton X-100 at room temperature overnight. Species-specific secondary antibodies (goat anti-mouse or anti-rabbit conjugated to FITC or rhodamine, 1:400; Jackson ImmunoResearch, West Grove, PA) were applied for 2h at room temperature, and the slides were cover-slipped with anti-fade Dapi-Fluoromount-G (SouthernBiotech, Birmingham, AL). Fluorescently stained slides were stored at 4°C.

Measurement of spared tissue and cyst volume

The volume of spared tissue, lesion, and cystic cavities were assessed in a blinded fashion using Stereo Investigator in coronal Nissl/myelin stained sections, similar to our previous study (Jin et al., 2014). For each animal, every 10th cryostat section (1mm interval) was used to determine the total volume of a 18mm long segment centered at the contusion epicenter and the spared tissue within this segment. Tissue was considered spared if areas contained healthy-looking spinal neurons and myelin, and lacked cysts. Conversely, tissue with unhealthy-looking neurons and demyelination, without cavities, was counted as lesion. Cyst volumes were determined as cavities within the lesion area. Using Stereo Investigator, spared tissue (including white and grey matter) and cysts can be marked with different colors on each section. The system then calculates the total volume and provides the fraction of each part for each animal. Data were presented as percentages of total cord volume for spared white and grey matter, lesion volume, and cyst volume. Lesion area was also obtained using Stereo Investigator and presented as millimeter distance in the rostral and caudal direction from lesion epicenter. Lesion volume and lesion area were calculated without including cavity volume or area, respectively.

Analysis of distribution of ED1, GFAP, PKCγ, and RECA1 positive staining

For analysis of ED1, GFAP, PKCγ, and RECA1 staining, images from the lesion epicenter and 3mm, 6mm, and 9mm rostral and caudal to the lesion epicenter were taken using a DC-330 CCD color video camera (DAGE-MTI, Inc., Michigan City, IN) attached to a Leica DMRBE microscope (Leica Microsystems; Bannockburn, IL) with a 10X objective. Images were converted using NIH Image J software to grayscale. In each image, a standard sized rectangle was overlaid on the dorsal column and ventral white matter. A standard sized oval was overlaid on the lateral white matter. The illumination intensity was held constant throughout the analysis of the images for all groups. The labeled areas within the rectangle or oval were measured, and presented as a percentage of the respective area.

Statistical analysis

A two-way analysis of variance (ANOVA) comparing 1 contusion versus 2 contusions over time, with time taken as a repeated measure, was used to analyze the behavioral data. Significance set at $p < 0.05$. Post-hoc analysis was performed using the Bonferroni test. One-way ANOVA was used for analysis of spared tissue, lesion volume, and cyst volume. Student independent unpaired t -test was used for distribution of ED1, GFAP, PKC γ , and RECA1.

Results

Behavioral changes

Open-field locomotion (BBB)—The baseline value for all animals prior to any procedures was 21 (Fig. 1). The minimal, unilateral contusion at C5 resulted in minor disruption of locomotion 2–3 days post injury (BBB: C1, 20.4 ± 0.6 ; C2, 19.6 ± 0.4). Animals regained near perfect scores during the following 3 weeks (BBB: C1, 21 ± 0.1 ; C2, 20.9 ± 0.0). At that time, animals were divided into two groups: C1, for animals receiving a single contusion, and C2, for animals receiving a second contusion in the identical location and with the same force as the initial contusion. To summarize, rats in the C1 group received a second surgery, in which the lesion site was re-opened, but no further contusion injury performed while rats in the C2 group received a second minimal contusion at same injury site.

Two to three days after the second surgery, animals in the C1 group demonstrated a small decrease in the BBB score (20 ± 0.9), which was not found to be significant when compared to BBB scores 3 weeks after the initial surgery. Animals in the C2 group demonstrated a greater deficit 2–3 days after the initial injury, with a BBB score of 19.0 ± 0.5 , which was found to be significantly lower than their baseline value ($p=0.0008$) as well as significantly lower than their score the week prior to the second surgery ($p=0.018$). One week after the second surgery, both groups regained near-normal function and demonstrated high BBB scores through the duration of experiment (BBB: 20.8 ± 0.2 in C1, 20.6 ± 0.1 in C2, 7 weeks after the second injury).

Cylinder Test (Paw Preference)—Baseline for all animals prior to injury (Fig. 2) was 50% use of the ipsilateral limb ($50 \pm 1\%$). Following the first injury, animals did not demonstrate major deficits at 1 week (C1, $46 \pm 2\%$; C2 $50 \pm 2\%$) or at 2 weeks (C1, $49 \pm 2\%$; C2 $50 \pm 1\%$). Following the second surgery, the C2 group demonstrated a major deficit in the use of the affected ipsilateral limb 1 week after the injury, with a score of $34 \pm 3\%$, which was found significantly lower than their baseline value ($p<0.001$) and the C1 group score ($45 \pm 5\%$, $p=0.003$). No further improvement in paw preference was observed through the duration of the experiment ($48 \pm 2\%$ in C1; $34 \pm 2\%$ in C2 at 7 weeks after the second surgery, $p<0.001$ C1 vs C2 as well as C2 vs baseline).

Mechanical Sensitivity (Von Frey filaments)—Von Frey filaments were used to assess mechanical allodynia below the level of injury (Detloff et al., 2012). Two weeks following the initial injury, there was no significant difference between both hind limbs

when comparing the C1 and C2 groups in terms of average threshold eliciting a paw withdrawal. Two weeks after the second surgery through the end of the study, there were no significant changes in the threshold for paw withdrawal compared to baseline for the ipsilateral hind limb (right) in both groups at any time point (Fig 3A). For the contralateral hind limb (right), the C2 threshold was significantly lower than the C1 at 7 weeks after the second injury, but no differences compared to its baseline value or any other time points were observed (Fig 3B).

Animals demonstrating variation of threshold from baseline greater than 50% were defined as “hyper-sensitive” (Detloff et al., 2012; Detloff et al., 2013). It was also observed that the percentage of animals demonstrating more than 50% variation from their baseline was similar in both groups and for both limbs (44% in the C1, 50% in the C2), which was consistent with other findings (Detloff et al., 2013). Following the second injury the percentage of animals with more than 50% of variation increased to 60% in the C2 for the left hind limb while the C1 ratio was reduced to 20% (Fig. 3C). The right hind limb was not affected by the second injury in either group at any time point (Fig. 3D).

CatWalk™ gait analysis—Analysis of locomotion using the CatWalk™ system showed that basic parameters did not change following the initial injury. Speed, dominant step pattern, phase dispersion (not shown), and regularity index were not affected by the initial contusion, confirming the results obtained using the BBB open field locomotion scale. The base of support for both pairs of limbs was unchanged in both groups 3 weeks following the initial injury (Table 1). However, the hind limb base of support was decreased 5 and 7 weeks after the second surgery in the C2 group compared to 3 weeks after the 1st surgery ($p=0.038$ and $p<0.001$, respectively). There was also a significant difference in the analyses between the C1 and C2 groups 7 weeks after 2nd surgery ($p=0.026$).

The parameters associated with the intensity of contact were used to analyze changes in sensory functions (Vrinten and Hamers, 2003). Following the first injury, no significant variation in the maximum intensity or mean intensity was recorded for all paws across both groups. In contrast, while the C1 group maintained its baseline level for both parameters for all paws 7 weeks after injury, the maximum and mean intensities significantly dropped below their baseline and week 3 post first-injury levels in all paws in the C2 group, indicating that less pressure was applied by all paws during locomotion in the C2 group (Table 2). Within the C1 group, no difference was observed in the maximum or mean intensity across each paw throughout the duration of the experiment. These results, taken together with the increase of swing and the shift in duty cycle, indicate an increase in sensitivity in the C2 group compared to baseline.

A more detailed analysis of locomotion parameters for each individual limb is demonstrated in Table 2. Data from 3 weeks following the initial injury showed that the initial injury had little effect on overall locomotion, with animals developing compensatory mechanisms early on and continuing throughout the duration of the study. Stand (time spent in stand, average in seconds), stand index, swing speed (average of all 3 runs in cm/s), and print area (area of the paw in contact with the glass in cm²) were not affected by the injury. There were no differences in these parameters in the C1 and C2 groups at all time points except 7 weeks

after the second surgery. Seven weeks after the second surgery, some parameters such as swing time (s) of individual paws were found to be significantly different from their baseline values, but no significant differences were found for any parameters between the C1 and C2 groups.

Anatomical changes

Analysis of spared tissue—Cross sections stained with Nissl/myelin showed that a minimal contusion caused minor damage, limited to the lesion epicenter with a small lesion cavity in some animals that demonstrated limited extension in both rostral and caudal directions (Fig. 4). Spared tissue was analyzed in two ways: the total volume and area of lesion at the epicenter as well as in different locations extending from the epicenter, for a total coverage of 9mm both rostral and caudal directions. Measurement of the volume of spared tissue indicated that there were no differences in spared white and grey matter on the ipsilateral side when comparing the C1 with C2. Similar results were found on the contralateral side (Fig. 5). Lesion volume was less than 1% with $0.44\% \pm 0.06$ in the C1 and $0.66\% \pm 0.01$ in the C2. There was no significant difference in lesion volume when comparing the C1 with C2. Analysis of the area of spared tissue showed that most damage was located within the grey matter, with a low percentage of damaged tissue area at the lesion epicenter as well as 1mm rostral and caudal to the lesion with no significant difference between the C1 and C2 groups. There were no changes in the area of spared grey matter from 2mm rostral and caudal to the lesion epicenter with no difference between two groups except for more percentage of grey matter at 3mm caudal to the lesion in the C2 compared to the C1 group (Fig. 6A). The area of spared white matter indicated that spared white matter was significantly decreased at 1 and 2 mm rostral to the epicenter in the C2 compared to the C1 group (Fig. 6B). The majority of the lesioned area was at the epicenter and 1mm rostral and caudal to the epicenter (about 6% in the C1 and 7% in the C2 group at the epicenter). No lesion was found beyond 1mm caudal to the epicenter in the C1 and 2mm in the C2 group. Minimal lesion area was found at 2 and 3mm rostral to the epicenter in both the C1 and C2 groups and at 5mm in the C2 group (Fig. 6C). $*p < 0.05$, C1 vs C2.

Macrophage expression—Macrophages, identified by ED1 staining, accumulated at the lesion epicenter and reduced with the distance from the epicenter (Fig 7A). Analysis of ED1 distribution at different regions showed significant increases of ED1 expression in the C2 group at 2 and 3mm caudal to the epicenter in the dorsal part on the ipsilateral side of the lesion. Within the lateral part, a significant increase of ED1 expression in the C2 group was found in the epicenter and 3mm caudal the epicenter. Within the ventral part, there was a significant increase of ED1 expression in the C2 group at the epicenter and 2mm caudal to the epicenter (Fig. 7B). $*p < 0.05$, C1 vs C2.

Glial scar formation—Astrocytes were stained with GFAP. GFAP⁺ astrocytes were found located within lesion epicenter as well as 1mm rostral and caudal to the epicenter in both the C1 and C2 groups (Fig. 8A), with minimal GFAP positive staining found beyond 1 mm rostral and caudal to the epicenter. Area fraction of GFAP expression demonstrated that most GFAP positive staining was located at the lesion epicenter as well as 1mm rostral and

caudal to the epicenter with no significant difference between the C1 and C2 groups (Fig. 8B).

PKC γ and RECA1 expression at the dorsal column corticospinal tract location

—The proximity of the lesion to corticospinal tract (CST) within the dorsal columns caused us to examine the changes in the CST using antibody PKC γ . In addition we also examined angiogenesis using the RECA1 antibody to identify blood vessels. CST axons positive for PKC γ were stained red and blood vessels stained green at the same location (Fig. 9A). Area fraction of both PKC γ and RECA1 at CST location was analyzed using Image J. On the ipsilateral side, low PKC γ expression was seen at the epicenter in the C1 group with no significant difference compared to the C2 group. There was also no difference in PKC γ expression when comparing the C1 to C2 group at all locations. Similar PKC γ expression was found on the contralateral side (Fig 9B, I). Area fraction of RECA1 staining indicated increased blood vessels at the epicenter in the C2 group in both ipsilateral and contralateral sides, but no significant differences when compared to the C2 group. There was no significant difference in RECA1 expression at any location when comparing the C1 to C2 in both sides of the spinal cord (Fig. 9B).

Discussion

A concussion, defined as a mild traumatic brain injury, is induced by biomechanical force, which is caused by a direct blow to the head, face, neck, or elsewhere on the body with impulsive force transmitted the head (Cantu et al., 2007). Concussion can be difficult to identify at the onset and may go unrecognized, which places individuals at risk for future and more complicated injury (Cantu and Register-Mihalik, 2011; Echlin et al., 2010). Repeated concussion is responsible for neurodegenerative diseases such as Alzheimer disease (AD), Parkinson disease (PD), and chronic traumatic encephalopathy (CTE) (Baugh et al., 2012; Fleminger et al., 2003; Goldman et al., 2006; Stern et al., 2011). While brain concussion has been studied at the experimental and clinical levels, few studies have focused on spinal cord concussion. Spinal cord concussion is a minimal contusion injury that results in transient neurologic disturbances with sensory and/or motor deficits, with complete neurological recovery occurring within 24 to 48 hours. The long term effects of one or more spinal cord concussions on spinal cord function are unknown. Using decerebrated frogs, Parkinson and colleagues found a minimal force necessary to abolish impulse transmission down the spinal cord, and that single and repeated concussing blows can transiently modify spinal cord reflex function (Parkinson et al., 1981; Parkinson et al., 1983). Despite these findings, there are still no vertebrate animal models to elucidate the long-term effects of single and multiple concussions on the consequences of anatomy and behavior.

Our previous study on mild thoracic contusion (Jin et al., 2014) indicated that repeated mild contusion resulted in an accumulated effect on functional deficits. However, due to the large amount of tissue damage at the lesion epicenter and slow locomotion recovery, the mild thoracic contusion model using the NYU impactor did not represent the clinic syndrome of spinal cord concussion. In this study we developed a new model for a minimal cervical contusion that mimics spinal cord concussion.

Behavioral changes after single and repeated injury

The minimal cervical contusion was generated unilaterally by an IH impactor with a low force (50kdyn). Several tests were selected to analyze functional outcome, including open field locomotion (BBB), paw placement (cylinder), Catwalk, and Von Frey. BBB test showed rapid locomotor recovery 2–3 days after injury and reached normal levels by 3 weeks, with BBB scores of 21. CatWalk™ data further confirmed near-normal locomotor function by 3 weeks after initial injury. Cylinder test, which is specific for forelimb paw use, demonstrated no changes compared to the baseline. Sensory function, assessed by CatWalk™ and Von Frey, indicated no significant changes compared to the baseline after initial injury. Taken together, the behavioral data suggests that the injury to the spinal cord is minor, with no effects on motor and sensory function, indicating that this new model closely resembles the clinical syndrome. Three weeks after initial injury, some rats received a second contusion at the same location as the initial injury (C2) and others received a sham surgery with the lesion area reopened (C1). Following the second injury, BBB scores were lower in the C2 group 2–3 days after 2nd injury compared to both its baseline and 3 weeks after 1st injury, but no significant differences between the C1 and C2 groups at 2–3 days after 2nd surgery and all tested time points were observed. CatWalk™ showed similar results for rat locomotion except at the last data point, 7 weeks after the second injury, at which time the hindlimb base of support changed significantly in the C2 group compared to the C1, and C2 at 3 weeks after the first injury. Sensory function derived from the CatWalk™ analysis showed significant changes in all 4 paws in pressure applied during stance as well as increase of the swing phase during the stepping episode in the C2 group compared to its baseline and post-first injury levels. This indicates changes in weight bearing and an increased sensitivity of not only the affected limb but also contralateral forelimb and both hind limbs (Vrinten and Hamers, 2003). It has been shown that more severe cervical lesion has major detrimental sensory effect below the level of injury (Detloff et al., 2013). Several studies also showed similar results in different pain models (Medhurst et al., 2002, Tabo et al., 1998, Coderre and Wall, 1988). Our study showed that while gross locomotion is not disturbed, fine locomotion and sensory pathways at and below level the injury are affected. While Von Frey showed no significant changes in the two groups compared to their baseline, the contralateral left hind limb in the C2 group revealed subtle changes in the percentage of rats that expressed more than 50% variation from their baseline compared to the C1 group, indicating minor changes in the pain threshold after the second injury and further confirming results obtained with the CatWalk™. The significant change in motor function is reflected in the use of the affected limb. Use of the affected paw dropped 1 week after the second injury and did not recover to the baseline, with a significant decrease in use compared to the C1 group at all-time points after the second injury. These results indicate that repeated minimal injuries result in an accumulation of functional deficits after full recovery from the initial injury, similar to our previous finding in repeated mild thoracic contusion (Jin et al., 2014).

Anatomical changes after single and repeated injury

Sections from cervical spinal cord were stained with Nissl/myelin to visualize the neurons and myelin in gray and white matter of the spinal cord. Nissl/myelin staining showed a small cyst at the lesion epicenter in some rats in both groups, which was located at the middle of

the grey matter or the white matter close to the grey matter. Analysis of the volume and area of spared tissue indicated some differences. For the measurement of the volume of spared tissue, there were no significant differences in grey matter and white matter between the C1 and C2 groups. In both groups, the lesion volume was less than 1% of the total volume. For the analysis of the area of spared tissue, both groups showed reduced spared grey matter that appeared only at the lesion epicenter as well as 1mm rostral and caudal to the epicenter without further extension. There was a significant decrease in area of spared white matter in the C2 group at 1 and 2 mm rostral to the lesion compared with the C1 group. This morphological data confirms that the contusion at the cervical level is very minor and repeated injury did not significantly change the anatomy of the cord. Further molecular analyses demonstrated that the numbers of ED1 macrophages increased in the C2 group, but that scar formation at/near the lesion center did not show significant differences between the C1 and C2 groups. Single and repeated injury did not influence the corticospinal tract (CST) nor blood vessel architecture in the injured cord, with no changes in PKC γ expression for CST and RECA1 expression for blood vessel, respectively.

Correlation between behavior and anatomy

The minimal cervical contusion caused by IH impactor caused minimal tissue damage that was limited to the lesion epicenter and resulted in no obvious functional deficits. Repeated injury did not significantly change the extent of tissue damage, but significantly altered effected paw performance. Analysis of the correlation between anatomy and behavioral function by Spearman test indicated no correlation between BBB and spared tissue as well as paw placement and spared tissue. Since not all rats had a cavity at the lesion epicenter, we further examined correlations between rats with cavity at epicenter and behavior changes in each group, but we failed to find any correlation between them. This differs from our previous study on repeated mild thoracic contusion (Jin et al., 2014). One possibility for the differences between these two different injury models may be the minor tissue damage that is located mostly within middle of grey and white matter in the minimal cervical contusion model. This model may have spared descending axons such as the CST within dorsal column and rubrospinal and reticulospinal tracts within the lateral funiculi. The rubrospinal tract is involved in skilled limb movement (Liu et al., 1999; Whishaw and Gorny, 1996; Whishaw et al., 1992) as well as plays a role in ongoing overground locomotion (Muir and Whishaw, 2000). The reticulospinal tract plays an important role in control of open-field locomotion (Schucht et al., 2002). The corticospinal tract is involved in controlling movements that require the greatest skill and flexibility (Martin, 2005). However, without obvious damage in these descending tracts, it is not clear what causes the affected forelimb deficit after repeated injury. Another possible underlying etiology may be due to the process of inflammation occurring within the injured spinal cord. A recent study showed that repeated mTBI causes deficits in hippocampal function and changes in excitatory synaptic neurotransmission, which are associated with chronic neuroinflammation and neurodegeneration (Aungst et al., 2014). Our previous study also demonstrated that repeated mild thoracic contusion caused increase of macrophages along the white matter (Jin et al., 2014). In this study, accumulated macrophages along the injured spinal cord may contribute to functional deficits.

Comparison of new model with mild thoracic contusion and other cervical contusion

The behavioral tests performed in this study confirmed that this new minimal contusion, spinal cord concussion model did not result in any obvious gross functional deficits. Rats recovered rapidly within 2–3 days after cervical contusion compared to thoracic contusion utilizing the NYU impactor. Three weeks after contusion, motor function in thoracic contusion rats recovered to close to normal levels, while motor function achieved normal levels in cervical contusion rats. The lesion area in thoracic contusion was over 30% while the lesion area was less than 8% in the cervical model, and the rostral and caudal extension of this lesion area was greater in the thoracic contusion model. Increased apoptotic staining of the white matter was found in the repeated thoracic contusion with minimal staining in the single and repeated cervical contusion. The only similar result in both injury models was an increased macrophage staining at the lesion epicenter with extension in rostral and caudal directions after repeated injury (Jin et al., 2014). Compared to other cervical contusion studies, our model also demonstrates rapid functional recovery and minor tissue damage. Mild cervical contusion using different devices demonstrated more severe functional deficits and decrease of spared white matter and grey matter (Pearse et al., 2005; Soblosky et al., 2001). Mild unilateral cervical contusion by NYU impactor resulted in nearly completely loss of grey matter and partial white matter loss within the dorsal and lateral funiculus (Gensel et al., 2006). Therefore, this new mild cervical contusion model can be used to study spinal cord concussion in different conditions.

In summary, we have developed a new, minimal cervical contusion model that resembles clinical spinal cord concussion. Repeated injury after full functional recovery resulted in some locomotor deficits that did not recover to normal levels. Future studies will investigate: 1) whether the window for repeated injury is important; 2) whether the level of repeated injury results in different outcomes, which includes rostral or caudal to the initial injury as well as ipsilateral or bilateral injury; 3) whether the consequences of repeated injury we described in adults is different in adolescents and aged animals; 4) whether the sequelae of repeated injury shows significant gender differences. Answers to these questions will provide detailed experimental data for clinical considerations not only on decisions regarding an athlete's return to play after spinal cord concussion, but also for the clinical care of non-athletes with spinal cord concussion.

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Figure 1. Locomotion recovery

BBB test shows that mild contusion resulted in minor functional deficits 2–3 days post injury with rapid recovery to near-normal levels by 1 week. The second injury showed a smaller deficit at 2–3 days post injury with significant differences compared to the baseline (* $p=0.0008$) and 3 weeks after the first injury (# $p=0.018$) in the C2 group, but no significant difference compared to the C1 group. Both groups regained near-normal function 1 week after the second surgery. There were also no significant differences in the C2 group compared to the C1 group in all time points following the second injury.

Figure 2. Paw preference

Cylinder test demonstrates that the baseline value for all animals was around 50% of use of the affected limb. Following the first injury, animals showed minor deficits with no significant differences compared to the baseline. Following the second surgery, the C2 group demonstrated major deficits in use of the affected limb 1 week after the injury with significantly lower scores than their baseline value ($p<0.001$) and the C1 group score ($p=0.003$), with no further improvement for the duration of the experiment. * $p<0.05$, C1 vs C2; $p<0.001$, C2 vs baseline. ** $p<0.001$, C1 vs C2 and C2 vs baseline.

Figure 3. Mechanical sensitivity

Von Frey shows that there were no significant differences in both hind limbs when comparing the C1 to C2 group in terms of average threshold eliciting a paw withdrawal 2 weeks following initial injury. Two weeks after the second 2nd surgery and until the end of the study, there were no significant changes in the threshold compared to baseline for the ipsilateral hind limb (right) in both groups at any time points (A). For the contralateral hind limb (left), the C2 threshold was significantly lower than the C1 seven weeks after the second injury, but no difference compared to its baseline value or any other time points (B). The percentage of animals demonstrating more than 50% variation from their baseline was similar in both groups and both limbs after the initial injury. Following the second injury, the percentage of animals with more than 50% of variation increased in the C2 for the left hind limb, while the C1 ratio was reduced (C). The right hind limb was not affected in both groups at any time points by the second injury (D).

Figure 4. Morphology of minimal C5 contusion

Nissl/myelin histology showed small changes at the lesion site. Some rats in both the C1 and C2 groups developed small cavities mostly within the grey matter (right panel in both the C1 and C2). Some rats did not show obvious damage at lesion epicenter. No lesion damage was found beyond 2mm rostral and caudal to the lesion epicenter.

Figure 5. Anatomical changes (volume)

Analysis of the spared white and grey matter, lesion and cavity along 18mm long spinal segments shows no significant differences in spared white matter, lesion volume, and cavity volume between the two groups in both ipsilateral and contralateral side of the spinal cord.

Figure 6. Anatomical changes (area)

Analysis of area of spared tissue showed that most damage was located in the grey matter at the lesion epicenter. There was very limited extension of damaged tissue from the rostral and caudal directions with no significant differences between the C1 and C2 groups (A and C). The distribution of spared grey matter was similar in both groups, except for the C1 group with a small decrease at 3mm caudal to the epicenter (A, $*p<0.05$). Spared white matter significantly decreased at 1 and 2mm rostral to the epicenter in the C2 group compared to the C1 group (B, $*p<0.05$).

Figure 7A

Figure 7B

Figure 7. Inflammation after minimal C5 contusion

A: Macrophages, identified by ED1 staining, accumulated at the lesion epicenter and reduced with distance from the epicenter. **B:** Significant increase of ED1 expression was found in the C2 group at 2 and 3mm caudal to the epicenter within the dorsal part of the ipsilateral side of the lesion. Within the lateral part, significant increases of ED1 expression in the C2 group was found at the lesion epicenter and 3mm caudal the epicenter. Within the ventral part, there was a significant increase of ED1 expression in the C2 group at the epicenter and 2mm caudal to the epicenter (* $p < 0.05$, C1 vs C2).

Figure 8. Scar formation

A: Astrocytes, stained by GFAP, accumulated within the lesion epicenter as well as mm rostral and caudal to the epicenter. **B:** Analysis of GFAP expression indicated higher percentage of GFAP expression at the epicenter, and reduced percentages at 1mm rostral and caudal to the lesion center, with no further extension beyond 1mm in both directions. There was no significant difference in GFAP expression when comparing the C2 to C1, although the C2 showed a trend of a higher percentage.

Figure 9A**C1-lesion center****C2-Lesion center**

Figure 9B

Figure 9. Expression and distribution of PKC γ and RECA1 at the CST location

A: CST axons can be identified by PKC γ staining (red) and blood vessels stained by RECA1 (green). Expression of PKC γ and RECA1 was captured at the most dorsal part of the dorsal columns. **B:** Analysis of both expressions (I: PKC γ ; II: RECA1) within the ipsilateral and contralateral spinal cords indicates no significant differences at any location when comparing the C2 to C1 group.

Table 1

Basic CatWalk Data

		Baseline	Week 3 after 1st surgery	Week 7 after 2nd surgery
Average Speed (cm/s)	C1	44.73 ± 4.14	52.56 ± 4.41	54.98 ± 4.41
	C2	41.40 ± 4.16	44.84 ± 4.16	50.49 ± 4.16
Step Pattern (% AB)	C1	51.04 ± 8.43	55.48 ± 8.43	51.34 ± 8.43
	C2	69.93 ± 7.94	62.38 ± 7.95	47.23 ± 7.98
Regularity Index (%)	C1	96.24 ± 1.15	98.90 ± 1.15	98.88 ± 1.15
	C2	95.65 ± 1.08	97.17 ± 1.08	96.98 ± 1.08
Base of Support (FL, cm)	C1	1.83 ± 0.08	1.66 ± 0.08	1.59 ± 0.08
	C2	1.71 ± 0.07	1.83 ± 0.07	1.56 ± 0.07
Base of Support (HL, cm)	C1	2.21 ± 0.07	2.24 ± 0.07	2.09 ± 0.07
	C2	2.03 ± 0.07	2.25 ± 0.07	1.78 ± 0.07 *

* p<0.001, C2 week 3 after 1st surgery vs week 7 after 2nd surgery. P=0.026, C1 vs C2 at week 7 after 2nd surgery.

Detailed CatWalk Data

Table 2

		Baseline				Week 3 after 1st surgery				Week 7 after 2nd surgery			
		Right Fore Limb	Left Fore Limb	Right Hind Limb	Left Hind Limb	Right Fore Limb	Left Fore Limb	Right Hind Limb	Left Hind Limb	Right Fore Limb	Left Fore Limb	Right Hind Limb	Left Hind Limb
Stand (s)	C1	0.178 ± 0.02	0.164 ± 0.02	0.195 ± 0.02	0.186 ± 0.02	0.159 ± 0.16	0.161 ± 0.02	0.162 ± 0.02	0.154 ± 0.02	0.171 ± 0.02	0.196 ± 0.02	0.184 ± 0.02	0.182 ± 0.02
	C2	0.193 ± 0.19	0.196 ± 0.01	0.198 ± 0.02	0.201 ± 0.01	0.223 ± 0.22	0.228 ± 0.02	0.217 ± 0.02	0.22 ± 0.02	0.19 ± 0.02	0.184 ± 0.01	0.179 ± 0.02	0.189 ± 0.02
Stand Index	C1	-9.73 ± 1.32	-9.79 ± 1.28	-5.87 ± 0.99	-6.11 ± 1.03	-10.01 ± 0.56	-10.66 ± 0.73	-8.09 ± 0.65	-8.37 ± 0.90	-9.20 ± 1.14	-10.62 ± 1.11	-8.58 ± 1.73	-10.22 ± 2.17 *
	C2	-8.52 ± 0.42	-8.42 ± 0.68	-5.32 ± 0.36	-4.89 ± 0.42	-8.29 ± 0.122	-9.01 ± 0.82	-7.09 ± 0.89	-7.07 ± 1.20	-8.58 ± 0.68	-10.48 ± 0.57	-11.73 ± 2.02 *	-11.03 ± 1.92 *
Swing (s)	C1	0.13 ± 0.008	0.13 ± 0.01	0.12 ± 0.006	0.12 ± 0.004	0.15 ± 0.008	0.14 ± 0.009	0.13 ± 0.004	0.14 ± 0.005	0.15 ± 0.009	0.16 ± 0.016	0.15 ± 0.004 *	0.14 ± 0.004 *
	C2	0.13 ± 0.004	0.13 ± 0.003	0.12 ± 0.51	0.12 ± 0.004	0.15 ± 0.006	0.14 ± 0.009	0.14 ± 0.67	0.14 ± 0.004 *	0.17 ± 0.006 *	0.16 ± 0.009 *	0.16 ± 0.17 *	0.15 ± 0.003 *
Swing Speed (cm/s)	C1	127.10 ± 8.17	124.30 ± 10.35	129.29 ± 8.01	124.27 ± 7.85	134.71 ± 8.17	138.10 ± 10.35	134.20 ± 8.01	139.42 ± 7.85	131.72 ± 8.17	132.38 ± 10.35	129.31 ± 8.01	134.21 ± 7.85
	C2	119.28 ± 7.71	128.23 ± 9.76	118.54 ± 7.53	126.78 ± 7.40	119.37 ± 7.71	129.86 ± 9.76	124.53 ± 7.53	126.58 ± 7.40	119.75 ± 7.71	120.31 ± 9.76	125.68 ± 7.53	126.54 ± 7.40
Stride Length (cm)	C1	14.73 ± 0.68	14.39 ± 0.72	14.70 ± 0.79	14.46 ± 0.70	18.80 ± 0.35 *	18.42 ± 0.68 *	17.89 ± 0.73	18.48 ± 0.58	18.39 ± 1.12 *	18.46 ± 0.96 *	18.56 ± 1.11 *	18.59 ± 1.12
	C2	14.45 ± 0.37	14.40 ± 0.48	14.32 ± 0.41	14.28 ± 0.56	16.58 ± 0.91	16.51 ± 0.94 *	16.53 ± 0.92	16.71 ± 0.78	18.69 ± 1.10 *	18.15 ± 1.01	19.06 ± 1.03 *	18.87 ± 0.96
Duty Cycle (%)	C1	56.42 ± 1.74	53.19 ± 1.94	59.23 ± 2.1	58.65 ± 2.34	52.35 ± 1.32	54.19 ± 0.96	54.02 ± 1.44	52.78 ± 1.67 **	52.98 ± 1.84	51.80 ± 1.07	52.48 ± 2.72	52.25 ± 2.76 *
	C2	58.78 ± 1.57	59.05 ± 0.95	60.48 ± 0.85	61.38 ± 1.29	56.26 ± 1.99	58.49 ± 1.89	57.94 ± 2.35	59.20 ± 2.08	53.53 ± 1.64	54.24 ± 1.32	51.71 ± 2.33 *	54.52 ± 2.38 *
Print Area (cm ²)	C1	0.94 ± 0.08	0.95 ± 0.06	1.27 ± 0.15	1.24 ± 0.14	1.17 ± 0.08	1.25 ± 0.08	1.22 ± 0.17	1.33 ± 0.11	1.30 ± 0.04 *	1.33 ± 0.06 *	1.40 ± 0.12	1.33 ± 0.11
	C2	1.10 ± 0.08	1.12 ± 0.06	1.43 ± 0.11	14.0 ± 0.09	1.19 ± 0.04	1.23 ± 0.05	1.44 ± 0.08	1.41 ± 0.11	1.30 ± 0.02	1.18 ± 0.05	1.17 ± 0.09	1.19 ± 0.09
Mean Intensity Mean	C1	89.10 ± 3.88	91.60 ± 4.30	92.3 ± 3.60	89.83 ± 3.64	102.84 ± 4.40	107.19 ± 4.32	98.93 ± 6.50	101.93 ± 5.82	82.43 ± 2.77	85.14 ± 3.83 *	83.73 ± 4.26	76.64 ± 3.82
	C2	98.28 ± 3.64	100.30 ± 2.51	99.11 ± 3.618	96.73 ± 3.46	106.23 ± 2.40	109.72 ± 2.48	108.96 ± 2.27	101.507 ± 4.91	82.02 ± 2.61 *	80.39 ± 2.79 *	73.46 ± 2.25 *	70.84 ± 2.99 *
Max Intensity Mean	C1	185.60 ± 5.21	187.28 ± 5.73	195.41 ± 7.40	192.35 ± 7.28	201.33 ± 5.84	205.68 ± 4.21	194.09 ± 14.87	201.15 ± 11.77	174.84 ± 9.05	169.92 ± 8.78	187.44 ± 10.40	178.01 ± 12.08
	C2	197.31 ± 6.31	199.93 ± 3.40	206.21 ± 5.46	205.72 ± 4.88	205.97 ± 1.93	204.66 ± 3.72	214.77 ± 2.79	212.05 ± 4.95	166.35 ± 6.44 *	166.45 ± 8.60 *	166.25 ± 9.72	160.72 ± 11.14 *
Max Contact Intensity (%)	C1	41.53 ± 1.87	40.52 ± 1.53	23.19 ± 1.38	24.83 ± 2.64	39.22 ± 1.34	43.47 ± 1.46	23.21 ± 2.30	23.29 ± 1.90	38.50 ± 1.14 *	41.92 ± 1.98	24.91 ± 3.63	30.51 ± 3.87
	C2	44.87 ± 2.04	43.23 ± 1.90	23.30 ± 1.76	21.92 ± 0.81	37.89 ± 1.34	45.37 ± 1.62	26.41 ± 2.28 *	28.59 ± 3.88 *	37.74 ± 1.51 *	45.89 ± 1.69	37.36 ± 3.16 *	39.74 ± 3.45 *

* p<0.05, compared to baseline;
** p<0.05 compared to baseline as well as C1 vs C2;