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Aberrant sensory responses are dependent on lesion severity after spinal cord contusion injury in mice

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Abstract

Following spinal cord injury (SCI), individuals lose normal sensation and often develop debilitating neuropathic pain. Basic research has helped to elucidate many of the underlying mechanisms, but unanswered questions remain concerning how sensation changes after SCI and potential negative consequences of regenerative therapies. Mouse models provide an opportunity to explore these questions using genetic markers and manipulations. However, despite the increasing use of mice in pain and sensory research, the responses to sensory stimuli after SCI are poorly characterized in this species. This study evaluated behavioral responses to mechanical and nociceptive stimuli applied to the hindlimbs and the dorsal trunk in C57BL/6 mice after mid-thoracic SCI. Adult mice were subjected to laminectomy, contusion injuries of different severities, or complete transections to test the hypothesis that the patterns of sensory pathology depend on the extent of tissue damage at the injury site. In the hind paws, hyper-responsiveness to a heat stimulus developed independent of injury severity, while mechanical sensitivity decreased, except after the most severe contusion injuries sparing less than 2% of the white matter at the injury site, when enhanced sensitivity was observed. On the trunk, mechanical and pin prick testing revealed diminished sensitivity at and below the injury level, while responses above the level of the injury were unchanged. The contrast in injury severity threshold for thermal and mechanical hypersensitivity in the hind paws suggests that these responses have different underlying mechanisms. These results establish essential baseline information for murine studies of pain and changes in sensation after SCI.

Keywords

hyperalgesia; nociception; Hargreaves' method; allodynia; von Frey; dysesthesia

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1. Introduction

Spinal cord injury (SCI) results in profound motor and sensory dysfunction (1,97,107,108). Individuals typically suffer from a loss of sensation below the level of the injury, but an average of 65% of the population with SCI also lives with spontaneous or evoked central neuropathic pain that can be localized below, girdling, or above the level of direct spinal cord damage (57,58,73,89,90,96). While a great deal has been gleaned from basic research studies about the cellular mechanisms that contribute to neuropathic pain after peripheral nerve or spinal cord injuries (112,113), for many individuals, the sensory abnormalities and pain remain intractable to treatment (30,31,32). In addition, current research in regenerative and rehabilitation therapies after SCI raise the concern of potential unwanted sensory abnormalities that could arise by enhancing sensory fiber growth or activity (24,48).

Animal models provide an opportunity to investigate mechanisms and potential treatments for sensory loss and neuropathic pain. Rats, cats and primates have proven beneficial in unraveling some of the causes of neuropathic pain (27,40,84) including anatomical correlates (21,35,63, 64), and rats have been used extensively to study underlying correlates of pain after SCI (37, 38,39,40,41,42). In addition, mice provide a unique and valuable potential to extend this knowledge and test specific hypotheses of the molecular mechanisms of pain and sensory dysfunction through genetic manipulations (4,13,14,17,22,43,55,67). While the use of mice in peripheral sensory research has increased dramatically over the last decade (75,84) this species has been underutilized in SCI pain research, in part because their responses following injury remain incompletely characterized.

The current study was conducted to establish mouse contusion SCI as a potential tool for elucidating the mechanisms underlying central pain and to examine the relationship between lesion severity and changes in sensory responses following SCI in mice. Responses were measured following mechanical and nociceptive stimuli applied to the hind paws and trunk after receiving mid-thoracic (T9) contusion injuries ranging from very mild to very severe, or complete transection injury in C57BL/6 mice (54,71). Nylon monofilaments were used to examine responses to mechanical stimuli, and a focused radiant heat source (45) was used to establish hind paw withdrawal latency from a thermal stimulus (49). Sensate and insensate regions on the haunch and dorsal trunk of the mouse were verified using pinprick. Following SCI, hyper-responsiveness in response to a thermal stimulus developed in all injured animals, including transected mice, independent of injury severity. In contrast, responses to mechanical sensation were decreased in hind paws after all lesion severities except for subjects with the most severe contusions, which developed increased sensitivity to the non-noxious stimulus. Responses to mechanical and pin prick stimuli on the dorsal trunk were diminished below the level of the injury on the trunk while mechanical and prick responses above the level of the injury remained constant or decreased slightly after injury, with no indication of above-level allodynia.

2. Methods

2.1 Mice

All procedures were performed in accordance with the Ohio State University Animal Care and Use Committee and the NIH Guide to Care and Use of Laboratory animals. A total of 71 adult, female C57BL/6 mice (8-12 weeks of age at the beginning of the experiments) were obtained from The Jackson Laboratory (Bar Harbor, Maine, USA) and Harlan Laboratories (Indianapolis, Indiana, USA). Of these, 44 mice were subjected to contusion SCI, 13 received a complete transection injury, and the remaining 14 served as laminectomy or naïve controls. Three injured mice were removed from the study because of overgrooming and autophagia occurrence on the dorsal trunk below the level of the injury (20,60,114), and two mice from

the complete transection group were excluded because of incomplete transections determined histologically. Mice were housed three to four per cage and were maintained on a 12-hour light-dark cycle with food and acidified water (pH 6.5) *ad libitum* for the duration of the study.

2.2 Experimental Design

The results from two separate experiments are reported. In Experiment 1, we compared responses to sensory testing in mice after they were subjected to a laminectomy (n=6) or to moderate (0.5mm displacement, n=4) or severe (0.7mm displacement, n=5) contusion injury. These injuries have been well characterized in our laboratory with regard to motor recovery (54,56,71). Laminectomy and naïve control groups were combined after no difference was found in any behavioral measure. Experiment 2 was performed to extend important findings of effects of lesion severity from Experiment 1 and to accommodate improvements in the sensory testing procedures. Specifically, both groups of injured mice in Experiment 1 showed similar responses to hind paw stimuli, and thus it was not possible to provide a definitive test of our hypothesis. In addition, the trunk testing procedures (see below) uncovered ceiling and floor effects in sensory responsiveness that could be addressed using a slightly modified protocol. Therefore, in Experiment 2, we used a full range of injuries from very mild to very severe contusion and transection. We took advantage of the fact that a new Infinite Horizons contusion injury device (Precision Systems and Instrumentation, LLC, Lexington, KY) was being used in the laboratory, which allowed us to choose a wide range of force settings and to mix up the animals so that our sensory testing was done with no knowledge of the true or intended injury severity. Mice received a severe injury on the OSU ESCID (0.7 mm displacement, n=6), or a 40 (n=1), 50 (n=4), 60 (n=4), 70 (n=4), 80 (n=2), 90 (n=2), 100 (n=2), or 120 kD (n=1) impact with the IH device, with six mice serving as laminectomy controls. Finally, an additional group mice was prepared with complete transection injuries (n=13) to allow unequivocal interpretation of the ability of segmental systems to mediate hind paw responses in the absence of any ascending or descending continuity.

2.3 Spinal Cord Injury

All mice were anesthetized prior to surgery with ketamine (80mg/kg) and xylazine (10 mg/kg; i.p.), and the spinal cord was exposed by laminectomy at the T9 spinal cord level. Contusion injuries were performed with either the OSU electromagnetic spinal cord injury device (ESCID) (54,71) or the Infinite Horizons (IH) device (3,87), as described above. To prepare complete transection, mice received a laminectomy, the dura was opened with iridectomy scissors and a scalpel was used to completely sever the spinal cord. The complete nature of the lesion was verified by visual confirmation and retraction of rostral and caudal stumps. After hemostasis was established, a small pledget of gelfoam (Surgifoam gelatin sponge, Owens and Minor, Mechanicsville, VA, USA) was placed in the transection gap to discourage apposition of the stumps or regeneration. After all injury or laminectomy procedures, the muscles were sutured in layers, the skin incisions were closed, and mice were allowed to recover in a warmed cage overnight. Post-operative care included subcutaneous saline injections and antibiotics (gentocin; 5 mg/kg, s.q.) for five days, and bladder expression twice a day for the duration of the study (49).

2.4 Behavioral Testing and Analyses

All behavioral testing was performed by investigators that were not knowledgeable of injury severity, and in climate-controlled settings. For sensory testing, ambient temperature was maintained at 25±3°C. Time of day that the tests were administered was consistent for each test to control for circadian variations in behavior.

Motor Testing

Basso Mouse Scale (BMS) testing: The Basso Mouse Locomotor Scale is a 0 to 9 point scale (0=hind limb paralysis, 9=normal locomotion), which was developed to describe recovery of function after thoracic spinal cord contusion or transection injury in mice (5). The BMS scale is known to correlate with injury severity and provides a reference for comparison with past and future contusion injury studies. Testing sessions were conducted by a team of two trained investigators prior to surgery, at one, three, seven, and 14 days afterward, then weekly for four more weeks. BMS scores were calculated for left and right hind limbs and averaged to obtain a single value per mouse per test.

Sensory Testing

Hind Paw Thermal Sensitivity Test: Thermal sensitivity of the plantar hindpaws was tested according to Hargreaves' method (30,32,47,49, Plantar Test Apparatus, Ugo Basile, Comerio, Italy). Mice were placed unrestrained in individual clear plastic compartments (11 cm × 17 cm × 14 cm). When the mouse was stationary and not attending to the tester or stimulus, an infrared radiant heat source (25 watts) was applied through a glass floor to the middle of the plantar surface of the hind paw, between the foot pads. A photocell automatically stopped the heat source and the timer when the mouse lifted its paw. For transected mice that were not capable of hind paw plantar placement, the mice were held gently to assist the plantar placement of the hind paws. Each mouse was tested for five trials on each hind paw, with at least one minute between trials, and a randomized order of testing to minimize windup or avoidance behaviors (69). A 20 second maximum cutoff was established to prevent tissue damage. Latency (in seconds) to withdrawal from the heat source was recorded, together with any other behavior indicating attendance to the stimulus, including sniffing, licking, or looking at the affected paw or attacking the stimulus. High and low latencies were dropped for each paw, and the remaining six latencies were averaged for each mouse.

Hind Paw Mechanical Sensitivity Test: The hind paw withdrawal threshold to tactile stimulation was determined using factory-calibrated Touch Test filaments (von Frey, Semmes-Weinstein monofilaments) (Stoelting, Wood Dale, IL USA, 49,76). Mice were placed under a small, clear compartment (8 cm × 12 cm × 5.5 cm) on an elevated wire mesh screen to allow the investigator free access to the plantar surface of the paws. For transected mice that were not capable of hind paw plantar placement, the mice were held gently to assist the plantar placement of the hind paws. Left and right hind paws were tested in a random order using the up-down method when the mouse was not attending to the tester or the stimulus (15,23). The 50% withdrawal threshold was defined as the lowest stimulus evoking withdrawal in at least half the trials. Each hind paw had 15 consecutive trials. Supraspinal movements associated with the stimulus, including sniffing, licking, or looking at the affected paw or attacking the stimulus were noted with each trial (12,16,70). Since this test stimulated the same area of skin as the plantar heat test, the two tests were conducted on different days.

Pin Prick Nociceptive Sensitivity Test: Testing sites were chosen 1 cm lateral to midline, in line with the axilla of the mouse and about 1 cm below the T9 vertebral level (see Figure 3A). The fur was shaved at least one day prior to testing to expose and mark the testing site. Each pin prick was administered such that the pin was perpendicular to the surface of the skin. Pressure was applied so that the skin dimpled, but the pin did not penetrate or damage the skin (82). The type of response or absence of response was noted for each trial. Responses included turning the head to look at the stimulus (turn), body flinch withdrawal (flinch, not moving feet), moving away/escape (with feet), scratching, licking, and biting/attacking the stimulus. In Experiment 1, bilateral pin pricks were administered, five rostral to the lesion and five caudal to the lesion with 30 seconds to one minute intervals between trials. In Experiment 2, bilateral pin pricks (1cm from midline) were applied at seven sites beginning on the plantar hind paw,

and ascending up the dorsal trunk from the haunch to the shoulder (See Figure 9B). Stimulus location was altered between trials, and 30 seconds-1 minute elapsed between trials on the same animal so that avoidance and wind-up were minimized. Stimulation was not delivered if the mouse was attending to the tester or the stimulus.

Dorsal Trunk Mechanical Sensitivity Test: To test mechanical sensitivity after SCI, calibrated nylon monofilaments (Touch Test) were also applied 1cm lateral to midline on level with the axilla and 1 cm below the T9 vertebral level (see Figure 3A). The same responses to stimulus as with pin prick were noted. In Experiment 1, the withdrawal threshold was established using the up-down method, as described above for the plantar hind paw. We encountered a ceiling effect caudal to the injury (absence of responses at the highest useful force) and a floor effect prior to injury and rostral to the SCI (naïve mice respond to the lightest force filament) with the up-down method. Therefore, in Experiment 2, we selected three filament forces and calculated percent response. We selected 2.44 (0.04g force) as the highest force filament because it was the median response threshold in Experiment 1. The lightest force filament in the set, 1.65 (0.008g force), was applied until it bent, thereby delivering the calibrated force. Finally, to ensure a stimulus level that was below a response level for normal mice, the 1.65 filament was also applied in such a manner that it touched the skin without bending so that lower forces were delivered (<0.008g force). Stimulus location was altered between trials, and 30 seconds-1 minute elapsed between trials. Stimulation was not delivered if the mouse was attending to the tester or the stimulus.

2.4 Anatomical Lesion Severity Analysis

Tissue Preparation—Six to seven weeks after SCI, mice were deeply sedated until fully unresponsive using a 1.5× surgical dose of ketamine/xylazine (120 mg/kg : 15 mg/kg) (36), and were transcardially perfused with phosphate buffered saline (PBS) followed by 4% paraformaldehyde. The spinal cords were removed and post-fixed for two hours. After an overnight rinse in phosphate buffer and cryoprotection in 30% sucrose, they were blocked and frozen in optimal cutting temperature (OCT) compound (Sakura Finetek U.S.A., Inc; Torrance, CA, USA) in 8mm blocks centered on the injury site. Each block was cut on a cryostat in 10 µm transverse sections, mounted on slides in 10 alternating sets, and stored at -20° C until they were stained (71). Control animals and differing injury severities were mounted and stained on the same slides. Lesion analysis was done with coded sections and the investigator was blind to severity.

Histology—One set of sections spaced 100µm apart was stained with Eriochrome Cyanine (EC) to define the extent of damage based on the distribution of myelin (53). The epicenter was identified as the section of tissue with the smallest area of blue-stained white matter in the rim. Computer assisted imaging with the MCID Analysis System (Imaging Research; St Catherine's, Ontario, Canada) was used to measure the cross-sectional area of white matter sparing (WMS) and the total cross-sectional area of the tissue section (TCA), and then the proportional cross-sectional area at the lesion epicenter was calculated (WMS/TCA).

2.5 Data and Statistics

Data from Experiments 1 and 2 are analyzed and presented separately when testing procedures differed because of the evolution of methods from Experiment 1 to Experiment 2. For locomotor (BMS) and hind paw thermal testing, where the methods remained the same for both experiments, the results were combined to create a data set spanning the complete range of injuries.

Because the contusion injuries covered a wide range of lesion severities and the IH injuries were not yet validated in our laboratory, we used proportional white matter sparing as the

measure of injury severity and this served as the independent variable. To bin groups in an unbiased manner, each subject was ranked using the proportional area of spared white matter at the injury epicenter, and subjects were binned into three injury groups with an equal number of subjects per group. Control animals and animals with a complete surgical transection were grouped into a fourth and fifth bin. When Experiment 1 and 2 data were combined, the Experiment 1 data was added into the same bins based on white matter sparing limits established in Experiment 2 so that the subjects remained in the same group for comparison across testing modalities.

Two-way ANOVA with repeated measures was used to compare behavioral outcomes over time between groups. To visualize overall post-injury performance after the behavioral plateau, each animal's behavioral score was averaged from weeks 3-6, and one-way ANOVA with Tukey's post hoc was used to compare groups. Differences were considered significant at $p < 0.05$. Graphed values represent mean $\pm$ standard error of the mean.

Receiver Operator Curves (ROCs) are used clinically to determine a threshold to differentiate and diagnose normal versus abnormal values (26,68,78). An ROC was performed to assess the efficacy of using white matter sparing as a predictor of increased mechanical sensitivity, the measureable animal representation of clinical allodynia (pain due to a stimulus which does not normally provoke pain) (see Supplemental Tables 1 and 2). For ROC analysis, injured mice were designated as having decreased sensitivity to mechanical stimuli when 3-6 week post injury withdrawal threshold averages were above the averaged control value. Injured mice were designated as having increased sensitivity when 3-6 week post injury withdrawal thresholds were below the control average. Graphing and analyses were performed with Graphpad Prism 4 (Graphpad Software, San Diego, CA, USA).

3. Results

3.1 Experiment 1: Sensory changes after injury

Experiment 1 was designed with distinct moderate and severe injury groups, as established by previous studies of motor recovery, to examine the differences in sensory changes that may have been due to the extent of the lesion (8,63). As expected, the 0.5mm and 0.7mm displacement injuries resulted in two groups with tightly clustered locomotor function (Figure 1A). The severe group recovered to 3.5 ± 0.3 on the BMS, which represents plantar placement, dorsal stepping, or occasional plantar stepping. The moderate group recovered frequent or consistent stepping with no coordination or stepping with some coordination and rotated paws (5.0 ± 0 on the BMS). There were significant differences in BMS scores between the moderate group, the severe group, and the control group over time ($p < 0.0001$ by injury group, over time, and interaction, 2-way ANOVA). Data combined from both injury groups showed that locomotor performance (BMS) for injured animals and proportional white matter sparing were highly correlated ($p = 0.002$, $r = 0.88$, $r^2 = 0.77$) (Figure 1B). Representative histology of epicenter sections from severe, moderate, and laminectomy groups are shown in Figure 1C,D,E. Both moderate and severe groups had limited white matter sparing, with severe injuries between 1% and 3% and moderate injuries between 4% and 7% white matter sparing (where proportional white matter sparing = the area of white matter/total cross sectional area, and the percent white matter sparing is proportional white matter sparing $\times 100$).

Thermal sensitivity is increased in the hind paws after SCI—We examined hind paw response to a radiant heat source to test for loss of thermal sensation or hyperalgesia as a result of SCI. Both severe and moderate injuries caused a similar decrease in latency to withdrawal compared with pre-injury or control values, traditionally interpreted as hyperalgesia (21) (group $\times$ time ANOVA of moderate vs control with significant main effects of group $p = 0.0004$, time $p = 0.0002$, and interaction $p < 0.0001$, post hoc $p < .001$ at all time points post-injury, see

Figure 2A). Data averaged from weeks 3-6 post-injury revealed nearly identical withdrawal latencies for severe and moderate groups, which were both significantly lower than control latencies ($p < 0.0001$, 1-way ANOVA, Figure 2B,C). Thus, there was no correlation between withdrawal latency and white matter sparing in injured mice (Figure 2C).

Mechanical sensitivity in the hind paws is variable after moderate and severe injuries—Hind paw withdrawal from mechanical sensation was tested to evaluate either decreased sensation or allodynia (increased sensory responsiveness compared to normal) (16). Mice were randomly assigned to injury and laminectomy groups, and were tested blindly. Unexpectedly, when pre-injury data were analyzed, there were significant differences between groups. However, there were no differences due to injury severity or changes in threshold measures over time (2-way ANOVA, effects of time $p = 0.93$, group $p = 0.84$, interaction $p = 0.11$, Supplemental Figure 1A). Data normalized to baseline also showed no indication of emerging sensory changes in mechanical response thresholds or differences between groups (Supplemental Figure 1B).

Dorsal trunk nociceptive sensitivity is all-or-none after SCI—Pin prick testing is used clinically in humans to evaluate nociceptive function and establish the functional sensory level of injury (86), and has also been used to measure hyperalgesia (82). To test the effects of SCI on sensory responsiveness to noxious stimuli applied to the trunk, pin prick testing was conducted on the dorsal trunk rostral and caudal to the T9 injury site, as shown in Figure 3A. Regardless of location, uninjured mice responded to a trunk pin prick stimulus nearly 100% of the time. After injury, there was no change in the percent response above the level of the injury (Figure 3B). We also saw no apparent change in the types of responses (Supplemental Figure 3). Below the level of the injury, both moderate and severe mice showed a significant, dramatic decrease in the percent response to pin prick (Figure 3C). The ability of these same mice to respond to mechanical and heat stimuli applied to the hind paws confirms that all of the mice had sufficient motor capacity to flinch and/or withdraw from the stimulus.

Mechanical sensitivity of the dorsal trunk is reduced caudal to SCI—Evaluation of the changes in responsiveness to mechanical stimulation on the trunk was conducted with nylon monofilaments. Using the up-down method to estimate the 50% withdrawal threshold, we encountered a floor effect in uninjured mice and rostral to the injury in all groups. Some mice responded consistently to the lowest force filament available (0.008 g), not allowing a true estimation of the 50% withdrawal threshold (Figure 4A,B). In injured mice, a profound loss of responsiveness was evident when filaments were applied caudal to the level of the SCI. These thresholds were not precise because the testing paradigm reached a ceiling effect when injured mice were unresponsive to all but high-force filaments (> 5 g bending force) (Figure 4B). A cut-off was established when the force required to elicit a response resulted in direct movement of the underlying muscle and the body of the mouse, possibly resulting in proprioceptive or deep tissue stimuli. While these effects limited the precise detection of the 50% withdrawal threshold at the extremes of sensitivity and insensitivity near the level of the injury, a dramatic increase in withdrawal threshold, or a decrease in apparent sensitivity, was evident below the level of the injury (Figure 4C).

3.2 Experiment 2: Sensory responses over a wide range of lesion severities

Both moderate and severe injuries from Experiment 1 resulted in extensive damage with a range of 1-7% WMS. Experiment 2 was designed to evaluate sensory changes induced by a wider and more complete range of injuries. Locomotor scores averaged over the recovery plateau (weeks 3-6 after SCI) were used to compare the severity of injury with historical and future studies. The injuries spanned the full range of functional deficits, from 1.25 on the BMS scale (1 signifies slight ankle movement) to 8.75 (9 represents normal locomotion). In

comparison, mice with complete transections recovered minimally, attaining BMS scores of 1.6 ± 0.12 (Figure 5). Anatomically, the percent of the spared white matter in proportion to total cross-sectional area (%WMS) ranged from 0.3% to 61%. For reference, white matter accounts for 63–69% of the cross sectional area of the uninjured spinal cord. The specimens that were subjected to complete surgical transection were also cut and stained with EC to confirm the absence of any myelinated profiles at the injury site (Figure 6A). By rigorous microscopic examination, 2 of 13 complete transection specimens contained a very small number of fibers; the behavioral data from these 2 subjects were excluded from all analyses.

Across Experiment 2, the locomotor scores and the white matter sparing were highly correlated ($p < 0.0001$, $r^2 = 0.64$, $r = 0.80$) (Figure 5A). The combined data from Experiments 1 and 2 offer a full range of injuries, ranging from complete transections and minimal sparing to near normal preservation of white matter (Figure 5B,C; Figure 6). Dividing the ranked specimens into equal groups for comparisons (the binning also used for behavioral analyses, described in the “Data and Statistics section”), the most severe injury bin ranged from 0.3 to 1.2% WMS, termed “minimal sparing,” more moderate injuries ranging from 1.5 to 20.8% WMS, and the mildest injuries included 21.6 to 60.7% WMS, termed “maximal sparing.” Where no myelin was evident in the transection group (Figure 6A), in the minimal sparing group, a few myelinated axons could still be seen in the region of the anterolateral-spinothalamic tract (AL-STT) of the spinal cord (105, see Figure 6B,E,F,H). The moderate sparing group contained a continuous rim of spared white matter (Figure 6C). In the maximal sparing group, the center of the spinal cord was damaged, including the dorsal corticospinal tract, the medial limits of the lateral funiculus, and the dorsal limits of the ventral funiculus (105, Figure 6D,G).

Thermal sensitivity is increased in the hind paws independent of lesion severity

—Thermal hind paw testing across the wider range of injury severities revealed hyper-responsiveness across all injury groups (Figure 7A, Supplemental Figure 2A), demonstrating that even mild injuries are sufficient to produce reduced withdrawal latencies. 2-way ANOVA (injury over time) shows a significant injury effect ($p < 0.0001$), and post hoc analysis demonstrates that all injury groups (including the complete transection group) had significantly reduced withdrawal latencies compared to sham injured animals (Supplemental Figure 2A). When data were averaged from weeks 3–6 post injury, all injured groups had significantly lowered withdrawal latencies compared to controls, and the group with moderate sparing had lower latencies than the maximal sparing group and the transection group ($p < 0.0001$ 1-way ANOVA, Figure 7A and B).

Mechanical sensitivity in the hind paws is enhanced only in contusion injuries with minimal sparing

—Hind paw 50% thresholds to withdrawal from mechanical stimulation in Experiment 1 were highly variable. This contrasts with published studies from peripheral injury and inflammation models in mice (100,111), raising concerns about the longevity and reliability of smallest filaments (115). A new set of calibrated filaments was purchased for Experiment 2 and the data were not combined.

Hind paw mechanical testing results from the very mild and very severe animals included in Experiment 2 revealed clear differences in withdrawal thresholds across injury groups that were not apparent in Experiment 1. Injured mice with maximal and moderate sparing had higher withdrawal thresholds (blunted sensitivity) compared with uninjured controls. Similarly, withdrawal thresholds of mice with complete surgical transection were also greater than that of controls. In contrast, the withdrawal thresholds for the group of mice with minimal sparing were significantly lower than those of the maximal sparing, moderate sparing, and transection groups (Figure 8A,B). The mean threshold for the minimal sparing group was not significantly different from controls; however, all but 2 of the subjects from the minimal sparing group were more sensitive than the sham-injured controls. To determine if the increased responsiveness

to mechanical hind paw stimuli in severely injured mice could be clearly distinguished from mechanical thresholds of animals with more moderate or mild lesions, a Receiver Operator Curve (ROC) was plotted for all contusion mice using white matter sparing as a predictor. We found that a cutoff value of <1.9% white matter sparing predicts a response threshold below the mean threshold of the uninjured mice with 88.9% accuracy (100% sensitivity, 88.9% specificity, where sensitivity indicates the likelihood of detecting a true positive result and 1.0 minus the specificity reflects the likelihood of detecting a false positive result) (Figure 8C; Supplementary Table 1,2). Thus, severe contusion injuries with minimal white matter sparing in mice predict a modest but consistent decrease in withdrawal threshold, representing increased hind paw mechanical sensitivity after SCI (7,9). This increased sensitivity disappears in completely transected mice.

Nociceptive sensitivity on the dorsal trunk—To enable a mapping of responsiveness to a nociceptive stimulus, a series of pin prick stimuli were applied at 7 sites including the plantar hind paws, the haunch, and the trunk of the mice. In agreement with results from the first experiment, we found no change in the likelihood to respond to pin prick site 1 cm rostral to the lesion level and a dramatic loss of responsiveness just below the level of the lesion. When mapped over the wide range of injury severities in Experiment 2, the pin prick test revealed a zone of reduced responses to sensory stimuli which varied in size and completeness with injury severity (Figure 9A-C). Severely injured mice respond rarely or never to pin prick just below the lesion level and the severe contusion and transection groups even showed a similar lack of responsiveness that extended above the injury level. In contrast, the most mildly injured mice were able to respond to the pin prick near the lesion site nearly half the time. For all injury severities, the least responsive zone (Zone 4) was just below the lesion level and gradually gave way to 100% responsiveness to a pin prick stimulus on the plantar hind paws (Zone 7). No change in type of response was obvious at any zone.

Mechanical sensitivity on the dorsal trunk—Responses to mechanical stimuli were determined in this experiment using three von Frey filaments as described above. Control subjects and pre-injury testing showed approximate response percentages of 50% to stimuli using the 0.04 g filament, 30% using the 0.008 g filament, and 25% using the 0.008 g filament without bending. Despite the large window available to detect increased sensitivity, none of the groups had altered response percentages to the 0.008 g and 0.04 g filaments administered rostral to the lesion level. In response to the unbent 0.008 g stimulus, the transected group had a reduced mean percentage response, suggesting an expansion of the injury region as revealed with the pin prick test (Figure 10 A,C,E). Caudal to the injury, sensory responses were blunted for all stimulation forces, and all groups of injured animals had a significantly lower percent response to the 0.008 g and 0.04 g filaments (Figure 10 B,D,F).

Suprasegmental responses to hind paw testing in injured mice—Some research efforts have suggested that pain or sensation following a stimulus in rodent SCI models can be detected by identifying responses that require movement of muscle groups above the injury level in a manner that is directed toward the site of stimulation. To answer the question of whether the responses to sensory stimuli included these suprasegmental components in addition to reflexive, segmental withdrawal responses, behaviors that are thought to indicate attention to and/or awareness of the stimulus such as sniffing, licking, or looking at the affected paw or attacking the stimulus were noted during all plantar hind paw testing sessions (thermal and mechanical) (12,16,70). Surprisingly, the results show that intact mice only exhibit these suprasegmental responses accompanying 7.8% of withdrawals in response to heat stimulation and 26% of withdrawals in response to mechanical stimulation. These potential suprasegmentally mediated responses were not as frequent in injured mice, but a majority of contusion-injured mice (53%) did exhibit at least one such indicator in response to hind paw

stimulus during the course of testing (Figure 11). Notably, none of the transected mice exhibited suprasegmental responses in association with nociceptive or mechanical stimuli.

4. Discussion

SCI can initiate a wide range of abnormal sensory symptoms, including analgesia, dysesthesias and paresthesias (96). Basic researchers have studied changes in responses to sensory stimulation, especially in rat models of SCI, in an effort to understand the complex underlying anatomical, cellular, and molecular mechanisms and to identify novel treatment strategies for these conditions (21,38,40,42,44,51). This research indicates that in addition to loss of ascending information, the initial trauma and secondary events evoke lasting changes in excitability of the sensory pathways at the level of the dorsal roots, the spinal cord, and supraspinal regions to contribute to the development of central neuropathic pain (4,52,74,92, 103). However, a number of questions remain unanswered to date, such as how the signaling pathways associated with abnormal sensation are altered and how these changes are maintained chronically. In related fields, including peripheral nerve, visceral, and cancer pain research, transgenic mice have proven to be valuable adjuncts in this pursuit because they allow the use of subjects with genetic deletion, overexpression, or the identification and tracking of cells, receptors, or signaling molecules (8,13,14,22,33,72). However, the use of transgenic mice to extend the study of sensory dysfunction after SCI has not yet been realized. In addition, mice are being used increasingly in studies of neural regeneration (17,61,93, reviewed by 116), but to date, reliable tests of beneficial or deleterious sensory changes that might occur after these treatments have not been fully described. To enable a greater contribution of mice to the future study of sensory dysfunction and recovery after SCI, we have characterized the patterns of responses to sensory stimuli after mid-thoracic contusion injury. The results establish important baseline data and reveal similarities and differences between the effects of injury severity on changes in thermal and mechanical sensitivity, as well as between mice and rats, and between clinical findings and the rodent models.

People who suffer from dysesthesias and pain after SCI often report spontaneous and evoked pain above, at and below the lesion level (91). Diffuse pain located below the injury level is the most common and most severe condition, occurring primarily in areas of functional loss (58,91). While we cannot directly measure the perception of pain in rodent models (75,76, 109), we have made use of commonly used surrogates, including responses to mechanical and nociceptive stimuli on the hindlimbs and the trunk below the lesion level. Both mice and rats show reproducible hypersensitivity to both thermal and mechanical stimuli applied to the hind paws after SCI. Based on peripheral neuropathy models (9,88,62) and the presence of corresponding changes in nociception related supraspinal regions including the thalamus and cortex after rat SCI (50,77,103), these behaviors are thought to reflect a testable component of clinical below-level pain, and are commonly referred to as correlates of thermal hyperalgesia and mechanical allodynia (16,94).

In this study, we tested the relationship between the severity of injury and incidence of withdrawal to thermal and mechanical stimuli. We show that thermal hypersensitivity in the plantar heat test occurs uniformly, and is even prominent in completely transected mice. In contrast, hypersensitivity to a light touch stimulus on the hind paws was only found in a small group of mice with a minimal amount of tissue sparing. These results contribute to the growing body of literature that indicates differences in potential mechanisms of thermal and mechanical hypersensitivity (8,49,80,99). Following mid-thoracic SCI, thermal hypersensitivity appears to be mediated entirely by reorganization at the spinal cord level. Indeed, we and others have shown that small-diameter nociceptive primary afferent fibers in the remote lumbar cord sprout in response to SCI (49,66,81). The expansion or strengthening of nociceptive input to

withdrawal reflex pathways may be sufficient for invoking the reduced latencies to thermal stimuli (7,83).

In contrast to thermal responses, mechanical hypersensitivity is highly dependent on lesion severity and occurs only after the most severe contusion injuries. Notably, a few remaining fibers found continuing through the epicenter of severely injured mice were consistently localized in the region of the anterolateral-spinothalamic tract (AL-STT) in the animals with hind paw mechanical hypersensitivity. This suggests that the near-complete damage to the AL-STT may contribute to conveying aberrant sensory signals that mediate below-level mechanical allodynia (10,18,19,25,101,102,46,104). Alternatively, the heightened activation of withdrawal to touch may be mediated by subtle alterations in residual descending modulation (6,85). Recent evidence has mounted for the role of injury-activated microglia and other inflammatory mediators in neuropathic pain (79,80,99). Such effectors are heightened both at the site of trauma and in the spared cord distal to the injury and inflammatory signals can exacerbate the excitability of nociceptive neurons (106,110). Severely injured rats that develop hind paw allodynia after mid-thoracic contusion have significantly increased microglial activation in the lumbar spinal cord, while those with less severe contusions do not (21). Taken together, these results suggest that mechanical hypersensitivity after SCI is a selective outcome measure which has the potential for invoking supraspinal interpretation and may reflect a rodent correlate of below-level allodynia.

The most common sensory abnormality in clinical SCI is the loss of sensation below the level of injury (28,65). Somewhat surprisingly, little emphasis has been placed on describing rodent correlates to below level sensory loss in recent studies. The inability to directly measure sensation and the ubiquitous nature of heightened evoked reflexes contribute to this reporting pattern, although some investigators have used hotplate withdrawal latencies (34) or sensory evoked potentials (59) to measure sensory loss and recovery and have shown a loss of ascending tract function after SCI (2). In the present study, we mapped the distribution and frequency of evoked responses to touch and pinprick below the lesion. We have characterized reliable and severity-dependent diminished responsiveness to mechanical and nociceptive stimuli applied to the trunk below the level of the injury, and shown that the area of diminished responsiveness extends even rostral to the injury level following severe contusions or transection. This loss of sensitivity only recently been reported in models of contusion SCI in rats (2). Instead, rats with contusive SCI injuries frequently exhibit enhanced responsiveness to light touch applied above and at the lesion level (16,50,70) This is thought to model above-level SCI pain in the clinical population, but it is worth noting that “above-level” allodynia in people is fairly rare (96).

Perhaps the most surprising finding in this characterization was the absence of heightened responses to stimuli applied above or at the level of injury in the mice. Both at-level and above-level pain are described by SCI patients, and pain at the injury level is common (90,96). In regard to this, a number of investigators have also described allodynic responses on the trunk above and at the level of contusion injuries in rats (16,70). The current findings suggest an important species difference. In a recent report examining the responses of two mouse strains to mechanical stimuli applied to the trunk (60), the authors were unable to establish a threshold of response to mechanical stimulation in C57BL/6 mice due to a very high baseline response. We discovered a similar “floor” effect when applying the up-down method to intact mice in Experiment 1. However, when we adapted our procedures to identify percent responses at the mean threshold stimulus level and below threshold levels, the average intact response was between 15 and 45% for all stimulus strengths and testing locations. Using this paradigm, we have firmly established that adult female C57BL/6 mice do not exhibit evoked above or at-level mechanical allodynia on the trunk after contusion injuries ranging from mild to severe. The reasons for these observations are not yet clear. Given that at-level pain may be mediated

directly by processing of second order neurons at the lesion level, we can speculate that the unique wound healing in mice may somehow alter the excitability of those neurons (71,95).

In summary, murine models and the genetic tools they offer are a critical component of ongoing SCI research. We have characterized sensory changes after SCI in mice, furthering their utility as a tool in SCI research. We have demonstrated the separate development of hypersensitivity to mechanical and thermal stimuli depending on extent of damage to the spinal cord and the location and modality of testing, showing that severe damage to spinal cord with some remaining intact ascending fibers induces allodynic behavior, where as hypersensitivity to heat can be demonstrated after very minimal damage, and can even occur in completely transected mice. Improved understanding of residual sensory transmission in severe “discomplete” injuries is necessary in order to develop better therapies for the devastating symptoms of sensory dysfunction and SCI induced evoked and spontaneous neuropathic pain (29). Mouse contusion injury provides a model where the complex molecular mechanisms of these divergent phenomena can be studied in further detail.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Controlled moderate and severe contusion injuries in mice produce specific and consistent locomotor and histological results

A) Severe and moderate injury groups demonstrate locomotor recovery to distinct levels. Severely injured mice recovered to 3.5 ± 0.3 , and moderately injured mice recovered to 5.0 ± 0 on the BMS, while control animals remain at 8 ± 0 throughout the study ($p < 0.0001$ by injury group and time, 2-way ANOVA). Laminectomy and naïve mice were combined to form the control group. Dotted line represents time of injury. *** $p < 0.001$ versus laminectomy controls, ### $p < 0.001$ versus severe injury group, Bonferroni post tests. **B)** Scatter plot of the average of week 3-6 BMS scores for each animal plotted against proportional white matter sparing (area of spared white matter/total cross sectional area of the spinal cord). Locomotor performance and proportional white matter sparing are highly correlated ($p = 0.002$, $r = 0.88$, $r^2 = 0.77$). **C, D, E** Representative Eriochrome Cyanine-stained sections from the injury epicenter of severe (C), moderate (D), and control (E) groups. Scale bar is 200 μ m.

Figure 2. Mice demonstrate similar increased responsiveness to thermal hind paw stimulation after moderate and severe SCI

A) Moderate and severe groups demonstrate a significant decrease in latency to withdrawal from a focused heat stimulus to the plantar hind paw after SCI. 2 way ANOVA of moderate vs control: $p=0.0002$ over time, 0.0004 by group, with an interaction $p<0.0001$. *** $p<.001$ compared to control group, post hoc. This hypersensitivity to thermal stimuli was evident by 3 weeks post injury, and continued throughout the remainder of the study. Dotted line represents the injury. **B)** Bar graphs representing the 3-6 week averages of thermal withdrawal latencies for severe, moderate, and control groups. $p<0.0001$, 1-way ANOVA. *** $p<.001$ compared to control group, post hoc. **C)** Scatter plots of white matter sparing from moderate

and severe injury groups and 3-6 week average hind paw withdrawal latency. Withdrawal latency was not significantly correlated with injury severity (proportional white matter sparing). Dotted line represents control group average withdrawal latency for comparison.

Figure 3. Responses to thoracic pin prick immediately rostral and caudal to the SCI

A) Sites used in dorsal trunk testing. Gray line represents the T9 injury level. **B)** Percent response to a prick stimulus rostral to the level of the injury. Mice responded to near 100% of stimuli both before and after injury, with no group differences. **C)** Percent response to a prick stimulus caudal to the level of the injury. Mice responded to nearly 100% of stimuli before injury. After injury, moderate and severe injury group responses decreased to less than 20% ($p < 0.0001$, 1-way ANOVA). Control group was significantly different from both injured groups at both post-injury time points, $p < .001$, Tukey's post-hocs.

Figure 4. Responses to thoracic mechanical stimuli immediately rostral and caudal to the SCI
A) Experiment 1 mechanical sensory testing using the up-down method to establish the 50% response threshold encountered a floor effect rostral to the SCI, with mice responding to the lowest gram-force filament (0.008g). There were no differences by group or over time, 2-way ANOVA. **B)** Testing caudal to the SCI revealed a ceiling effect. Mice responded only to high-force filaments, and a cut-off was established to prevent proprioceptive or deep tissue responses. 2-way ANOVA: $p < 0.0001$ over time, $p = 0.0002$ by group, $p < 0.0001$ interaction. Post hocs: *** $p < 0.001$ versus control, * $p < 0.05$ versus control, # $p < 0.05$ versus severe group. **C)** Response thresholds averaged from 3-6 weeks post injury show a dramatic decrease in responsiveness below the level of SCI. 1-way ANOVA $p < 0.0001$, caudal responses in severe and moderate groups are different from all rostral responses and control caudal responses, $p < 0.001$, Tukey's post hoc.

Figure 5. Wide-ranging contusion injuries produce an array of locomotor abilities that correlate tightly with white matter sparing

A) A scatter plot of locomotor performance versus proportional white matter sparing from Experiment 2 reveals a nonlinear range of functional and anatomical injury severity. Proportional white matter sparing and locomotor performance are highly correlated ($p < 0.0001$, $r^2 = 0.64$, $r = 0.80$). Filled circles to the right are laminectomy controls. **B)** Combined data from Experiments 1 and 2 and transected mice represents a more complete range of functional and anatomical lesion severity. Proportional white matter sparing correlation ($p < 0.0001$, $r^2 = 0.71$, $r = 0.85$). Vertical lines represent binning. **C)** Selected data from (B) expanded to show detail.

Data from the lowest region of proportional white matter sparing scale (0-0.05) were replotted for clarity.

Figure 6. Range of anatomical sparing in Experiment 2

A-D Representative sections from the injury epicenter for the transection (**A**), minimal sparing (**B**), moderate sparing (**C**), and maximal sparing (**D**) injury groups in Experiment 2. Sections shown are from the median specimen from the range included in each bin. **E** and **G** are epicenter sections showing the extent of the lesion in the most severe (**E**) and mild (**G**) contusion injuries. Scale bars are 200 μm . **F** Enlargement of boxed area in (**E**). Scale bar is 30 μm . **H** A diagram of the location of major tracts from the rat (67) for comparison. Note the location of the spared fibers from the most severe injuries correspond with the AL-STT (shaded).

Figure 7. SCI induces increased responsiveness in the plantar hind paw

A) Latency to withdrawal from a thermal stimulus averaged from weeks 3-6. 1-way ANOVA $p < 0.0001$. Post hoc: *** $p < 0.001$, ** $p < 0.1$ versus control group, ^ $p < 0.05$ versus maximal sparing group, ++ $p < 0.01$ versus transected group. **B)** There was no correlation of hind paw thermal withdrawal latency and proportional spared white matter for injured animals (all mice shown on graph for reference). Dotted line represents averaged control group latency.

Figure 8. Responses to mechanical stimulation of the hind paws reveal a complex pattern of blunted sensory responses and hypersensitivity depending on injury severity

A) Data averaged from weeks 3-6 show a clear picture of injury-induced sensory responses. Mice with maximal and moderate sparing at the epicenter have a higher withdrawal threshold than normal animals, while mice with minimal sparing have thresholds slightly below those of controls ($p < 0.0001$, 1-way ANOVA), post hoc: * $p < 0.05$, ** $p < 0.01$ versus control; ## $p < 0.01$, ### $p < 0.001$ versus minimal sparing. A scatter plot of injured and control mice **B)** shows the same response patterns without binning. Withdrawal thresholds of injured mice did not correlate with the extent of injury (proportional white matter sparing). **(C)** A receiver-

operator curve (ROC) demonstrates that <1.9% WMS predicts allodynia with 100% sensitivity, 88.9% specificity (graph shows 100% - %specificity).

Figure 9. Trunk and hindlimb frequency of response to pin prick stimuli

A) Graphical representation of the percent response for all seven zones tested in Experiment 2 for the different bins of injury severity. Decreased response to pin prick in injured mice was focused in Zones 4 and 5, caudal to the SCI. Injured groups' responses were not different from controls rostral to the lesion (Zone 1) or in the plantar hind paws (Zone 7) at any injury severity. 2 way ANOVA: differences due to injury $p < 0.0001$, due to time $p < 0.0001$, interaction $p = 0.002$. Post hocs: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ with reference to control group, $+p < 0.05$ versus transection group. **B)** Schematic of pin prick testing used in Experiment 2. Testing was conducted in the planter hind paws (Zone 7) ascending up the haunch to the upper trunk. Colored boxes are used to represent percent response to pin prick in the different zones in **(C)**. **C)** Visual representation of responsiveness to pin prick at different severities of injury. Pin prick response was decreased just below the injury level, but remained intact rostral to the injury site and in the plantar hind paws. This pattern was consistent across injury severities, but the effect was more pronounced at the more extensive injuries.

Figure 10. Responses to mechanical stimuli on the trunk just rostral and caudal to SCI

A) Stimulation with a 0.04g filament rostral to the lesion (white bars) results in a response near 50% of the time at all lesion severities, with no difference in response between injury groups. Frequency of responses below the level of the injury (black bars) to the 0.04 g filament decreased significantly after injury, with more severe injuries having a more dramatic decrease in percent response (1-way ANOVA comparing caudal responses across control and all injury severities, $p<0.0001$). **B)** 0008 g stimulation rostral to the lesion (white bars) causes a response around 30% of the time, with a significant difference in responsiveness due to injury level ($p=0.04$, 1 way ANOVA of rostral responses). Frequency of responses below the level of the injury (black bars) to the 0.008 g filament decreased significantly after injury (1-way ANOVA comparing caudal responses across control and all injury severities, $p=0.0003$). **C)** A light

touch with the 0.008 g filament rostral to the injury (white bars) also elicited a response differing by group, with transected mice showing significantly less responsiveness than controls ($p=0.01$, 1 way ANOVA). Responses to an unbent 0.008 g filament applied below the injury level (black bars) decreased after injury (1-way ANOVA $p=0.03$). Refer to Figure 3 for diagram of testing location. Post hocs for all graphs: * $p<0.05$, ** $p<0.01$, *** $p<0.001$ with reference to control).

A Proportion of Supraspinal Responses to Hind Paw Thermal Stimuli

B Proportion of Supraspinal Responses to Hind Paw Mechanical Stimuli

Figure 11. Mice demonstrate suprasegmental responses to mechanical and thermal hind paw stimuli at all lesion severities

The proportion of positive responses to thermal (A) and mechanical (B) stimuli to the hind paws that resulted in suprasegmental responses after injury. Responses included a sustained withdrawal of the hind paw, licking the hind paw, looking at it, sniffing it, or attacking the stimulus. Shaking the paw was not included, as transected mice were observed with this response. There was only a weak ($p=0.005$, $r=0.49$, $r^2=0.24$ for thermal stimuli and $p=0.005$, $r=0.46$, $r^2=0.21$ for mechanical stimuli) correlation between injury severity and proportion of supraspinal responses in injured mice. While uninjured mice have more suprasegmental

responses, even the most severely injured mice can exhibit them, suggesting the transmission of information from the stimulus to levels above the injury.