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14. ABSTRACT Essentially all spinal cord injured patients receive stretching therapies beginning within the first few weeks post-injury. Despite this fact, almost nothing is known about how stretching might influence the neural circuitry in the spinal cord that is responsible for controlling the motor and locomotor activities of the legs. Recently, while studying activity-based rehabilitation in a rat model of spinal cord injury, we observed that stretching actually worsened locomotor recovery. The goal of this project is to investigate how the timing and intensity of a stretch-based therapy influences locomotor recovery after moderate and severe spinal cord injuries. In this, the first year of this award, we have found that stretching negatively influences locomotor function in animals with both acute (within days) and chronic (after 3 months) spinal cord injuries. We have also determined that stretching for short periods of time (4-5 weeks) allows substantial recovery to occur once stretching is stopped, and both acute and chronic animals show a similar time course of recovery. Finally, in very preliminary studies, we have found that the torque being applied during stretching of the rat hindlimb is roughly similar to that applied to human lower extremities relative to body weight.					
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Final Report:

SC110169 – “Directing Spinal Cord Plasticity: The Impact of Stretch Therapy on Functional Recovery after Spinal Cord Injury”.

Principle Investigator: David S. K. Magnuson, PhD. University of Louisville.

1. Introduction:

This research focuses on the impact of stretching (physical therapy maneuvers involving force or torque applied to specific muscle groups) on functional recovery after spinal cord injury in a rat model. We have undertaken these studies because of an observation we made a few years ago during a study where the hindlimbs of rats with spinal cord injuries were being immobilized in a wheelchair. We found that immobilization dramatically influenced locomotor recovery, presumably by reducing the sensory input associated with movement (Caudle et al., 2011). In that study we employed a stretching procedure designed to prevent reductions in joint range-of-motion. The stretching didn't prevent contractures, but it did have a negative impact on locomotor recovery. Thus, in the proposal just completed, specific Aim (SA) 1 focuses on the timing of stretching relative to the injury and whether or not there is a window of susceptibility to a stretching-based therapy. SA2 focuses on the pattern and forces of the actual stretching protocol and if the negative influence of stretching is due primarily to the length of each maneuver or to the forces applied during stretch. An overarching goal in the project is to develop a computer model of the stretching to allow direct comparison of rat and human.

2. Key Words: spinal cord injury, locomotor recovery, physical therapy, muscle stretch, joint range-of-motion, rat.

3. Accomplishments:

Major goals of the project.

Specific Aims:

1. To determine if there is a window of opportunity within which an intense stretch-based therapy disrupts locomotor recovery, and what that window of opportunity is for moderate and moderately-severe spinal cord injuries.
2. To determine if there is a minimum intensity threshold for stretching below which a stretching/ROM therapy does not disrupt locomotor recovery.

This proposal consists of 2 specific aims plus an in-parallel effort to model and quantify stretching being done in the rat model for comparison with published values for human patients.

Task 1. For the first specific aim, a total of 120 animals will be studied in 4 groups of 20 plus 40 control animals. We will start with the 4 day acute stretching groups (4d, 12.5 and 25g-cm) and the 8 week stretching groups that will serve as controls for the acute stretching groups (Figure 1, grps 1 & 2). The experiments themselves will take 8 and 16 weeks for the two groups, plus an additional 4 months for histology and analysis.

We estimate that Task 1 will take approximately 8 months.

Task 2. We will then move to the 2w and 4w groups that will be done separately (consecutively) and will include a control group of 10 injured animals for each. These studies will take a little over 4 months each and should be completed by month 20 of the granting period. These are very labor-intensive studies and we anticipate that completion of the experiments in specific aim 1 will take approximately 20 months. Our preliminary data suggests that we will find that stretching effects locomotor function at 4 and 8 weeks post-injury, so we anticipate that all the proposed experiments will need to be completed.

Task 3. In parallel with the studies in specific aim 1, we will begin our efforts to model the forces applied during stretching maneuvers. This work will involve modeling based on the kinematics and forces measured from animals being stretched for specific aim 1; no additional animals will be required. The forces and kinematics will be used to calculate moment about each joint during stretching maneuvers. This data will allow us to quantitatively compare the stretching we use in rats with that published for human patients with SCI.

Task 3a. Develop dynamic stick figures of each stretching maneuver

Task 3b. Establish vectors of forces applied during stretch

We estimate that Tasks 3a & 3b will take approximately 3 months.

Task 3c. Estimate forces and calculate torques about each joint. Develop theoretical torque-angle standard curves.

We estimate that Task 3c will take approximately 2 months.

Task 3d. Compare calculated torques with published values for hindlimb stepping.

Task 3d should take approximately 1 month.

Task 3e. Measure forces being generated during stretching, calculate torques, create actual torque-angle curves and compare with theoretical curves.

Task 3f. Estimate forces being generated during stepping. Calculate torques, create stepping torque-angle curves. Compare stepping and stretching torque-angle curves.

Tasks 3e & 3f should take approximate 12 months.

Task 3g. Compare rat data with published human stretching data. Model with additional parameters to address questions/unknowns.

Task 3g should take approximately 6-12 months.

Task 4. For specific aim 2 we will use groups of 10 animals that will receive one of three different stretching maneuvers, in addition to a group receiving our current high-intensity stretch and hold maneuvers. We will run three groups of animals simultaneously for each study, two experimental and one unstretched control for a total of 60 animals.

For tasks 1 and 3 above, the experiments themselves will take between 4 months each to complete, however, collecting and analyzing all of the histological, electrophysiological, kinematic and behavioral data will take an additional 4 months for each study.

We estimate that Task 4 will take 16 months.

Task 5. Manuscripts will be prepared and submitted as appropriate. We anticipate that one paper will contain the results of experiments in task 1 where acute and chronically stretched rats are compared head-to-head for two different injury severities. We anticipate that task 2 will result in a paper primarily because the 2 and 4 week groups represent post-injury time periods when most patients begin to receive regular stretching sessions. This would be a logical place to include our initial efforts of modeling and measuring the forces generated during stretching from task 3. We also anticipate that we will prepare and submit for publications papers describing the different stretching maneuvers investigated in task 4. If we find that the experimental groups separate out based on intensity and pattern, rather than one or the other, this work might result in more than one manuscript to describe it.

Task 6. Results will be prepared and presented as posters or talks at national, international and DOD/SCRIP meetings as appropriate. We will most likely target the National Neurotrauma and Society for Neuroscience meetings.

Task 7. Progress and financial reports will be prepared and submitted annually as required.

What was accomplished under these goals.

I will report our accomplishments by referring to and summarizing our findings reported in two published papers (paper 1 and 2) and two prepared manuscripts (papers 3 and 4). These are included in the appendix as shown below.

Paper 1: Appendix 1, pages 1 – 10. Disruptions of locomotion in response to hindlimb muscle stretch at acute and chronic time points after a spinal cord injury in rats. *J. Neurotrauma*, 2017. 34: 661-670.

Paper 2: Appendix 2, pages 11 – 16. Dynamic “Range of Motion” hindlimb stretching disrupts locomotor function in rats with moderate subacute spinal cord injuries. *J. Neurotrauma*, 2017. Epub ahead of print.

Paper 3: Appendix 3, pages 17 – 30. Electromyographic patterns of the rat hindlimb in response to muscle stretch after spinal cord injury. In preparation.

Paper 4: Appendix 4, pages 31 – 54. Nociceptor-dependent locomotor dysfunction after stretching in adult rats with spinal cord injury. In preparation.

Task 1 resulted in paper 1, and due to the findings that showed clearly that stretching reduced locomotor function both acutely and chronically, we did not have to undertake Task 2. As a result we were able to take the time and had the resources to do the study discussed in the original grant and requested by the reviewers, but not proposed, which was to investigate the role of c-fibers. This resulted in paper 4.

Task 4 resulted in paper 2. Again, the results were unambiguous and we were able to clearly answer the primary question without doing all of the groups, but we extended some of the analysis to get deeper into what was going on with the muscles and responses to stretching (see below).

Task 3 resulted in paper 3. Within the framework of that paper we talk about forces and modeling the torques being applied and comparing them with published work for humans. Due to having two of the students graduate and move on, and the different skill sets of the students who came into the lab, we were unable to fully “model” the hindlimbs of rats for comparison with humans. However, what was accomplished was sufficiently in-depth to demonstrate what torques were being applied, and how these changed for the different muscles being stretched and over time. We believe that these findings will be critical for further translation of the stretching phenomenon to the clinic.

Paper 4 was accomplished due to the time and money “saved” in tasks 1 and 4, and was a direct follow-up to concepts introduced in the initial grant proposal and most importantly, requested by the reviewers. The decision making-process was discussed in the quarterly and annual reports.

Key Results as reported in papers 1-4.

Paper 1 (Appendix 1): Using our 2minutes per muscle group stretching protocol and female SD rats with T10 moderately-severe contusion injuries we show that daily (5d/wk) stretching reduces locomotor function acutely (Fig. 2A) and chronically (Fig. 2B). Figure 2A also shows that the negative impact of stretching is force and position dependent because early post-injury the therapists were not pushing to end-range-of-motion and the negative impact was lower. Figure 3 shows that stretching influences hindlimb kinematics and gait, as objective measures to supplement the subjective BBB scale. Importantly, Figure 3 shows that while the BBB scores recover when stretching is stopped, some kinematic deficits persist, in particular for the hip (IHA

angle; Fig. 3A and B). We also show in this paper that the drop in hindlimb locomotor function is accompanied by a drop in distance traveled overnight (nocturnal in-cage activity) for the chronically stretched animals only (Figure 4 B). Using the magnetically-evoked tail reflex, we show in Figure 5 that stretching reduces hindlimb motoneuron excitability, again primarily for the chronically stretched animals, and that during this chronic period stretching results in a small reduction in hip and knee functional (passive) range of motion as measured using kinematics (Figure 6). We also report that stretching has little influence on muscle fiber size (Paper 1, table 1) although we found that gastrocnemius and biceps femoris muscle fiber size was significantly reduced in the chronic animals. Table 1 shows clearly that stretching did not increase the number of centralized nuclei nor did it increase the amount of collagen in or around the muscle supporting the concept that stretching has not caused any frank muscle damage when applied acutely or chronically. Centralized nuclei are a persistent measure of muscle regeneration after damage. So, in summary, paper 1 shows that the negative effects of stretching are force dependent (at least acutely) and are evident both acutely and chronically in the absence of frank muscle damage. Dramatic drops in locomotor function are accompanied by decreases in motoneuron excitability and even though the BBB scores recover there are persistent decreases in hip range of motion after stretching.

Paper 2 (Appendix 2). In this work we used a “dynamic” stretching protocol where stretches were applied using a 2 second on, 1 second off rhythm (to a metronome), again for 2 minutes for each muscle group. As before we used female SD rats with 25g-cm T10 contusion injuries and for this study we initiated stretching at week 6 post-injury. In addition to BBB scores we used 3D kinematics and gait analysis. Based on the concept that the spinal cord responds to rhythms and patterns we hypothesized that dynamic stretching would be less disruptive to locomotor function than our standard stretch and hold pattern. Figure 1 in paper 2 shows that dynamic stretching is very disruptive to locomotor function based on the BBB scores (Fig. 1A) and both kinematic and gait measures (Fig. 1B and C). For these animals there was an apparent persistent change in hip kinematics following stretching, but this was not statistically significant. Keep in mind that we stretched for 4 weeks only. In this study we also began to record (kinematically) and quantify responses of the contralateral limb to stretches being applied ipsilaterally. We report in Figure 2 that the contralateral limb responded to stretching with “vibrations” that are very clonus-like in nature. We report in Table 1 that the frequencies recorded were between 12 and 17Hz, which is significantly higher than clonus reported in people with SCI. As expected, we did not observe any increase in muscle centralized nuclei following stretching suggesting that neither stretching pattern (tonic or dynamic) is causing frank muscle damage.

Paper 3 (Appendix 3). In this work we employed a combination of approaches to really understand what is happening during the hindlimb muscle stretching that results in locomotor deficits after SCI. We used female SD rats with 25g-cm T10 contusion injuries that were also instrumented with DSI telemetry devices that allowed us to record EMG patterns from two hindlimb muscles, wirelessly. In addition we utilized our “force glove” based on small FSRs (force sensitive resistors) to measure the forces being applied by the rat physical therapist during the stretching. Finally, we did 3D kinematic measurements of the limbs during the stretching and of the contralateral limb during the EMG recordings (done during the stretching) to understand how the stretch, movements and EMG patterns all fit together. All this information was used in a crude model of the rat hindlimb to calculate the torques being generated by the stretching around each joint and how those torques changed over time post-injury for each muscle. We purposely stretched only 1 limb (un-instrumented) over the first 8 weeks to avoid accidentally moving the EMG wires that were implanted into the knee flexors and extensors. We also purposely stretched only twice a week, primarily in an effort to avoid causing dramatic drops in hindlimb function. In

many ways this was a technical and engineering “tour de force” (pun intended) that had fantastic outcomes in our efforts to move this concept to the clinic. Paper 3, Figure 1 shows examples of the three different EMG patterns we encountered. These coincided with movements described as “vibrations”, “air stepping” and “spasms” and were recorded from knee flexors and extensors. Often, these EMG responses were seen overlapping or simultaneously during stretching (of the opposite limb), so in Figure 2 we show some examples to illustrate how messy the responses could be. Then, in Figure 3 we show output from both EMG recordings and the force transducers over the 55 seconds or so of an individual stretch, again in the opposite limb. This shows that force changes are sometimes accompanied by EMG responses, but not always. We believe this data is important because it allows physical therapists who work with patients to relate what they feel while maneuvering the limb of a patient to what our rat therapists experience. It is also incredibly important because of the relatively low torques being applied. In Figure 4 we show several important things including that the amplitude of clonic-like responses tended to increase from week 2 through 8 and were generally higher in the ipsilateral limb (being stretched). It also shows that the frequency of EMG bursts in clonic-like responses were centered around 6Hz, which is very close to what is reported for clonus in the clinic. This distinguishes it from the kinematic vibrations reported in Paper 2, due to the fact that clonus is reported as peak to peak while the vibrations are reported peak to trough (doubling the frequency). In Figure 5 we show that the torques required to achieve an end range of motion when stretching the tibialis anterior muscle tends to decrease from week 2 through 7, whereas they significantly increase over the same period for the quadriceps muscle group. Figure 6 shows that stretching only one limb, twice a week, did not cause dramatic drops in open field locomotor function (BBB, Figure 6A) but did have modest effects on kinematics and gait when comparing function immediately before and 1-2 hours after stretching.

Paper 4 (Appendix 4). In this study we took advantage of a collaborator's expertise and an ongoing study to acquire female SD rats that were depleted of TRPV1 positive C-fibers. These are the thermal/chemical nociceptors that normally signal heat/tissue damage nociceptive stimuli. At 2 days post-injury, these animals were treated with Capsaicin (50mg/Kg, ip), a well described model that has been used for many years. We used the cutaneous trunk muscle reflex (a nociceptor specific reflex) to confirm that these animals no longer respond to thermal nociceptive stimuli, but retain the capacity to respond to mechanical nociceptive stimuli (Figure 1). We also confirmed that loss of TRPV1+ c-fibers did not disrupt normal locomotion using kinematic assessment (Figure 2). We then gave all the C-fiber depleted animals, and a group of vehicle controls (received saline without capsaicin) and some untreated controls T10 25g-cm contusion injuries. Prior to injury, the C-fiber depleted animals had longer onset latencies and lower amplitude EMG responses to magnetic stimulation of the tail afferents (Figure 3) whereas after injury all the groups had similar reduction in EMG responses that did not recover. After injury we allowed the animals to recovery for 6 weeks and then initiated our standard daily stretching protocol where each muscle group is stretched twice for 1 minute each. In Figure 4 the VEH group is C-fiber intact and showed the expected drop in hindlimb function. The CAP group is C-fiber depleted and showed a very small drop in locomotor function while the CON group was not stretched (to serve as a histology/immunohistochemistry control). Figure 4B and C show that the CAP animals had significantly different (better) hindlimb function during stretching compared to the VEH animals. For this paper we used stepping in shallow water as a body-weight-supported assessment of stepping. We did immunohistochemistry for CGRP (a marker of c-fibers) in sections from the rostral segments of the lumbar spinal cord (L1-L3) and found that indeed CAP animals had dramatically reduced CGRP fibers (but not entirely depleted). We also discovered that stretching appears to increase CGRP fibers in the dorsal horn as compared to the CON (injured but not stretched, C-fiber intact). This is important because it further illustrates a

potential link between C-fibers and stretching. We also did immunohistochemistry for cFOS, an immediate-early gene commonly used as a marker for neuronal activity. We stained sections from L1-L5 and counted 3 sections per segment per animal. We found that stretching dramatically increased the number of cFOS positive nuclei throughout not just the dorsal horn but also the intermediate gray matter where the critical locomotor interneurons reside (Figure 6). Figure 7 shows some example sections of lumbar spinal cord stained for cFOS with labeled nuclei throughout the intermediate gray matter. Importantly, even the CAP animals (C-fiber depleted) had some cFOS positive nuclei indicating that some afferents other than C-fibers are also likely be activated by stretching, but that this activation did not result in as drastic a loss of locomotor capacity. In figure 8 we show that most of the changes in cFOS nuclei numbers are occurring in the intermediate and ventral gray matter, which is in some ways counter-intuitive. It is important to note that, due to the labor-intensive nature of the terminal assessments, some animals had one or two additional days of stretching before being sacrificed 2 hours after the last stretching session. The animals with one or two additional days of stretching had many more cFOS activated nuclei (figure 6, “outliers”). In this study we also assessed the hindlimb muscles and found no significant differences in centralized nuclei, but did find that the TA muscle showed a hypertrophic response to stretching in both the VEH and CAP groups. Thus, our stretching is inducing hypertrophy without doing frank damage, with the caveat that some animals obviously had increases in centralized nuclei (Figure 9A) despite there being no statistically significant changes. Finally, figure 10 shows that the relationship between prevalence of clonic-like vibrations of the limb and cFOS positive neurons is reversed for the VEH (c-fiber intact but stretched) as compared to the CAP animals (c-fiber depleted and stretched). These graphs suggest that intact c-fibers result in an increase in the number of cFOS positive nuclei and an increase in the “vibration score”. In the c-fiber depleted animals, it appears that the number of cFOS positive neurons is inversely related to the prevalence of clonic-like vibrations.

Observations from the two as-yet unpublished studies: Paper 3

1. Stretching of one hindlimb results in at least three distinct EMG patterns in the contralateral hindlimb. Two of these patterns are related to movement (air stepping and a “clonus” like response). One appears to be unrelated to movement but involves high frequency but low amplitude co-activation of antagonist muscles characterized as spasm. Figure 4 shows examples of each from the rectus femoris (knee extensor) and biceps femoris (knee flexor) muscles.
2. The highest levels of force during stretching sometimes exceed 400g (well beyond body weight of 250g), but these peaks of force are rare and occur during movement or briefly when the stretch position is being established. Importantly, the torques being applied are equivalent to, or less than the torques applied to patients when scaled for body weight.
3. Torques necessary to achieve end range-of-motion increased for the quadriceps (primary load during stance) but were otherwise similar over time post-injury. The tibialis anterior required the highest torques and the gastrocnemius muscles required the lowest torques.
4. Stretching one limb has a temporary negative impact on hindlimb function bilaterally (few hours), and a slightly longer negative impact on hindlimb function ipsilaterally (stretched limb, up to a day). We determined in this study that stretching one limb only a few times a week did not result in lasting decreases in locomotor function.
5. We have identified an “EMG signature” of stretching. We will use this signature in future studies to determine if stretching in patients is having a negative impact on function that has been heretofore unrecognized.

Observations from the two as-yet unpublished studies: Paper 4

1. The stretching phenomenon, where locomotor function declines after stretching, is C-fiber dependent. This implies that the stretching activates C-fibers even though we see no indication of muscle damage.
2. Stretching activates large numbers of neurons throughout the spinal cord gray matter, something that is very surprising. In turn, this implies that neurons in the intermediate gray matter that we believe would normally be involved in locomotion, are being strongly activated by stretching (sufficiently to induce cFOS) but these neurons are thereafter unable to participate in locomotion. This is a finding that is among the most important to follow-up in future work.
3. The irrefutable involvement of C-fibers is a very important observation because this activation is all about nociception; information getting into and effecting spinal circuitry without being perceived as pain because of the spinal cord injury. It is possible that other sources of nociceptor activation (contractions, skin abrasions, pressure sores) might also have a negative impact on locomotor function.

Conclusion: Overall Research Accomplishments.

The results described above show that the majority of the research goals originally proposed were completed. The primary research accomplishments overall for this project are:

1. The forces being applied during stretching of the rat are roughly similar to those being applied to humans clinically, based on a body-weight comparison.
2. That daily stretching, whether applied in the acute or chronic phase, can be devastating to motor function, and in the light of our previous work, if stretching is continued for 8 weeks or more, the deficits may be long-lasting.
3. That daily stretching is devastating to locomotor function after mild, moderate or severe low-thoracic contusion injuries.
4. That deficits resulting from both acute and chronic stretching can be viewed as temporary if the stretching is only done for a few weeks. This finding has to be viewed with the caveat that rats are more active than people (post-injury) and that this activity may be what allows them to recover after several weeks of stretching.
5. Stretching of one hindlimb is strongly influencing spinal cord circuitry bilaterally, resulting in several distinct patterns of muscle activation in the limb not being stretched. Some of this activation is via circuits involved in movement, seen as airstepping and clonus, and some of this activation is via other circuits or is more general resulting in high-frequency, low amplitude co-activation patterns that are clearly abnormal and appear as spasms.
6. Stretching that achieves an end-range of motion is sufficient to induce locomotor decline. If forces insufficient to achieve maximal end range-of-motion are used, then locomotor declines are less. Peak forces during stretching to maximal end range-of-motion are the result of muscle activation, not the force being applied by the therapist.
7. Despite the peak forces of more than 400g, neither daily acute nor daily chronic stretching (for 4-5 weeks) resulted in muscle damage as assessed by counting centralized nuclei and quantification of collagen deposition.
8. Dynamic stretching applied in a rhythmic fashion is just as devastating to locomotor function as is tonic stretching despite the fact that the total time spent at end range-of-motion is less. Thus, attempting to employ dynamic stretching clinically will not allow any locomotor decline be avoided.
9. Stretching effects on locomotor function are dependent on the activation of nociceptive C-fibers. This is an incredibly important observation that will be important to follow-up in future studies/proposals.

Specific questions asked during initial review of the rejected final report:

(1) What has come out of the effort to develop a computer model for effective comparison between rats and patients?

Answer: As described above, due to the loss of one graduate student and one undergraduate student who had the skills to perform the modeling, we were unable to fully pursue this line of work. However, we did accomplish the primary goals of determining what forces and torques were being applied to our rats and how they compare to the forces published for humans. When scaled based on body weight (which seems logical) the forces are very similar and in fact the highest forces or torques applied to human are higher than the highest forces we recorded for the rats.

(2) The discrepancy between your observation that stretching leads to muscle atrophy and the "clinical phenomenon that stretching can lead to muscle fiber hypertrophy" is of interest and concern. Please discuss what could be the potential reason for the discrepancy and how this may affect the translatability of your results.

Answer: We agree that this is an extremely important observation. To clarify, in paper 4 (appendix 4) figure 9 we show that stretching induced an increase in the TA muscles (non-weight bearing) mean fiber cross sectional area despite also reducing the locomotor function of the animals. This suggests that the "muscle hypertrophy" effect of stretching is independent of the negative impact of stretching on locomotor function and is independent of TRPV1+ nociceptors which are depleted in the capsaicin treated animals. This has important implications for translation by suggesting that it may be possible to induce muscle hypertrophy (a good thing) without inducing a drop in locomotor function (a bad thing). We will hope to tackle this question in a future grant.

(3) By "EMG signature", do you mean the 3 EMG patterns shown in Fig 4? Where are the data showing that these patterns are unique to stretching?

Yes, we mean the 3 EMG patterns shown in Figure 4 of Appendix 3. We call it a "signature" for two reasons. First of all, there have been very few reports of clonus in rats despite it being a very common clinical feature and observation. Further, we can find no reports of clonus in rats recorded in the contralateral limb (not being stimulate or manipulated) which means that the circuitry being activated by stretching crosses the midline efficiently. If similar EMG patterns can be observed in the contralateral limbs of patients being stretched, then we believe an argument can be made that the influence of stretching on spinal cord circuitry is similar in rats and patients. In otherwords, activation of these 3 patterns in the contralateral limb may be indicative of the process that occurs during stretching that leads to a drop in locomotor function, ie. the signature. We hope to pursue this idea of an EMG signature in a future grant.

Opportunities for training and professional development.

The student involved, Anastasia Keller, is now within 10 days of defending her PhD dissertation (June 9). She was able to attend 6 national and 5 regional meetings during her time in the laboratory. She presented her results from these studies at many of these meetings (see below).

How were results disseminated to communities of interest.

In addition to the publications and abstracts shown later in this report, I presented results from this study 8 different times over the period of the grant and EWF. This included departmental/center seminars given nationally, internationally (in Canada, the UK and Sweden) and of course locally.

Plans for next reporting period.

This is the final report.

4. Impact.

What was the impact on the development of the principle disciplines of the project?

Our results are extremely important and have already had an impact on medical treatment of spinal cord injury. Within our center (Frazier Rehab Institute and the Kentucky Spinal Cord Injury Research Center), the amount of stretching being done on patients has been reduced. So far there are no reports of problems. We have not begun to study this, clinically, but hope to soon. Our results demonstrate that the forces we are using while stretching the rats are not causing overt muscle damage and that the peak forces are occurring in response to nervous system activation, and not the stretching force itself. We are building up a picture of how the spinal cord is responding to the afferent input caused by the stretching and that at least some of this response involves co-activation of antagonist muscles at high-frequency and low amplitude. We are the first to report (once the paper is published) the expression of clonus after spinal cord injury in the rat model. None of our results are pointing towards the development of a product, but will lead to the suggestion that our current stretching practices in the clinic will need to change.

What was the impact on other disciplines.

While this is hard to predict/measure, I have been invited to speak at a number of meetings/universities with the request that I focus on this aspect of our work. Interest in our results is very high in particular for physical therapists and rehabilitation specialists.

What was the impact on technology transfer.

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report.

5. Changes/Problems.

Changes in approach: Nothing to report.

Actual or anticipated problems or delays: Nothing to report.

Changes that had a significant impact on expenditures: Nothing to report.

Significant changes in use or care of humans or animals: Nothing to report.

6. Products.

Publications, abstracts and presentations:

Publications:

Caudle KL, Atkinson DA, Brown EH, Donaldson K, Seibt E, Chea T, Smith E, Chung K, Shum-Siu A, Cron C, **Magnuson DSK**. Hindlimb stretching alters locomotor function post-spinal cord injury in the adult rat. *Neurorehab and Neural Repair* 29(3): 268-77, 2015. PMID 25106555

Appendix 1: Keller AV, Wainwright G, Shum-Siu A, Prince D, Hoeper A, Martin E, Magnuson DS. Disruption of locomotion in response to hindlimb muscle stretch at acute and chronic time points after a spinal cord injury in rats. *J Neurotrauma* 2016. PMID: 27196003

Appendix 2: Keller AV, Rees K, Prince D, Morehouse J, Shum-Siu A, Magnuson DSK. Dynamic “range of motion” hindlimb stretching disrupts locomotor function in rats with moderate subacute spinal cord injuries. *Journal of Neurotrauma*, in press. 2017.

Papers in preparation:

Appendix 3: Keller AV, Wade A, Rees K, Prince D, Morehouse J, Shum-Siu A, Magnuson DSK. Electromyographic patterns of the rat hindlimb in response to muscle stretch after spinal cord injury. In preparation.

Appendix 4: Keller AV, Krupp S, Wade A, Morehouse J, Shum-Siu A, Petruska JC, Magnuson DSK. The negative impact of stretching on locomotor function after SCI is C-fiber dependent. In preparation.

Links to journal publications:

Link to Caudle et al., 2015. *Neurorehab and Neural Repair*.

<http://nnr.sagepub.com/content/early/2014/08/07/1545968314543500.long>

Link to Keller et al., 2017. *Journal of Neurotrauma*

<http://online.liebertpub.com/doi/pdf/10.1089/neu.2015.4227>

Link to Keller et al., in press. *Journal of Neurotrauma*

<http://online.liebertpub.com/doi/10.1089/neu.2016.4951>

Presented abstracts/posters:

Keller AV, Nord K, Wade A, Shum-Siu A, and Magnuson DSK. Electromyographic patterns in the contralateral limb in response to muscle stretch in rats with moderate spinal cord injuries. Society for Neuroscience 2015.

Keller AV, Nord K, Wade A, Shum-Siu A, and Magnuson DSK. EMG patterns of the contralateral limb in response to muscle stretch in rats with mild SCIs. American Spinal Injury Association Meeting, Montreal, PQ. 2015.

Keller AV, Shum-Siu A, Wainwright GN, Seibt S and Magnuson DSK. Hindlimb muscle stretch reduces locomotor function after a Spinal Cord Injury: Implications for Physical Therapy and Rehabilitation. National Neurotrauma Society Meeting, August 2014. San Francisco, CA.

Prokopenko (Keller) AV, Shum-Siu A, Wainwright GN, Seibt E. D.S.K. Magnuson. Muscle stretch reduces locomotor function after a spinal cord injury: Acute and chronic time points. International Society for Neural Regeneration Symposium, Asilomar Conference Center, December 11-15, 2013.

Seibt E, Prokopenko (Keller) AV, Shum-Siu A, Wainwright GN, D.S.K. Magnuson. Force sensing glove for quantification of joint torques during stretching after SCI in the rat model. National Neurotrauma Society meeting, Nashville, TN, 2013.

Invited presentations: David S. K. Magnuson.

For Better and Worse: Activity, Exercise and Rehabilitation after SCI. Spinal Cord Summit, The Ohio State University, May 18-19, 2017.

Directing Spinal Cord Plasticity: The impact of stretch therapy on functional recovery after spinal cord injury. In-Progress Reviews of SCIRP funded research (DOD/CDMRP). Fort Detrich, MD, October 26, 2016.

Neurorehabilitation – insights from animal studies. Spinal Cord Medicine – How it may evolve. 50th Anniversary Symposium, Spinal Cord Injury Center at University of Heidelberg, Germany. June 24-25, 2016.

Bench to Bedside to Bench in Upper and Lower Extremity Rehab. American Spinal Injury Association. Philadelphia, PA. April 14-16, 2016. With Edelle Field-Fote, D. Michele Basso and Karim Fouad.

What is “Clinically Relevant” in spinal cord injury research using animal models. Neuroscience Grand Rounds, University of Louisville. October 8, 2015

Of Mice and Men: The bench to bedside efforts to improve the quality of life of people with spinal cord injury. (with Dr. Kris Rau). “Scientific Proofs” series at Goodwood Brewery, Louisville, KY. August 11, 2015

Stretching, exercise and functional recovery after SCI: Update from the Magnuson Lab. Frazier Rehab Institute, Physical Therapy In-Service. April 2, 2015.

The Evolving Theory of Everything: An update from the Magnuson Lab. KSCIRC Research Update Seminars. October 10, 2014.

So how bad is stretching, really? KSCIRC Research Update Seminars, October 4, 2013.

Inventions, Patents and Licenses: Nothing to report.

Reportable Outcomes: Nothing to report.

Other Achievements: Nothing to report.

7. Participants & other collaborating organizations

Individuals who have worked on the project. Please see submitted budget/personnel.

Nothing else to report for section 7.

8. Special Reporting Requirements.

Collaborative Awards: Nothing to report.

QUAD Charts: Please see attached QUAD chart.

Appendices: Please see links above to published journal articles. Appendices 1-4 to follow.

Directing Spinal Cord Plasticity:
The Impact of Stretch Therapy on Functional Recovery after Spinal Cord Injury
CDMRP Investigator-Initiated Research Award

PI: David S. K. Magnuson

Org: University of Louisville, Louisville, KY



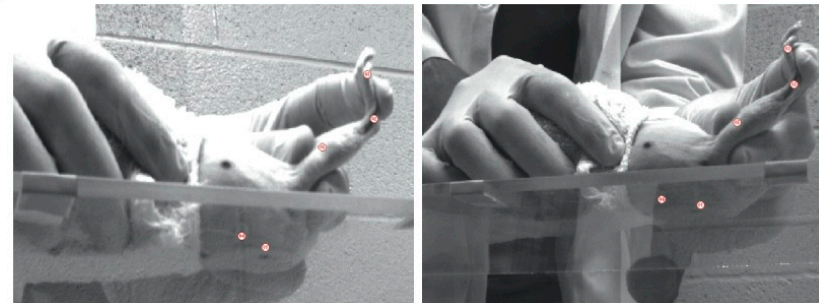
Stretch-based physical therapy may impede recovery after spinal cord injury.

In a rat model of moderate contusive SCI:

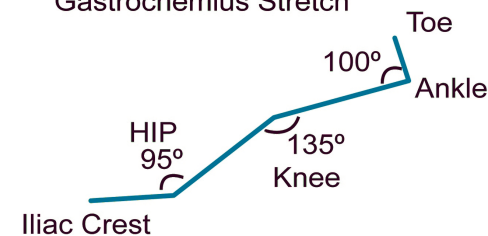
- Daily stretching reduces hindlimb function
- Single 30-min sessions induce change acutely
- Repeated sessions induce cumulative dysfunction

Clinically, in Military and Civilian Populations:

- All SCI patients receive stretch-based therapy
- Stretching is initiated acutely and continues to chronic phase



Gastrocnemius Stretch



Questions to be addressed:

What aspects of stretching are detrimental?

- Timing post-injury
- Intensity
- Pattern

What injury severities are most at risk?

- Moderate
- Moderately-severe

Is the stretching similar to that used clinically?

- Applied forces will be measured and modeled.

The stretching being used routinely in the clinic is likely (or unlikely) to be detrimental, and if simple changes to standard practice could reduce the negative impact on recovery.

Timeline and Total Cost (direct and indirect)

Activities	FY	12	13	14	15
Timing post-injury. Stretch started a 2-12 weeks post-injury.					
Intensity & pattern. Does reduced intensity or different pattern alter impact?					
Measuring and modeling forces applied during stretching.					
Estimated Budget (\$K)		15	155	155	130

Directing Spinal Cord Plasticity:
The Impact of Stretch Therapy on Functional Recovery after Spinal Cord Injury
CDMRP Investigator-Initiated Research Award

PI: David S. K. Magnuson

Org: University of Louisville, Louisville, KY



Award Information

Log Number/Contract Number: SC110169

Period of Performance: FY 2013-2015

Award Amount: \$456,597.00

GOR: Originally, Dr. Iddil Bekirov

GOR: Dr. Tian Wang

Collaborators: None.

Problem Areas

No major problems were encountered.

Early results ruled-out the need to perform some of the experiments proposed (see report). Thus, we completed an additional experiment focused on C-fibers that was suggested in the original proposal but not fully described. It was extremely successful.

Key Research Accomplishments

Additional questions answered:

Stretching has a negative impact on locomotor function after mild or moderately-severe injury, when applied acutely or chronically, and when applied tonically or in a rhythmic pattern.

Muscle damage is absent. Forces used are equivalent to or less than those used clinically.

EMG patterns observed during stretch are similar to those observed clinically.

Negative impact of stretch is force (intensity) and C-fiber (nociceptive) dependent.

Next Steps

- Three manuscripts have been published.
- Two more manuscripts are nearing submission.
- Clinical collaborators have been sought and clinical studies are being considered/designed.

Conclusion

The stretching being used routinely in the clinic may be detrimental, and simple changes to standard practice could reduce the negative impact on recovery.

Disruption of Locomotion in Response to Hindlimb Muscle Stretch at Acute and Chronic Time Points after a Spinal Cord Injury in Rats

Anastasia V.P. Keller,^{1,2} Grace Wainwright,⁵ Alice Shum-Siu,^{1,3} Daniella Prince,^{1,3}
 Alyssa Hoeper,⁵ Emily Martin,⁵ and David S.K. Magnuson^{1,3,4}

Abstract

After spinal cord injury (SCI) muscle contractures develop in the plegic limbs of many patients. Physical therapists commonly use stretching as an approach to avoid contractures and to maintain the extensibility of soft tissues. We found previously that a daily stretching protocol has a negative effect on locomotor recovery in rats with mild thoracic SCI. The purpose of the current study was to determine the effects of stretching on locomotor function at acute and chronic time points after moderately severe contusive SCI. Female Sprague-Dawley rats with 25 g-cm T10 contusion injuries received our standard 24-min stretching protocol starting 4 days (acutely) or 10 weeks (chronically) post-injury (5 days/week for 5 or 4 weeks, respectively). Locomotor function was assessed using the BBB (Basso, Beattie, and Bresnahan) Open Field Locomotor Scale, video-based kinematics, and gait analysis. Locomotor deficits were evident in the acute animals after only 5 days of stretching and increasing the perceived intensity of stretching at week 4 resulted in greater impairment. Stretching initiated chronically resulted in dramatic decrements in locomotor function because most animals had BBB scores of 0–3 for weeks 2, 3, and 4 of stretching. Locomotor function recovered to control levels for both groups within 2 weeks once daily stretching ceased. Histological analysis revealed no apparent signs of overt and persistent damage to muscles undergoing stretching. The current study extends our observations of the stretching phenomenon to a more clinically relevant moderately severe SCI animal model. The results are in agreement with our previous findings and further demonstrate that spinal cord locomotor circuitry is especially vulnerable to the negative effects of stretching at chronic time points. While the clinical relevance of this phenomenon remains unknown, we speculate that stretching may contribute to the lack of locomotor recovery in some patients.

Keywords: locomotor function; rehabilitation; spinal cord injury

Introduction

SEVERE BUT INCOMPLETE SPINAL CORD INJURY (SCI) results in partial paralysis below the level of injury because of the loss of descending inputs onto voluntary motor and the more automated locomotor circuitry of the spinal cord. Partial paralysis, in turn, results in prolonged periods of immobility accompanied by significant alterations in the musculoskeletal components.¹ Over time, the spinal cord circuitry below the level of injury undergoes a number of adaptations that lead to an increase in alpha motoneuron excitability.^{2,3} This increase can be associated with improvements in locomotor function.^{2,4} The system, however, lacks appropriate modulation by inhibitory circuitry because of impaired control from supraspinal centers^{5–7} and local maladaptive plasticity.⁸ These changes combine to result in exaggerated motor output

(hypertonia and spasticity) in response to muscle stretch and/or other sensory input.⁹ As a result, some level of spasticity develops in approximately 78% of patients with chronic SCI.¹⁰

Although spasticity has potential benefits for some patients, indirectly contributing to their ability to stand or perform daily tasks such as transfers, it also presents a multitude of unwanted consequences that presumably contribute to more than half of the patient population with spasticity reporting it as a major obstacle to resumption of activities of daily living.¹¹ Unmanaged spasticity, where muscles remain in shortened positions for prolonged periods can lead to the development of joint and muscle contractures that manifest as dramatically decreased range of motion (ROM)¹² about affected joints.

Preservation of a functional ROM is not only important for timely initiation of rehabilitation,¹³ but it also can significantly improve

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independence in some patients with SCI. For example, an elbow contracture in patients with cervical level 6 injury rendered them unable to perform transfers and maintain bed mobility, functionally making them similar to patients with cervical level 5 injury.

Stretching remains the cornerstone for the treatment by physical therapists (PTs) of both spasticity and muscle contractures^{11,14,15}; it is encouraged even in the absence of contractures to maintain soft tissue extensibility, because it is believed that preventing contractures is easier than treating them.¹⁶ Stretching provides a potent mechanical stimulus for the induction of protein synthesis¹⁷ and has been shown to result in serial addition of sarcomeres within muscle fibers,¹⁸ which can potentially prevent atrophy¹⁹ and decreases in muscle length. These observations suggest that muscle stretch should be an effective method for achieving desirable changes in muscle length or preventing undesirable changes in soft tissues; thus the rationale to include stretching therapy in the rehabilitation program for patients with SCI appears sound. Evidence that most commonly used stretching techniques actually improve symptoms of spasticity, ROM and/or prevent contracture formation in subjects with SCI is mixed at best, however.⁵

Previously, we found that wheelchair hindlimb immobilization in rats with mild-moderate spinal cord injuries resulted in the loss of locomotor function and, in addition, contractures developed in some animals, grossly similar to human patients.²⁰ Thus, we invited PTs who work with human patients with SCI to guide us in the development of a clinically relevant stretching protocol for the treatment and prevention of contractures in hindlimb-immobilized animals as part of their daily care. The protocol was standardized for stretching muscles around the major hindlimb joints: ankle flexors/extensors, knee flexors/extensors, hip flexors/extensors, and hip abductors/adductors. Surprisingly, when the same stretching protocol was applied to control (nonimmobilized) injured rats, we found that it caused a decrease in their locomotor function.²⁰

The goal of the current study was to extend these observations, using the same stretching protocol, to a more clinically relevant moderately severe spinal cord contusion model and to examine the effects of stretching at both acute and chronic time points. Based on our previous findings, we hypothesized that stretching would have detrimental effects on locomotor function in rats with spinal cord injuries at both acute and chronic time points.

Methods

SCI and study design

Twenty-two adult female Sprague-Dawley rats (190–230 g) were used in the study. All experimental procedures involving animals were approved by the University of Louisville Institutional Animal Care and Use Committee. On arrival, animals underwent a weeklong acclimatization protocol that involved daily gentling and introduction to the experimental apparatus used for kinematic and behavioral assessments. Figure 1 shows a timeline for the entire experiment, including collection of the primary outcome measures. Rats received a T9 laminectomy followed by a T10 moderately severe spinal cord contusion (25 g/cm, NYU Impactor) as described previously.²¹

Animals were randomly assigned to one of three experimental groups after the first behavioral locomotor assessment on day 4 post-injury: Acute Stretch (AcS, $n = 10$), Chronic Stretch (ChS, $n = 9$), or injury control for muscle histology ($n = 3$). The stretching protocol was implemented as described previously²² for the AcS group starting at 4 days post-injury and continuing for 5 weeks and for the ChS group starting at 10 weeks post-injury and continuing for 4 weeks. Stretching in the ChS group was stopped after 4 weeks be-

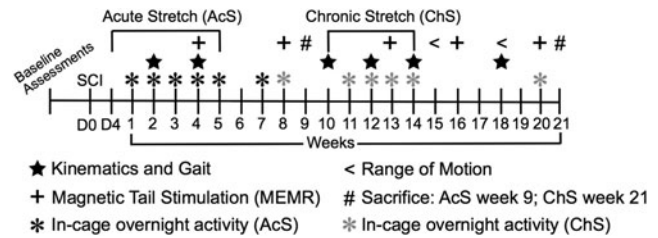


FIG. 1. Timeline for the primary experimental procedures including functional assessments. SCI, spinal cord injury.

cause of the development of severe contractures in some of those animals and the inability of our PTs to achieve full end ROM during stretching.

Briefly, the stretching protocol consisted of two 12-min sessions of six static stretches (each held for 1 min at the end ROM) performed bilaterally of major hindlimb muscle groups: ankle flexors and extensors, quadriceps, hamstrings, and hip abductors/adductors (each muscle group received 2 min of stretch per day). There were seven PTs who participated in stretching sessions. Animals were rotated among the PTs such that no animal was stretched twice by the same therapist in any given week. PTs were trained on proper animal handling and hindlimb positioning for each stretch in a pilot study that is not reported. We did not measure the forces applied during stretching; thus, PTs were instructed to closely monitor the limb position and to achieve normal end ROM for each stretch. Stretching was performed 5 days a week (Monday–Friday). After 5 weeks of stretching, the AcS group survived for an additional 13 weeks and the ChS group for 7 weeks.

Locomotor functional assessments

Overground stepping was assessed using the Basso, Beattie, and Bresnahan (BBB) Open Field Locomotor Scale²³ as described previously. BBB assessments were performed three times per week: Monday am (pre-stretch), Monday pm (>1 h post-stretch), and Friday pm (>1 h post-stretch) during the weeks of stretching and once weekly (Monday am) when stretching ended. During the first 5 weeks, the ChS group served as a behavioral control for the AcS group. The BBB scores of the ChS group plateaued at 4 weeks post-injury, and they served as their own controls.

In addition, hindlimb kinematics and gait were assessed using digital video (sagittal and ventral views) acquired while the animals stepped in 2" (5 cm) of water sufficient to supply approximately 60% body weight support, as described previously.²⁴ We used a three-segment, two-angle model of the hindlimbs for kinematic analysis with the hip-ankle-toe (HAT) representing ankle and knee excursions and the iliac crest-hip-ankle (IHA) representing hip and knee excursions.

Gait analysis was derived from ventral view videos that allowed accurate identification of paw placement and timing, and each paw placement was determined to be plantar (paw and toes appropriately placed with dorsal surfaces up) or dorsal (with one or more toes curled and/or the paw itself oriented such that dorsal surface was observable in the ventral view). Three indices of gait were determined: the central pattern index (CPI), calculated as the number of correctly patterned plantar and dorsal steps over the total number of steps, the regularity index (RI), calculated as the number of correctly patterned plantar steps over the total number of steps (dorsal and plantar), and the plantar stepping index (PSI), calculated as the number of hindlimb plantar steps over the total number of forelimb steps. Kinematic and gait analyses were performed every other week using MaxTraQ & MaxMate software (Innovision Systems Inc., Columbiaville, MI) and custom designed Excel macros.^{21,22}

Nocturnal in-cage activity recordings

Overnight in-cage activity was measured once a week (Thursday) during the weeks of stretching using overhead cameras (Basler, acA645-100 gm) and infrared lights. A 2-cm tracking dot was drawn with a Sharpie pen on the shaved lower back of each animal. The recordings were made using custom software that acquired high-resolution video at 4 Hz for 1 min of every 10 for 12 h. The video recordings were analyzed using MaxTraQ software and the distance travelled determined using a custom-designed Excel based add-in program. Discreet movements of the tracking dot that were less than 2.0 cm were not counted, thus removing much of the movement associated with grooming and sleeping.

Magnetically evoked potentials from tail stimulation

Previously, it was established that stretching affects both the mechanical properties of muscles and motoneuron excitability²⁵; 30 sessions of stretching resulted in a temporary reduction in H-reflex amplitude.²⁶ We thus wanted to determine whether our stretching protocol similarly affects the excitability of the gastrocnemius (GM) motoneuron pool after SCI. Unanaesthetized animals were restrained on a pine board using a cloth stockinette as described previously.²⁷ Afferents in the base of the tail were stimulated using a 25-mm figure-8 magnetic coil attached to a MagStim 200 (MagStim Ltd., Whitland, U.K.); stimuli were delivered at 80% of maximum intensity, sufficient to induce a plateau response but avoid direct muscle/motor axon activation.²⁸ GM muscle responses (EMGs) were recorded bilaterally using 26-gauge needle electrodes.^{22,27} EMGs were analyzed for onset latency and peak-to-peak amplitude. The assessments were performed every 4 weeks. During the weeks of stretching, the tail stimulation was performed at least 1 h after the last stretching session for each animal.

Histological procedures

Animals were overdosed with a ketamine (50 mg/kg)/xylazine (0.024 mg/kg)/acepromazine (0.005 mg/kg) cocktail and transcardially perfused with phosphate buffer, followed by 4% paraformaldehyde (PFA).²⁹ The spinal cord and hindlimb muscles (biceps femoris [BF], GM, and tibialis anterior [TA]) were dissected out, post-fixed in 4% PFA overnight, and cryoprotected with 30% sucrose. The fixed spinal cords were carefully examined to confirm the injury level (T10) and 1-cm long pieces containing the injury epicenter were prepared and placed in tissue freezing medium. Transverse sections were cut at 30 μ m and stained for spared white matter using eriochrome cyanine (EC).²⁷ Photomicrographs were acquired at 4X magnification. Cross-sectional area of compact, darkly stained white matter was traced and measured using ImageJ software (NIH) as described previously.¹⁶

Muscles were divided in two at the midbelly, placed in tissue freezing medium, and transverse sections were cut at 18 μ m. Sections were stained with Masson Trichrome for collagen as a marker of tissue fibrosis and hematoxylin and eosin (H&E) for identification of centralized nuclei as a marker of regenerating muscle fibers. For collagen quantification, two 10X images were taken from midbelly sections in specified areas oriented to consistent vascular landmarks (branches of the posterior tibial artery) in the posteromedial portion of the TA muscle (or to fascia separating the two heads of the GM and BF muscles that were present in all animals. Area (mm²) of collagen was measured using ImageJ. Manual tracing of 150 random midbelly muscle fibers was performed, per muscle, and cross-sectional area (CSA) was determined using ImageJ. It was previously established that a sample size of 150 fibers is sufficient to accurately estimate the mean muscle fiber CSA.³⁰ Muscle fibers with centralized nuclei were counted from three 40X images and normalized to the total number of muscle fibers analyzed for each

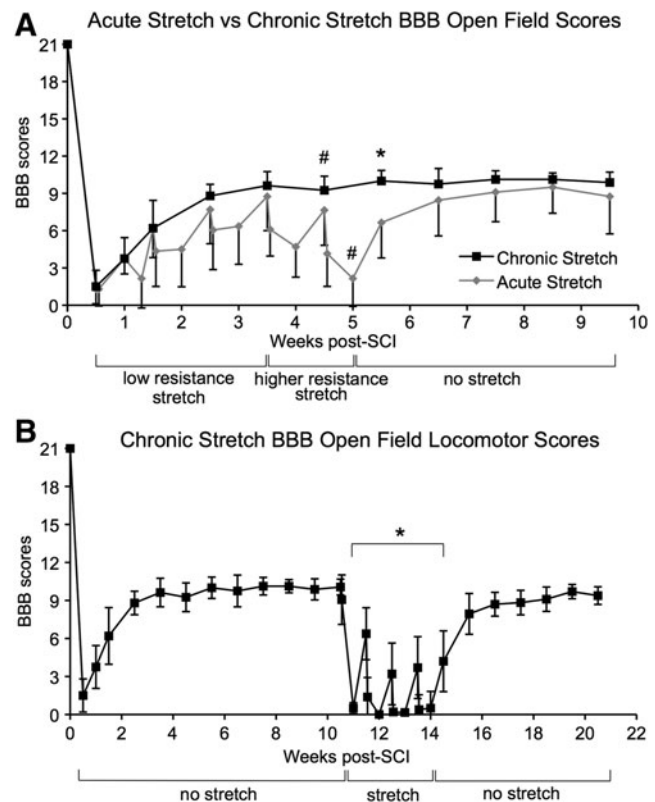


FIG. 2. Acute and chronic Basso, Beattie, and Bresnahan (BBB) Open Field Locomotor Scores. (A) BBB scores are shown for the acute and chronic (ChS) stretch groups over the first 10 weeks post-injury. Drops in BBB scores were modest and not significant during the first 4 weeks but became significant at 5 weeks after higher perceived forces were applied starting at week 4. #Indicates significant differences between Monday morning and Friday afternoon BBB scores. *Indicates significant differences in BBB scores for stretched and unstretched groups. (B) BBB scores of the ChS group dropped dramatically after only 1 week of stretching. *Indicates significant differences between pre-stretch (week 10 Monday morning) and stretch BBB scores. SCI, spinal cord injury.

animal (reported as a percentage). Histological analysis was performed by a person blinded to the experimental groups.

ROM assessment

After 4 weeks of stretching therapy, it was noted that ChS animals had reduced ROM about the hip and knee joints. To confirm this observation, we measured passive ROM around those joints at week 15 post-injury. The animals were restrained as for stretching, and each hindlimb was moved into hip/knee extension/knee flexion until passive resistance was felt. A goniometer was used to measure the joint angles as described previously.¹² ROM assessment was repeated at week 18 post-SCI when locomotor function had recovered to pre-stretch levels.

Statistical analysis

Data are presented as group means $\pm$ standard deviation. Mixed model repeated measures analysis of variance (RM ANOVA), fixed and random effects, followed by Bonferroni *post hoc t* tests were performed on all outcome measures except gait indices. Nonparametric one-sample *t* tests were performed to analyze the CPI, PSI, and RI in chronic stretch animals and PSI and RI of the acute stretch

group. A binomial proportions nonparametric test was performed to analyze CPI in the acute stretch group. Differences between groups and/or time points were considered statistically significant when $p \leq 0.05$.

Results

Open field locomotor function assessments

Stretching caused a drop in the BBB scores of both the AcS and ChS groups. Overall, RM ANOVA showed significant difference between groups ($F=27.9$, $df=1,12$, $p<0.001$) and within group time points ($F=37.5$, $df=20,12$, $p<0.001$). During the first 2 weeks of stretching, the hindlimbs of the AcS group were flaccid, and end ROM was achieved easily during the stretching sessions, with a low perceived force being applied by the PTs. Muscle tone

returned, however, by week 3, and the PTs noted that the perceived force needed to achieve the desired stretching positions had increased. Figure 2A shows that the BBB scores were apparently more influenced by stretching at weeks 4 and 5, when additional perceived force was used, and a significant difference was observed at week 5 ($p<0.005$). Two weeks after the stretching protocol ended the BBB scores of the AcS group recovered to the level of the unstretched ChS animals, albeit with high variability.

Locomotor function of the ChS group reached a plateau at post-injury week 4 and remained consistent for the next 6 weeks until the start of stretching at 10 weeks post-SCI (Fig. 2B). After only 1 week of stretching, BBB scores of the ChS group dropped to an average of 0.5 ($p<0.005$); most animals had only slight movement of one joint on one side. By week 13, all nine animals had BBB scores of 0. The vulnerability of locomotor function to stretching at chronic

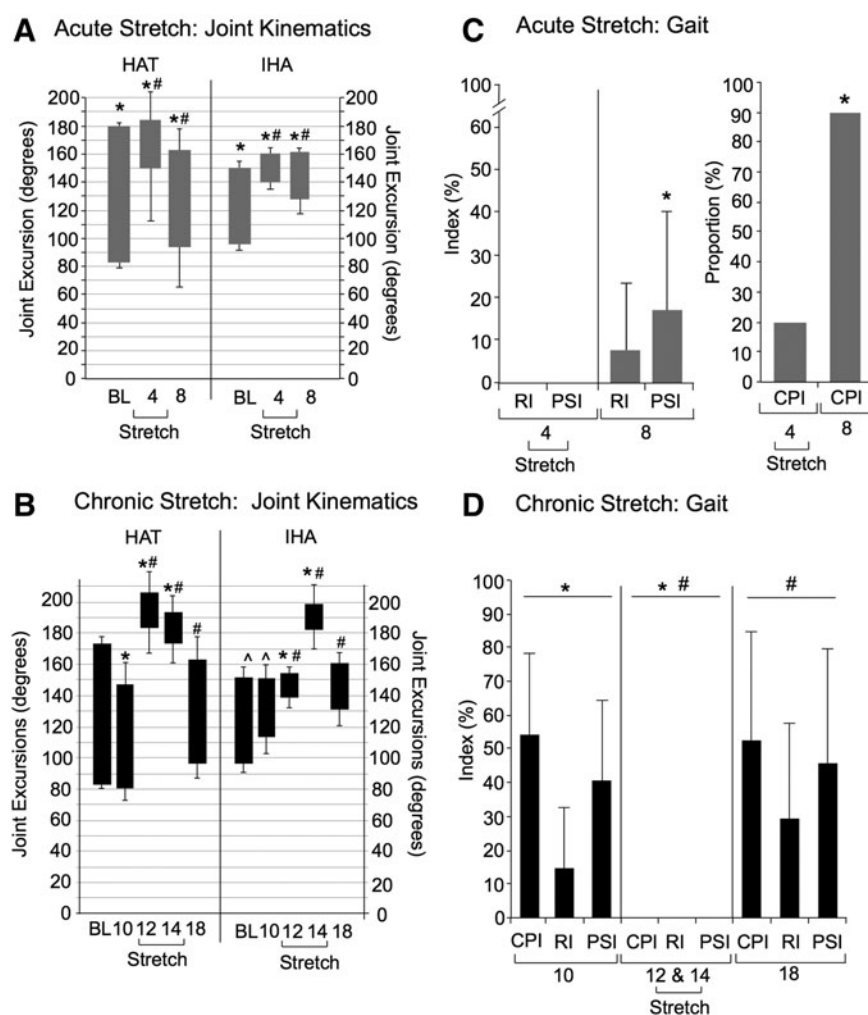


FIG. 3. Kinematic and gait analysis of shallow water walking (SWW). Distal (hip-ankle-toe, HAT) and proximal (iliac crest-hip-ankle, IHA) joint excursions are indicated by the bars: top and bottom of the bar represents the mean peak extension and flexion, respectively, of the joint angles $\pm$ standard deviation, and thus bar length represents the mean angular excursion or range of motion (ROM). (A) Acute Stretch (AcS): Significant differences are indicated by (*) for HAT and IHA joint excursions of AcS animals compared with the Chronic Stretch (ChS) group. Significant differences are indicated by (#) for IHA and HAT excursions of AcS animals at week 4 (during stretching therapy) compared with week 8 (3 weeks after the last stretching session). (B) ChS: Significant differences indicated by (*, #) for ChS animals when comparing weeks 10 or 18 with weeks 12 and 14. In addition, IHA excursions remained significantly lower at week 10 compared with baseline (^). (C) AcS: Significant differences (*) were seen when comparing central pattern index (CPI), plantar stepping index (PSI), and regularity index (RI) for week 4 and week 8. (D) Significant differences indicated by (*, #) for ChS animals when comparing weeks 10 or 18 with weeks 12 and 14.

time points was also revealed by the fact that one stretching session (Monday am) was enough to undermine any recovery that took place over the weekend when the animals were not stretched. Within 2 weeks of the last stretching session, the BBB scores for the ChS group recovered sufficiently to be not different from pre-stretch levels.

Kinematic and gait analysis of the locomotor function

Kinematic (HAT and IHA excursions) and gait analysis (CPI, PSI, RI) of the AcS animals are summarized in Figure 3A and C. RM ANOVA showed significant time point differences in AcS HAT ($F=42.7$, $df=2,27$, $p<0.001$) and IHA excursions ($F=137.4$, $df=2,27$, $p<0.001$). Specifically, HAT and IHA “joint” excursions were significantly lower at both week 4 and 8 compared with pre-SCI baseline ($p<0.05$). In addition, HAT and IHA excursions were significantly lower at week 4 (during stretching therapy) compared with week 8, 3 weeks after the last stretching session ($p<0.05$). At week 4, only 2 of 10 AcS animals achieved some dorsal stepping in shallow water and thus had a measureable CPI above 0; the remaining 8 did not step, and therefore had PSI and RI indices of 0. By week 8, AcS rats achieved significant recovery revealed by the PSI ($p<0.05$) and CPI ($p<0.001$).

Kinematics and gait analyses of the ChS group are shown in Figure 3, B and D. Overall, RM ANOVA showed significant time point differences in ChS HAT excursions ($F=275.9$, $df=4,40$, $p<0.001$) and ChS IHA excursions ($F=376.1$, $df=4,40$, $p<0.001$). At week 10 post-SCI, before stretching, HAT excursions had recovered sufficiently to not differ significantly from baseline (pre-injury), whereas IHA excursions were still significantly lower ($p<0.05$). At weeks 12 and 14, during the stretching therapy, ChS animals dragged their hindlimbs resulting in very low angular excursions likely reflecting only passive movements of the ankle as the animal moved in shallow water. Figure 3 shows the significant differences ($p\leq 0.005$) between pre-stretch IHA and HAT excursions (week 10), stretch (12 and 14) and week 18 (4 weeks after the

last stretching session) when ChS animals regained locomotor function to a pre-stretch level.

By week 10 (before the beginning of stretching), CPI and PSI for the ChS group recovered to about 50% of normal, whereas RI remained poor, indicating that both the forelimbs and hindlimbs were achieving plantar stepping, but the two girdles were decoupled (poor or no hindlimb/forelimb coordination). During the weeks of stretching (weeks 12 and 14) RI, PSI, and CPI were all equal to zero, indicating a complete lack of stepping, which was significantly different ($p<0.05$) from pre-stretch (week 10) values. Four weeks after the last stretching session (week 18), ChS animals regained the ability to step, indicated by the return of RI, PSI, and CPI to pre-stretch levels.

Nocturnal in-cage activity

In-cage, overnight activity was monitored, and the distances traveled were estimated using a 1 in 10 min sampling rate (Fig. 4). Even though mixed model RM ANOVA analysis of the AcS overnight distance traveled showed significant differences across time points ($F=4.9$, $df=6,45$, $p<0.001$), *post hoc t* tests did not (Fig. 4A). Post-injury distances averaged approximately 150 m per animal per night by 4 weeks post-injury. Thus, stretching did not have an apparent effect on the in-cage activity of this group.

RM ANOVA showed significant differences in distance traveled of the ChS animals ($F=7.8$, $df=7,48$, $p=0.001$). ChS animals averaged 149.3 m/night at week 8 (before stretching intervention) and 67.7 at week 13 (during the third week of stretching), which was significantly lower than week 8 ($p<0.05$), while at week 14, the ChS group averaged 71.7 m/night (approaching significance, $p=0.061$) (Fig. 4B). Further, overnight activity of the ChS animals was significantly lower ($p<0.05$) at weeks 12, 13, and 14 (3 of the 4 weeks of stretching) compared with week 20, 4 weeks after the last stretching session. They recovered to 134.7 m/night by week 20, which was not different from pre-stretching (week 8) levels.

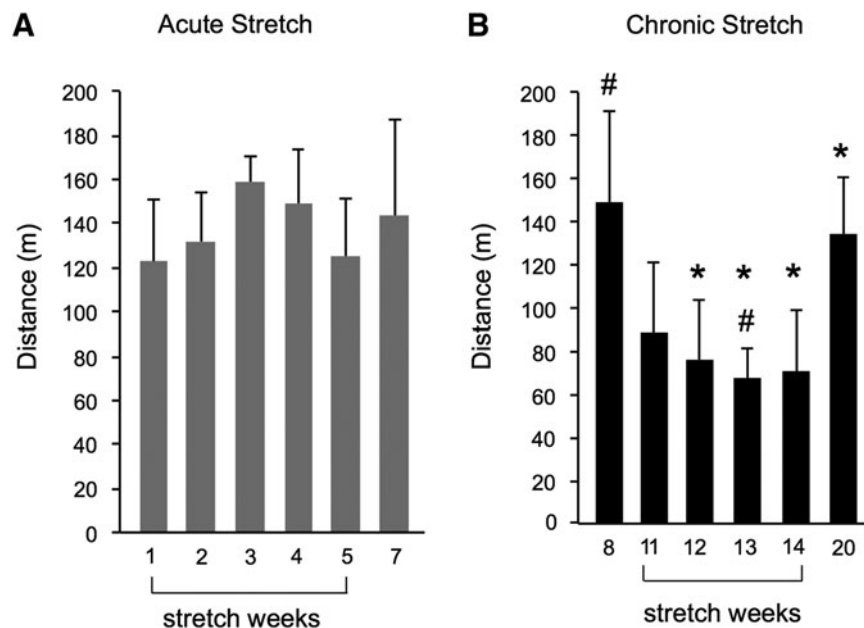


FIG. 4. Nocturnal in-cage activity. (A) Acute Stretch: There were no statistically significant differences in distance traveled in the acute animals at any time points. (B) Chronic Stretch (ChS): Significant differences in overnight activity of ChS animals are indicated by (#, *) for ChS animals when comparing weeks 8 or 20 with weeks 11–14.

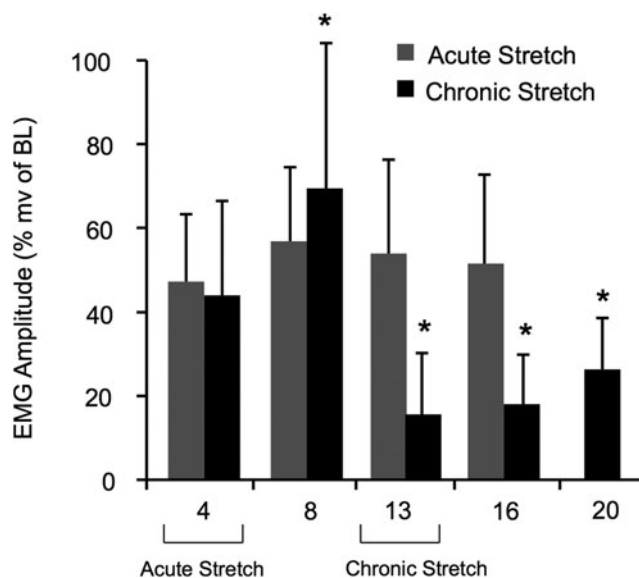


FIG. 5. Amplitude of magnetically evoked muscle responses (MEMRs). Significant differences are indicated by (*, #) for Acute Stretch (AcS) and Chronic Stretch (ChS) animals when comparing normalized MEMRs at weeks 13, 16, or 20 to weeks 4 or 8. There was no difference in MEMRs when comparing AcS and ChS animals at week 4. EMG, gastrocnemius muscle responses; BL, baseline.

Magnetically Evoked Muscle Response (MEMR):

Responses of the GM muscle to magnetic stimulation of the base of the tail (MEMRs) were assessed every 4th week of the experiment. These short-latency (5–6 msec), presumed mono- or disynaptic responses, reflect excitability of the GM motoneurons and any changes in the primary afferents and muscles themselves that could influence the EMG signal. Figure 5 shows MEMR data (post-SCI time points normalized to baseline). RM ANOVA showed significant group ($F=10.8$, $df=1,3$, $p<0.005$) and time point ($F=5.16$, $df=4,3$, $p<0.005$) differences in normalized MEMR.

SCI resulted in a significant decrease in EMG amplitude for both acute (AcS) and chronic (ChS) groups (by about 50%). Stretching had no additional effect on MEMR in the AcS group, because the amplitude of the EMG responses did not differ significantly from the unstretched group (ChS) at week 4. The normalized response amplitude of the ChS animals dropped significantly at week 13 (measured during the 4th week of stretching), however, a significant decrease from the week 8 (pre-stretch) values. Responses remained significantly decreased at weeks 16 and 20 (2 and 5 weeks, respectively, after the last stretching session) even though BBB scores returned to control levels. The onset latency remained unchanged (5–6 msec) throughout in both groups (data not shown).

ROM

Significantly reduced locomotor function in the ChS at the end of the 4-week stretching protocol resulted in the development of

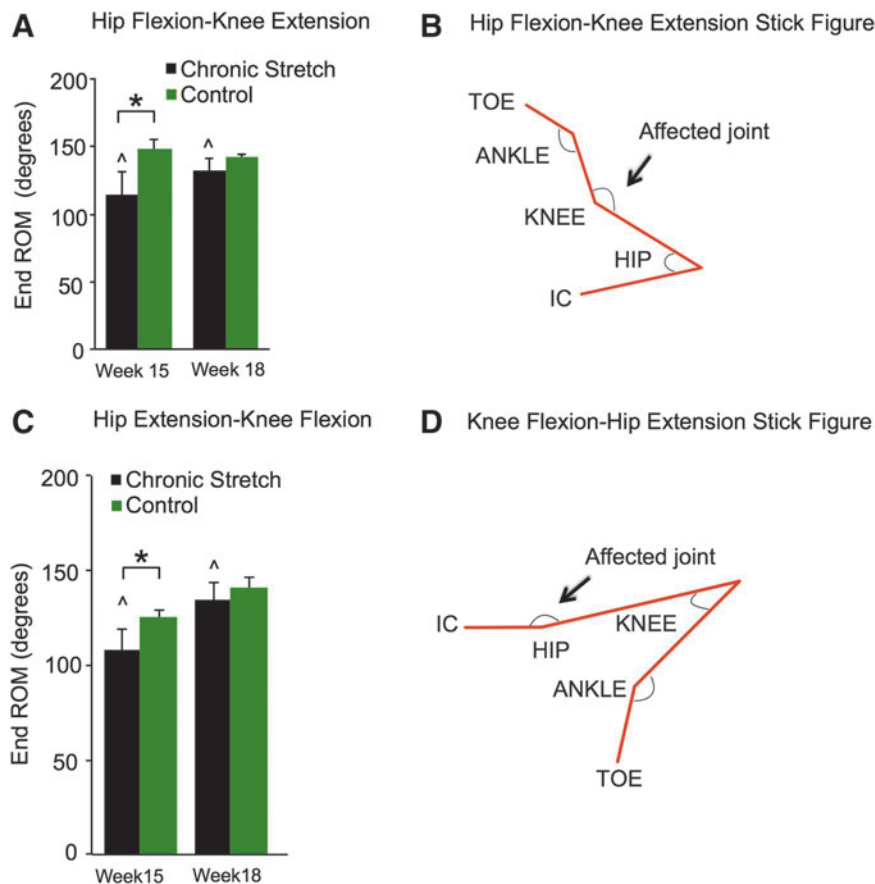


FIG. 6. Passive end range of motion (ROM). Significant differences are indicated by (*) for knee (A,B) and hip (C,D) angles at end ROM when comparing the Chronic Stretch (ChS) animals with the unstretched control animals at weeks 15 and 18. By week 18, the ChS animals had recovered significantly (^) when compared with week 15. IC, iliac crest. Color image is available online at www.liebertpub.com/neu

TABLE 1. SKELETAL MUSCLE HISTOLOGICAL ANALYSIS

	Muscle Fiber CSA (μm^2)		
	<i>Tibialis Anterior</i>	<i>Gastrocnemius</i>	<i>Biceps Femoris</i>
Acute Stretch ($n=10$)	573.05 $\pm$ 99.00	730.04 $\pm$ 139.75	616.89 $\pm$ 154.77
Chronic Stretch ($n=9$)	531.52 $\pm$ 116.70	598.83 $\pm$ 122.42*	468.38 $\pm$ 100.84^
Control ($n=3$)	521.55 $\pm$ 82.26	871.96 $\pm$ 261.74	684.55 $\pm$ 118.28
	Centralized nuclei count (% of MF analyzed)		
	<i>Tibialis Anterior</i>	<i>Gastrocnemius</i>	<i>Biceps Femoris</i>
Acute Stretch ($n=10$)	3.53 $\pm$ 0.02	3.457 $\pm$ 0.02	1.669 $\pm$ 0.01
Chronic Stretch ($n=9$)	2.9 $\pm$ 0.01	2.738 $\pm$ 0.01	3.294 $\pm$ 0.03
Control ($n=3$)	2.01 $\pm$ 0.01	3.456 $\pm$ 0.01	4.283 $\pm$ 0.03
	Collagen area (mm^2)		
	<i>Tibialis Anterior</i>	<i>Gastrocnemius</i>	<i>Biceps Femoris</i>
Acute Stretch ($n=10$)	0.093 $\pm$ 0.04	0.071 $\pm$ 0.03	0.127 $\pm$ 0.03
Chronic Stretch ($n=9$)	0.121 $\pm$ 0.5	0.073 $\pm$ 0.02	0.146 $\pm$ 0.03
Control ($n=3$)	0.112 $\pm$ 0.17	0.064 $\pm$ 0.01	0.116 $\pm$ 0.03

CSA, cross-sectional area; MF, muscle fiber.

*Chronic Stretch (ChS) gastrocnemius CSA significantly different from Control.

^ChS biceps femoris CSA significantly different from Control.

contractures around the knee and hip joints. A reduced ROM around those joints was confirmed using a goniometer. RM ANOVA showed significant group differences in the knee ($F=14.34$, $df=1,1$, $p<0.005$) and hip ($F=7.5$, $df=1,1$, $p<0.05$) ROM values as well as significant time point difference in hip ROM ($F=21.3$, $df=1,1$, $p<0.001$). At week 15, the ROM around the knee (Fig. 6A) and hip (Fig. 6B) joints of the ChS animals were significantly lower compared with control animals ($p<0.01$; $p<0.05$, respectively). At week 18, when locomotor function of the stretched animals recovered back to pre-stretch levels, the ROM for both previously affected joints had improved significantly ($p<0.01$) from week 15 and was no longer different from the controls. This suggests that in-cage activity was sufficient to ameliorate the muscle contractures when the animals were not being stretched.

Spinal cord and muscle histology

There were no significant differences in the percent spared white matter at the injury epicenter, reported as mean $\pm$ standard deviation: AcS 3.97 ± 2.33 , ChS 2.64 ± 1.62 , control 3.77 ± 2.6 . TA, GM, and BF muscles were analyzed for persistent signs of overt injury induced by repetitive or excessive strain during stretching and that might contribute to the observed locomotor deficits. We also measured CSA of muscle fibers to assess whether muscle stretch resulted in hypertrophy because it is a potent mechanical stimulus for muscle growth in non-SCI models.¹⁷ The results of the histological analysis are summarized in Table 1.

The percentage of muscle fibers that contained centralized nuclei was also not significantly different for any group. We could not identify any areas of fibrosis (tissue scarring) in the three muscles examined, and quantitative analysis of collagen revealed no significant differences between the groups. CSA of TA, GM, and BF muscle fibers in the AcS group were not different from injured, unstretched controls. One-way ANOVA showed significant group difference in CSA of GM ($F=7.5$, $df=2,19$, $p<0.005$) and BF ($F=15.8$, $df=2,19$, $p<0.001$), indicating that the ChS group had significantly decreased GM and BF fiber CSA compared with either the AcS group or the controls; however, no differences in TA fiber CSA were found. These observations suggest that the period of very

low activity (weeks 12–15) for the ChS animals resulted in a decrease in muscle fiber CSA (disuse atrophy) in the extensor muscles analyzed.

Discussion

These results extend our previous observations of the stretching phenomenon^{20,22} into a more clinically relevant moderately severe animal model of contusive SCI. There are four primary findings in this study. First, locomotor function of animals with chronic SCIs is highly vulnerable to the effects of stretching (BBB scores of 0 for all nine animals after the second week of stretching) despite the fact that locomotor recovery of the ChS group had plateaued for more than 4 weeks before the initiation of the stretching protocol. Second, 4 or 5 weeks of stretching did not result in persistent, long-lasting changes in either overground locomotion in the chronic and acute SCI groups as shown by the BBB scores or by shallow water walking because the HAT and IHA “joint” excursions achieved significant recovery. Third, in the chronic group, EMG responses to magnetic stimulation of the base of the tail (MEMRs) remained low for as long as 5 weeks after the last stretching session suggesting a persistent decrease in motoneuron or circuit excitability. Finally, we could find no signs of muscle damage in either AcS or ChS animals that could explain, even in part, the deficits in locomotor function, suggesting that stretching does not result in overt muscle damage that could explain its negative impact on locomotor function; thus, neurological mechanisms are likely underlying the locomotor deficits.

Unlike human subjects with SCI, animals with only 5–10% spared white matter achieve significant locomotor recovery.²⁴ One of the biggest differences between human subjects and rats with SCIs is the state of mobility after the traumatic event. Experimental animals are returned to their home cages (usually with a cage mate) where they move about freely. Flaccid paralysis resolves, in-cage activity increases, and the functional forelimbs provide a built-in training mechanism leading to significant locomotor recovery.²⁴ On the other hand, animals immobilized in wheelchairs after SCI have significant deficits in locomotor function compared with unrestrained SCI rats.²⁰

In this study, we found that the overnight, in-cage activity (which does not indicate hindlimb involvement in stepping) of the AcS animals remained consistently high throughout the 5 weeks of stretching, potentially contributing to their quick recovery from the stretch-induced deficits on the weekends. Overnight activity of the ChS animals, on the other hand, significantly decreased during the weeks of stretching, most likely because these animals used only their forelimbs for propulsion because stretching temporarily negated the hindlimb recovery achieved in the first 10 weeks after SCI. These results support the concept that hindlimb function associated with higher BBB scores facilitates in-cage distance traveled.

After the last stretching session, animals in both stretching groups recovered significant locomotor function back to control/pre-stretch levels within 2 weeks. In our previous stretching study, the animals also achieved substantial locomotor recovery; however, persistent and significant deficits in function remained.²² The previous study involved 8 weeks of stretching as opposed to 4 or 5 weeks in the current study. This suggests that longer periods of immobility within the perceived critical window of opportunity for functional locomotor recovery may have resulted in an inability to achieve "full" recovery when returned to standard double-housing. In addition, animals in the previous study had milder SCIs and higher average BBB scores of 14 compared with 11 in the current study. Thus, the persistent deficits observed in the previous study may be reflected only in the finer aspects of locomotion, such as hindlimb-forelimb coordination (BBB scores of 11 vs. 14), which would not have been discerned in the current study because of the more severe injury and lower functional plateau.

The focus of PTs who employ stretching for patients with SCI is to maintain the extensibility of soft tissues and preserve or regain ROM in joints vulnerable to the development of contractures.³¹ Based on a systematic review of stretching for the treatment and prevention of contractures in persons with neurological conditions, however, stretching therapies did not result in clinically important improvements in joint mobility.³² The authors suggest that a potential limitation in the practice of stretching is a lack of standardized protocols or specific recommendations for the frequency, intensity, and duration of stretch to achieve the desired outcomes.

One animal study investigated the effectiveness of different stretching characteristics (manipulation of torque intensities and duration) to manage the development of knee contractures in animals with complete thoracic spinal cord transections.³³ They found that maximizing both the torque and the duration of stretch resulted in the most significant improvements in contractures (increased ROM). Thus, the authors suggest that high intensity-long duration static stretching may be an effective modality to investigate in clinical trials. This study, however, did not consider the potential adverse neurological effects of intense, long-duration stretch.

In the present study, when a higher perceived force was needed to achieve a normal end ROM during the 4th and 5th weeks of stretching in the AcS group, the negative impact on locomotor function became significant. It appears that the optimal stretching characteristics for treatment and prevention of muscle contractures might also be the most negative for locomotor function after an incomplete contusive SCI.

Despite the fact that we carefully monitor the hindlimb position for each stretching maneuver and received consultation from experienced human PTs, we wanted to investigate whether the negative effects of stretching might be partly attributed to overt and persistent muscle trauma. Muscle fibers injured by strain undergo

robust regeneration via the activation of satellite cells.^{34,35} Because the nuclei are located peripherally in the myocyte, it is easy to identify fibers that have been injured, because regenerating/regenerated muscle fibers have centralized nuclei, which persist for at least 4 months.³⁶ We found no significant group differences in the number of muscle fibers containing centralized nuclei.

Collagen deposition in the extracellular matrix around muscle fibers is another indication of trauma, in particular after repetitive muscle strain.³⁷ In addition, myocytes that experience persistent or repetitive trauma will eventually fail to regenerate, and the debris of dead muscle fibers will be removed and replaced with collagen, resulting in easily recognizable and permanent fibrosis or scarring.³⁴ Thus, we quantified collagen in the muscles but again found no differences between the three groups. We therefore concluded that our stretching protocol does not result in frank and persistent muscle damage that can help explain the significant locomotor deficits that we observe in our animals.

The severe locomotor dysfunction, significantly reduced in-cage activity, and contractures (reduced passive end ROM of the knee and hip) in the ChS rats were accompanied by significant decreases in the CSA of GM (ankle extensor) and BF (knee flexor/hip extensor) muscles, suggesting disuse atrophy. The TA (ankle flexor) muscle, however, did not undergo significant atrophy. Previous studies found a similar pattern of muscle atrophy as a result of disuse affecting extensor muscles more than flexors.^{38,39}

It is worth considering, however, that muscle stretch does not affect only the muscles, tendons, and ligaments. Stretching will also affect group Ia and II muscle spindle afferents, potentially increasing their threshold for firing (decreasing their sensitivity to stretch). Given their importance for the generation/recovery of locomotion after SCI,⁴⁰ changes to muscle spindle sensitivity is one potential explanation for the stretching phenomenon. In addition to Ia and group II muscle spindle afferents, muscles are innervated by small diameter thinly myelinated and unmyelinated group III and IV afferents that are activated by mechanical stimuli such as contraction and muscle stretch.⁴¹⁻⁴⁴ Specifically, Cleland and colleagues⁴³ have identified stretch-sensitive free nerve endings of group III and IV fibers that mediate powerful reflex inhibition of the homonymous muscles in an animal model of clasp-knife reflex. This same group has also found force-sensitive interneurons that receive input from group III and IV afferents and that produce rapid and sustained inhibition of motoneuron output.⁴⁵

Group III and IV afferents have also been implicated in the inhibition of motor output (central fatigue) during exercise in humans.^{46,47} Thus, it is probable that the stretching protocol we administer to animals with SCI activates group III and IV afferents that in turn should have an inhibitory affect on motor circuitry resulting in dramatic drops in locomotor function. Whether or not this mechanism is responsible, in whole or in part, for our current observations remains to be determined.

After an incomplete SCI in rats, the sprouting of spared descending axons is thought to mediate at least some of the remarkable functional recovery.⁴⁸ On the other hand, primary afferent sprouting, particularly of C and A δ fibers, has been associated with neuropathic pain and autonomic dysreflexia.^{49,50} It is possible that increased arborization of these primary afferents also leads to the more robust inhibitory effects of group III and IV afferents over motor output, thus explaining why animals with stabilized locomotor recovery (ChS group) are so vulnerable to the negative effects of stretching. If this hypothesis is confirmed, it is likely that the stretching phenomenon we observe in our rats has high clinical relevance.

Harvey and associates⁵¹ investigated the amount of torque PTs apply during regular stretching sessions and found that some therapists applied torques that were two to six times higher than what is tolerated by sensate individuals. In addition, the authors discuss the possibility that patients with SCI regularly generate very high torques around their hip joints while dressing in a seated position. For the majority of patients with SCI, stretching that activates nociceptive afferents would not result in the perception of pain because of the loss of sensory function below the level of injury. Existing evidence suggests, however, that transmission of nociceptive signals results in a multitude of unfavorable effects over the locomotor circuitry^{52,53} that has an otherwise high capacity for retraining, given the appropriate proprioceptive feedback.^{54–57}

Conclusion

Stretching has been adopted as a therapy in the rehabilitation regime for patients with SCI based in part on past evidence from animal studies that focused on soft tissues undergoing maladaptive changes as a result of immobilization.¹⁶ While more recent clinical investigations into stretching therapy reveals its general ineffectiveness for the treatment of muscle contractures in patients with SCI,^{14,32} current and previous findings from our laboratory suggest that stretching is detrimental to locomotor function in animals with mild to severe SCIs at both acute and chronic time points. The clinical relevance of our results is yet to be determined, but these findings suggest strongly that the neurological effects of muscle stretch warrants consideration as being potentially detrimental to the function of locomotor circuitry after SCI.

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Author Disclosure Statement

No competing financial interests exist.

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Dynamic “Range of Motion” Hindlimb Stretching Disrupts Locomotor Function in Rats with Moderate Subacute Spinal Cord Injuries

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Abstract

Joint contractures and spasticity are two common secondary complications of a severe spinal cord injury (SCI), which can significantly reduce quality of life, and stretching is one of the top strategies for rehabilitation of these complications. We have previously shown that a daily static stretching protocol administered to rats at either acute or chronic time points after a moderate or moderate-severe T10 SCI significantly disrupts their hindlimb locomotor function. The objective of the current study was to examine the effects of dynamic range of motion (ROM) stretching on the locomotor function of rats with SCI as an alternative to static stretching. Starting at 6 weeks post-injury (T10 moderate contusion) eight adult Sprague–Dawley rats were subjected to hindlimb stretching for 4 weeks. Our standard stretching protocol (six maneuvers to stretch the major hindlimb muscle groups) was modified from 1 min static stretch-and-hold at the end ROM of each stretch position to a dynamic 2 sec hold, 1 sec release rhythm repeated for a duration of 1 min. Four weeks of daily (5 days/week) dynamic stretching led to significant disruption of locomotor function as assessed by the Basso, Beattie, Bresnahan (BBB) Open Field Locomotor Scale and three-dimensional (3D) kinematic and gait analyses. In addition, we identified and analyzed an apparently novel hindlimb response to dynamic stretch that resembles human clonus. The results of the current study extend the observation of the stretching phenomenon to a new modality of stretching that is also commonly used in SCI rehabilitation. Although mechanisms and clinical relevance still need to be established, our findings continue to raise concerns that stretching as a therapy can potentially hinder aspects of locomotor recovery.

Keywords: dynamic stretching; locomotor function; physical therapy; rehabilitation; SCI

Introduction

SPINAL CORD INJURY (SCI) that causes paralysis below the level of the lesion results in a multitude of secondary complications that commonly include joint contractures and spasticity.¹ Both complications can significantly reduce the quality of life of SCI patients.² Currently, stretching remains the first line therapy to treat and prevent contractures and spasticity.^{3–5} Previously in our laboratory, we showed that a 24 min protocol of “static” stretching of the major hindlimb muscles of rats with mild to moderately acute and chronic SCI resulted in significant declines in locomotor function.^{6,7} The effects of therapeutic stretching on motor function of SCI patients is currently not known; the main outcome measures of clinical studies on stretching are range of motion and/or spasticity.⁸ However, a multitude of studies have investigated static stretching in uninjured athletic populations and have observed

negative effects on some aspects of motor function.⁹ Specifically, static stretching results in decrements in vertical jumping and running performance,^{10–12} muscle strength endurance,¹³ and isometric strength^{14,15} as well as isokinetic torque production.^{16,17} Dynamic stretching is an alternative technique to improve range of motion (ROM) and flexibility that has been recommended as part of the warmup routine for athletic events; it is not thought to cause detriments in performance, and may actually improve it.^{18,19} Dynamic stretching involves multiple repetitions of limb movement through its entire range to the end ROM.²⁰ Such ROM exercises are also commonly performed during the rehabilitation of SCI patients.²¹ Therefore, we wanted to determine if a dynamic stretching protocol involving all major hindlimb muscle groups in rats with SCI disrupted locomotor function similarly to our static stretching protocol. For a variety of reasons including a reduction in the total time at end ROM and the involvement of a rhythm, we

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hypothesized that dynamic stretching would not be detrimental to locomotor function in rats with moderate SCI.

Methods

Eight young adult female Sprague–Dawley rats were used in this experiment. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Louisville. After a week of acclimatization and baseline data collection, the animals received moderate spinal cord contusion injuries (NYU, 12.5 g/cm) at T10 as described previously.²² Locomotor function was assessed at least weekly using the Basso, Beattie, Bresnahan (BBB) Open Field Locomotor Scale,²³ and video-based kinematic and gait analysis was performed biweekly. As described previously,⁷ we used a three segment, two angle model of the hindlimb, and “joint” excursions were measured as the hip–ankle–toe (HAT) and iliac crest–hip–ankle (IHA) angles. Two gait indices were also used, the regularity index (RI) and the plantar stepping index (PSI), based on ventral-view video allowing paw placement order, timing, and location to be determined for every step taken (from initial contact to lift-off) using MaxTraq software (Innovision Systems Inc., Columbiaville, MI) and custom-designed Excel macros. The RI is calculated as the number of correctly patterned plantar steps over the total number of steps (dorsal and plantar).²⁴ The PSI is

calculated as the number of plantar hindlimb steps over the total number of forelimb step.²⁵

At 6 weeks post-injury, the animals entered a 4 week dynamic stretch protocol that was performed 5 days a week for 4 weeks. Dynamic stretching consisted of the same six bilateral stretches of major hindlimb muscle groups as our previous static stretching protocol;^{6,7} however, instead of holding the limb at the end ROM of each stretch for 1 min, each stretch was held for only 2 sec, followed by a 1 sec release, repeated 20 times over a 1 min period. A metronome (at 1 Hz) was set to the 2-1 ratio, using different tones, in order to give an audible clue to the rat physical therapists (PT) allowing them to maintain the rhythm and ensuring that timing was consistent and accurate between therapists for each animal every day. During the 4 weeks of stretching, BBB scores were assessed three times weekly (Monday a.m., Monday p.m., and Friday p.m.).

Tonic stretching triggered several observable responses in both the stretched and unstretched limbs that rat PTs took note of.⁶ Dynamic stretching resulted in a new and highly consistent response that was observed as paw “vibrations”: high frequency and low amplitude fluctuations in one or two joints. Because of its resemblance to human clonus, which can be triggered by sudden stretching or upon the release of stretch,^{26,27} we sought to better understand this phenomenon. To quantify this response, single stretching sessions for four animals were recorded (digital video at

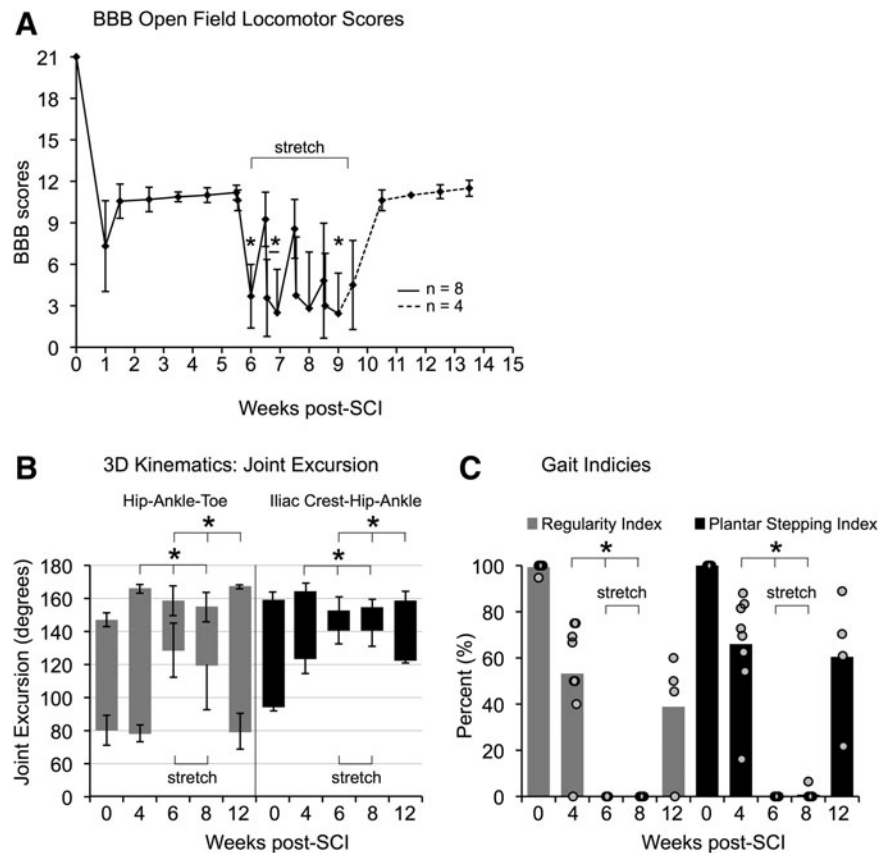


FIG. 1. Locomotor function. (A) Dynamic stretching protocol began 6 weeks after spinal cord injury (SCI) when locomotor function had reached a stable plateau and continued on for 4 weeks. At the end of the first week of stretching, the animals had significantly lower Basso, Beattie, Bresnahan (BBB) scores as compared with their pre-stretch values. Over the weekend animals achieve significant recovery; however, the first session of the 2nd week of stretching induced deficits in the locomotor function that persisted to the end of the week. Similar pattern of recovery/disruption followed for the remaining 2 weeks of the stretching protocol. Four of the eight animals were euthanized after 2 h of stretching, for histological muscle assessment and the other four were allowed to recover for 4 weeks before being euthanized. Their locomotor function was assessed weekly (dotted line). (B,C) Analysis of biweekly kinematic and gait recordings showed that the animals had significantly reduced joint excursions (B) and gait indices (C) indicative of lack of stepping or even sweeping in some animals during the weeks of the stretching therapy (weeks 6 and 8), as compared with the pre-stretch stepping ability and hindlimb joints excursions (week 4). By week 12, the locomotor function of the remaining four animals had achieved significant recovery back to pre-stretch levels.

100 Hz frame rate with the same PT stretching all animals). Most often vibrations occurred in both limbs simultaneously; however, kinematic analysis was performed on the contralateral limb, as more joint markers were easily viewable and could be tracked with the software for analysis. The toe, ankle, knee, hip, and iliac crest were marked with a black marker for kinematic analysis of the movement using MaxTraQ software. Three vibration responses per stretch per animal were analyzed for ROM (excursion of movement) and frequency (peaks per second). Averages of these outcome measures from the four animals for each stretch are reported in the Results.

After the last week of stretching (week 4), four of the eight animals were euthanized 2 h after the last stretching session, and the other four animals were maintained for an additional 5 weeks. The spinal cords and major hindlimb muscles (tibialis anterior [TA], medial gastrocnemius [MG] and biceps femoris [BF]) were dissected out, post-fixed in 4% paraformaldehyde (PFA) and cryo-protected in 30% sucrose. Muscles (mid-belly) were sectioned at 10 μ m and stained with Hematoxylin and Eosin. Muscle fibers (MF) were analyzed for the presence of centralized nuclei, a marker of regeneration.²⁸ Spinal cords were sectioned at 50 μ m and stained with eriochrome cyanine to determine the percentage of white matter sparing at the injury epicenter using ImageJ software as described previously.²²

Statistical Analysis

Each animal served as its own control for the locomotor outcome measures, and the data were analyzed for changes over time using a mixed model analysis of variance (ANOVA) (BBB scores and joint excursions) followed by Tukey *post-hoc*. Nonparametric Wilcoxon signed ranks test was used to make comparisons between time points in the gait indices RI and PSI. One way ANOVA followed by independent *t* tests were used to compare the percentages of MF containing centralized nuclei between the animals euthanized 2 h after the last stretching session (group 1, $n=4$) and the animals euthanized 5 weeks later (group 2, $n=4$). Significant differences were established at $p < 0.05$.

Results

Based on a dense, compact appearance on eriochrome cyanin stained sections, moderate SCI at T10 resulted in an average spared

white matter (SWM) at the injury epicenter of $7.35\% \pm 3.36$. The average SWM for group 1 was $6.08 \pm 2.54\%$ and $8.61 \pm 3.96\%$ for group 2. There was no significant difference in SWM between the groups euthanized at two different time points ($p = 0.322$). Despite the low percentage of spared white matter, the animals achieved substantial locomotor recovery: BBBs plateaued by week 6 with an average score of 11, which is indicative of consistent weight-supported stepping without forelimb–hindlimb coordination (Fig. 1). One week of dynamic stretching starting at 6 weeks post-SCI resulted in a significant decrease in locomotor function as average BBB scores dropped to 3.7 ± 2.3 ($p = 0.004$). Thereafter, at the end of each week of stretching, BBB scores hovered near 2.5 (indicative of slight movement of one or two joints only). As in previous studies, these animals achieved some locomotor recovery over the weekends when they were not stretched.^{6,7} However, one stretching session (Monday morning) was enough to again reduce the BBB scores to below 4 (Fig. 1). Lack of hindlimb movement was further confirmed with the more objective kinematic and gait analysis. The animals had significantly reduced excursions (ROM) of both IHA and HAT hindlimb angles during the weeks of stretching (weeks 6 and 8). RI and PSI were reduced to 0 at week 6 (measured on the 3rd day of the 1st week of stretching) indicating a complete lack of plantar stepping (Fig. 2B). These gait indices remained at 0 until stretching ceased. Figure 2 depicts some characteristics of the vibration response. In B, a stick figure is shown representing a single episode of the vibration response upon the release of stretch (in this case of the hamstring stretch): black line showing a starting position and gray lines showing a full ROM that the limb goes through during the response. As the foot “vibrates,” (multiple flexion/extension phases) the positions of the toe, ankle, and knee change, and Figure 2A shows the velocity profile of these points. Table 1 shows the average frequencies (calculated as number of peaks in knee–ankle–toe [KAT] per second), and averaged ROM (degrees) in the KAT angle during the vibration response for each stretch. The percentages of muscle fibers containing centralized nuclei for each muscle analyzed are listed in Table 2. Animals euthanized 2 h after stretching had a significantly higher percentage of muscle fibers with centralized nuclei in the MG muscle than the group euthanized 5 weeks later ($p < 0.05$). There were no significant differences for the BF and TA muscles.

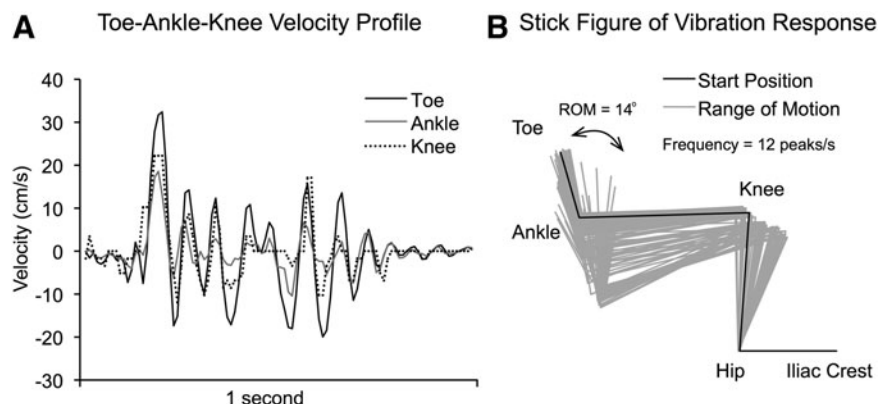


FIG. 2. Characteristics of vibration response. Kinematic analysis was performed on the contralateral limb vibration response during a release of 2 sec hamstring stretch of the opposite limb. Vibrations occurred in both limbs simultaneously; however, kinematic analysis was performed on the contralateral limb, as more points were visually available and could be tracked with the software for analysis. (A) During a vibration response, the limb goes through multiple clonic-like contractions, as evidenced by the multiple peaks in velocity of the toe–ankle–knee angle in 1 sec. (B) Stick figure of the contralateral limb during a vibration response. The black line shows a steady position of the limb during the 2 sec stretch hold period of the opposite limb. Upon release of stretch, the clonic-like contractions (gray stick figures) are initiated as the limb is pulled in toward the body (knee and hip are flexed). Most of the movement occurs in the ankle joint, with an average range of motion of 14 degrees, and contractions occurring at high frequency (~ 12 peaks per second).

TABLE 1. CHARACTERISTICS OF CLONIC-LIKE MUSCLE RESPONSES TO STRETCH

<i>Muscle/Stretch</i>	<i>Clonic-like stretch response characteristic</i>	
	<i>Frequency (peaks/sec)</i>	<i>Range of Motion (degrees)</i>
Tibialis anterior	12.86 ± 3.44	16.24 ± 12.29
Gastrocnemius	13.29 ± 5.55	11.74 ± 7.81
Quadriceps	16.35 ± 1.41	7.04 ± 2.29
Hamstring	15.38 ± 1.72	8.56 ± 3.55
Hip abductors	15.48 ± 2.65	8.33 ± 8.28
Hip adductors	13.14 ± 2.51	2.96 ± 0.93

Data reported as means ± SD.

Discussion

In this study, we sought to determine if an alternative dynamic hindlimb stretching protocol would result in disrupted locomotor function in rats with SCI. Based on the simple concept that phasic or rhythmic afferent input would be less detrimental to spinal cord circuitry function as it relates to locomotor function than tonic afferent input,²⁹ we hypothesized that dynamic stretching would not induce locomotor deficits. Contrary to our hypothesis, dynamic stretching dramatically disrupted locomotor function, reducing BBB scores to levels similar to those of static stretching, even though the total amount of stretch that the animals received on a daily basis was less (20 sec less/stretch) than in our previous studies.^{6,7,29} Histological analysis of the muscles revealed that for the ankle extensor MG, the group euthanized 2 h after stretching had elevated numbers of centralized nuclei (8%) as compared with the rats euthanized 4 weeks later (~4%), suggesting that more muscle fiber regeneration was occurring caused by the strain resulting from multiple stretching sessions. It is important to note however, that the additional muscle fiber regeneration was apparently complete by 4 weeks, suggesting that the stretching did not cause damage beyond what the muscle repair system is equipped to handle, and is, therefore, unable to explain the locomotor deficits. Nevertheless, this is the first evidence suggesting some degree of muscle damage caused by stretching, potentially because our previous studies had animals surviving for several weeks after the last stretching session.

During stretching, our rat PTs kept a thorough record of all observable hindlimb responses, among which are contralateral limb kicking, air stepping, and spasms (contralateral and ipsilateral

limbs). A new response was uncovered with dynamic stretching, which we refer to as “vibrations.” These were induced by the onset and the release of stretch, and were seen in the both the stretched and the contralateral unstretched limb. Although we did not record electromyography (EMG) during these responses, we did determine the kinematics of the vibrations. We found that the vibrations had a frequency range of 10–19 Hz, with a mean of 14.42 for all stretches combined, and a very low amplitude, ~10 degrees (in the KAT angle). Onset of muscle contractions when the limb was being stretched and the muscles lengthened, or in other words, eccentric muscle contractions, were being generated during our stretching protocol. Eccentric muscle contractions that occur at the ground contact phases of normal locomotion are a part of stretch-shortening cycle, one of the fundamental functions of the skeletal muscle.³⁰ This type of muscular activation serves to decelerate the limb for smooth and controlled contact with a surface,³¹ and is often utilized in athletic training because it provides an excellent stimulus for muscle hypertrophy and strength gains.^{32–34} However, eccentric contractions have been also recognized to result in a greater amount of disruption in myofibrillar structure,³⁵ which could potentially explain why we see an increased number of regenerating fibers in the muscles of the animals that were euthanized within hours of the last stretching session. Delayed onset muscle soreness (DOMS), which is a type of mechanical hyperalgesia, occurs 1–2 days after eccentric contractions as a result of nociceptive afferents sensitization in response to release of bradykinin and nerve growth factor.³⁶ Consistent with the DOMS phenomenon is the following observation: we have never observed any decrements in locomotor function following the initial stretching session in this (Fig. 1) or any of our earlier studies.⁷ However, in the 2nd week of stretching, one session did cause the deficits, presumably because of the sensitization of afferents, which now were functioning at lower thresholds.

Nociceptive afferents have complex modulatory effects on locomotor circuitry. Kniffki and coworkers have observed that stimulation of group III and IV afferents can either accentuate an ongoing fictive locomotor rhythm or block the rhythmic output by inducing a period of tonic hyperactivity.³⁷ In healthy humans, activation of nociceptive afferents may only have mild inhibitory effects on locomotor function, as supraspinal modulation may limit their activity. Electrical stimulation of mesencephalic locomotor region in the cat inhibits group III and IV transmission in the dorsal horn.³⁸ After SCI, however, central pattern generators in the lumbar cord become much more reliant on peripheral inputs for their activation,³⁹ and, therefore, locomotor circuitry is also likely to be more vulnerable to inhibitory effects of group III and IV afferents. Moreover, nociceptive afferents undergo substantial plasticity after SCI, which has been implicated in the development of neuropathic pain^{40,41} and autonomic dysreflexia.⁴² Activation of nociceptive afferents impairs locomotor recovery after SCI in rodents, and spinal learning in rats^{43–45} and humans.⁴⁶ Therefore, it would not be surprising that nociceptive afferents are also involved in mediation of the negative effects of stretching on locomotor function.

Conclusion

In conclusion, muscle stretching is a commonly used technique aimed at increasing/maintaining joint ROM, and is used across multiple populations from the athletic to the clinical. We have now shown that both static and dynamic stretching applied to the hindlimbs of spinal cord injured animals result in the dramatic but temporary disruption of their locomotor function. The physiological

TABLE 2. PERCENT OF MUSCLE FIBERS WITH CENTRALIZED NUCLEI

<i>Muscle</i>	<i>Percent of muscle fibers with centralized nuclei</i>	
	<i>Group 1 (n=4)</i>	<i>Group 2 (n=4)</i>
Tibialis anterior	3.77% ± 1.62	4.7% ± 1.81
Medial gastrocnemius	8.21% ± 2.06 ^a	4.18% ± 1.12
Biceps femoris	4.49% ± 2.45	4.65% ± 1.53

Data reported as means ± SD.

Group 1, animals euthanized 2 h after the last stretching session; Group 2, animals euthanized 5 weeks after the last stretching session.

^aGroup 1 had a significantly greater percentage of muscle fibers with centralized nuclei in the medial gastrocnemius muscle than Group 2 ($p < 0.05$).

mechanism of this phenomenon is not yet known, although based on our observations and histological findings it is unlikely to be caused by frank muscle damage, but rather may involve activation of nociceptive afferents. The clinical relevance of our findings needs to be established, but given that static stretching has been shown to have detrimental effects on performance in healthy humans,^{9,14,47} it is conceivable that after SCI, the motor circuitry is even more vulnerable to the negative effects of stretch.

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Author Disclosure Statement

No competing financial interests exist.

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Electromyographic patterns of the rat hindlimb in response to muscle stretch after spinal cord injury.

Introduction.

Stretching remains a leading therapy for the treatment of muscle contractures as well as spasticity, two of the most common complications after a severe spinal cord injury (SCI). Moreover, stretching is a rehabilitation strategy often employed acutely post-SCI in an effort to prevent the development of muscle contractures because they are much harder to treat once established [1]. The rationale for using stretching in the rehabilitation of soft tissue contractures arose initially from animal studies that showed stretching to be effective at maintaining joint range of motion (ROM) due to immobilization [2]. However, stretching for the treatment or prevention of contractures in SCI patients does not have the same efficacy [3]. We determined recently that a daily protocol involving either static or dynamic application of stretch, disrupts hindlimb locomotor function in rats with SCI [4]. The mechanisms underlying the stretch-induced loss of locomotor function are not known but based on our previous findings they do not involve overt muscle damage [4]. It is well documented that in healthy subjects static stretching decreases strength and maximum performance [5-8]. Avela et al., have shown that repeated and prolonged stretching results in decreased EMG amplitude during maximum voluntary contraction, suggesting a central mechanism [9]. Interestingly, Cramer et al., observed strength loss not only in the stretched limb but also in the contralateral non-stretched limb, further implicating a circuitry-based mechanism [10]. Given these observations and widespread use of stretching in the rehabilitation/physical therapy of patients with SCI it is important to establish if the negative effects of stretching on locomotor function in rats with SCI are clinically relevant. Therefore, we designed and performed an experiment using tools that would allow direct comparison with human studies, such as electromyography, force and torque measurements and limb kinematics during stretching. We hypothesize that EMG patterns represent the response of the spinal cord to the afferent input generated by stretching and thus can serve as a translational cue: if responses are similar for rats and humans, then it is likely that the effect of stretching on motor circuitry are also similar. Force measurements will provide context for the EMG responses and will help set the parameters for future clinical studies on stretching after SCI.

Methods.

Animals, EMG transmitter instrumentation and spinal cord injury.

Four adult Sprague Dawley rats were used for this experiment. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Louisville. After 1 week of standard gentling procedures and baseline assessments the animals underwent an initial surgery for instrumentation with a telemetry-based 2 lead EMG transmitter (F20-EET, Data Sciences International®, St. Paul, MN;). The animals were anesthetized with a ketamine cocktail and a surgical level of anesthesia was confirmed by the absence of paw withdrawal reflexes to strong foot pinch. The upper back of each animal was shaved and cleaned and a small skin incision was made between the shoulder blades where the body of the transmitter was placed. The wires of the transmitter were tunneled subcutaneously down to the right hindlimb by separating the skin from the muscle layer using blunt dissection. A small incision was made over the thigh of the animal allowing the wire electrodes to be inserted into and through the belly portion of the Rectus Femoris and Biceps Femoris muscles. Sutures were placed on each side of the wire to hold it in place. The incisions were closed with sutures and animals were allowed to recover, receiving our standard post-operative care with antibiotics (gentamicin sulfate for 7

days (20 mg/kg)) and pain management (buprenorphine 0.03 mg/kg twice a day for 3 days). Two weeks later the animals underwent a second surgery and received a mild-moderate spinal cord contusion injury at thoracic level T10 (12.5 g/cm, NYU contusion device) as previously described [11]. The animals were then allowed to recover for two weeks prior to the beginning of stretching.

Stretching and recording procedures.

In this study we used 6 stretches (1 minute static hold) of the major hindlimb muscle groups from our previously described stretching protocol [12]. For the first 6 weeks the stretching protocol was carried out twice a week (Monday and Wednesday) only on the left (non-implanted) limb, while recording the responses of the contralateral (implanted) limb. For weeks 7 and 8 we switched to daily stretching and included two ankle stretches (gastrocnemius and tibialis anterior) of the implanted limb. For the final stretching and recording session (week 8 Friday) all 6 stretches were performed on the implanted limb. EMG data was acquired through the DataQuest Acquisition hardware (Data Sciences® International, St. Paul, MN) and PONEMAH® 5.0 software package (sampled at 1000 Hz). The data was exported for further analysis to LabChart (ADInstruments Colorado Springs, CO). EMG was band-pass filtered @ 50-2000 Hz. The analysis of clonic-like responses for amplitude and frequency (in one second) was performed on rectified and smoothed (101 samples) EMG using LabChart's peak analysis feature. Videos of the implanted limb were linked to EMG recordings and each analyzed clonic-like response was confirmed visually in the video.

The forces applied during stretching were measured using a custom designed force glove (FlexiForce A301 sensors connected to a USB-6210 data acquisition system) operated by LabView software. Force sensors were calibrated to standardized weights of 105, 305 and 505 grams prior to each stretching session. Force sensor data was sampled at 1000 Hz. Sensors for the thumb (TS) and index finger (IS) were used to measure forces during the four stretches. They were placed as follows: for the tibialis anterior stretch (ankle flexor) TS was placed on the dorsal portion of the foot and IS on the heel; for gastrocnemius stretch we used only one sensor (TS), placed on the mid plantar portion of the foot; for quadriceps TS was placed on top of the knee (which was bent during this stretch), while IS was placed at the mid dorsal portion of the foot; and for the hamstring stretch TS was placed at the heel and IS - on the thigh slightly superior to the knee which was extended for this stretch. The forces were not measured during hip abduction and adduction stretches due to difficulty of locating precise points of force application. The positions of the toe, ankle, knee, hip and iliac crest, along with the sensors were digitized using MaxTraQ software (Innovision Systems, Inc., Columbiaville, MI) to determine the distances between the joints and sensors allowing torque to be calculated ($\text{torque} = \text{force} \times \text{distance}$ (cm) $\times 9.8 \text{ (m/s}^2\text{)} / 1000 \text{ (g/kg)}$, where 9.8 is the gravitational acceleration constant).

Locomotor function was assessed using 3D kinematics and gait analysis based on paw placement order. Horizontal and ventral view recordings were made pre- and post-stretching for each session. As previously described [11, 13] we used a 3 segment 2 angle model of the hindlimb for kinematics – iliac crest-hip-ankle (IHA angle) and hip-ankle-toe (HAT angle), digitized using MaxTraQ software with final excursions determined using a custom designed excel macro. Gait was assessed using the regularity index which is calculated as the number of correctly patterned plantar steps over the total number of steps [13]. BBB open field locomotor assessments [14] were done once a week on Fridays.

Animals were sacrificed using a ketamine (50 mg/kg)/xylazine (0.024 mg/kg)/acepromazine (0.005 mg/kg) cocktail and transcardially perfused [15] with phosphate buffer. The spinal cord was dissected

out and postfixed in 4% PFA overnight and cryoprotected in 30% sucrose for at least 4 days. The injury level (T10) was confirmed visually using a dissection microscope, and the injury epicenter was blocked in tissue freezing medium. Transverse sections were cut at 30 μ m on a cryostat for histological assessment of spared white matter (SWM) at the SCI epicenter. The sections were stained with eriochrome cyanine and imaged at 4X using a light microscope. Dark blue compact white matter was traced and measured in ImageJ and the section with the lowest percentage of SWM was determined to be the epicenter [16].

Data is shown as mean $\pm$ standard deviation. The outcome measures were analyzed for changes over time using RM ANOVA, followed by a Bonferroni post hoc t-test. One-sample t-test was used to compare regularity index values between baseline (100%) and all time points post-injury.

Results.

EMG responses to stretch.

Figure 1 shows representative EMG responses to stretch in the contralateral limb: clonic-like contraction (between 3 to 9 Hz), air-stepping and spasms. Clonic-like responses and spasms were also present in the ipsilateral limb when it was stretched at week 8, but air stepping could not be observed because the limb was held in a stretched position. These three responses were observed in all animals and are consistent with our previous observations [12, 17]. The responses can be observed while the limbs are being positioned (“pre-stretch”) and stretched and then typically decline while the limb is maintained in the stretch position for 1 minutes. The frequency and amplitude of these responses to stretch increased over time after SCI. Figure 2 shows EMG responses recorded while the limb was being positioned at week 2 (A) and week 8 in the contralateral limb (B) and ipsilateral limb (C). Figure 3 shows the EMG responses and recorded torque during a 1 minute quadriceps stretch of the contralateral limb at week 2 (A&B) and in the ipsilateral limb at week 8 (C&D). EMG response amplitude and the frequency of clonic-like contractions were quantified for weeks 2 to week 8 (Figure 4). Although there were no significant differences over time there was an overall trend of increased amplitude and frequency in clonic-like responses from week 2 to week 8 during most stretches in both contralateral and ipsilateral limbs. Clonic-like contractions were particularly robust during quadriceps and tibialis anterior stretches.

Torques applied during stretching.

The torques applied to achieve and end range-of-motion varied dramatically for the four muscles stretches, with the gastrocnemius requiring about 0.8 N*cm and the tibialis anterior requiring about 6 N*cm. Overall the required forces were consistent over time for the Gastrocnemius, Hamstring and TA stretches (with the exception of week 7 for the TA) however the forces required for quadriceps stretches gradually increased over time and were significantly greater at weeks 5, 6 and 8 as compared to week 2.

Locomotor function.

Figure 6 shows the weekly BBB score (A), regularity index (B) and joint excursions for the hip-ankle-toe (C) and iliac crest-hip-ankle (D). The EMG implant appeared to have no adverse effects on the locomotor function of the animals as they moved in the open field as all the animals had BBB scores of 21 post-implant. Spinal cord injury resulted in significant disruptions to locomotor function, however by week 2 post-SCI the animals were able to generate consistent weight supported stepping. The gait was significantly impaired for the first 5 weeks post-SCI as compared to baseline (Fig. 6B). The excursion of the HAT angle, representing the ankle and knee, significantly increased compared to baseline (Fig. 6C),

while excursions of the IHA angle, representing the hip and knee, significantly decreased (Fig 6D). Stretching one limb twice a week had no obvious effect on the locomotor function of the animals over the initial 6 weeks. During weeks 7 and 8 when we began stretching the non-implanted limb and added ankle stretches of the implanted limb, BBB scores still remained stable, however, the BBB subscores dropped from an average of 4 to 2.2 during weeks 7 and 8 (data not shown). Pre and post-stretch HAT and IHA angular excursions of the stretched limb were not significantly different. In addition, there was a trend towards gait impairment; the regularity index dropped from pre- to post stretch for each stretching session. These effects were only statistically significant at week 6, when pre-stretch RI values were identical for all animals. This low variability most likely allowed statistically significant differences to be detected despite the small sample size.

Discussion.

Previously (Caudle et al., 2012, 2016, Keller et al., 2016) we observed that stretching hindlimb muscles after an incomplete thoracic spinal cord injury induces motor responses in both the stretched (ipsilatera) and unstretched (contralateral) hindlimb. These responses have been noted and referred to as air stepping, spasms and kicking. In the current experiment we determined that the “kicking” responses have a robust EMG pattern occurring at high frequencies (3-9 Hz) that is similar in appearance and frequency to clonus in humans [18]. We observed these clonic-like contractions in response to stretch in both contralateral and ipsilateral limbs. Although clonus in humans with neurological impairments is most frequently seen in the stretched limb one study observed clonus also in the contralateral leg in SCI patients [19]. In our current study the clonic-like responses could be evoked at 2 weeks post-SCI, when we began stretching, however the number of responses and their amplitude and frequency increased by week 8 post. These increases were not significant due to the small sample size and variability. Clonic-like responses were most often observed simultaneously in both the knee flexor (biceps femoris) and knee extensor (rectus femoris), appearing as co-contractions, although less frequently we also observed clonic-like responses in one but not the other muscle. Co-contractions have also been reported during clonus in human subjects with SCI. Clonus in SCI patients can occur in response to stretch or other cutaneous inputs and significantly interferes with the activities of daily living [20]. We did not observe any spontaneously occurring clonic-like contractions while animals were in their cages or during kinematic and gait analysis, although spasms are sometimes observed during open field locomotor assessments. In general, it has been difficult to create a clinically relevant model of spasticity because animals with incomplete injuries develop only mild forms of hypereflexia that is difficult to detect without using velocity-dependent measures [21]. However, animals with complete transections at the sacral spinal cord level develop spasticity and tail clonus [22]. More recently, in a study by van Gorp et al., animals exhibited bilateral kicking in response to nociceptive mechanical stimuli applied to the unloaded hindpaw [23]. The authors suggest that these responses could be clonus. In the present study we demonstrate that stretching activates nociceptive afferents, thus it is possible that the clonic-like responses are the result of nociceptive signaling. Although clonus in humans is thought to be mediated through activation of Ia afferents [24], a study of human subjects with SCI showed that myoclonus could no longer be elicited when the underlying pathology (in spinal nerve roots, hip joint, thigh muscle) was resolved [25]. Therefore, activation of nociceptive afferents should be considered as a potential physiological trigger for at least some types of clonus.

Air stepping is another consistent pattern that we see during stretching. Frequently, robust clonic-like movements at the initiation of stretch were replaced with air stepping that varied in intensity and the

number of hindlimb segments involved. With more intense air stepping we saw the EMG bursts with greater activation in Rectus Femoris, but in most cases, air stepping although rhythmic and frequent did not result in detectable EMG in the knee muscles. Consistently with previous observation, spasms frequently occurred at the end of a 1 minute stretch. Sometimes clonic-like contractions had spastic like appearance, with wider less consistent peaks within the clonic episode. Early on after injury we also observed spasms that were quite subtle in appearance, almost like an isometric contraction – increased EMG in the knee muscles of the contralateral limb but only slight movements in the toes were apparent while a static stretch was maintained on the opposite limb.

In this experiment we used a custom designed force glove to measure forces during the four major stretches. We found that the highest forces applied during tibialis anterior stretch were on average around 6 N*cm, while the lowest forces of 0.8 N*cm were applied during the Gastrocnemius stretch. One of the limitations of the force sensors used in the current experiment is that the accuracy of their force measurement was somewhat dependent on the precise localization of the center of the sensor to the point of the highest force application. Tibialis Anterior stretch was perhaps the easiest to achieve such precise positioning of the force sensors, as the index finger sensor was placed on the heel of the rat (a very distinct landmark which was smaller than the sensor itself) and the thumb was placed on the dorsal part of the foot. In addition, end ROM of the ankle plantarflexion is achieved more readily as compared to the other stretches and therefore there is higher resistance from the tissues at the end ROM of the ankle plantarflexion which could also explain the highest forces were achieved during this stretch. While the forces during TA, Gastrocnemius and Hamstring stretches were consistent throughout the weeks, there was a gradual increase in forces during the Quadriceps stretch. As animals recovered from SCI, greater number of clonic-like contractions, particularly during the quadriceps stretch, likely contributed to the increase in torque over time, since the force sensors are sensitive to the resistance coming from the animal during the stretch.

To our knowledge, the current experiment is first animal study to conduct force measurements during stretching in rats (besides the previous experiment in the lab for a master thesis project – the development of the force glove, unpublished data). A few studies have addressed the issue of forces applied during stretching in human patients with SCI. Harvey et al., quantified the torques applied during hamstring stretch by twelve different PTs [26]. The range of forces varied substantially with the median torques in the range of 30-68 N*m, while some therapists applied torque over 100 N*m. If we compare torques about the hip for human and rat based strictly on body weight (average weight of subjects from Harvey's study – 73kg, and average weight of the rats in our study - .25 kg) the ratio would approximately be 290:1. The equivalent range of torques for rats during the hamstring stretch would be 10-23 N*cm. In this study, the average torque during hamstring stretch was around 3 N*cm. In another study by Harvey et al., examining a 4 week stretching intervention patients' ankles were stretched (gastrocnemius stretch) using a set torque of 7.5 N*cm while ROM assessment was performed using 10N*cm torque [27]. Once again, equivalent torques for rats would be 2.8 and 3.8 N*cm. The average torques applied during gastrocnemius stretch in the current experiment were 0.8 N*cm. This comparison has obvious limitations, for example, it is not known if rat's sensitivity to forces is comparable to humans, however, the torques applied in this study are still well under those reported for human patients for these stretches.

The primary goal of the current study was to determine the EMG responses of the animals to stretch. However, some observations regarding the effects of stretch on locomotor function were made.

Stretching of one limb for the first 6 weeks did not result in overall disruption to the locomotor function as in previous studies [4, 12, 17, 28]. There was a drop in BBB subscores (reduction in toe clearance and increased paw rotation) at the end of the weeks when stretching was done every day (week 7 and 8), however. In addition there were slight drops in the regularity index after every stretching session which occurred most likely due to increased number of dorsal steps. This pattern of subtle disruption taken together with drops in BBB subscores suggests that the “low” dose of stretching used in this experiment had an effect on finer motor control aspects of locomotion. This data provides small but informative details to help us further understand the stretching phenomenon. Perhaps, these effects of intermittent stretching in high functioning animals with mild-moderate injuries (20% SWM) are comparable to the negative effects of stretching on some aspects of motor performance (isometric strength, muscle strength endurance, isokinetic torque production, etc) seen in intact human subjects [5, 7, 8].

In conclusion, we have identified at least two similar EMG responses elicited by hindlimb stretching in rats as reported for human subjects: clonic-like contractions and spasms. We also determined that the torques used during stretching of rat hindlimbs are comparable and likely even lower than those PTs apply to human patients. Although it would be ideal to design a similar experiment in patients with SCI in order to directly compare the findings between human and rat, the existing evidence strongly suggests that stretching might have similar effect on the nervous system in humans as it does in rats. Therefore, further investigation of stretching and its effects on motor function in human subjects is strongly warranted.

Figure Legends.

Figure 1. Three commonly occurring EMG responses during hindlimb stretching in the Rectus Femoris (top trace) and Biceps Femoris (bottom trace) of the contralateral limb: clonic-like contractions at frequencies 3-10 Hz (A), air stepping with predominantly extensor activity (B) and spasms (C)

Figure 2. Contralateral and ipsilateral hindlimb responses start as the limb is being positioned into the end range of motion for a given stretch. The responses became more pronounced with time post-SCI. An example of EMG responses during a 10 second positioning period prior to quadriceps stretch in the contralateral limb at 2 weeks post-SCI (A), contralateral limb at 8 weeks post-SCI (B) and ipsilateral limb at 8 weeks post-SCI (C).

Figure 3. EMG responses during a 1 minute quadriceps stretch in the contralateral limb at week 2 post-SCI (A-C) and ipsilateral limb at week 8 post-SCI (D-F). C and F show forces applied over the duration of the stretch. In F forces increase when there is a spike in EMG activity, demonstrating that the force sensors were sensitive to the resistance from the animal when hindlimbs were activated.

Figure 4. The forces remained consistent through the weeks of stretching for Gastrocnemius, Hamstring and Tibialis Anterior (except at week 7, when forces were significantly lower as compared to week 2, $p < .05$). The forces gradually increased with weeks post-SCI and were significantly greater at weeks 5, 6 and 8 as compared to week 2.

Figure 5. Quantification of amplitude and frequency of clonic-like EMG responses to stretch in the contralateral limb at week 2 and 8, and ipsilateral limbs at week 8. Although there were no statistically significant differences due to small sample size ($n=4$) and high variability, there was a trend in increased

amplitude (A,C) and frequency (B,D) of clonic-like contractions from week 2 to 8, most pronounced in responses to quadriceps and tibialis anterior stretch.

Figure 6. Spinal cord injury resulted in significant disruption of locomotor function. By 5 weeks the animals achieved significant recovery as the regularity index was no longer different from baseline (except week 6 post-stretch). There were no statistically significant difference in pre- and post- stretch gait regularity index (B) although there was a trend in slight decreases in RI post-stretch. The BBB scores remained stable for the duration of the study and stretching of one limb twice a week for 6 weeks and then daily stretching (all 6 stretches of the non-implanted limb and 2 ankle stretches of the implanted limb) during week 7 and 8 did not overall disrupt locomotor function (A). After SCI Hip-Ankle-Toe (HAT) (C) excursions were significantly increased while Iliac Crest-Hip-Ankle (IHA) (D) excursions were significantly decreased for the duration of the study. Pre- and post-stretch HAT and IHA excursions were not different (assessed using 3D kinematics).

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Figure 1. Typical EMG responses to stretch.

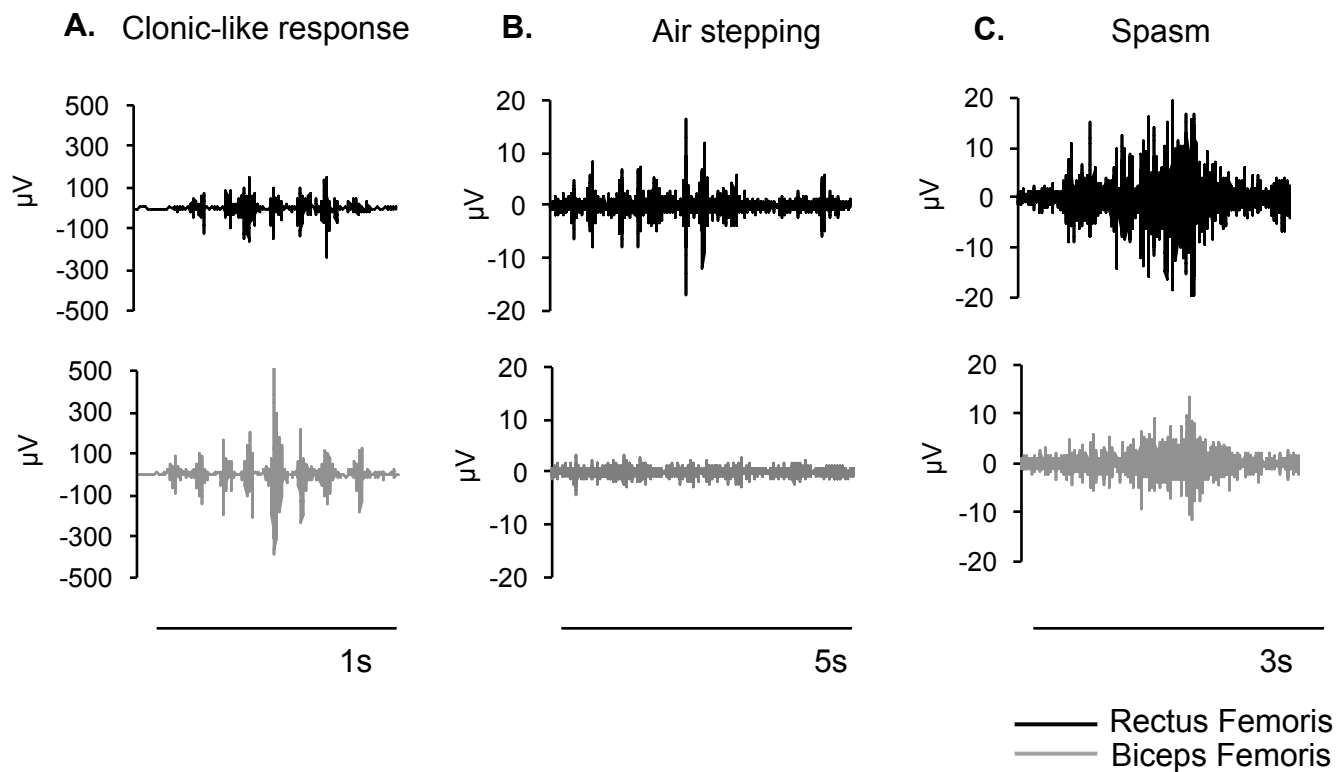


Figure 1. Three commonly occurring EMG responses during hindlimb stretching in the Rectus Femoris (top trace) and Biceps Femoris (bottom trace) of the contralateral limb: clonic-like contractions at frequencies 3-10 Hz (A), air stepping with predominantly extensor activity (B) and spasms (C)

Figure 2. EMG responses prior to quad stretch.

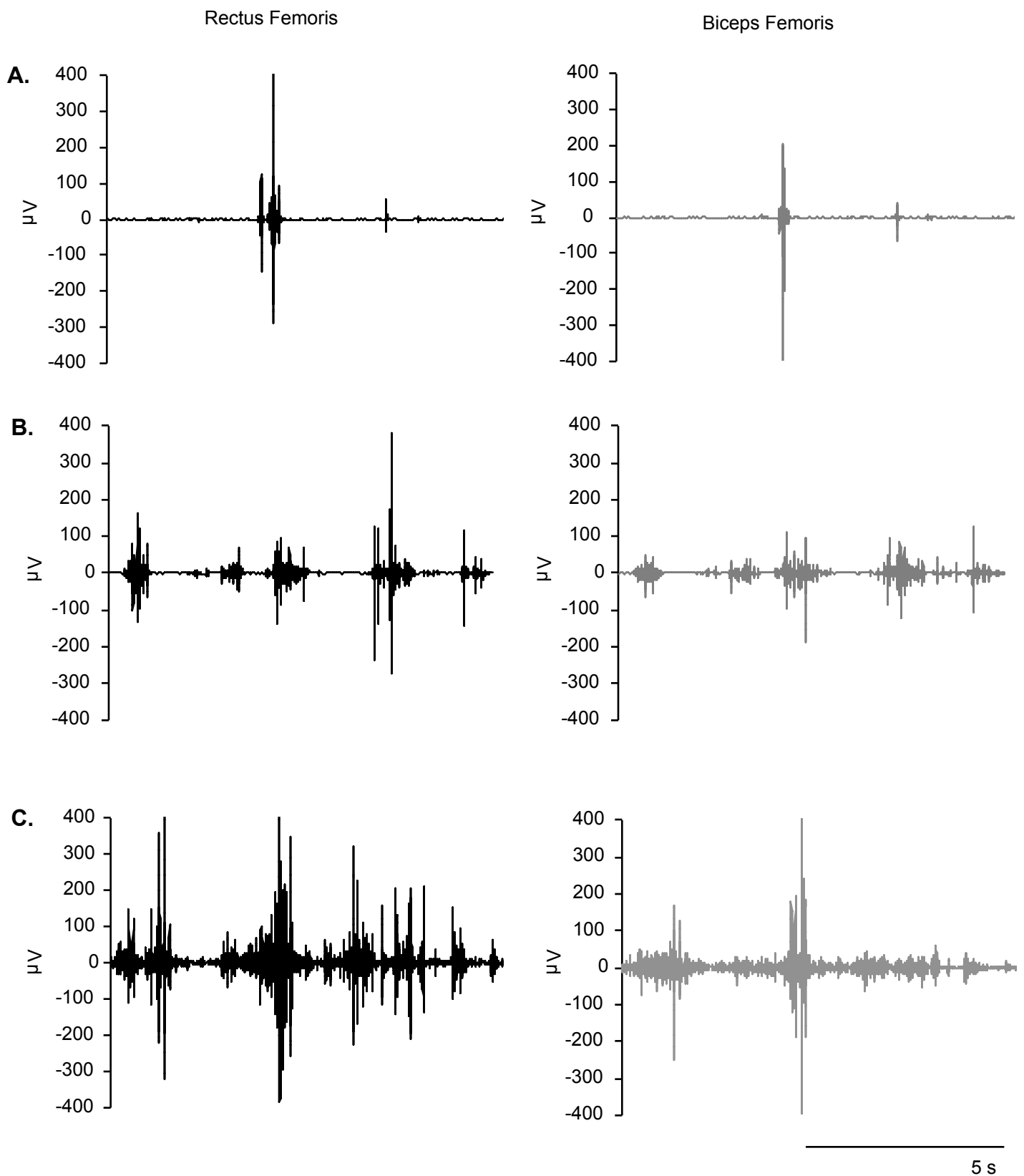


Figure 2. Contralateral and ipsilateral hindlimb responses start as the limb is being positioned into the end range of motion for a given stretch. The responses became more pronounced with time post-SCI. An example of EMG responses during a 10 second positioning period prior to quadriceps stretch in the contralateral limb at 2 weeks post-SCI (A), contralateral limb at 8 weeks post-SCI (B) and ipsilateral limb at 8 weeks post-SCI (C).

Figure 3. Forces and EMG responses during quad stretch.

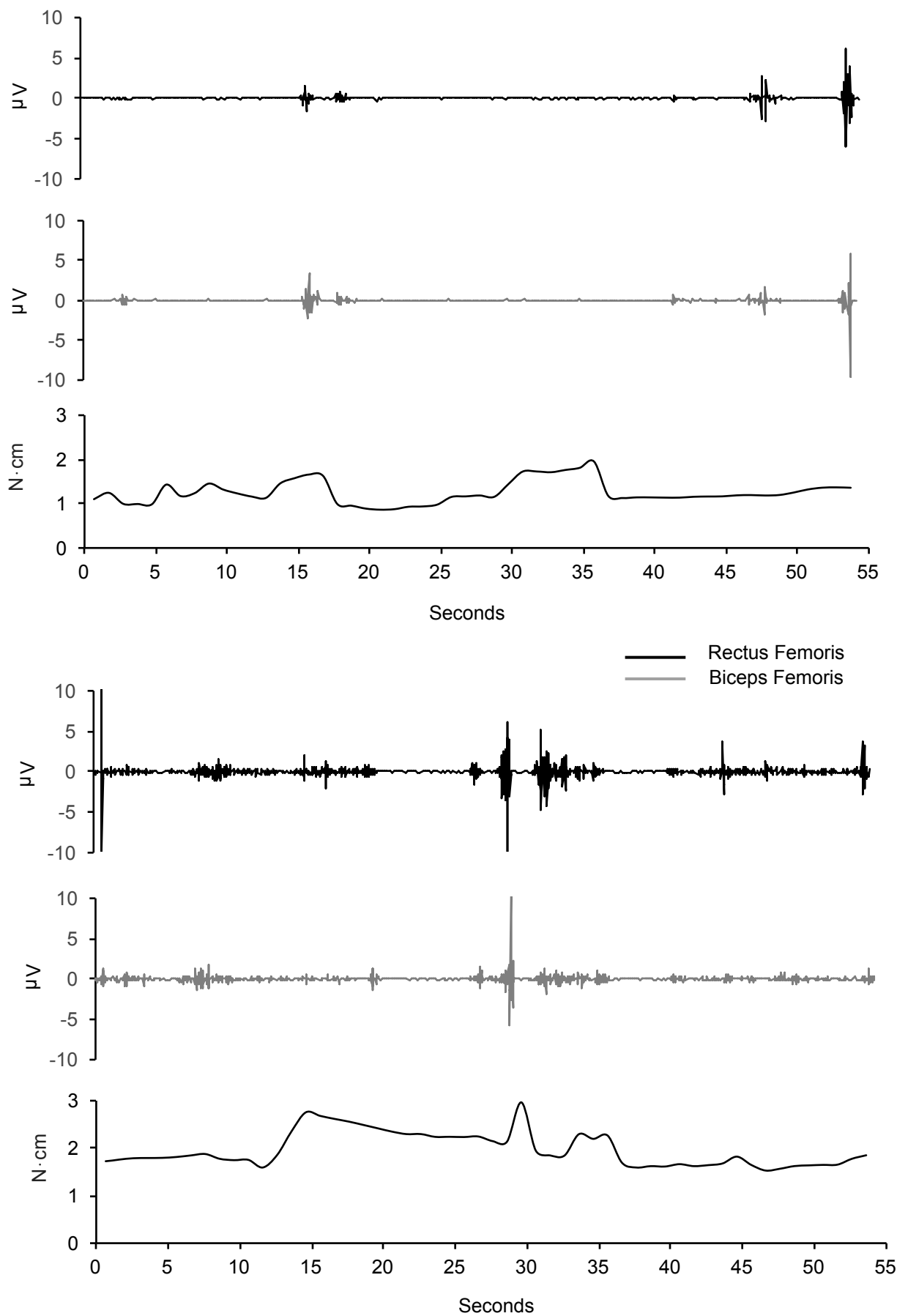


Figure 4. Characteristics of clonic-like responses to stretch.

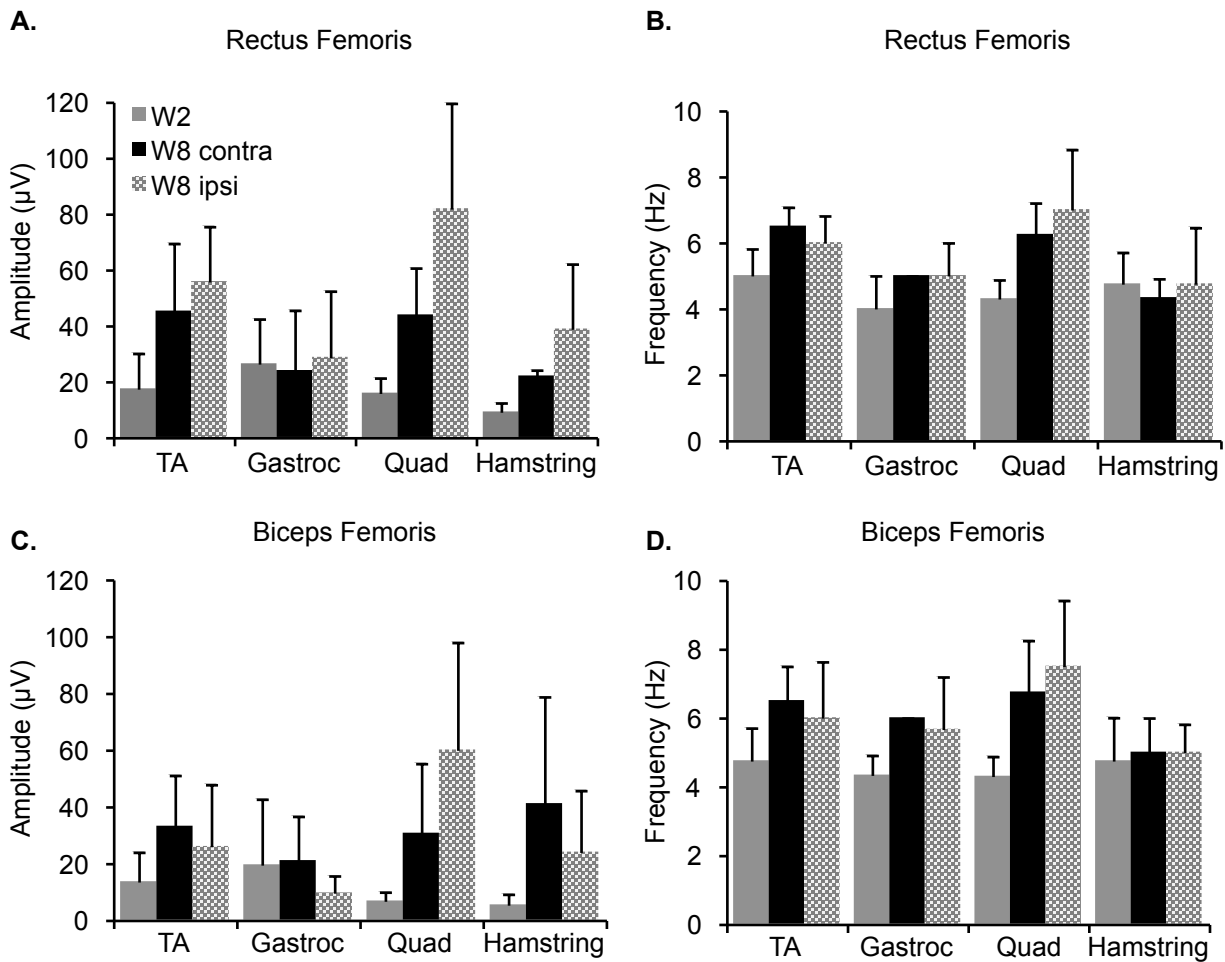


Figure 3. EMG responses during a 1 minute quadriceps stretch in the contralateral limb at week 2 post-SCI (A-C) and ipsilateral limb at week 8 post-SCI (D-F). C and F show forces applied over the duration of the stretch. In F forces increase when there is a spike in EMG activity, demonstrating that the force sensors were sensitive to the resistance from the animal when hindlimbs were activated.

Figure 4. The forces remained consistent through the weeks of stretching for Gastrocnemius, Hamstring and Tibialis Anterior (except at week 7, when forces were significantly lower as compared to week 2, $p<.05$). The forces gradually increased with weeks post-SCI and were significantly greater at weeks 5, 6 and 8 as compared to week 2.

Figure 5. Quantification of forces applied weekly during stretching.

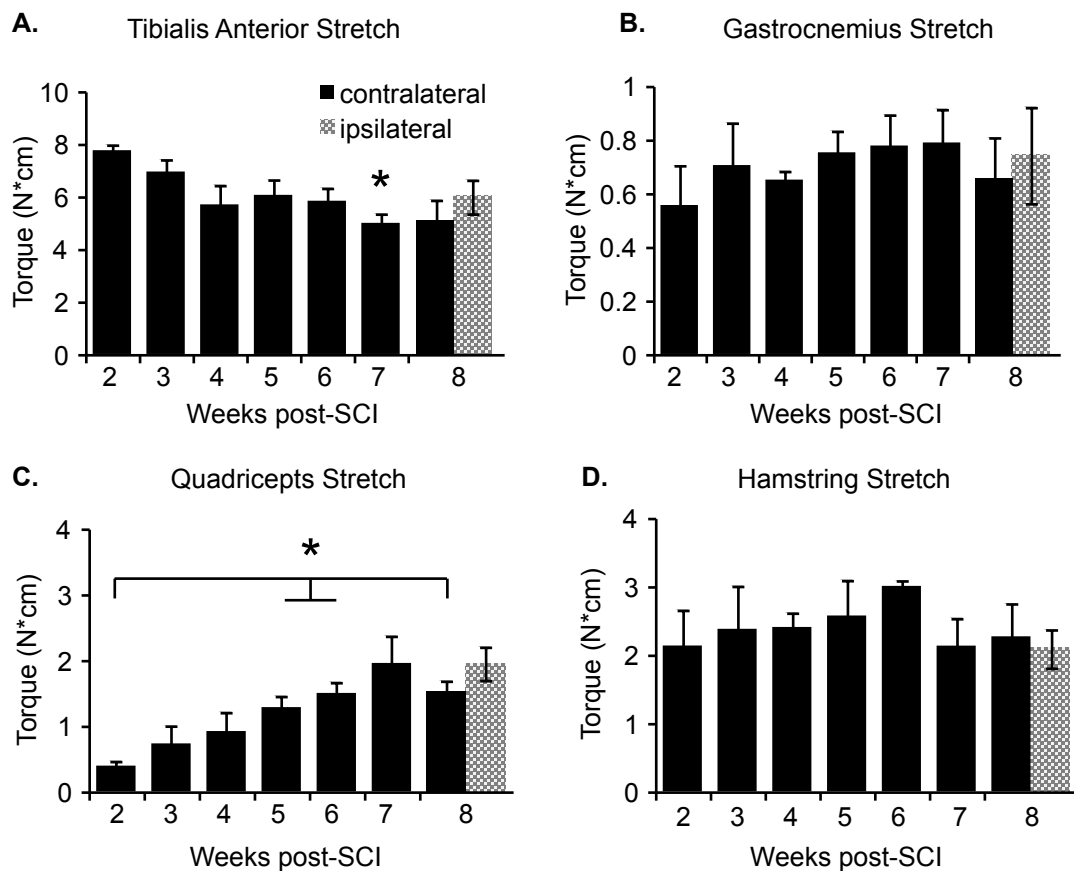


Figure 5. Quantification of amplitude and frequency of clonic-like EMG responses to stretch in the contralateral limb at week 2 and 8, and ipsilateral limbs at week 8. Although there were no statistically significant differences due to small sample size ($n=4$) and high variability, there was a trend in increased amplitude (A,C) and frequency (B,D) of clonic-like contractions from week 2 to 8, most pronounced in responses to quadriceps and tibialis anterior stretch.

Figure 6. Locomotor function.

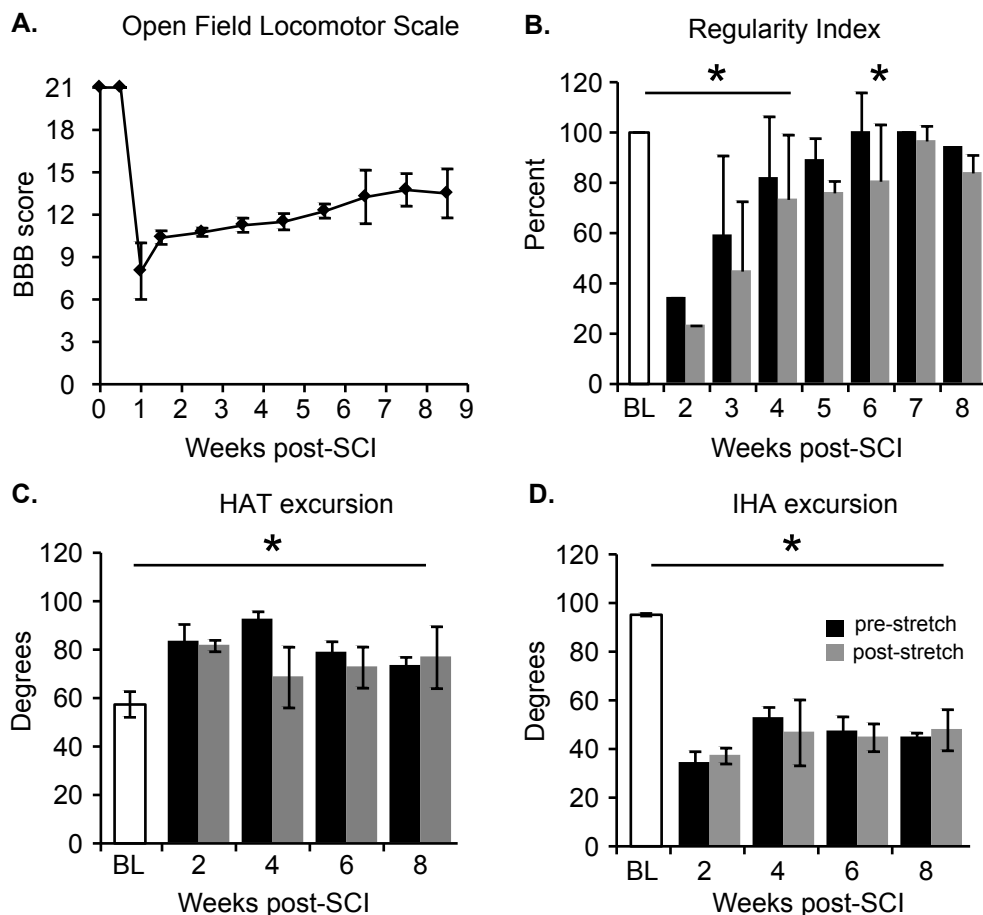


Figure 6. Spinal cord injury resulted in significant disruption of locomotor function. By 5 weeks the animals achieved significant recovery as the regularity index was no longer different from baseline (except week 6 post-stretch). There were no statistically significant difference in pre- and post- stretch gait regularity index (B) although there was a trend in slight decreases in RI post-stretch. The BBB scores remained stable for the duration of the study and stretching of one limb twice a week for 6 weeks and then daily stretching (all 6 stretches of the non-implemented limb and 2 ankle stretches of the implanted limb) during week 7 and 8 did not overall disrupt locomotor function (A). After SCI Hip-Ankle-Toe (HAT) (C) excursions were significantly increased while Iliac Crest-Hip-Ankle (IHA) (D) excursions were significantly decreased for the duration of the study. Pre- and post-stretch HAT and IHA excursions were not different (assessed using 3D kinematics).

Nociceptor-Dependent Locomotor Dysfunction After Stretching in Adult Rats with Spinal Cord Injury.

Introduction.

The most obvious manifestation of a severe spinal cord injury (SCI) is an immediate paralysis of musculature below the level of lesion and subsequent loss of motor function. Patients with severe SCIs become dependent on wheelchairs for their mobility. Lack of active loading and/or weight bearing in the limbs results in muscle atrophy and pathological changes in joints and their supporting structures that with time can result in muscle contractures – dramatically decreased range of motion (ROM) and increased stiffness [1, 2]. In addition, neurobiological changes within the spinal cord below the level of injury leads to the development of spasticity [3]. Stretching remains one of the leading therapies for the prevention and treatment of muscle contractures as well as spasticity [4, 5]. Rationale for therapeutic stretching largely comes from animal studies of joint contractures that develop as a result of cast immobilization of muscles in shortened positions of otherwise neurologically intact animals. Intermittent stretching in these studies was effective at preventing ROM decreases during cast-based limb immobilization [6-8]. In addition, stretching is the cornerstone of any flexibility training program and is effective at increasing ROM acutely after a single 30-90 second stretch [9, 10] and chronically, after multiple weeks of flexibility training utilizing various frequencies and durations of stretching in healthy intact human subjects [11-13]. Unfortunately, several systematic reviews found no evidence to support efficacy of stretching at preventing muscle contractures or improving ROM after SCI and other neurological conditions [14, 15].

With the help of trained and licensed physical therapist (PTs) we developed a hindlimb stretching protocol in order to treat muscle contractures in wheelchair-immobilized rats with mild SCIs [16]. Surprisingly, non-immobilized rats with SCIs that received stretching as a control measure had significantly reduced locomotor recovery as compared to unstretched, non-immobilized controls. We now have demonstrated that stretching at chronic time points results in dramatic reductions in locomotor function in rats with more severe injuries [17]. Furthermore, we showed that dynamic (ie. rhythmic) hindlimb stretching also disrupts locomotor function. However, the physiological mechanisms underlying the stretching phenomenon are not known. We have been unable to find signs of overt muscle damage [17] that could account for disruption in locomotor function. Animals show a robust and rapid (1-2 weeks) recovery when brief periods (1-4 weeks) of daily stretching cease. Thus, it appears feasible that the stretch-induced locomotor deficits likely have a sensory-dependent neurologic basis. Interestingly, skeletal muscles receive a robust sensory innervation. For example, the sciatic nerve, which is a major source of hindlimb innervation, is predominantly composed of sensory axons, 48% of which are unmyelinated sensory afferents (group IV)[18]. A study of 15 cat skeletal muscles determined that group IV sensory afferents comprise about 66% of the total sensory innervation [19]. Group III and IV afferents have been shown to mediate decreased motor output during fatiguing exercise in humans [20], and Cleland et al., showed that activation of stretch sensitive free nerve endings (group III and IV afferents) produces rapid inhibition of motor output [21]. Therefore, we hypothesized that the negative effects of stretching are mediated wholly or partly by nociceptive afferents. We propose that stretching will not be detrimental to the locomotor function of spinal cord injured rats that are systematically depleted of unmyelinated afferents using neonatal capsaicin treatment.

Methods.

Ethical statement concerning animals research.

All experimental procedures involving animals were approved by the University of Louisville Institutional Animal Care and Use Committee.

Neonatal capsaicin injections.

In order to test our hypothesis we turned to the well-established method for depleting nociceptive afferents, neonatal capsaicin injections [22, 23]. Six pregnant Sprague-Dawley rats of known gestational age were checked several times a day to ensure an accurate record of birth time. Every pup in two of the litters received capsaicin injections, pups in two other litters received vehicle injections and the remaining two received no injections. Injections were done at 2 days of age. The rat pups were taken out of their cages (half of the litter at a time, along with some bedding) and received intraperitoneal injections of either capsaicin (50 mg/kg) dissolved in 10% Tween 80 and 10% ethanol (v/v) in 0.9% saline or vehicle injections (same solution without capsaicin). Anesthesia was achieved by wrapping pups in gauze and placing them on ice for approximately 5 minutes. The animals were monitored continuously and the injections were done when all movements ceased and animals were not responsive to touch or pinch. After the injection, the animals were placed on the removed bedding until they warmed up and regained movement. The animals were returned to their mothers for the next 4 weeks after which they were weaned. The weaned animals were sexed so that males and female could be housed separately. Only female rats were used in the current study. The animals were allocated to 3 groups: capsaicin-treated (CAP, n=8), vehicle-treated (VEH, n=8) and control animals that received no injections (CON, n=8). All three groups received SCI; CAP and VEH rats received stretching, while animals in CON group served as controls and were not stretched. Gentling procedures and baseline assessments began when animals were 3 months old.

Baseline sensory assessments.

To assess the effectiveness of TRPV1+ C-fiber depletion by capsaicin, animals were tested for withdrawal thresholds to painful stimuli using electro-von Frey (mechanical) and Hargreaves (thermal) sensory tests, as described in detail previously [24]. Briefly, for von Frey testing, the animals were placed on a metal grid and an electro-von Frey rigid filament was applied to the plantar portion of the foot. The force recorded was that registered at the point of paw withdrawal when followed by a stereotypic behavioral response of attending to the stimulated foot. For the Hargreaves test, the animals were placed on a heated glass surface and a laser (32°C) was shone onto the plantar surface of the foot. The latency of paw withdrawal from the stimulus was recorded.

C-fiber depletion was also assessed using the cutaneous trunci muscle reflex (CTMR), which is purely nociceptive in rats [25]. CTMR testing was performed following administration of a sub-surgical dose of sodium pentobarbital (35mg/kg). A five by three centimeter grid of black dots was drawn on the skin of the back starting from the midline (3 cm from the base of the tail). The midline dots were 1 cm apart (rostrocaudally) and 5 mm apart mediolaterally (a schematic drawing of the grid is shown in Fig. 1C). Mechanical (forceps pinch) and thermal (metal probe heated to 65°C) were applied at two sites bilaterally. Digital video recordings of the skin were made with a camera placed directly above the animal. MaxTrack software was used to digitize the dots and to quantify contraction distance and time to minimal contraction using two dots rostral to the stimulation sites. Measurements were made using a custom-designed excel macro. Assessments were done by individuals blinded to the experimental groups.

Baseline locomotor function assessment.

Hindlimb 3D kinematics were recorded while animals walked in the narrow Plexiglass tank using two side view cameras. Within the three major hindlimb segments (hip, knee and ankle) we analyzed the excursions of two angles: hip-ankle-toe and iliac crest-hip-ankle using MaxTrack program and custom-designed excel macro As previously described [26], in an effort to avoid inaccuracies associated with the knee, we analyzed the limb as three segments and two angles: the iliac crest-hip-ankle and the hip-ankle-toe angles.

Magnetically evoked muscle potentials.

Electromyographic (EMG) responses were recorded from gastrocnemius muscles following magnetic stimulation of afferent fibers at the base of the tail as described previously [17, 27]. Unanesthetized animals were gently but securely pinned to a wooden board using a cloth stockinet. Twenty six gauge needle electrodes were inserted into the gastrocnemius muscles bilaterally. The stimulus (80% intensity) was delivered to the base of the tail using a 25 mm figure 8 magnetic coil powered by a MagStim 200 (MagStim Ltd., Whitland, U.K.). The EMG responses were analyzed for onset latency and peak-to-peak amplitude. Assessments were performed pre-injury and at week 4 and 11 post SCI.

Spinal cord injury.

Spinal cord injury was performed as previously described [28]. Animals were anesthetized with ketamine/xylazine and a midline incision was made over the lower thoracic spine, followed by partial laminectomy at T9 to expose the spinal cord. Moderately-severe spinal cord contusions (25g/cm) were delivered using the NYU “MASCIS” Impactor (W. Young, Rutgers University, Piscataway, NJ).

Locomotor assessment after SCI and stretching.

Locomotor function was assessed using the BBB Open Field Locomotor Scale [29] weekly for the first 6 weeks to demonstrate a functional plateau. During the weeks of stretching, BBB scores were assessed 3 times a week, Monday am (pre-stretch), Monday pm (after one stretching session) and Friday pm (after 5 days of stretching), as previously described [17, 27]. In addition, partial weight-supported stepping was assessed using shallow water (2 inches) every second week. Eight to ten passes (uninterrupted movement the full length of the tank) were recorded using horizontal and ventral views for each animal. Hindlimb steps, defined as a movement involving clear foot contact (either dorsal or plantar) with the pool surface at initiation followed by an active propulsion phase (body movement forward relative to a planted and stationary foot). In other words, a full step cycle consisting of both swing and stance phases had to be present for a step to be included in the analysis. For each identified step we calculated the excursions of the HAT and IHA angles using MaxTraQ software. The data is presented as the ratio of steps to passes for each animal, normalized to their pre-stretching (week 4) ability.

Stretching protocol.

Our standard hindlimb stretching protocol [27] was initiated at 6 weeks post SCI when BBB scores had plateaued and locomotor function was stable for 3 weeks. The protocol consists of two 12 minute sessions of 6 stretches (each held at the end range of motion for 1 minute) applied to major hindlimb muscle groups bilaterally (ankle, knee, hip flexors/extensors and hip adductors and abductors). For the stretching sessions, animals were gently wrapped in a towel, leaving their hindlimbs exposed, and placed on their backs. Seven rat PTs, who were blinded to experimental groups participated in the daily stretching sessions and animals were rotated through the PTs so that they were not stretched by the same PT more than twice in a week. During each stretch the PTs closely monitored the animals and took notes of any responses that the animal displayed during each stretch in the “stretch response” form. Some of the most common responses are kicking (vigorous movement of the entire hindlimb), vibrations (high frequency, low range of motion movement mostly around the ankle) [30], pull back (hindlimb

resistance to stretch) and air-stepping (slow rhythmic step-like movement of the hindlimb). The responses were given a score of 1, 2 or 3 based on intensity and frequency during each stretch: 1=mild/infrequent (5-20% of the stretch time), 2=moderate, frequent (20-70%), 3=severe/very frequent (70-100%). Control animals were handled daily: they were wrapped in a towel and placed on their backs, but not stretched. Animals were stretched 5 days a week for 3 weeks and then were allowed to recover for 2 weeks. The rats were then stretched for an additional 2, 3 or 4 days and were sacrificed 2 hours after the last stretching session. Three animals from each stretch group (CAP and VEH) were sacrificed each day. Control animals were all sacrificed 2 hours after being wrapped in a towel and handled. All the animals were assessed using the BBB Open Field Locomotor Scale between the final stretching session and the time of sacrifice.

Euthanasia and tissue processing.

The animals were overdosed with ketamine (50 mg/kg)/ xylazine (0.024 mg/kg)/acepromazine (0.005 mg/kg) cocktail and transcardially perfused with phosphate buffer saline (PBS) followed by 4% PFA as previously described [31]. The spinal cord including the injury epicenter and lumbar enlargement was dissected out and post-fixed in 4% PFA for one hour and transferred to 30% sucrose for cryoprotection for at least 4 days. The spinal cords were examined under the dissection microscope confirming that all of the lesions were at the T10 level. A 12 mm length of each cord containing the injury epicenter was isolated, placed in a block with tissue freezing medium completely covering the sample and rapidly chilled to freezing on dry ice. Transverse 30 μ m sections were cut and stained with eriochrome cyanine (EC) and were photographed at 4X as described previously. Spared white matter (SWM) was assessed using the cross-sectional area of darkly stained, compact tissue, traced using ImageJ (NIH) and compared to uninjured (control) sections. The section with the least SWM was assigned as the injury epicenter. The lumbar spinal cord (L1-L5) was cryoprotected and sectioned at 20 μ m for immunohistochemical (IHC) analysis of c-Fos and CGRP. Three hindlimb muscles (Tibialis Anterior, Medial Gastrocnemius and Biceps Femoris) were also dissected out for histological analysis. Muscle length (origin to insertion) was measured, the muscle was divided exactly in half and a 5 mm length (proximal to origin) was post-fixed in 4% PFA and cryoprotected in 30% sucrose. Each muscle sample was sectioned at 10 μ m and stained with hematoxylin and eosin (H&E). The muscle was analyzed for the presence of centralized nuclei and fiber cross sectional area as previously described [17]. Photomicrographs of four muscle areas from each region (proximal, distal, medial and lateral within the cross section) were acquired at 20X. The number of muscle fibers (MF) containing centralized nuclei (CN) were counted and expressed as a percentage of the total number of MFs analyzed. In addition, over 150 muscle fibers were traced from each muscle and CSA area determined using ImageJ software.

c-Fos immunohistochemistry and analysis.

Nuclei positive for c-Fos were immunolabeled using avidin-biotin peroxidase [32]. Slides with sections of lumbar spinal cord were warmed and washed with PBS. Endogenous peroxidase activity was quenched using 3% hydrogen peroxide (15 min). The sections were then blocked with 10% normal donkey serum (NDS) and 10% bovine serum albumin (BSA) in 0.3% PBS-Triton (PBST) for 1 hour. The sections were incubated with c-Fos primary antibody (donkey-antimouse, 1:1000, Abcam) overnight at 4°C. Sections were then rinsed with PBS/PBST, incubated with biotinylated antibody (donkey-antimouse, 1:1000, Immunostar) for 1 hour, rinsed with PBS/PBST, incubated with horseradish-streptavidin (1:1000 in PBS) for 1 hour and rinsed with PBS/PBST. The reaction sites were visualized by applying DAB solution (Sigma) for 10 minutes. The reaction was stopped in distilled water and the slides were air dried for at least 10 minutes, followed by 3 minutes in Xylene. The slides were coverslipped using paramount. Images (10X) of each section were acquired on an inverted microscope using the "large image" option in the Elements software package. Using ImageJ the gray matter was divided into three regions of interest (bilaterally):

Substantia Gelatinosa (SG, border was clearly visible in stained tissue), dorsal horn (DH) and intermediate gray matter/ventral horn (IGM&VH). Darkly-stained, c-Fos positive nuclei were counted manually in ImageJ by an individual blinded to experimental groups. Three images for each lumbar level (L1-L5) were acquired and an average number of neurons from the section for each animal was taken into group average analysis. In addition, random, de-identified sections were analyzed by the primary investigators to confirm the definition of stained nuclei.

CGRP immunohistochemistry and analysis.

CGRP IHC was performed on sections of L3 spinal cord. Slides were warmed, rinsed and blocked (10% NDS, 10% BSA in 0.3% PBST) for 1 hour. Sections were incubated with CGRP primary antibody (donkey anti-rabbit, 1:2000, Millipore) overnight at 4°C, rinsed and incubated with secondary antibody (donkey-antirabbit with Alexa 467 fluorophore). The sections were coverslipped with fluoromount. The dorsal horns from three different sections for each animal were imaged at 20X. The area of CGRP-positive puncta within the dorsal horn was identified and differentiated using the threshold tool within Elements, and the same threshold range was then applied to the rest of the images. The area of CGRP was quantified for each dorsal horn area excluding the dorsal root entry zone that were removed manually from each image. All these analyses were performed by an individual blinded to the experimental groups.

Statistical Analysis.

Data with multiple comparisons was analyzed using ANOVA followed by Bonferroni or Tukey HSD post hoc test. For data analyzed using ANOVA is reported as means $\pm$ standard deviation (SD). For non-parametric analysis Mann-Whitney U and Spearman's rho tests were used. Significance was established when $p < .05$; Figure legends indicate which test was used for the represented set of data.

Results.

Baseline sensory and locomotor function.

Neonatal capsaicin treatment had no effect on responses to mechanical nociceptive stimuli. Paw withdrawal thresholds to von Frey filament stimulation were similar for the CAP, VEH and CON groups ($F = .065$, $df = 2, 21$, $p = .937$) (Fig. 1A). There were also no significant differences in contraction distance of the CTMR in response to forceps pinch at either site of stimulation, A ($F = 1.862$, $df = 2, 20$, $p = .181$) or B ($F = .798$, $df = 2, 20$, $p = .464$; Fig 1D). Unexpectedly, the CAP group had an increased speed to minimal contraction in response to the mechanical stimulus applied to site B ($p < .05$) (Fig. 1E). CAP animals had increased latency to paw withdrawal in response to the heat stimulus of the Hargreave's test as compared to VEH animals ($p < .05$) (Fig 1B). As expected, in the CAP group the CTMR in response to the thermal probe (65°C) was practically abolished: Contraction distance (Fig. 1F) was significantly decreased ($p < .001$) accompanied by significant reduction in speed of contraction (Fig. 1G) as compared to VEH ($p < .05$) and CON ($p = .005$).

All animals had normal locomotor function while moving in the open field (BBB scores of 21, subscores of 13). In addition, the hip-ankle-toe and iliac crest-hip-ankle angles showed the typical excursion patterns of normal stepping (80 to 160 degrees of motion) and there were no group differences (Fig.2)

Magnetically Evoked Muscle Potentials (MEMP)

The CAP group had significantly smaller MEMP amplitudes (Fig. 3A) as compared to the VEH ($p < .001$) and CON ($p < .01$), groups, as well as a significantly longer MEMP onset latency (Fig. 3B) as compared to the VEH ($p < .05$) group at baseline. SCI significantly reduced the MEMP amplitude ($p < .001$) and onset

latency in VEH and CON animals ($p < .001$), while the responses in the CAP animals remained unchanged. After SCI all three experimental groups had MEMPs with similar characteristics.

Spinal cord histology.

After spinal cord injury the CAP, VEH and CON groups had $9.4 \pm 5.3\%$, $8.4 \pm 4.2\%$ and $15.5 \pm 8.69\%$ spared white matter at the epicenter, respectively. Oneway ANOVA analysis showed no significant group differences ($F=2.82$, $df=2,20$, $p=.083$).

Locomotor function after SCI and during stretching.

Locomotor function, as assessed by BBB scores, plateaued at 3 weeks post-SCI at around 10 or 11 in all groups (Fig. 4A), although BBB subscores continued to increase slightly over the 6 weeks after injury but prior to stretching (Fig. 4B). By week 6 a significantly larger proportion of VEH and CON ($p < .05$) animals showed consistent weight supported stepping (a BBB subscore of at least 0) as compared to the CAP group (Fig. 4B). After the first 5 days of stretching the BBB scores of VEH animals decreased significantly ($p < .005$) and were significantly lower than those of CAP animals ($p = .005$). Stretching had only minor negative effects on the locomotor function of CAP animals; their BBB scores were not significantly different after 5 days of stretching. The locomotor function of VEH animals recovered back to pre-stretch levels over the weekend, however, just one stretching session in the following week negated that recovery ($p < .01$, as compared to pre-stretch values) and the next 4 days of stretching resulted in further detriments ($p < .001$). Some recovery was achieved over the second weekend without stretching, but this was not as robust as after the first week, with the BBB scores of VEH animals remaining significantly lower than those of the CAP and CON groups ($p < .005$). The same pattern of BBB score change persisted in the 3rd week of stretching. By the end of the 3rd week VEH animals were able to achieve BBB scores of only 2.5 corresponding to extensive movement of one of the joints. This is significantly different ($p < .001$) from their pre-stretch locomotor function when animals achieved consistent weight-supported stepping (BBB score of 11). On the contrary, BBB scores of the CAP rats fluctuated only slightly during the weeks of stretching (ranged from 8 to 10) and never dropped significantly below the scores of CON rats. Two weeks without stretching (weeks 9 and 10 post-SCI) allowed VEH animals to recover back to pre-stretch levels, but once stretching resumed at week 11, locomotor function again dropped to its lowest level.

Analysis of weight-supported stepping (in shallow water) is shown in Figure 4C shows the results of additional kinematic-based assessment of the animals stepping ability with partial weight support provided by 2 inches of water in the narrow ("runway") tank during the 1st (week 6) and 3rd (week 8) weeks of stretching (both time points normalized to pre-stretch stepping ability at week 4). Stretching resulted in a reduced ability of both CAP (62% of W4) and VEH (42% of W4) animals to step in shallow water after only 3 days of stretching at week 6 and there was no significant differences between the two groups. VEH animals had lower function than CON animals at week 6, the difference approaching statistical significance ($p = .053$). The stepping ability of the VEH animals was further reduced at week 8 to only (14% of W4), while CAP animals did not have further disruption in their function, remaining around 60% of W4, which was significantly greater different from VEH rats ($p < .05$). CON animals continued to improve stepping in shallow water throughout the weeks and by week 8 had significantly greater function as compared to CAP and VEH animals ($p < .01$).

CGRP analysis.

Representative images of CGRP IHC are shown in Fig. 5. CAP animals had very low numbers of CGRP positive puncta, significantly less area than the VEH and CON groups ($p < .001$). In addition, VEH animals

had noticeable levels of positive puncta revealed as significantly greater area of CGRP within the dorsal horn compared to CON ($p < .05$).

Activation of neurons in response to stretching.

Hindlimb stretching resulted in significant increases in the number of c-Fos positive nuclei throughout the lumbar enlargement gray matter in VEH animals as compared to the CON group, and also compared to the CAP group at L3. Figure 6 shows the mean numbers of nuclei for each lumbar segment (A) and a schematic representation of the distribution within each level for each group (B). Photomicrographs of c-Fos positive nuclei are shown in Figure 7. Figure 8 shows the c-Fos+ neuron count breakdown into bilateral areas of Substantia Gelatinosa (SG), dorsal horn (DH) and intermediate gray matter and ventral horn (IGM&VH). There was no significant differences in the number c-Fos+ neurons between groups in SG except at level L5 (VEH vs CON, $P < .05$) (A). VEH had significantly increased number of c-Fos+ neurons in DH compared to CON ($p < .01$) at L1-L5 and at L5 when compared to CAP ($p < .05$). CAP animals had significantly greater number of c-Fos+ neurons at L3 compared to CON ($p < .005$) (B). Similarly, the number of c-Fos+ neurons of VEH rats was significantly greater as compared to CON within IGM&VH at L1-L5 ($p < .05$) and at L2 ($p < .05$), L3 ($p < .01$) and L5 ($p < .05$) compared to CAP. CAP animals also had increased number of c-Fos+ neurons compared to CON at L2 ($p < .01$), L4 ($p < .01$) and L5 ($p < .005$) (C).

Hindlimb responses to stretch in correlation with the number of c-Fos+ neurons.

Based on the finding that the significant differences in the number of c-Fos+ neurons came from the IGM&VH region of the spinal cord which processes motor related information, we hypothesized that this upregulation was related to the hindlimb activation in response to stretch. To determine whether there is a relationship between the observed hindlimb responses and the number of c-Fos+ neurons within IGM&VH, an overall score for the four most common hindlimb responses (kicking, vibration, air-stepping and pull back) was generated by summing the grades of intensity/severity of the response recorded during each stretch of the last stretching session for each animal. First, we found that CAP animals had significantly higher number of kicking responses as compared to VEH ($p = .001$), whereas VEH rats had significantly higher number of vibration responses as compared to CAP rats ($p < .01$) (Fig. 9A). There was no significant differences in air-stepping or pull back responses between the groups. In addition, we found a significant correlation between the vibration hindlimb response and the number of c-Fos+ neurons at L5 for both VEH ($r_s = .793$, $p = .033$) and CAP group ($r_s = -.886$, $p = .019$) (Fig. 9 B,C). Interestingly, for the VEH group it was a positive correlation whereas CAP showed a negative correlation. Furthermore, the number of c-Fos+ neurons at L1 strongly correlated with the kicking response scores in the CAP group ($r_s = .841$, $p = .036$) (Fig. 9D), whereas there were no significant correlations between kicking and number of c-Fos+ neurons at any level in the VEH group.

Muscle histology.

The proportion of muscle fibers (MF) with centralized nuclei were not different between the groups and were very close to 3%, the percentage present in normal adult rat hindlimb muscles (A). VEH rats had significantly reduced MF cross sectional area (CSA) in the Tibialis Anterior muscle as compared to CON and CAP rats ($p < .05$). CAP rats had larger MF CSA in the Medial Gastrocnemius as compared to VEH ($p < .05$) (B).

Discussion.

Functional aspects of capsaicin treated animals pre- and post-SCI.

To our knowledge, this is the first report of a neonatal capsaicin-treated animal model of adult SCI. In order to confirm the success of C-fiber depletion by capsaicin treatment the animals were tested for thermal and mechanical thresholds to nociceptive stimuli prior to SCI. Consistent with previous findings [33], our CAP animals had intact mechanical nociception but significantly increased thermal nociceptive thresholds (Fig. 1). Lack of nociceptive afferents had no influence on baseline locomotor function. Hindlimb joint excursions assessed using 3D kinematics and gait were not different from nociceptor-intact rats. However, lack of nociceptive afferents did subtly influence locomotor recovery after SCI. At 6 weeks post-injury the proportion of CAP animals capable of consistent weight supported stepping (subscore of 0) was significantly lower than the VEH and CON groups despite BBB scores being similar. Epicenter spared white matter was also similar therefore injury severity could not account for the group differences in weight bearing stepping. Stimulation of nociceptive muscle afferents can modulate an ongoing fictive locomotor rhythm [34], however, activation of nociceptive afferents in freely moving animals during overground stepping is unlikely. Thus, at this time we have no explanation for this observation. Certainly, this finding is relevant to our overall understanding of the role of afferents in locomotor function after SCI and will need further investigation. An additional observation of interest is that nociceptor-depleted animals never recovered bladder function after SCI and required daily manual expression for the duration of the experiment. Zinck et al., showed that sprouting of lumbosacral CGRP positive primary afferents precedes reemergence of bladder function after complete spinal cord transection [35]. We confirmed that CAP animals had dramatically reduced CGRP signal in the dorsal horn of the L3 segment. These observations strongly support the notion that CGRP afferent sprouting is required for the reemergence of bladder activity post-SCI.

Stretching and locomotor function.

Stretching initiated at 6 weeks post-SCI resulted in a significant disruption of locomotor function in nociceptor-intact (VEH) rats, consistent with our previous observations. Also in keeping with our previous study [17] the first stretching session did not have a significant negative effect on locomotion. Daily stretching, however, had a cumulative effect; the post-stretch locomotor assessment on day 5 revealed significant impairment. The animals experienced significant recovery over the weekend following the first week of stretching, but one stretching session on the Monday of the second week dramatically reduced locomotor function. After three weeks of daily stretching locomotor function of the VEH rats dropped from the pre-stretch BBB score of 11 to around 2.5, even lower than their scores recorded at 1 week post-SCI. Two weeks without stretching allowed the animals to return to pre-stretch locomotor function, indicating that stretch-induced locomotor deficits are temporary, in agreement with our previous observation. However, after two weeks off re-initiating stretching induced a dramatic drop in locomotor function following a single stretching session. Overall, the pattern of locomotor disruption and recovery over time suggests that daily stretching sensitizes or primes the system and leads to the large functional declines.

Stretching had only a minor effect on locomotor function of nociceptor-depleted (CAP) rats. Throughout the weeks of stretching this group maintained, at a minimum, extensive movement in all three joints (an average BBB score of 7.5). The most noticeable effect of stretching on the locomotor function of CAP animals was during shallow water stepping. At weeks 6 and 8 (during the stretching protocol) CAP rats generated 40% fewer steps (per pass) as compared to week 4 which most likely resulted from a prolonged step cycle. The animals tended to keep their hindlimbs extended and dragging for longer periods of time when provided with the body weight support of buoyancy. Thus, instead of taking 3-4 steps per pass the animals took only 2 steps. This affect could potentially be mediated by the remaining nociceptive afferents since neonatal capsaicin treatment is rarely complete. Alternatively, this might

result from a nociceptor-independent mechanism such as stretching-induced muscle spindle insensitivity, suggested previously to explain reductions in H-reflex after prolonged stretching [36]. It is well-established that activation of locomotor generating spinal circuitry is heavily dependent on proprioceptive afferent input after SCI [37]. Nevertheless, all eight of the CAP rats maintained the ability to step through the weeks of stretching, while VEH rats had substantial reductions in their ability to step and by week 8 only 2 rats could produce some steps and in only one hindlimb.

It is interesting to note that while stretching is a mechanical stimulus, depletion of nociceptive afferents known primarily as “thermally responsive” (TRPV1 positive neurons) had the effect of protecting locomotor circuitry from stretch-induced disruption. In a previous study we discussed the possibility that eccentric muscle contractions during stretching may be key initiating factor leading to impaired locomotion. We reported that stretching evokes air-stepping and clonus-like vibrations in both the contralateral (unstretched) and ipsilateral (stretched) limbs. Eccentric contractions are very effective at inducing delayed onset muscle soreness (DOMS)[38], a phenomenon dependent on the sensitization of TRPV1 positive neurons [39]. We saw kicking and other motor responses in the current experiment which would have resulted in eccentric muscle contractions during stretching and subsequent sensitization of nociceptive afferents in VEH rats. Importantly, rats treated with capsaicin as neonates are known to be resistant to developing DOMS after eccentric muscle contractions [40]. Whether or not eccentric contractions/DOMS necessary and sufficient for stretch-induced locomotor deficits after SCI is currently unknown, but it remains a feasible mechanism for the stretching phenomenon.

Histological findings.

CGRP immunoreactivity was essentially negligible in lumbar spinal cord sections from CAP animals. This confirmed the effective depletion of CGRP positive (TRPV1 positive) afferents from the spinal cord. However, we discovered that VEH rats (nociceptor intact) showed increases in CGRP area in the dorsal horn when compared to CON (unstretched injured) animals. Although unexpected, this finding is consistent with the functional data, specifically the involvement of nociceptive afferents in stretch-induced locomotor deficits and the increase in sensitivity of the locomotor circuitry to the negative effects of stretching over time. It is well documented that certain kinds of repetitive activation of nociceptive afferents leads to their sensitization [41]. Moreover, plasticity within the nociceptive circuitry after SCI has been implicated in neuropathic pain [42] and autonomic dysreflexia [43] as commonly seen in SCI patients. We speculate that both the activation of nociceptive afferents during stretching and the lack of hindlimb activity following stretching contributes to the increased CGRP within the spinal cords of the VEH animals [44].

Using c-Fos as a marker for neuronal activation we determined that VEH (nociceptor intact, stretched) animals had significantly higher numbers of c-Fos⁺ nuclei, compared to CON animals, throughout the lumbar enlargement. In contrast, CAP (nociceptor depleted) animals had similar numbers of c-Fos⁺ neurons to both VEH and CON groups, except at L3 where they had significantly more c-Fos⁺ nuclei than the CON group. All the animals were sacrificed 2 hours after the last stretching session, however simply due to scheduling and manpower some animals were sacrificed after 2, 3 or 4 consecutive days of stretching. As a result we discovered that VEH animals sacrificed after 4 days of stretching had much higher numbers of c-Fos⁺ nuclei when compared to those sacrificed after 2 or 3 days of stretching (Figures 6 and 7). We then divided the gray matter counts into three general areas (Fig. 8) and found that VEH animals had significantly higher numbers of c-Fos⁺ nuclei in the intermediate gray matter and ventral horn as compared to CAP and CON rats. This finding suggests that afferents activated by stretching induce c-Fos expression in spinal cord interneurons throughout the lumbar gray matter and specifically in the intermediate gray matter, a region known to contain interneurons responsible for integrating and generating motor output. Since we indeed observe a plethora of motor responses during

stretching and have always kept a thorough record specifying the frequency/intensity of each response we were able to generate a stretch response score, compare the scores for each response between groups and determine whether there is a relationship between the hindlimb responses and the number of c-Fos⁺ neurons (Fig. 9). The first interesting finding was that the two major hindlimb responses – kicking and vibrations had a different, essentially opposite profile of expression between the groups. The vibration response, which we previously described in detail as having features close to those of a human clonus, was the most robust response in the VEH group and thus it is tempting to speculate that activation of nociceptive afferents greatly contributes to its observation. If in fact vibration we see in rats is functionally similar to the human clonus, then this observation also potentially implies a novel mechanisms for triggering of clonus – nociceptive afferent activation. Interestingly, in a series of human case studies on severe spinal myoclonus in SCI subjects, a subtle link between an existing painful condition and observation of clonus was established. After the resolution of a musculoskeletal pathology clonus could no longer be evoked in those SCI patients [45]. Recently, a robust nocifensive kicking behavior in response to painful mechanical paw stimulus was described in the rat referencing its similarity to clonus [46]. Description of the kicking response in the study by von Gorp is similar to the kicking we observe during stretching. It is possible that the robust kicking response in the CAP rats resulted from activation of other types of nociceptive afferents responsible for mechanonociception, for example. Thus, both vibration and kicking responses could be equivalent to human clonus even though they have some differences in appearance, presumably attributable to the type of afferent activation that drives that response.

Significant positive correlations identified between the vibration scores at L5 in VEH rats (Fig. 9B), kicking scores at L1 in CAP rats, (Fig. 9D) and the number of c-Fos⁺ neurons suggest that motor responses during stretching result in upregulation of c-Fos in the IGM&VH of the spinal cord. Ankle muscles which visually have the greatest activation during the vibration are innervated by motoneurons at L5, whereas L1 contains motoneurons innervating the hip musculature that visually appear to be the “driving” force behind the kicking response. It is possible that nociceptive signaling leads to increased excitability of the motor system that in long-term may have detrimental consequences, such as temporary loss of locomotor function in the stretched rats and when occurs in uncontrollable fashion it leads to the disruption of spinal learning and locomotor function as has been clearly demonstrated in studies by Grau et al [47].

We have determined that stretch-induced locomotor deficits are dependent on activation of nociceptive afferents and can occur in the absence of concurrent muscle tissue damage. Stretched limbs showed no increase in the number of regenerating muscle fibers. Some atrophy, presumably due to disuse, occurred in the Tibialis Anterior muscle of the VEH rats as they had smaller MF CSA as compared to CAP and CON animals. CAP rats had significantly greater CSA of the Gastrocnemius MF as compared to both VEH and CON. Whether this observation is consequential to neonatal capsaicin treatment is not known.

In conclusion, the findings of this study suggest that TRPV1+ “thermal” nociceptive afferents play a role in locomotor recovery after SCI and that their activation during a clinically-modeled daily hindlimb muscle stretch leads to the disruption of locomotor function. While the first observation could be explained by a complex modulatory effect of nociceptive afferents on locomotor circuitry through the flexor reflex afferent pathway [34], the latter effect might be the result of maladaptive plasticity resulting from “uncontrollable” nociceptive signaling in the already vulnerable post-injury spinal cord exhibiting a compromised inhibitory system [48]. While the clinical relevance of the stretching phenomenon still needs to be established, these findings nonetheless have significant implications for rehabilitation after SCI. Harvey et al., demonstrated that some physical therapists apply torques when stretching sensory complete SCI patients of sufficient magnitude to activate nociceptive afferent and

would thus be intolerable to sensate individuals [49]. Furthermore, in the field of sports performance it has been documented that static stretching impairs certain aspects of motor output [50]. These minor effects might have more severe consequences in SCI patients. Given the general ineffectiveness of stretching for its intended purposes after SCI [15], reports of its negative effects on performance in athletes and our data showing that stretching disrupts locomotor function after SCI in rats, the place of stretching as routine therapy in rehabilitation after SCI needs to be reevaluated.

Figure legends.

Figure 1. Baseline sensory function was assessed using von Frey (A), Hargreaves (B) and Cutaneous Trunci Muscle Reflex (CTMR, D-G). C. shows a schematic of CTMR markings on the dorsal skin for quantitative analysis of the reflex in response to mechanical (forceps pinch) or thermal (heated metal probe) stimulus. Dot A (star) and B (square) are two sites for stimulus application on either side of the midline. The data for contraction distance (mechanical – D, thermal – F) and speed of contraction (mechanical – E, thermal – G) between the two hollow dots rostral to the stimulation sites are shown. Data reported as means $\pm$ SD for A, D-G (Oneway ANOVA, Tukey HSD *post hoc*, $p < .05$), for B. bars represent means, dots each individual animal ($n=8$ in each group) (Mann Whitney U test, $p < .05$)

Figure 2. The graph shows results of 3D kinematic analysis of hindlimb angular excursions during overground stepping. The bars represent average excursions of the 2 angles (Hip-Ankle-Toe and Iliac Crest (IC)-Hip-Ankle) within the 3 hindlimb segments (hip, knee, ankle). Top error bars show standard deviation of the angular peaks, bottom – SD of the angular troughs.

Figure 3. The amplitude (A) and onset (B) of magnetically evoked muscle potentials (MEMP) were measured from the gastrocnemius muscle in response to magnetic stimulation at the base of the tail at baseline (BL), week 4 (W4) and 11 (W11) after SCI. Data shown as means $\pm$ SD (RM ANOVA, Bonferroni *post hoc*, $p < .05$)

Figure 4. BBB Open Field Locomotor assessment was performed weekly for the first 6 weeks after SCI (A). During the 5 weeks of the stretching protocol (beginning at 6 weeks post-SCI), BBBs were assessed three times a week (Monday am/pm and Friday pm). During the week 12 the sacrificing procedures began after the post-stretch BBB assessment: T-Tuesday, $n=8$ in each group; W-Wednesday, $n=5$, Th-Thursday, $n=2$. Data shown as means $\pm$ SD (RM ANOVA, Bonferroni *post hoc*, $p < .05$). Proportion of animals achieving a BBB subscore of at least 0 (for consistent weight support). Analyzed using Binomial proportions test ($p < .05$). Kinematic-based assessment of stepping ability in shallow water (for partial body weight support) was done biweekly. Weeks 6 and 8 data were normalized to week 4 (pre-stretch function) for each animal (C) Data shown as means $\pm$ SD (RM ANOVA, Tukey HSD *post hoc*, $p < .05$)

Figure 5. Effectiveness of neonatal capsaicin treatment was histologically confirmed with immunohistochemistry of CGRP in the dorsal horn of the spinal cord (L3). Data shown as means $\pm$ SD (Oneway ANOVA, Tukey HSD *post hoc*, $p < .05$)

Figure 6. c-Fos immunohistochemistry was performed on lumbar spinal cords (L1-L5). The bars represent an average number of c-Fos positive neurons from 3 sections at each level, while each dot represents individual animals (A). $n=8$ per group for each level except at L1 and L5 CAP: $n=6$ and at L1 VEH & CON $n=7$, each. Analyzed using RM ANOVA, Bonferroni HSD *post hoc*, $p < .05$. Traces of spinal cord sections representative of each group for each level with black dots corresponds to c-Fos+ neurons within the chosen sections (B).

Figure 7. Images of spinal cord sections stained for c-Fos. Arrows point to c-Fos+ cell nuclei. VEH (a) is an image from an animal representative of an average, sacrificed after 2 days of stretching in the last week of the stretching protocol. VEH (b) is an image from an outlier animal, sacrificed after 4 days of stretching that same week. CC – central canal.

Figure 8. c-Fos+ neuron count separated into three general regions of gray matter bilaterally, schematically shown next to the graphs SG- Substantia Gelatinosa (A), DH – dorsal horn (B), IGM&VH (C) – intermediate gray matter and ventral horn (shaded areas). Data shown as means $\pm$ SD (RM ANOVA, Bonferroni *post hoc*, $p < .05$). Two outliers (defined as ≥ 3 standard deviations) from VEH group were removed, so VEH $n=6$ for all intraregions and levels except at L2 (SG) $n=5$; CAP: $n=8$, except for L1 and L5 $n=6$; CON $n=8$, except for L1 $n=7$ and L4 (SG) $n=7$.

Figure 9. Four major hindlimb responses during stretching are kicking, vibrations, air-stepping and pull back (A); data shown as means $\pm$ SD, (independent t-test, $p < .05$); (B.) VEH group vibration score has a significant positive correlation with the number of c-Fos+ neurons at L5 ($r_s = .793$, $p = .033$) (C.) CAP group vibration score has a significant negative correlation with the number of c-Fos+ neurons at L5 ($r_s = .886$, $p = .019$) and a significant positive correlation between the kicking score and the number of c-Fos+ neurons at L1 ($r_s = .841$, $p = .036$) (D.) Significant correlations were determined using Spearman's rho test.

Figure 10. Three hindlimb muscles: Tibialis Anterior (TA), Medial Gastrocnemius (MG), Biceps Femoris (BF) were analyzed for number of muscle fibers (MF) with centralized nuclei (CN, marker of regeneration) (A) and muscle fiber cross sectional area (CSA) (B). Data shown as means $\pm$ SD (Oneway ANOVA, Tukey HSD *post hoc*, $p < .05$)

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Figure 1. Baseline Sensory Function

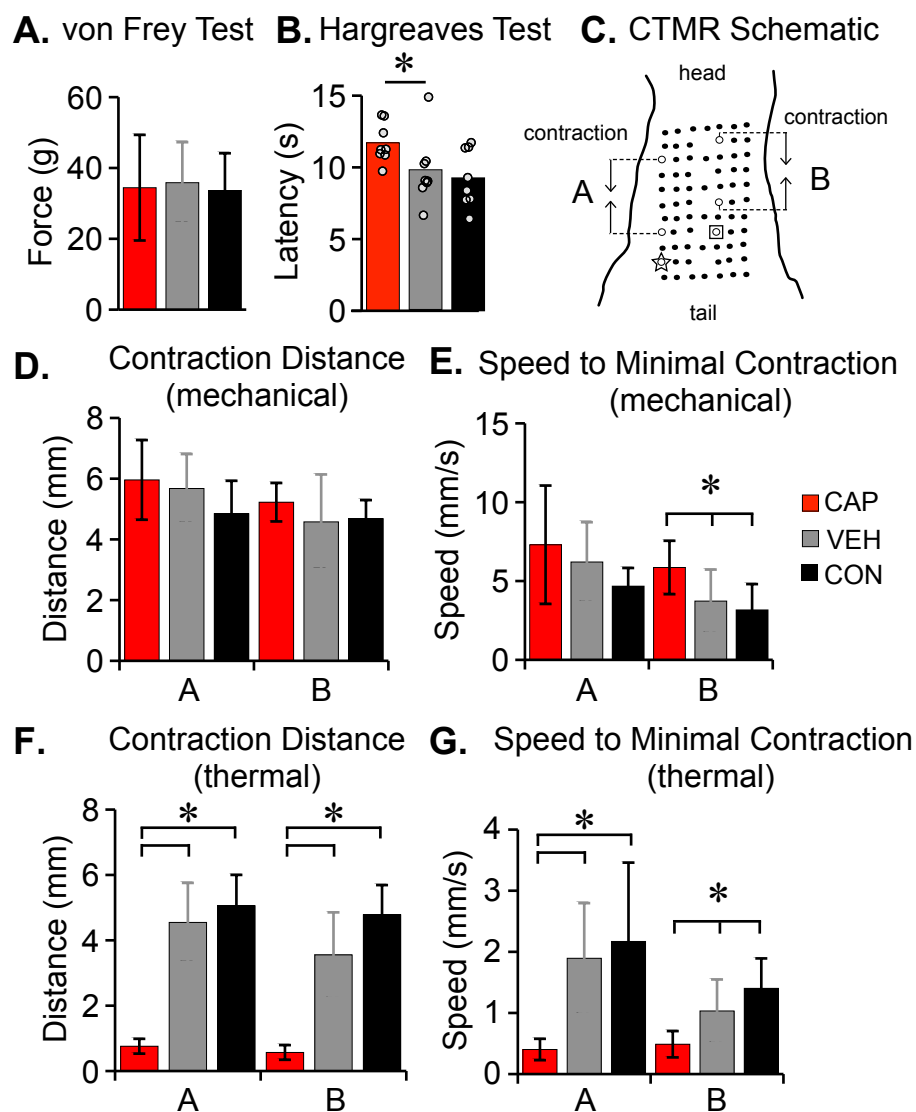


Figure 1. Baseline sensory function was assessed using von Frey (A), Hargreaves (B) and Cutaneous Trunci Muscle Reflex (CTMR, D-G). C. shows a schematic of CTMR markings on the dorsal skin for quantitative analysis of the reflex in response to mechanical (forceps pinch) or thermal (heated metal probe) stimulus. Dot A (star) and B (square) are two sites for stimulus application on either side of the midline. The data for contraction distance (mechanical – D, thermal – F) and speed of contraction (mechanical – E, thermal – G) between the two hollow dots rostral to the stimulation sites are shown. Data reported as means ± SD for A, D-G (Oneway ANOVA, Tukey HSD *post hoc*, $p < .05$), for B. bars represent means, dots each individual animal ($n=8$ in each group) (Mann Whitney U test, $p < .05$)

Figure 2. Baseline Joint Excursions.

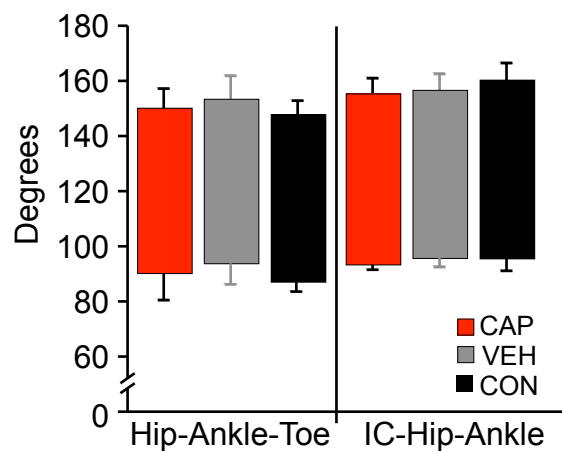


Figure 2. The graph shows results of 3D kinematic analysis of hindlimb angular excursions during overground stepping. The bars represent average excursions of the 2 angles (Hip-Ankle-Toe and Iliac Crest (IC)-Hip-Ankle) within the 3 hindlimb segments (hip, knee, ankle). Top error bars show standard deviation of the angular peaks, bottom – SD of the angular troughs.

Figure 3. Gastrocnemius MEMP.

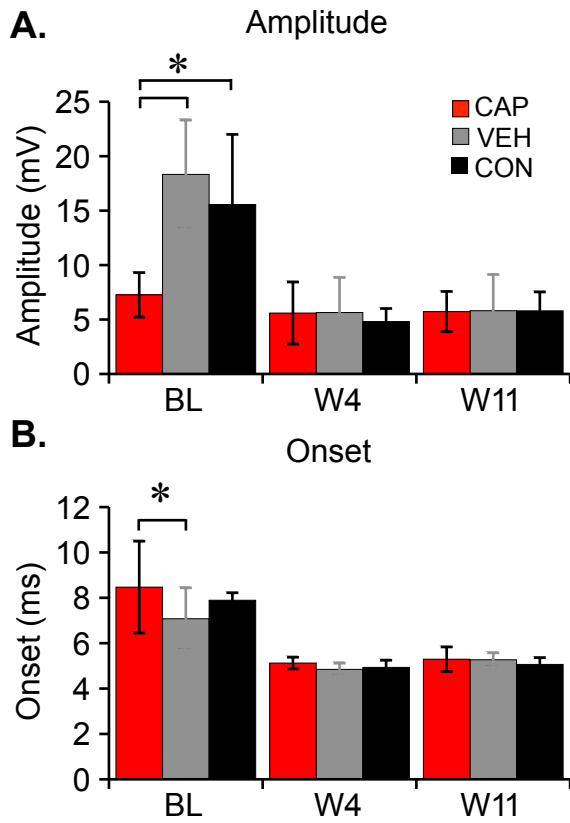


Figure 3. The amplitude (A) and onset (B) of magnetically evoked muscle potentials (MEMP) were measured from the gastrocnemius muscle in response to magnetic stimulation at the base of the tail at baseline (BL), week 4 (W4) and 11 (W11) after SCI. Data shown as means $\pm$ SD (RM ANOVA, Bonferroni *post hoc*, $p < .05$)

Figure 4. Locomotor Function after SCI

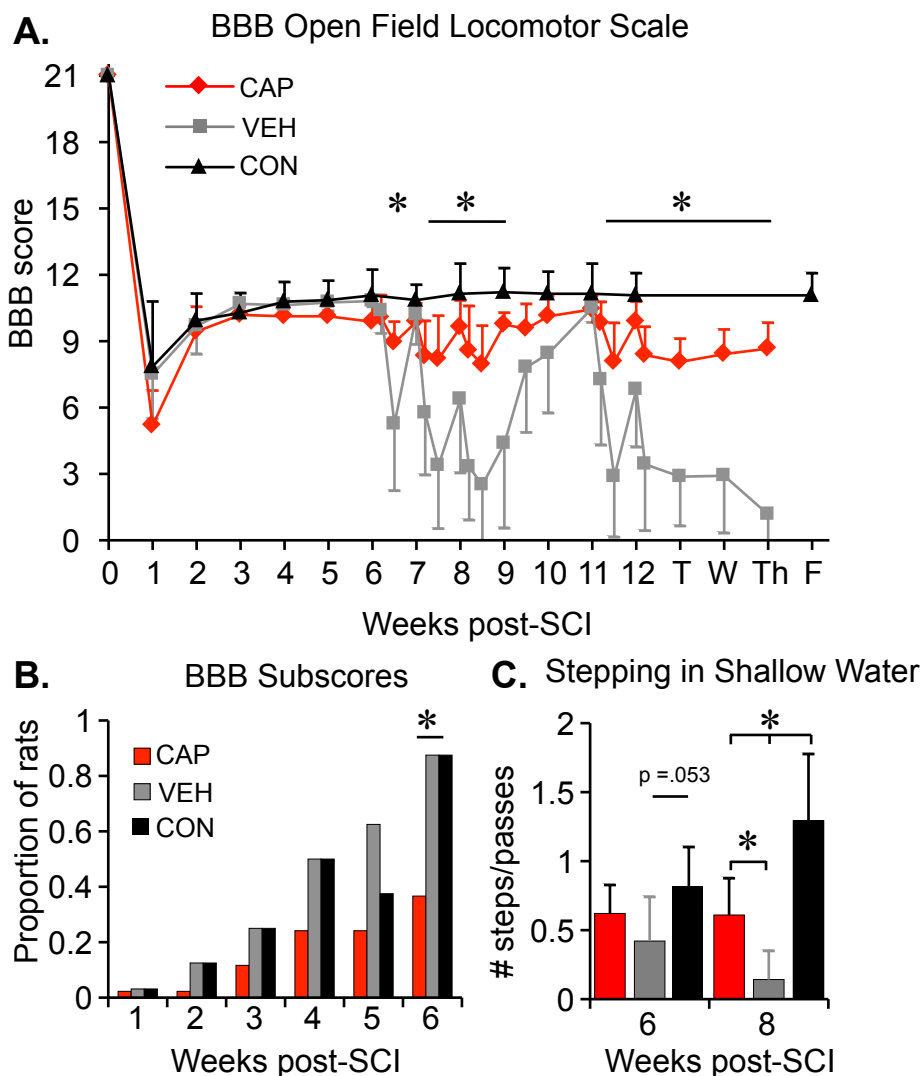


Figure 4. BBB Open Field Locomotor assessment was performed weekly for the first 6 weeks after SCI (A). During the 5 weeks of the stretching protocol (beginning at 6 weeks post-SCI), BBBs were assessed three times a week (Monday am/pm and Friday pm). During the week 12 the sacrificing procedures began after the post-stretch BBB assessment: T-Tuesday, n=8 in each group; W-Wednesday, n=5, Th-Thursday, n=2. Data shown as means $\pm$ SD (RM ANOVA, Bonferroni *post hoc*, $p < .05$). Proportion of animals achieving a BBB subscore of at least 0 (for consistent weight support). Analyzed using Binomial proportions test ($p < .05$). Kinematic-based assessment of stepping ability in shallow water (for partial body weight support) was done biweekly. Weeks 6 and 8 data were normalized to week 4 (pre-stretch function) for each animal (C) Data shown as means $\pm$ SD (RM ANOVA, Tukey HSD *post hoc*, $p < .05$)

Figure 5. Dorsal Horn CGRP area (L3)

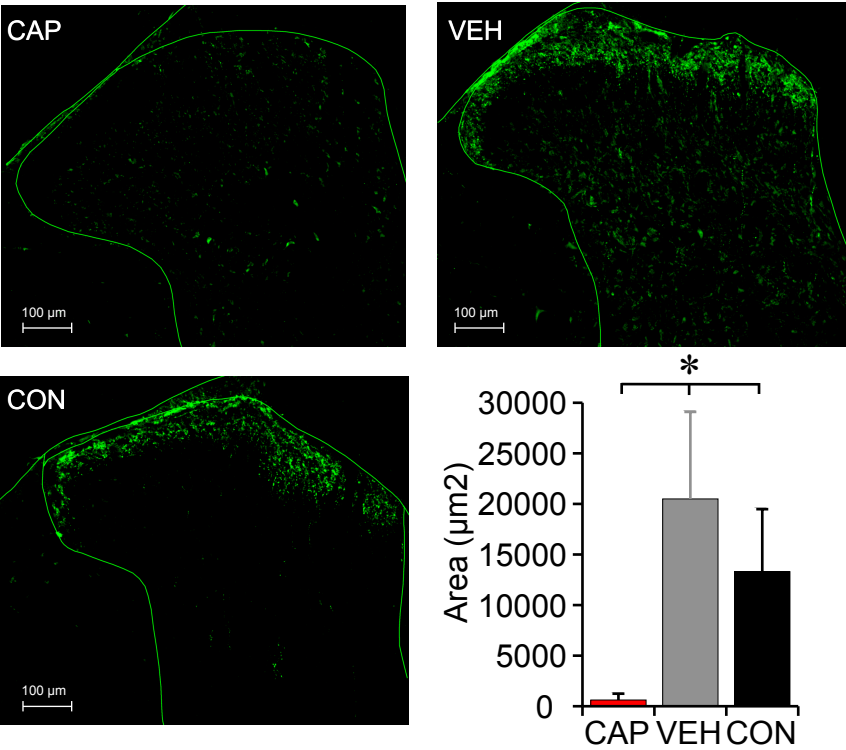


Figure 5. Effectiveness of neonatal capsaicin treatment was histologically confirmed with immunohistochemistry of CGRP in the dorsal horn of the spinal cord (L3). Data shown as means $\pm$ SD (Oneway ANOVA, Tukey HSD *post hoc*, $p < .05$)

Figure 6. Number and distribution of c-Fos positive neurons in the lumbar spinal cord.

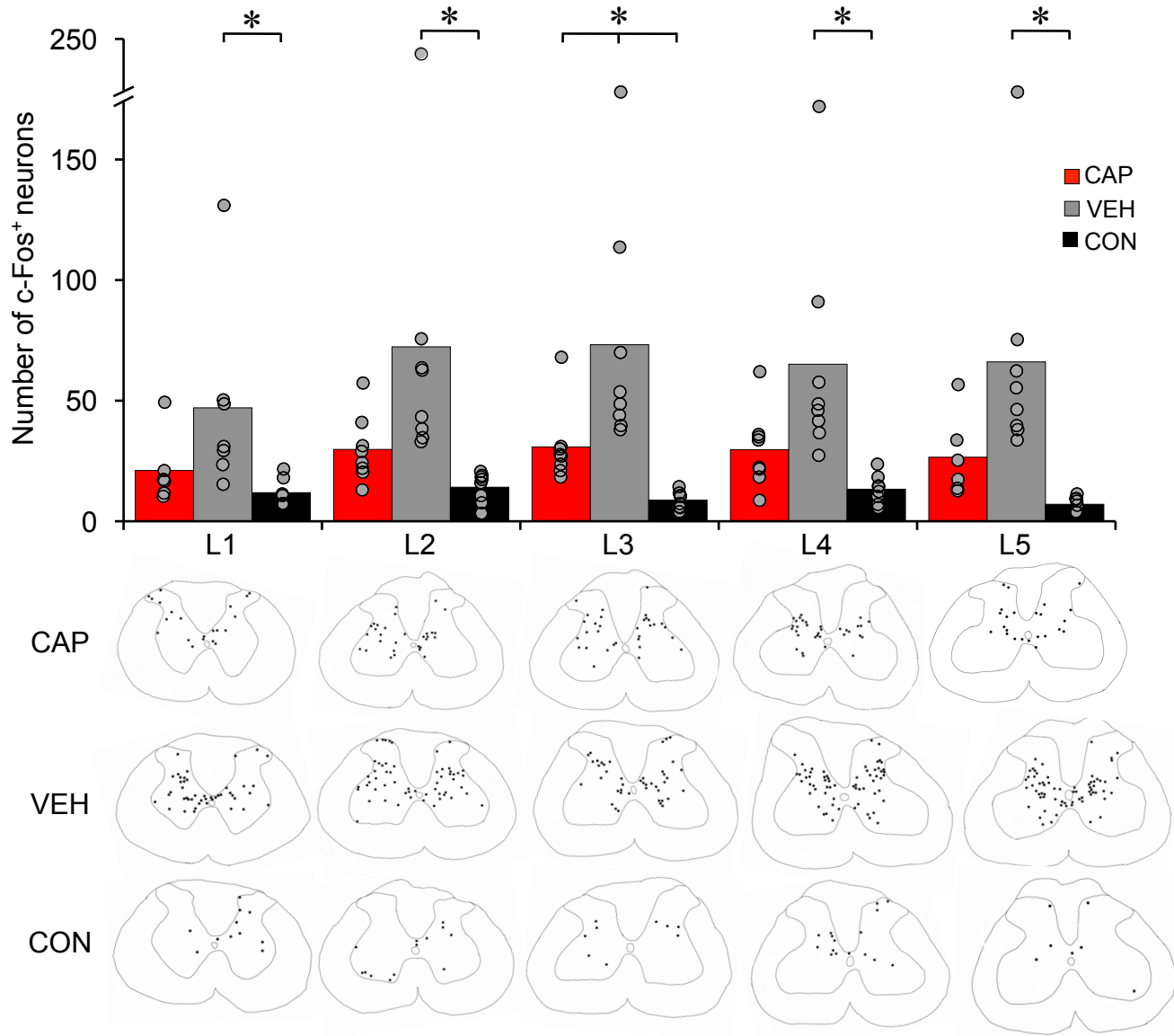


Figure 6. c-Fos immunohistochemistry was performed on lumbar spinal cords (L1-L5). The bars represent an average number of c-Fos positive neurons from 3 sections at each level, while each dot represents individual animals (A). n=8 per group for each level except at L1 and L5 CAP: n=6 and at L1 VEH & CON n=7, each. Analyzed using RM ANOVA, Bonferroni HSD *post hoc*, p<.05. Traces of spinal cord sections representative of each group for each level with black dots corresponds to c-Fos+ neurons within the chosen sections (B).

Figure 7. Spinal Cord Sections (c-Fos-HRP)

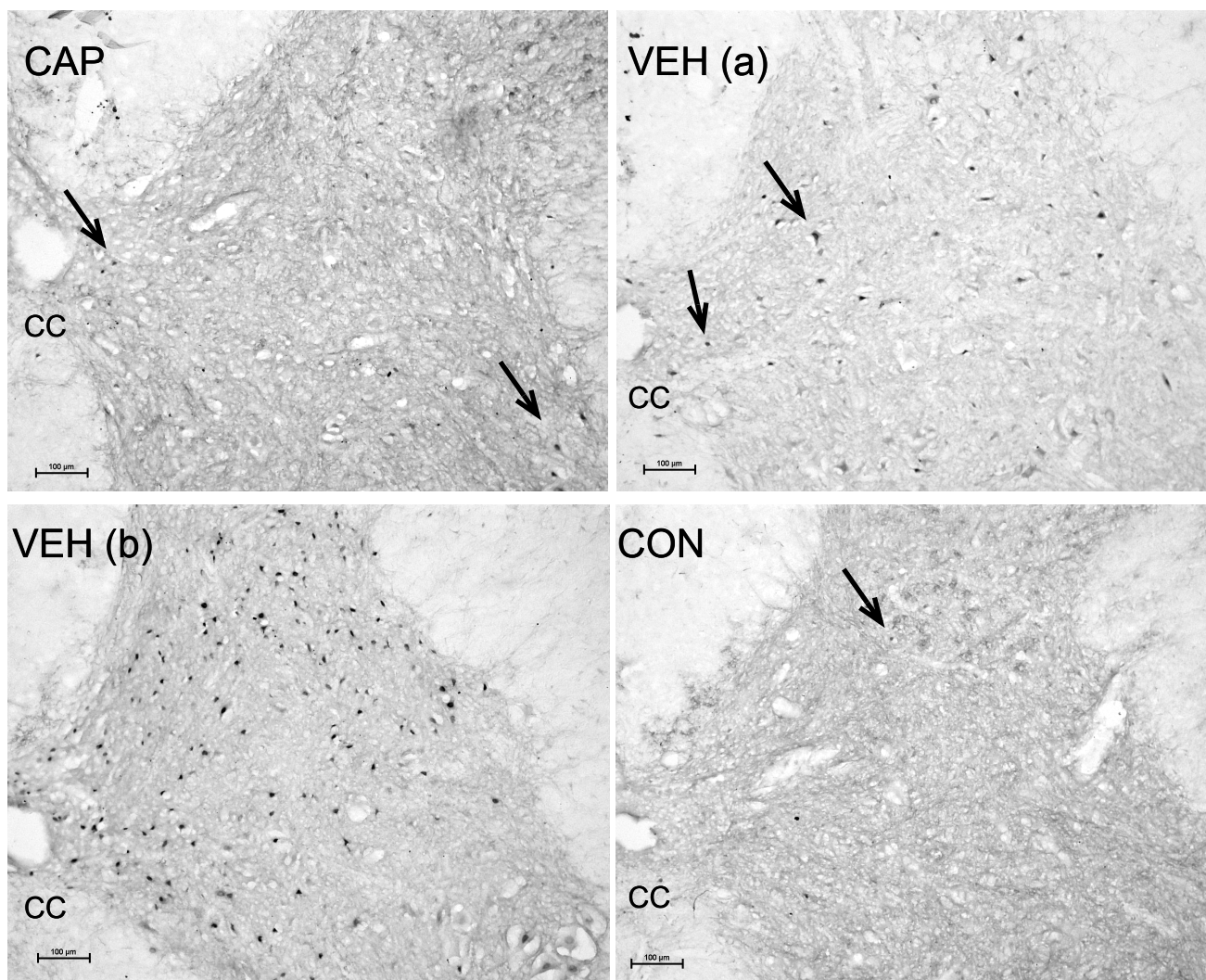


Figure 7. Images of spinal cord sections stained for c-Fos. Arrows point to c-Fos+ cell nuclei. VEH (a) is an image from an animal representative of an average, sacrificed after 2 days of stretching in the last week of the stretching protocol. VEH (b) is an image from an outlier animal, sacrificed after 4 days of stretching that same week. CC – central canal.

Figure 8. c-Fos Intraregional Distribution

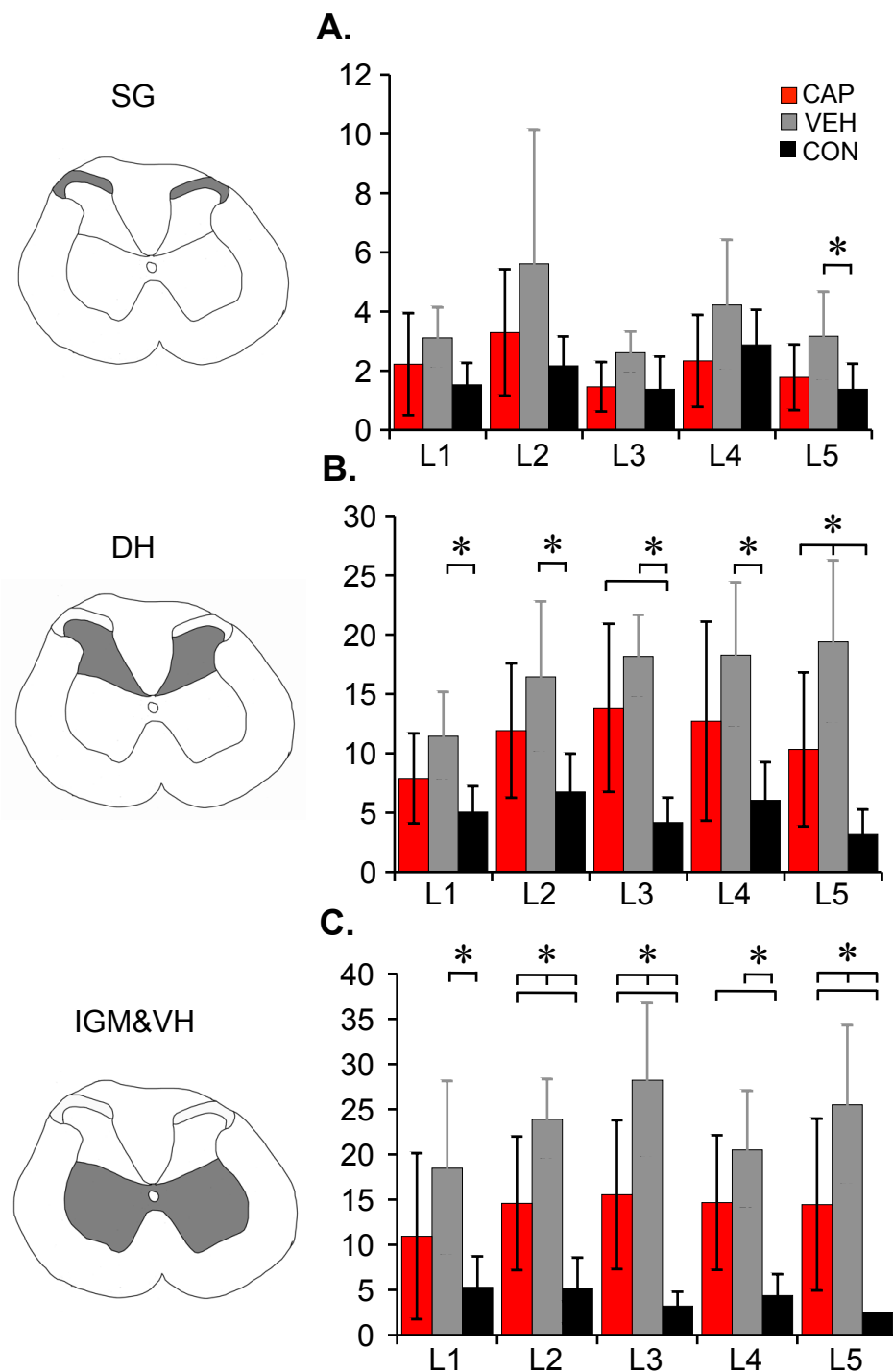


Figure 8. c-Fos+ neuron count separated into three general regions of gray matter bilaterally, schematically shown next to the graphs SG- Substantia Gelatinosa (A), DH – dorsal horn (B), IGM&VH (C) – intermediate gray matter and ventral horn (shaded areas). Data shown as means ± SD (RM ANOVA, Bonferroni *post hoc*, $p < .05$). Two outliers (defined as ≥ 3 standard deviations) from VEH group were removed, so VEH $n=6$ for all intraregions and levels except at L2 (SG) $n=5$; CAP: $n=8$, except for L1 and L5 $n=6$; CON $n=8$, except for L1 $n=7$ and L4 (SG) $n=7$.

Figure 9. Muscle Histology.

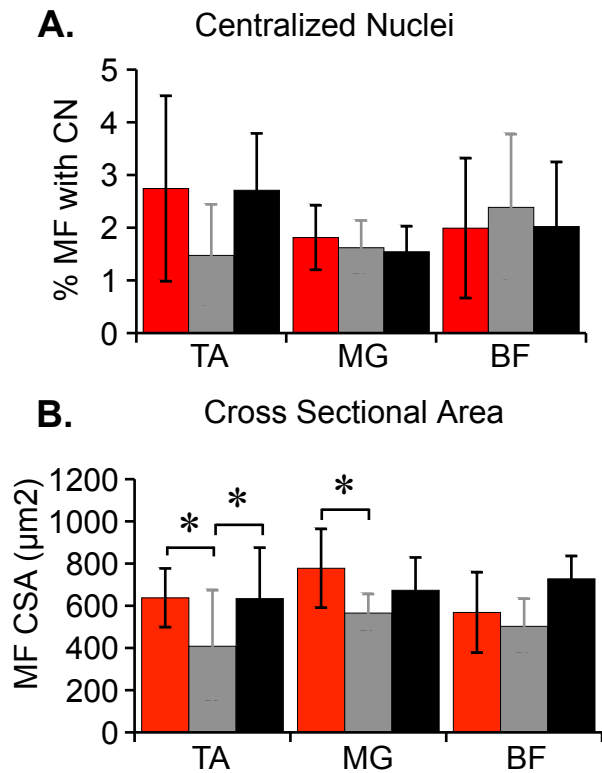


Figure 9. Three hindlimb muscles: Tibialis Anterior (TA), Medial Gastrocnemius (MG), Biceps Femoris (BF) were analyzed for number of muscle fibers (MF) with centralized nuclei (CN, marker of regeneration) (A) and muscle fiber cross sectional area (CSA) (B). Data shown as means $\pm$ SD (Oneway ANOVA, Tukey HSD *post hoc*, $p < .05$)

Figure 10. Hindlimb responses during stretch and in correlation with the number of c-Fos+ neurons

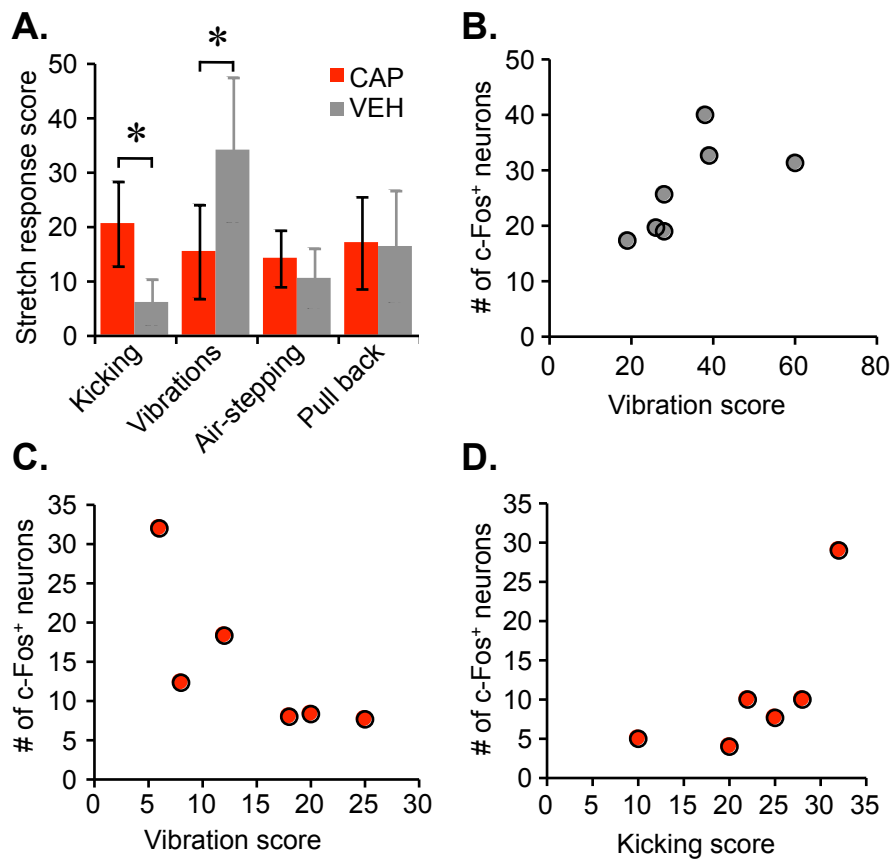


Figure 10. Four major hindlimb responses during stretching are kicking, vibrations, air-stepping and pull back (A); data shown as means $\pm$ SD, (independent t-test, $p < .05$); (B.) VEH group vibration score has a significant positive correlation with the number of c-Fos+ neurons at L5 ($r_s = .793$, $p = .033$) (C.) CAP group vibration score has a significant negative correlation with the number of c-Fos+ neurons at L5 ($r_s = .886$, $p = .019$) and a significant positive correlation between the kicking score and the number of c-Fos+ neurons at L1 ($r_s = .841$, $p = .036$) (D.) Significant correlations were determined using Spearman's rho test.