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3/30/12

Cutaneous Trunci Muscle Reflex as a Model for Neural Plasticity after Spinal Cord Injury

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A thesis submitted to the Faculty of Emory College of Arts and Sciences  
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## Abstract

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Spinal cord injury (SCI) often results in neuronal plasticity that causes alterations in sensory processing. A physiological model of neural plasticity that could be analyzed quantitatively after injury could greatly advance our understanding of plasticity after SCI. The cutaneous trunci muscle (CTM) reflex produces a skin shrug and is mediated by a 3 neuron circuit: C and A $\delta$  afferents in segmental dorsal cutaneous nerves (DCNs), ascending propriospinal interneurons, and the CTM motoneuron pool.

Female Long Evans rats were divided into 4 experimental groups and analyzed at 3 time points after right T10 hemisection: 1, 3, and 6 weeks, and uninjured controls. Animals were stimulated at C fiber stimulation strength (5 mA, 250  $\mu$ s pulse width, 1 and 5 Hz) at DCNs L01, T12, T08, and T06 bilaterally and both the A $\delta$  and C-fiber evoked left CTM neurogram signals were recorded and processed. The profile of the hemisection was reconstructed in the cross-sectional plane. Responses were compared between injured (contralateral to CTM recording site) and uninjured (ipsilateral) sides.

The contralateral/ipsilateral early response ratio was significantly higher 6 weeks after injury at L01 (1 Hz) and the late response ratio was significantly higher at T08 (5 Hz) 6 weeks after injury compared to 3 weeks after injury. Analysis of individual sides showed more significant changes on the left side (contralateral to hemisection) and above the level of injury. There was no correlation between extent of hemisection and neurogram, suggesting that anatomy does not easily predict physiology. An increased

stimulus response could be characterized as “nociceptive hyperreflexia” and raises the possibility of the CTM reflex as an animal model for neuropathic pain after SCI.

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## 1. Introduction

Spinal cord injury (SCI) often results in severe motor impairment. Additionally, patients typically lose bowel, bladder, and sexual function. Pain very often accompanies SCI and further contributes to decreased quality of life for patients. Alterations in pain perception can occur as the appearance of abnormal pain (hyperalgesia or allodynia) or loss of normal pain sensation (protective pain). Changes in motor function after SCI have received the most research attention but alterations in sensory processing are important as well. Both can manifest as loss of function (paralysis and sensory loss) and gain of function (spasticity and neuropathic pain) phenomena. Changes in sensory processing can often lead to chronic pain, a feature of SCI that persists well beyond the initial pain of injury, and is described as stabbing, burning, or electric like pain (Sidall et al, 2003). Many of these clinical outcomes are due to the expression of neuronal plasticity. Advances in our understanding of neural plasticity could greatly improve quality of life of SCI patients.

Previous research has failed to establish whether the neural plasticity leading to these changes is physiological (change in synaptic strength), anatomical (sprouting with altered connectivity) or a combination of both (Tansey *et al.* 2012). Many SCI studies to date have focused on detecting an anatomical change and correlating it with a behavioral alteration after injury. Often overlooked, however, is the underlying physiology. Quantitative physiology is essential to explain how the anatomy functions biologically and ultimately determines behavior.

Some previous studies have investigated synaptic and cellular physiology of sensory processing in the dorsal horn after spinal cord injury (Gwak and Hulsebosch,

2011; D’Mello and Dickenson, 2008). Our understanding of sensory processing in the spinal cord could benefit from studying a multi-synaptic neural circuit that changes after injury and that can be anatomically and physiologically quantified.

The cutaneous trunci muscle (CTM) is a thin wide sheet of skeletal muscle that begins bilaterally on the humerus and inserts beneath the dermis of the back skin. The CTM produces a skin “shrug” in response to nociceptive cutaneous stimulation. The CTM reflex is mediated by a 3 neuron circuit: C and A $\delta$  afferents in segmental dorsal cutaneous nerves (DCNs); ascending propriospinal interneurons; and the CTM motoneuron pool (Figure 1). The CTM was first characterized in rats and guinea pigs (Borgens *et al*, 1990; Nixon *et al*, 1984). Nixon *et al*. demonstrated that the reflex had both an A delta and a C fiber sensory afferent component. The reflex exhibits “local sign” character due to the segmental distribution of the DCNs that enter the spinal cord in the lower thoracic and lumbar levels. The motor output originates from the CTM motor neuron pool in a region of the cervical spinal cord. The CTM motor neuron pool is located in a thin column in the ventrolateral edge of the ventral horn from the caudal end of segment C6 to the rostral end of segment T1 (Therriault and Diamond, 1988).

The CTM reflex can be quantified by behavioral observation, by electromyography in the CTM itself or by recording the neurogram in a CTM nerve branch in response to electrical stimulation of individual DCNs. The neurogram demonstrates both an A $\delta$  and a C fiber mediated component. The CTM reflex has been used to study peripheral nerve sprouting (Nixon *et al*, 1984), dorsal root injury (Jiang *et al*, 2003), and spinal cord injury (Borgens and Blight, 1993; Bohnert *et al*, 2007). Few

studies, however, have focused on quantifying the CTM reflex using electromyography or neurography.

Several properties make the CTM reflex an attractive model for neural plasticity after spinal cord injury. First, the CTM motoneuron pool is advantageously located above and away from most sites of SCI in animal models. The motor output originating in the cervical cord should not be affected by an injury in thoracic segments. Further, the reflex can be quantified by recording the neurogram from a CTM muscle nerve branch in response to DCN electrical stimulation. DCNs can be stimulated to selectively activate A $\delta$  afferents or both A $\delta$  and C fibers and can be stimulated singly or with other DCNs. Selective activation of input axon type and number permits the determination of necessary and sufficient inputs to get a particular output. In the present work, 1 DCN at a time was stimulated at sufficient strengths to activate both A $\delta$  and C fibers.

Mid-thoracic SCI disrupts this neural circuitry, but the reflex may show some level of recovery. Additionally, the reflex is bilateral but not asymmetric such that there is a stronger contraction on the ipsilateral side of stimulation (Borgens and Blight, 1993). This asymmetry can be exploited to determine how the reflex changes after spinal cord injury by comparing contralateral/ipsilateral stimulus response ratios.

In a pilot study of 8 animals (unpublished data), a lateral hemisection of the T10 spinal cord was performed either ipsilateral (n=4) or contralateral (n=4) to the side of CTM nerve recording (left). One week later CTM neurograms were recorded during ipsilateral (left) and contralateral (right) L1, T12, T8, or T6 DCN stimulation. With ipsilateral hemisection, two patterns of injury to the CTM reflex were seen (data not shown). In 2 animals, no CTM reflex could be evoked from stimulation of T12 or L1

DCNs on either side. In 2 animals, only trace CTM reflexes could be evoked from ipsilateral DCN stimulation below the level of injury. There were no apparent marked differences in the extent of hemisection amongst these animals.

With contralateral hemisection, CTM reflexes could be evoked from both ipsilateral and contralateral DCN stimulation below the level of hemisection in all 4 animals. This indicates that DCN input on the right side of the body crossed the midline at segmental levels to ascend in the left side of the spinal cord to reach the left CTM motor nucleus as was reported by Blight *et al.* In 2 animals, the size of the CTM neurograms evoked by contralateral T12 and L1 DCN stimulation was smaller than expected relative to the neurograms evoked by ipsilateral T12 and L1 DCN stimulation (when compared to that ratio in uninjured animals). In the remaining 2 animals, however, the size of the CTM neurogram evoked by contralateral T12 and L1 DCN stimulation was actually larger than expected relative to the neurograms evoked by ipsilateral T12 and L1 DCN stimulation (when compared to that ratio in uninjured animals). This increase in CTM reflex recovery could be considered “hyper-reflexic” and raises the interesting possibility that the CTM reflex could be used as an animal model for neuropathic pain after SCI.

The present study expands on the pilot data by studying the CTM reflex in more animals, at more time points after injury, and with 2 stimulation frequencies. Further, our goal is to study the temporal dynamics of the CTM reflex using more sophisticated data analysis software.

## 2. Methods

### 2.1. Animals

Female young adult Long Evans rats (n=24, 220-240 g) were used in this study as approved by the institutional animal care and use committee. Animals were housed in pairs and were given food and water *ad libitum*.

### 2.2. Spinal cord hemisection surgeries

Animals were anesthetized with ketamine/dexmedetomidine (0.75 mL/kg/0.5 mL/kg) delivered intraperitoneally in the lower left quadrant. Next, the animals' backs were shaved and wiped with isopropyl alcohol and betadine 3 times each. The animals were then transferred to a heating pad on the surgery table where body temperature was maintained at 37°C via feedback control from a rectal probe.

An incision was made along the midline using a #15 surgical blade and paraspinal muscles at T9 were retracted to expose the T9 vertebra. A T9 laminectomy was then performed with rongeurs to expose the T10 spinal cord. A lateral hemisection on the right side (contralateral to the recording of the CTM neurogram) was performed at T10 with a sharp micro scalpel (Fine Science Tools). The blade was inserted at the midline and drawn laterally. Gel foam was placed over the hemisected spinal cord and that was then covered with a small piece of the dorsal fat pad. The paraspinal muscles were then approximated with absorbable Vicryl sutures and the skin edges rejoined with surgical staples.

Post-operatively, animals were given injections of atipamazole (2 mg/kg), a reversal agent, 0.03 cc of enrofloxacin, an antibiotic, and 3 cc of warm sterile saline.

Antibiotic injections were continued 3 days post-operatively and saline injections were continued 2 days post-operatively. The animals' bladders were expressed twice daily until spontaneous voiding recovered and the surgical staples were removed 10 days after surgery. Animal locomotion was characterized 24 hours after injury and weekly thereafter using the Basso, Beattie, and Bresnahan (BBB) Locomotor Rating Scale (Basso *et al.*, 1995). While the BBB scale was not designed for use with hemisection injury, BBB scores were useful to identify any animals with very severe or very mild behavioral deficit, concerning for an over or under hemisection. Two animals from each experimental group were discarded because of suspected over or under hemisection as estimated by BBB scores.

### 2.3. CTM recordings – Surgery

Four experimental groups (n=8 per group) were analyzed at 3 different time points after injury: 1, 3, and 6 weeks, and uninjured controls. Animals were anesthetized with pentobarbital (50 mg/kg) delivered intraperitoneally in the lower left quadrant and surgically prepared for electrophysiological recording. The animal's backs were shaved from the nape of the neck to the pelvic girdle and then transferred to a heating pad on the surgery table. Body temperature was maintained at 37°C with a heating pad and feedback control via a rectal probe. An incision was made from the nape of the neck to the lumbar spine with a #15 surgical blade. The skin was retracted and DCNs L1, T12, T08, and T06 on both the left and right side were exposed and freed from surrounding fascia as they exit the body wall to run into the skin (Figure 2). DCNs T1-5 were cut to expose the

underlying CTM muscle and nerve and a branch of the CTM motor nerve on the left side (usually the 3<sup>rd</sup>) was freed from the muscle belly using #5 forceps (Figure 3).

The animal was then transferred to the recording table (Figure 4) and the retracted back skin was used to form a pool for warm mineral oil to bathe exposed tissues. The DCNs were cut distally and draped over silver wire bipolar stimulating electrodes. The dissected branch of the CTM motor nerve was then cut and draped over silver bipolar recording electrodes. The experimental preparation is shown in Figure 4. Pentobarbital boosters of 10% of the initial dose were given intraperitoneally as needed to maintain stable anesthetic level.

#### *2.4. CTM recordings – Stimulation*

The DCNs were individually stimulated at C-fiber strength (5 mA, 250  $\mu$ s pulse width) at 1 Hz for 20 seconds and then at 5 Hz for 10 seconds to activate both A delta and C fiber afferents in the following order: L1-left, L1-right, T12-left, T12-right, T08-left, T08-right, T06-left, T06-right. At the end of several experiments the L01 DCNs were stimulated after T06 to confirm that there was no drift in the size of the L01 evoked responses over the course of the experiment. No difference in stimulus response was observed. The evoked CTM neurograms were amplified 10,000x (A-M Systems Model 3500 16 Channel Amplifier) and recorded on a USB data acquisitions board (National Instruments USB-6229). The stimulus signals and neurogram were recorded to the same file in LabVIEW (National Instruments) to assist with data analysis.

### *2.5. CTM recordings - Post processing*

The stimulus artifacts were zeroed in the stimulus response data. Next, the data was high pass filtered at 100 Hz and comb filtered at 60 Hz and 76.3 Hz (due to an ambient noise source). Wavelet denoising (Sym8, soft thresholding, Matlab) was performed on the data. The data was then separated into individual stimulus responses depending on the timing of the response, then rectified and windowed (Figure 5).

### *2.6. Stimulus response analysis windows*

For the earliest response, the analysis window from 3.5 to 25.5 ms was used and for the late response, 45.5 to 95.5 was used. These windows were chosen based on the location of the peaks of the respective responses. In all cases, the value of the signal in the window was calculated using the sum of the rectified signal. In simulations, this method performed better than the sum of the squared signal, or the square root of the sum of the squared signal in correlating to the total number of spikes in the neurogram (data not shown).

### *2.7. Hemisection characterization*

Following electrophysiological recording, uninjured animals were euthanized with sodium pentobarbital (“Euthasol” 200 mg/kg) overdose. Injured animals, still anesthetized animals from their terminal electrophysiological recordings were perfused trans-cardially with 300 cc of warm heparinized PBS and 500 cc of 4% Paraformaldehyde (PFA). The thoracic cord (T09-T11) was harvested and post-fixed for 12-24 hours in 4% PFA and cryoprotected for 6 hours in 10% sucrose and for 24 hours in



30% sucrose. Finally, the spinal cords were embedded in Optimum Cutting Temperature compound (Tissue-Tek) and sectioned into 20  $\mu\text{m}$  slices in the longitudinal plane on a cryostat (Leica CM1900).

The extent of hemisection was assessed in serial horizontal longitudinal sections spaced 100  $\mu\text{m}$  apart. Spinal cord sections were stained with a .1% solution of Luxol Fast Blue (Alfa Aesaer) in 95% alcohol and a .1% solution of Cresyl Violet (Sigma Aldrich) in deionized water. Sections were visualized under a light microscope (Nikon Eclipse 90i) at 2x magnification. The extent of hemisection was then measured using ImageJ software (National Institute of Health). Measurements of the width of the cord and of the injury were made and their ratio calculated. A representative image is shown in Figure 6A. A total percent hemisection value was calculated for each animal by averaging the ratio of injured to total cord width for each section throughout the spinal cord. Finally, the hemisection was reconstructed in the cross sectional plane in Adobe Illustrator (Adobe) (Figure 6B).

### *2.8. Statistical tests*

Standard parametric statistical tests were used to analyze the data. Stimulus responses in injured and uninjured animals were compared using one-way ANOVAs and Tukey's HSD post-hoc tests. Stimulus responses and hemisection measurements were correlated using Pearson's product-moment correlation coefficients. Results are presented as mean +/- standard deviation.

## 2.9. Contributions

Below is a list of everyone who assisted with present study and their primary contributions:

Patrick Malone: Animal care including pre-surgical and post-surgical preparations, operation of electrophysiology software during CTM recordings, bladder expressions, BBB testing, spinal cord harvesting, embedding, sectioning, staining, and imaging, hemisection analysis and reconstruction, design of MATLAB data analysis scripts, and electrophysiology and hemisection data analysis and statistics.

Keith Tansey: Hemisection surgeries and CTM recording surgeries.

Joon Li: BBB testing and perfusions.

Jason Tidwell: Perfusions and CTM recording surgeries.

Jason White: Design of LabVIEW electrophysiology recording software and MATLAB data analysis scripts.

### 3. Results

#### 3.1. Qualitative Analysis of CTM Neurogram after SCI

CTM neurograms for uninjured animals are displayed as false color images in Figure 7. The neurograms for all animals (N=8) were averaged. In each image, every row represents a single stimulus response (20 rows for 1 Hz and 50 rows for 5 Hz), with the first stimulation in the train is the top row and the last stimulation is the bottom row. There is a greater response with stimulation of the ipsilateral DCNs relative to contralateral ones regardless of stimulation frequency or DCN. There is virtually no C fiber response evoked from contralateral DCNs (with the possible exception of T06 at 5 Hz), and more C fiber response to 5 Hz stimulation of the ipsilateral thoracic DCNs relative to 1 Hz stimulation.

CTM neurograms of injured animals were compared to uninjured animals (Figure 8 A-D). There are several qualitative differences between the stimulus response of injured and uninjured animals. First, there is an emergence of a late response to contralateral DCN stimulation at 1 week and 6 weeks, and to a lesser extent, at 3 weeks after injury as compared to uninjured animals. The most obvious differences are in the responses evoked by stimulation of contralateral DCNs at T12, T08, and T06 at 1 and 5 Hz in both 1 and 6 week animals.

In response to ipsilateral stimulation, there is an increased late response at all DCNs and frequencies. The greatest increases in responses in 1 week animals occurred with stimulation below the level of injury at L01 and T12, in 3 week animals with stimulation at L01 and T08, and in 6 week animals with stimulation at all DCNs. Finally, there is an increase in background activity in 6 week animals as compared to uninjured

animals, especially after a 5 Hz stimulation train. This may represent an increased level of excitability in this pain circuit 6 weeks after SCI.

### *3.2. CTM reflex in uninjured animals*

The CTM reflex was first quantified in uninjured animals. Figure 9 shows the contralateral/ipsilateral early and late response ratios in uninjured animals (N=8) at 1 and 5 Hz. Ratios were calculated by dividing the maximum normalized response evoked with contralateral stimulation by the maximum normalized response evoked with ipsilateral stimulation at each DCN. The reflex is asymmetric such that there is a larger response to ipsilateral stimulation, and the contralateral evoked late response only grows close to 100% of the ipsilateral evoked response at T06. The reflex is highly variable, more so with T06 stimulation. The standard deviations of the contralateral/ipsilateral ratios range from +/- 0.10 to 0.33 for different stimulation parameters at L01, T12, and T08, and from +/- 0.35 to 0.44 at T06. Conversely, at L01, the early response to contralateral stimulation is only about 20% the early response to ipsilateral stimulation. The maximum response evoked from individual sides and DCNs is also numerically highly variable and more so at T06 with standard deviations at T06 ranging from +/- 1.21 to 3.65 for the ipsilateral side and from +/- 0.78 to 1.21 for the contralateral side.

### *3.3. CTM reflex in injured animals – Contralateral/ipsilateral response*

Contralateral/ipsilateral ratios in animals 1, 3, and 6 weeks after injury were calculated using the maximum normalized response to stimulation at each side and at each DCN (Figure 10 A-C). There was less variability in the ratios in the injured

compared to uninjured animals. The average standard deviation across all DCNs, frequencies, and windows was +/- 0.276 in uninjured animals, +/- 0.220 at 1 week, +/- 0.209 at 3 weeks, and +/- 0.176 at 6 weeks.

To determine how the ratios of responses to stimulation at each DCN were altered after injury, one-way ANOVAs were performed for each DCN and early and late responses. There were significant differences at L01 (1 Hz, early response) ( $F(3, 28) = 4.101, p = 0.016$ ) (Figure 11A), and T08 (5 Hz, late response) ( $F(3, 28) = 3.239, p = 0.037$ ) (Figure 11B). Trends towards differences were seen at L01 (5 Hz, early response) ( $F(3, 28) = 2.847, p = 0.055$ ), T06 (1 Hz, late response) ( $F(3, 28) = 2.733, p = 0.062$ ), T06 (5 Hz, late response) ( $F(3, 28) = 2.152, p = 0.116$ ), and L01 (5 Hz, late response) ( $F(3, 28) = 1.712, p = 0.187$ ). Tukey's HSD post-hoc comparisons were done to determine which time points after injury were different. Post-hoc analysis revealed that the ratio of contralateral/ipsilateral early response to stimulation at L01 (1 Hz) for 6 week animals ( $M = 0.5573, SD = 0.18$ ) was significantly higher than in uninjured animals ( $M = 0.2162, SD = 0.10, p = 0.009$ ). Additionally, the ratio of late response to stimulation at T08 (5 Hz) in 6 week animals ( $M = 0.7214, SD = 0.21$ ) was significantly higher than 3 week animals ( $M = 0.3797, SD = 0.26, p = 0.026$ ).

#### *3.4. CTM reflex in injured animals – Individual responses*

To determine which specific DCNs, and on which side, showed altered responses to stimulation after injury, one-way ANOVAs were performed for each DCN, side, frequency and window to compare injured animals to uninjured controls. The results of significant one-way ANOVAs and Tukey's HSD post hoc analysis are shown in Table 1.

The maximum stimulus response in each train was used for comparisons. Interestingly, all significant changes were on the left side (contralateral to hemisection, ipsilateral to recording site) and most alterations occurred with stimulation above the level of injury. Significant differences in the early response to stimulation on the left side at 1 Hz were observed at L01 ( $F(3,28) = 3.3134$ ,  $p = 0.041$ ) and T08 ( $F(3, 28) = 6.792$ ,  $p < 0.001$ ), in the late response at 1 Hz at T12 ( $F(3,28) = 3.695$ ,  $p = 0.023$ ), T08 ( $F(3,28) = 4.408$ ,  $p = 0.012$ ), in the early response at 5 Hz at T08 ( $F(3,28) = 5.553$ ,  $p = 0.004$ ) and T06 ( $F(3,28) = 4.006$ ,  $p = 0.017$ ), and in the late response at 5 Hz at T08 ( $F(3,28) = 11.282$ ,  $p < 0.001$ ).

Tukey's HSD post-hoc analysis was performed to determine which groups' stimulus responses were significantly changed. The late response to stimulation at 1 Hz at T12 on the left side was significantly higher 6 weeks after injury as compared to uninjured animals ( $p = 0.12$ ). Three weeks after injury, there was a significantly elevated response to stimulation at T08 on the left side in both the early ( $p = 0.022$ ) and late response ( $p = 0.033$ ) at 1 Hz and in the late response to stimulation at 5 Hz ( $p < 0.001$ ) as compared to uninjured animals. There was a decreased early stimulus response to stimulation at L01 on the left side at 1 Hz ( $p = 0.02$ ) 6 weeks after injury as compared to uninjured and to stimulation at T08 on the left side at 5 Hz as compared to 1 week ( $p = 0.037$ ) and 3 weeks ( $p = 0.005$ ) after injury. Finally, there was an increased early response to stimulation at T06 on the left side 3 weeks after injury as compared to 1 week animals ( $p = 0.39$ ).

### *3.5. CTM reflex and hemisection – Anatomy vs. Physiology*

To discern relationships between the extent of the hemisection injury and the CTM reflex physiology, the hemisection of each animal was reconstructed in the cross sectional plane and the ratio of overall hemisection calculated from the reconstructions was compared to neurogram false color plots. Example false color plots and reconstructions for two animals 1 week after injuries are shown in Figure 12 A-B, respectively. Hemisections in animal 7 and 8 were 50.7% and 47.5%, respectively. Hemisection data for all animals and all experimental groups is shown in Table 2. Despite similar patterns of hemisections, the animals have different stimulus response patterns and no clear trend was seen in false color plots relative to slight over or under hemisections. Examples of qualitative differences include a greater late response to stimulation at 1 Hz on the left side at T08 in animal 7 compared to animal 8, and a greater late response to stimulation on the right side at T12 in animal 8 than in animal 7. Systematic evaluation of the relationship between extent of hemisection injury and CTM reflex physiology across all animals failed to show a correlation between injury area and reflex size. The lack of a clear relationship between extent of hemisection and stimulus response indicates that anatomy may not easily predict physiology after SCI.

#### 4. Discussion

We demonstrate here a multi-faceted neural circuit that shows changes in physiology after SCI. The analysis of the neurogram after injury allows a fine grain view of changes in sensory processing caused by neural plasticity not easily afforded by current anatomical methods. The reflex is anatomically distributed (rostral vs. caudal) and bilateral but asymmetric (ipsilateral vs. contralateral DCN activation). It results from the activation of both A $\delta$  and C-fiber afferents but these two afferent populations contribute to different portions of the reflex response. Finally, activation of these pain related afferents results in a quantifiable output. Any of these features can be altered after SCI and the input/output relationships in the reflex can characterize changes in the sensory processing of pain following SCI.

Previous studies utilizing the CTM reflex have studied it simply as a behavioral test (as either present or absent) after SCI (Muguet-Chanoit *et al*, 2011; Borgens *et al*, 2002). Nixon *et al*. and others have used the CTM neurogram to assess the reflex but have not performed any quantitative analysis. The present study is the first to quantitatively characterize the CTM reflex evoked by activation of A-delta or C fibers in dorsal cutaneous nerves at different spinal levels before and after SCI.

Qualitative analysis of CTM neurograms at various time points after SCI revealed several important changes. There was an increased late response to stimulation at several DCNs at 1 and 6 weeks after hemisection. The late response to stimulation on the ipsilateral side increased at all DCNs and frequencies after injury. There was also an increase in background activity in 6 week animals as compared to uninjured animals, especially after a 5 Hz stimulation train.



Statistical analysis revealed that the maximum normalized contralateral/ipsilateral early response ratio to stimulation at L01 (1 Hz) increased over the course of 6 weeks after injury, and the response ratio was significantly higher in animals 6 weeks after injury as compared to uninjured controls. There was also an increase in the late response ratio to stimulation at T08 (5 Hz) in 6 weeks animals as compared to animals 3 weeks after injury.

Analysis of individual sides and DCNs revealed that most of the changes were occurring in response to stimulation on the ipsilateral side (relative to the recording site, contralateral to hemisection) and above the level of injury. Most of the changes in physiology occurred in both the early and late response to stimulation at T08 after both 1 Hz and 5 Hz stimulation. Correlational analysis showed that the extent of hemisection might not easily predict physiology.

The fact that qualitative analysis of CTM neurograms suggests changes in reflex response to stimulation on the contralateral side, yet statistical tests show no significant differences, raises the possibility that using the maximum normalized response may not be the best way to compare the data. The maximum response is especially sensitive to outliers. Other possible dependent variables that could be analyzed include the mean response, all stimulus responses in a train, or an average of each stimulus responses across all animals within an experimental group.

Of particular interest are responses to stimulation at DCNs in injured animals that showed an increased response as compared to uninjured controls. An increased response to stimulation of sensory afferents could be characterized as “nociceptive hyperreflexia.” This hyperreflexia might be explained by several possible mechanisms, including loss of

supraspinal modulation of reflex activity below the hemisection (Ossipov *et al*, 2000; Porreca *et al*, 2002), physiological changes in synaptic strengths between the DCN afferents and the propriospinal interneurons of the CTM pathway (Zimmerman 2001), or anatomical changes such as sprouting in either DCN afferent projections into the spinal cord (Mannion *et al*, 1996; Seltzer *et al*, 1990) or in the propriospinal interneurons of the circuit. We are developing a technique in our current studies (Figure 13) that will allow us to test if any anatomical plasticity of DCN afferents in the dorsal horn is related to any physiological plasticity measured in the neurogram of the reflex.

It is possible that an increased response to stimulation of DCNs after injury could be related to the development of neuropathic pain after SCI. The mechanisms responsible for the plasticity in CTM afferents could play a role in the more complex process that is perceived as pain. In order to characterize an increased stimulus response as neuropathic pain, future studies should correlate changes in physiology with the Von Frey assay, a classical behavioral test to detect behaviorally neuropathic pain (Le Bars *et al*, 2001).

The CTM reflex may have some advantages over other models of neuropathic pain after SCI. Most current tests measure paw withdrawal in response to sensory inputs in the limbs below the level of a SCI. It is difficult to determine, however, whether an increased sensitivity in paw withdrawal is due to changes in sensory processing (hyperalgesia or allodynia) or motor changes (spasticity). The CTM motor output is advantageously located above the injury site and is likely unaffected, meaning that changes in sensorimotor function below the level of an injury are likely due to sensory afferent or interneuronal changes.

A significant limitation of the present study is the variability of the CTM reflex. The variability could be due to several factors. Animals received anesthetic doses according to their weight, but a different number of 10% boosters were given to each animal to maintain a full level of anesthesia. Animals may respond to the anesthetic dose differently due to biological differences in how the drug is metabolized or the time of day of surgery. There is also variability in the experimental preparation. A different number of axons may be stimulated or recorded according to how the electrodes are positioned on the DCNs or CTM motor nerve.

The distribution of A $\delta$  and C-fiber afferents in DCNs could also contribute to the inconsistency of the CTM neurogram. We have histologically characterized the numbers and types of peripheral axons across the DCNs (unpublished results not shown). The total number of afferents from animal to animal is consistent, however the segmental distribution of A delta and C fibers across DCNs is variable. This variability could contribute to the variability of the CTM neurogram.

An important future direction of our current avenue of research is combining the CTM reflex as a physiological model of neural plasticity with anatomical changes after SCI. Correlating changes in CTM reflex physiology and plastic changes in afferents is essential to paint a full picture of plasticity after SCI. There is an ongoing set of investigations in our lab to determine if the central projections of A-delta and C fibers are different at each DCN or if their connections change differentially in response to injury. Preliminary results of this line of research are shown in Figure 13. The central projections of isolectin B4+ (IB4) positive C fibers in uninjured animals are shown following injection of the tracer into the T7 and T13 DCN. IB4 marks approximately half of the C

fiber population (Fang *et al*, 2006). T7 and T13 C fiber projections are found in laminae I and II but their location in a medial/lateral dimension are different (left side of the figure), as are their density and rostral/caudal distribution (right side of the figure for a n=2). Current studies are focusing on the characterization of A-delta fiber projections with cholera toxin subunit B (CTB) tracing (data not shown) and to determine how many of these projections are making synapses (using synaptic markers, data also not shown).

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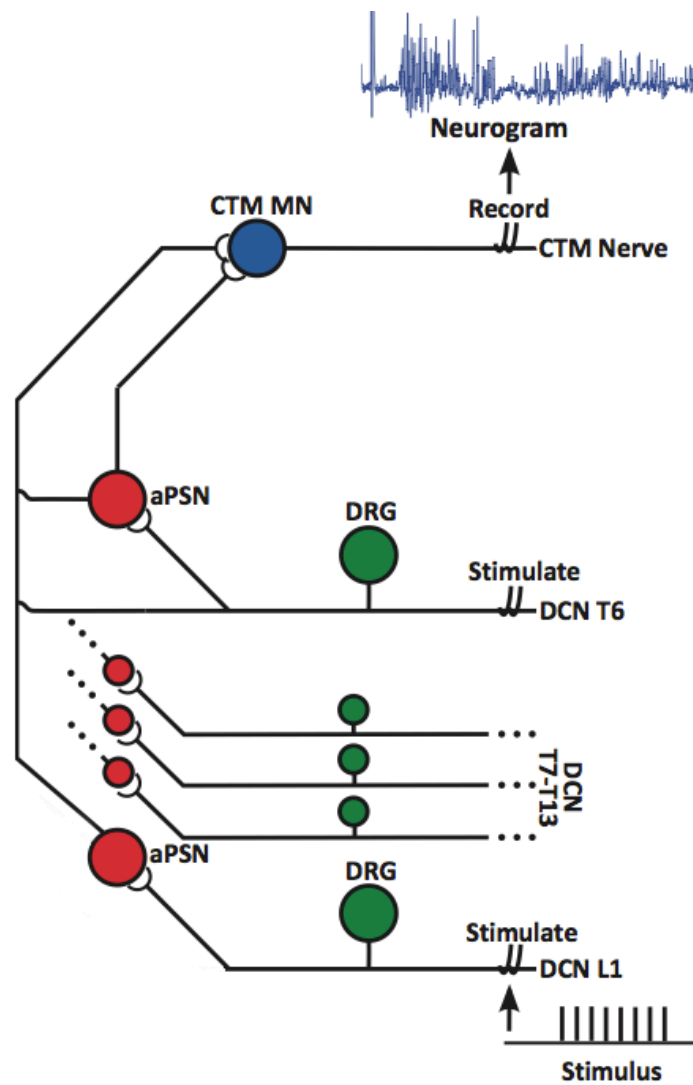
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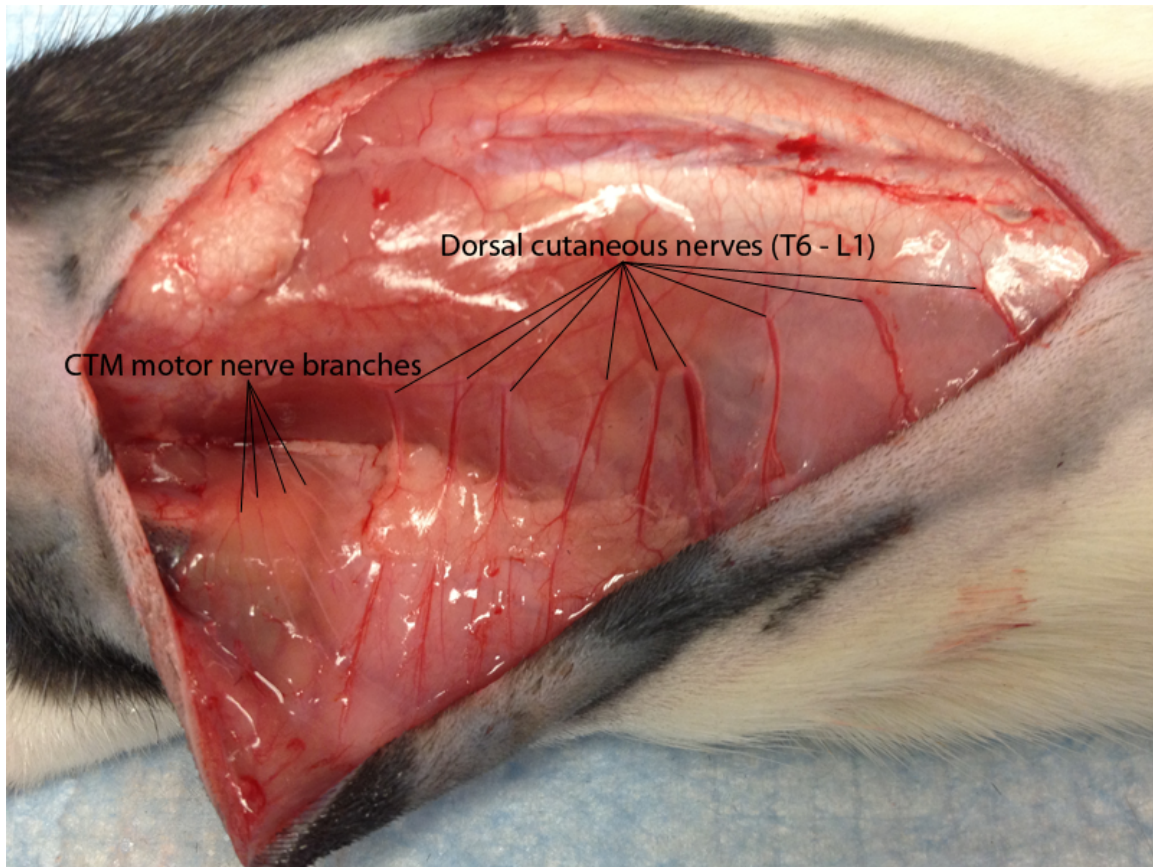
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## 6. Tables and Figures

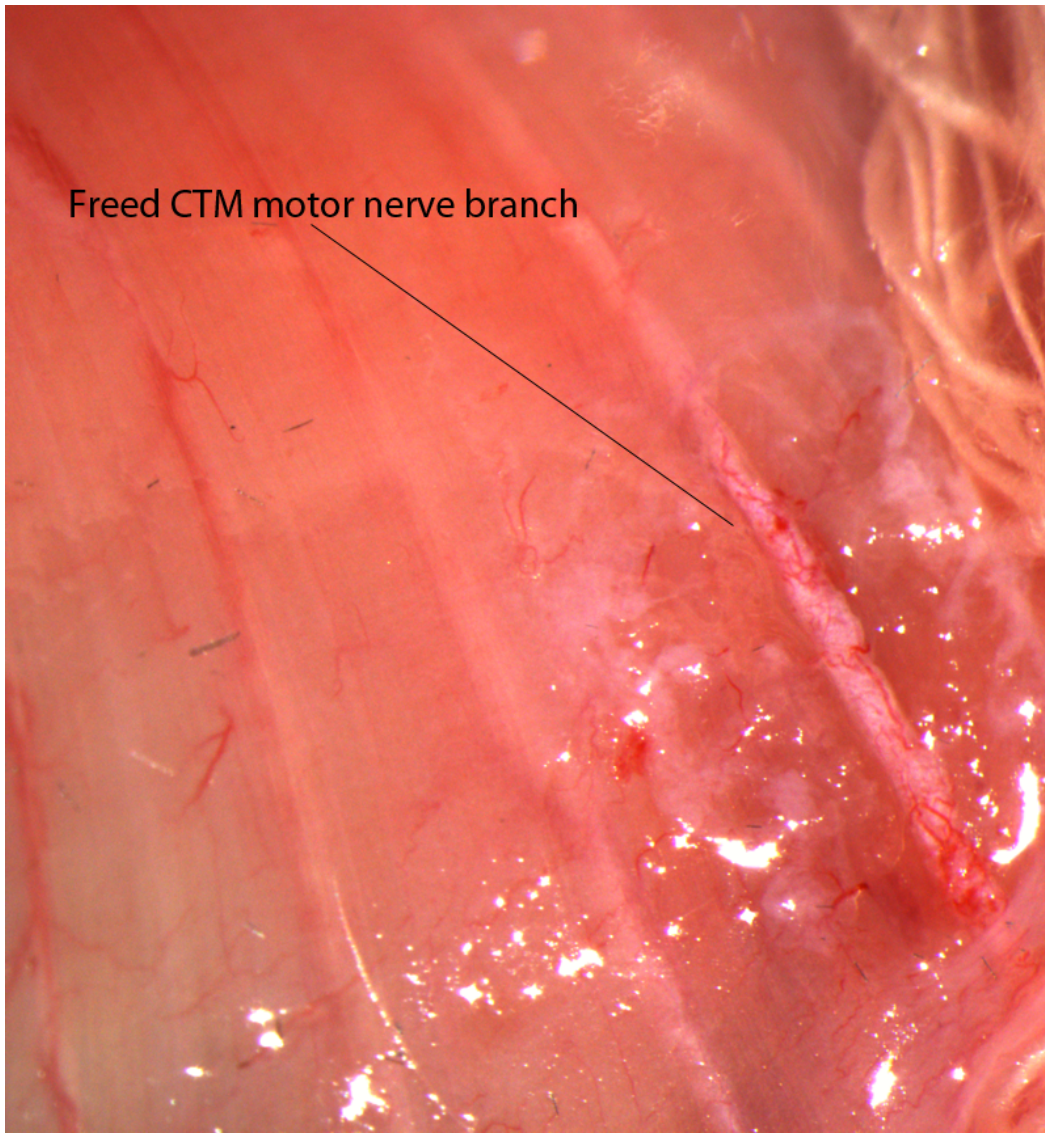


**Figure 1 CTM reflex circuit diagram.** The reflex is mediated by C-fiber and A $\delta$  afferents in segmental DCNs, ascending propriospinal interneurons, and the CTM motoneuron pool. DCNs were stimulated at each segment and the neurogram was recorded from the CTM motor nerve.

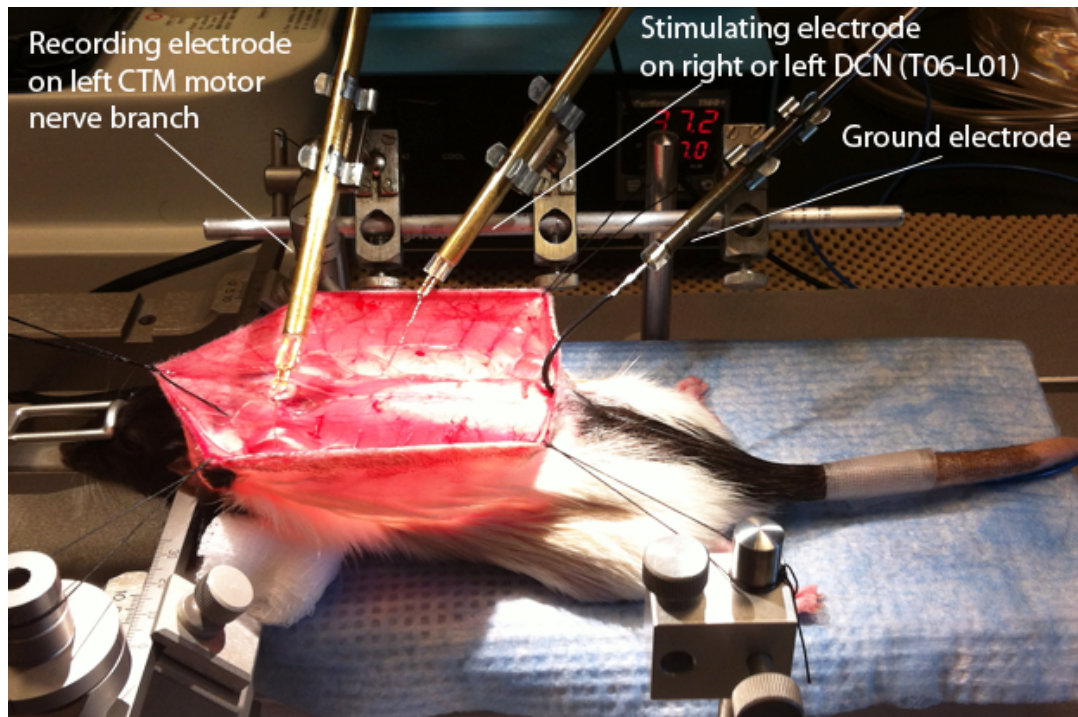




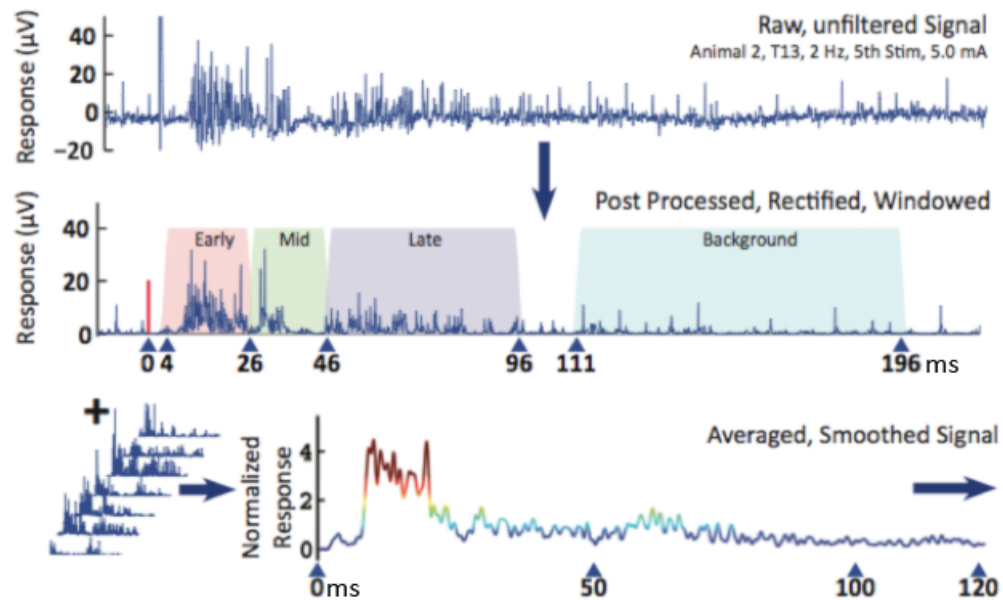
**Figure 2** Dorsal cutaneous nerves (DCNs) and CTM motor nerve branches. DCNs T6-L01 and CTM motor nerve branches 1-5 are shown.



**Figure 3** Dissected branch of CTM motor nerve. Usually the third branch of the CTM motor nerve was freed.

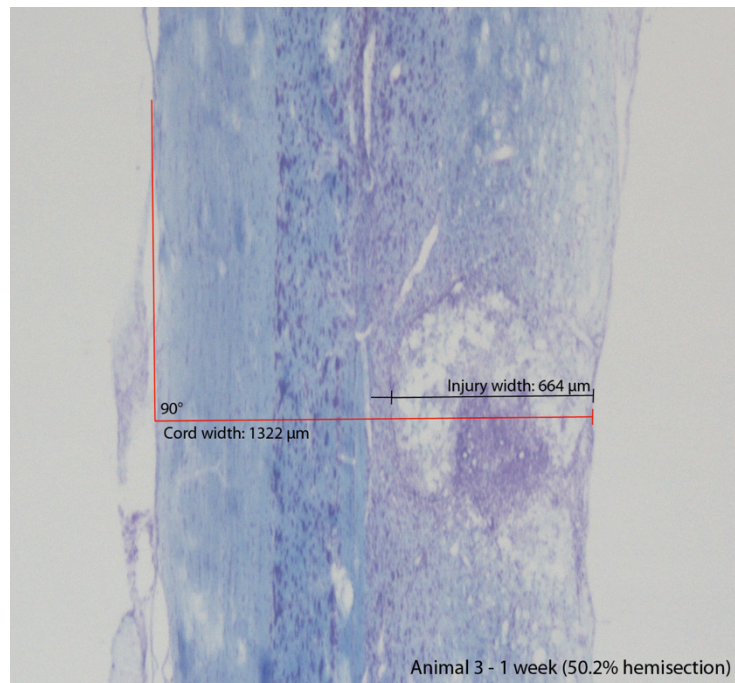


**Figure 4** Experimental preparation. Animals were stimulated at the left (ipsilateral to the recording site) or right (contralateral to the recording site) at T06, T08, T12, or L01 with a silver bipolar stimulating electrode. The neurogram was recorded from the left CTM motor nerve branch with a silver bipolar recording electrode. The retracted back skin was used to make a mineral oil pool to bathe the exposed tissues.

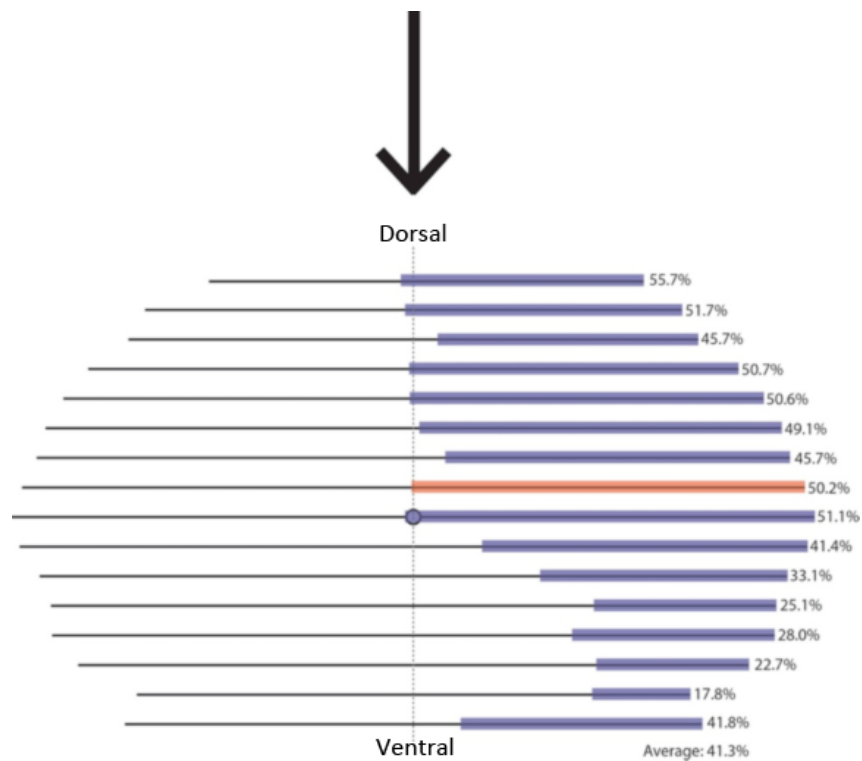


**Figure 5** Neurogram processing. Top shows the unfiltered, unprocessed stimulus response. The artifacts were zeroed, the signal was filtered and wavelet denoised, then the signal was rectified. Then the data was either windowed and integrated or normalized and averaged with the other animals to produce a mean response.

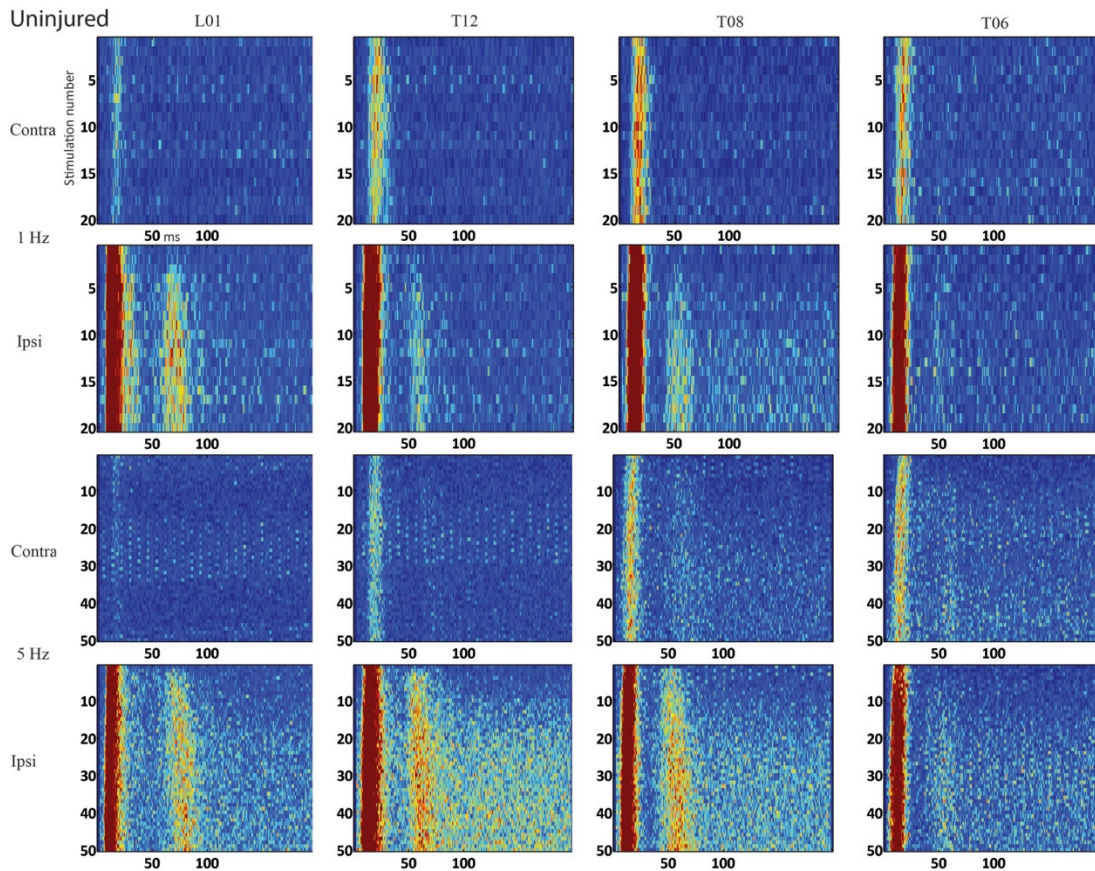
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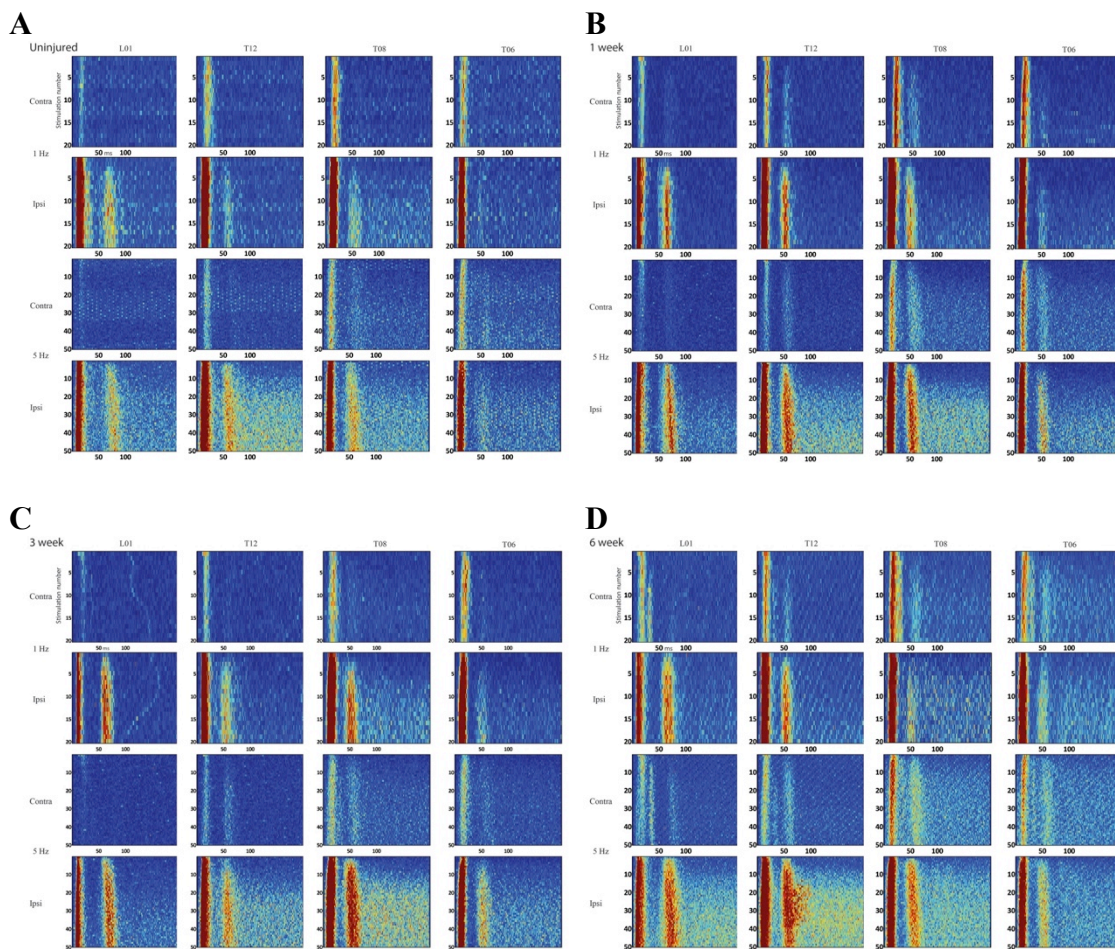
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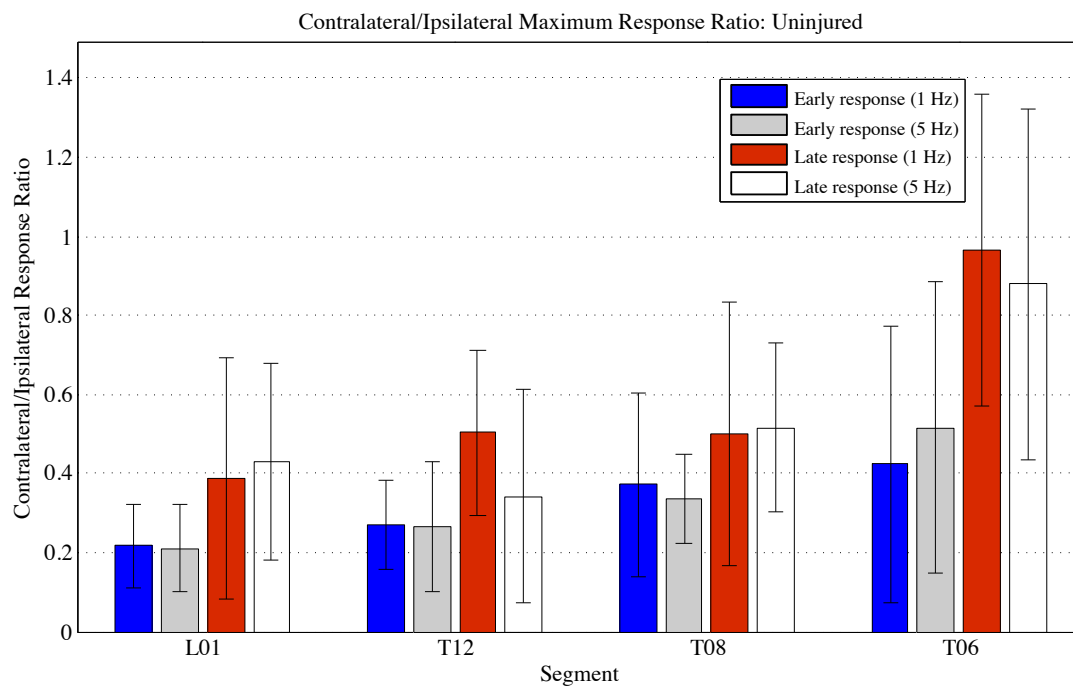
**Figure 6 A-B** Representative image of hemisection staining and measurement. (A) Spinal cords were sectioned in the horizontal longitudinal plane and stained with Luxol Fast Blue and Cresyl violet. (B) Spinal cords were reconstructed in the cross sectional plan. Slices are 100  $\mu\text{m}$  apart.



**Figure 7** False color plots of the averaged CTM neurograms from uninjured animals. Red represents the maximum normalized response and blue represents the minimum. Each row within each image represents a single stimulus response (20 for 1 Hz and 50 for 5 Hz). The first stimulation in the train is the top row, the last stimulation is the bottom row within each image. Each column of images are the responses from stimulation of a specific DCN. The top two rows show responses from 1 Hz stimulation and the bottom two rows from 5 Hz stimulation. Within each stimulation frequency the top row shows responses from stimulating DCNs contralateral to the recording site and the bottom row from stimulating DCNs ipsilateral to the recording site. Shown is a greater response with stimulation of the ipsilateral DCNs relative to contralateral ones regardless of stimulation frequency. There is virtually no C fiber response evoked from contralateral DCNs, and more C fiber response in response to 5 Hz stimulation of the ipsilateral DCNs.



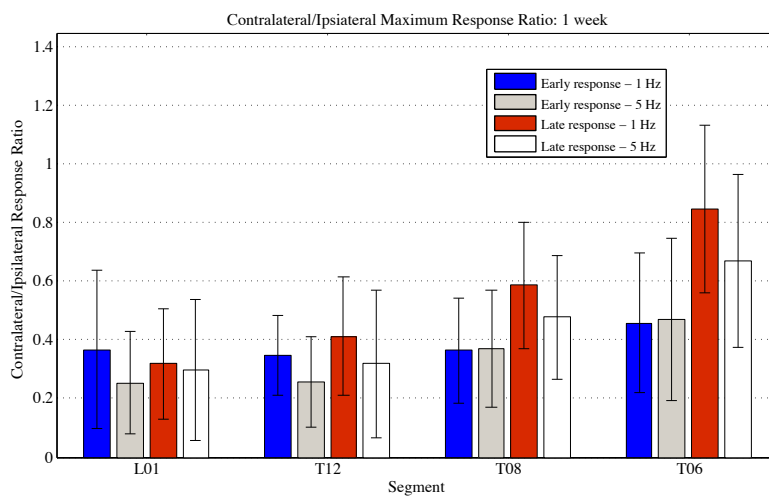
**Figure 8 A-D** False color plot of normalized stimulus responses (as per Figure 7) in uninjured (A), 1 week (B), 3 week (C), and 6 week (D) animals. In general, there appears to be an increase in the CTM reflex following SCI. Shown is an emergence of a late response evoked from contralateral DCNs after injury, as well as an increase in the late response evoked from stimulation of ipsilateral DCNs. There also appears to be an increase in background activity in 6 week animals as compared to uninjured animals, especially after 5 Hz stimulation of DCNs.



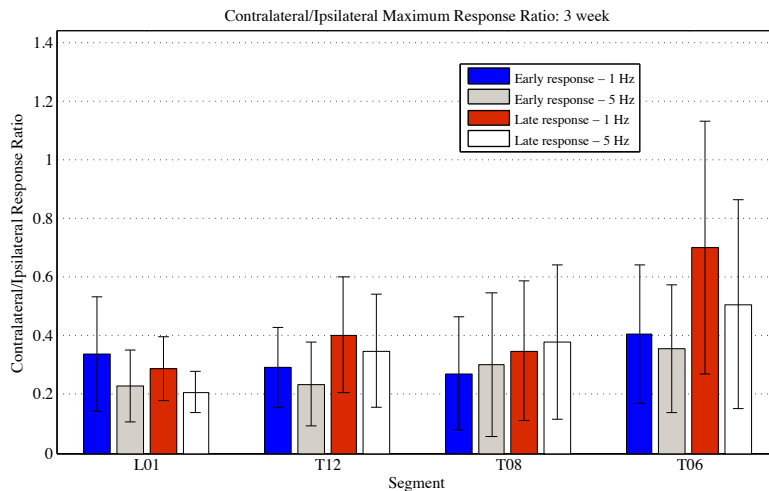
**Figure 9** Contralateral/ipsilateral maximum normalized early and late response ratio in uninjured animals (N=8) at 1 and 5 Hz. Error bars are standard deviation. Response ratios are numerically highly variable and approach 1 at T06. Conversely, at L01, the early response to contralateral stimulation is only about 20% the early response to ipsilateral stimulation.



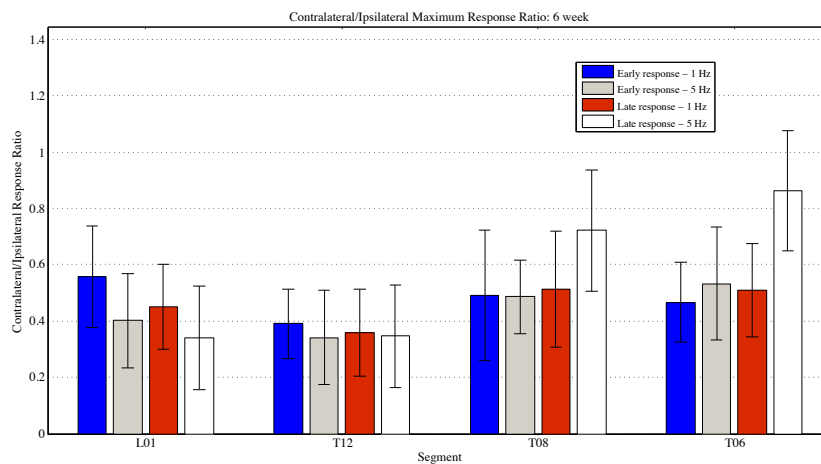
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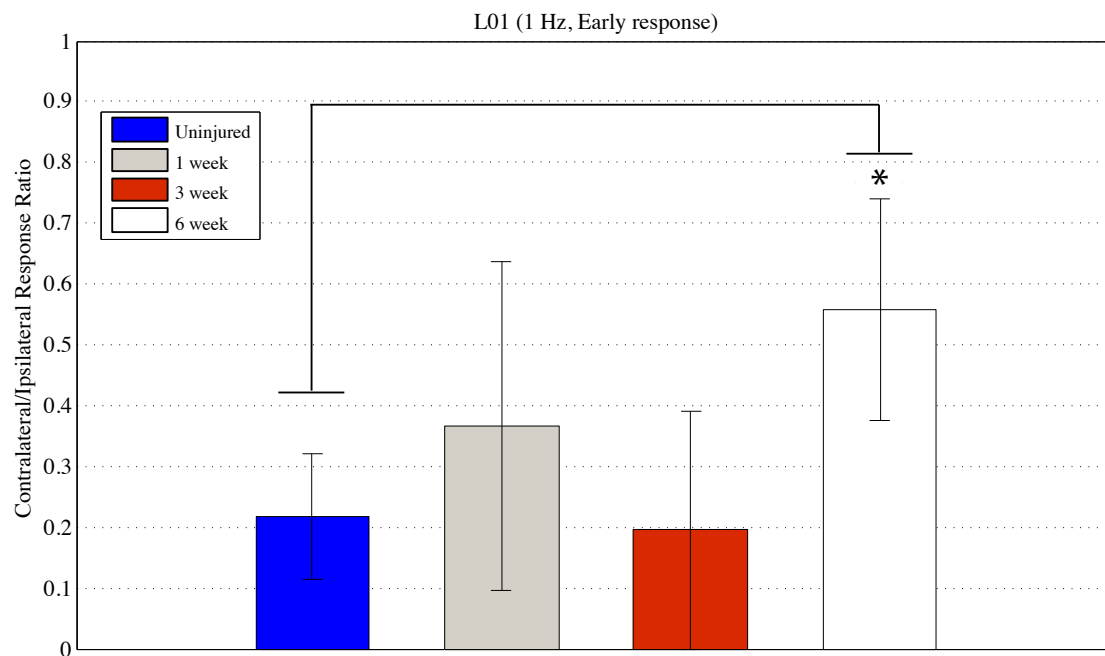


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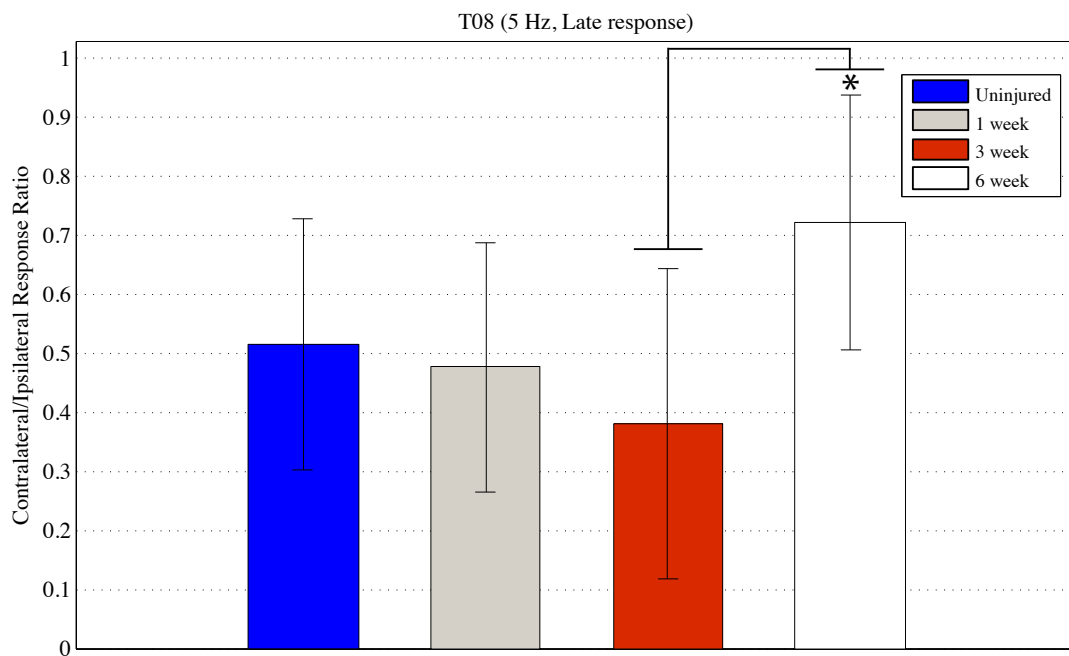


**Figure 10 A-C** Normalized contralateral/ipsilateral early and late response ratios in injured animals (N=8) 1 week (A), 3 weeks (B), and 6 weeks (C) weeks after injury at 1 and 5 Hz. Error bars are standard deviation.

A



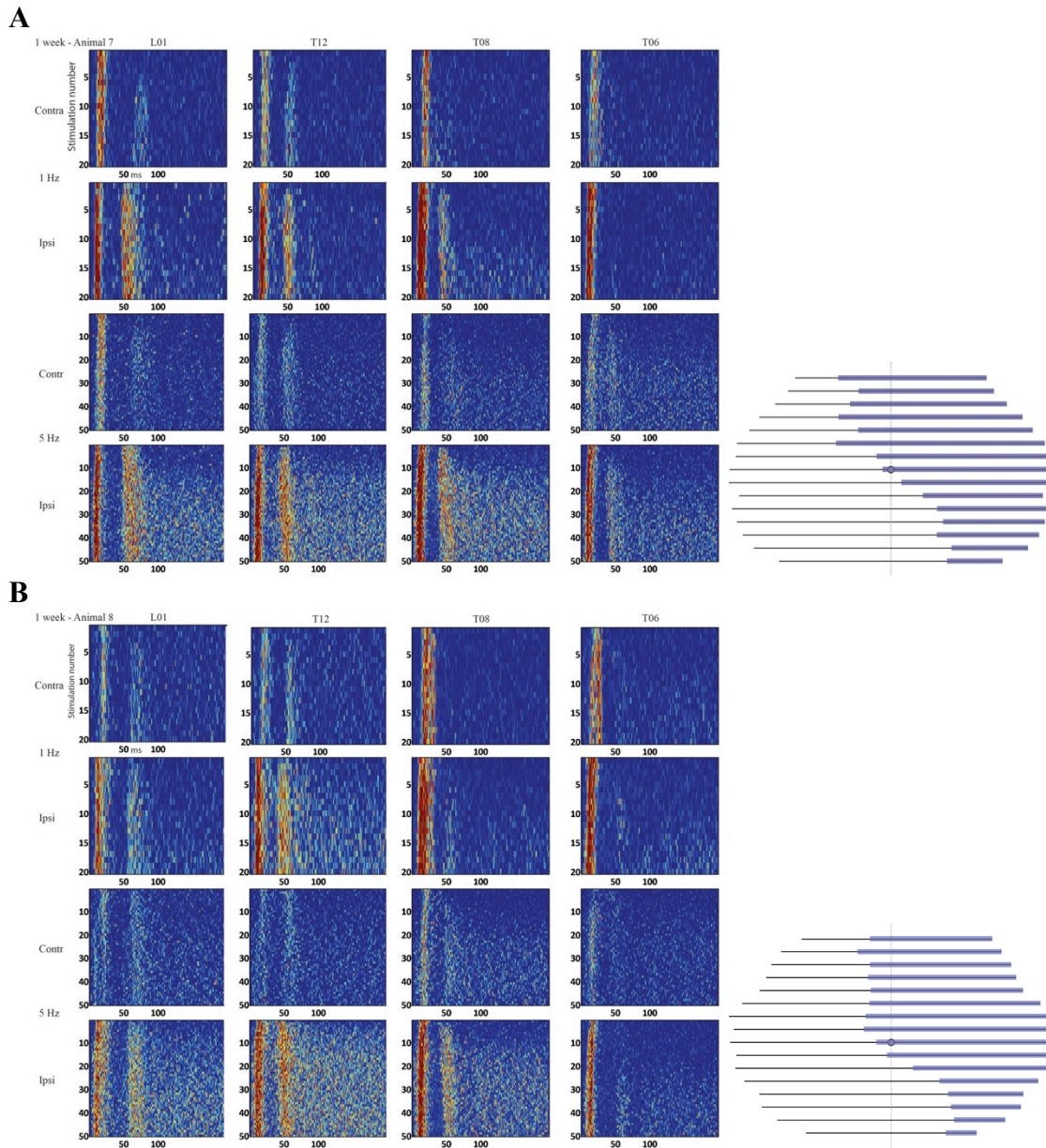
B



**Figure 11 A-B** Contralateral/ipsilateral maximum normalized response ratios at L01 (1 Hz, early response, A) and T08 (5 Hz, late response, B). Responses were significantly higher at L01 at 6 weeks as compared to uninjured animals and at T08 at 6 weeks as compared to animals 3 week after injury. Error bars are standard deviation.

<b>DCN</b>	<b>One-way ANOVA</b>	<b>Post hoc test</b>
<b>L01-left (1 Hz, early response)</b>	$F(3,28) = 3.3134, p = 0.041$	6 week (M = 2.710, SD = 1.16) is less than uninjured (M = 5.239, SD = 1.71, $p = 0.02$ )
<b>T08-left (1 Hz, early response)</b>	$F(3, 28) = 6.792, p < 0.001$	3 week (M = 8.362, SD = 2.45) is greater than uninjured (M = 5.489, SD = 1.37, $p = 0.022$ ) and 6 week (M = 4.697, SD = 1.19, $p = 0.003$ )
<b>T12-left (1 Hz, late response)</b>	$F(3,28) = 3.695, p = 0.023$	6 week (M = 4.413, SD = 1.11) is greater than uninjured (M = 2.428, SD = 1.00, $p = 0.012$ )
<b>T08-left (1 Hz, late response)</b>	$F(3,28) = 4.408, p = 0.012$	3 week (M = 4.540, SD = 1.47) is greater than uninjured (M = 2.765, SD = 1.13, $p = 0.033$ )
<b>T08-left (5 Hz, early response)</b>	$F(3,28) = 5.553, p = 0.004$	6 week (M = 4.267, SD = 0.60) is lower than 1 week (M = 6.676, SD = 2.00, $p = 0.037$ ) and 3 week (M = 7.367, SD = 2.31, $p = 0.005$ )
<b>T06-left (5 Hz, early response)</b>	$F(3,28) = 4.006, p = 0.017$	1 week (M = 5.637, SD = 2.17) is higher than 6 week (M = 3.430, SD = 0.71, $p = 0.039$ )
<b>T08-left (5 Hz, late response)</b>	$F(3,28) = 11.282, p < 0.001$	3 week (M = 7.132, SD = 1.59) is higher than uninjured (M = 4.341, SD = 1.03, $p < 0.001$ ), 1 week (M = 5.436, SD = 1.24, $p = 0.045$ ) and 6 week (M = 2.372, SD = 0.62, $p < 0.001$ )

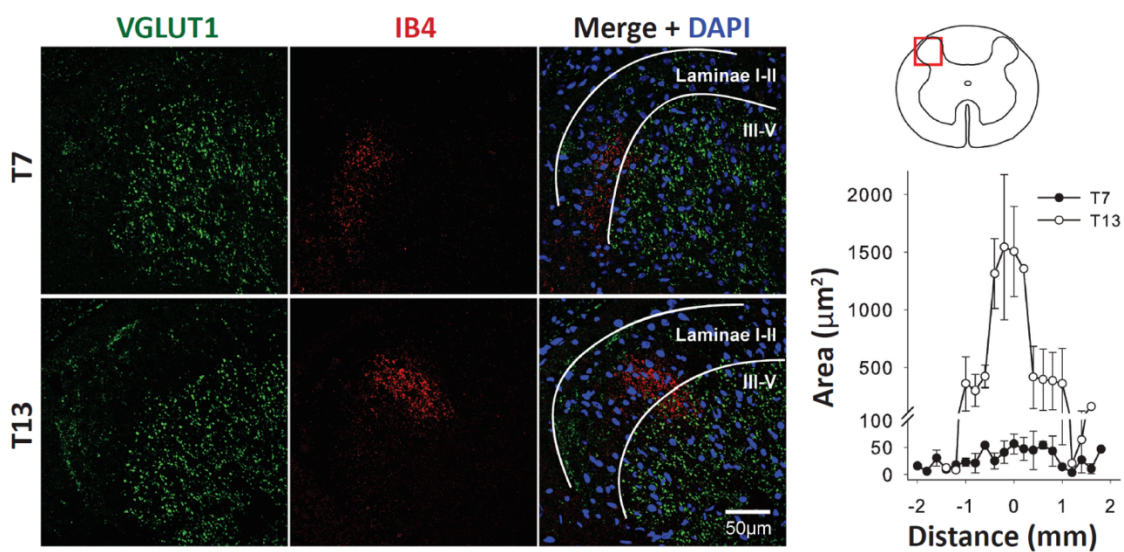
**Table 1** Statistical tests of individual DCNs and stimulation parameters comparing injured to uninjured animals. All post hoc tests are Tukey's HSD.



**Figure 12 A-B** False color plot of stimulus responses and hemisection reconstructions in the cross sectional plane for Animal 7 (A) and Animal 8 (B) one week after injury. Animal 7 is 50.7 % hemisected, and Animal 8 is 47.5 % hemisected. Despite similar patterns of hemisection the animals have different stimulus response patterns. Examples of qualitative differences include a greater late response to stimulation at 1 Hz on the left side at T08 in animal 7 compared to animal 8, and a greater late response to stimulation on the right side at T12 in animal 8 than in animal 7.

<b>Group</b>	<b>Animal</b>	<b>Hemisection (%)</b>
<b>1 week</b>	1	59.6
	2	50.0
	3	41.3
	4	44.6
	5	44.6
	6	51.6
	7	50.7
	8	47.5
	<b>Average:</b>	<b>48.7</b>
<b>3 week</b>	1	51.7
	2	42.2
	3	41.5
	4	42.1
	5	55.8
	6	45.3
	7	45.0
	8	60.1
	<b>Average:</b>	<b>48.1</b>
<b>6 week</b>	1	30.5
	2	34.4
	3	34.5
	4	48.9
	5	37.8
	6	45.1
	7	37.9
	8	39.4
	<b>Average:</b>	<b>38.6</b>

**Table 2** Hemisection data. Percent hemisection was calculated by averaging the hemisection of all sections within an animal.



**Figure 13** Central projections of IB4 positive C fibers in uninjured animals in the T7 and T13 DCN. The central projections of isolectin B4+ (IB4) positive C fibers in uninjured animals are shown following injection of the tracer into the T7 and T13 DCN. T7 and T13 C fiber projections are found in laminae I and II but their location in a medial/lateral dimension are different (left side of the figure), as are their density and rostral/caudal distribution (right side of the figure for a n=2).