Sensory Stimulation Prior to Spinal Cord Injury Induces Post-Injury Dysesthesia in Mice

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Abstract

Chronic pain and dysesthesias are debilitating conditions that can arise following spinal cord injury (SCI). Research studies frequently employ rodent models of SCI to better understand the underlying mechanisms and develop better treatments for these phenomena. While evoked withdrawal tests can assess hypersensitivity in these SCI models, there is little consensus over how to evaluate spontaneous sensory abnormalities that are seen in clinical SCI subjects. Overgrooming (OG) and biting after peripheral nerve injury or spinal cord excitotoxic lesions are thought to be one behavioral demonstration of spontaneous neuropathic pain or dysesthesia. However, reports of OG after contusion SCI are largely anecdotal and conditions causing this response are poorly understood. The present study investigated whether repeated application of sensory stimuli to the trunk prior to mid-thoracic contusion SCI would induce OG after SCI in mice. One week prior to SCI or laminectomy, mice were subjected either to nociceptive and mechanical stimulation, mechanical stimulation only, the testing situation without stimulation, or no treatment. They were then examined for 14 days after surgery and the sizes and locations of OG sites were recorded on anatomical maps. Mice subjected to either stimulus paradigm showed increased OG compared with unstimulated or uninjured mice. Histological analysis showed no difference in spinal cord lesion size due to sensory stimulation, or between mice that overgroomed or did not overgroom. The relationship between prior stimulation and contusion injury in mice that display OG indicates a critical interaction that may underlie one facet of spontaneous neuropathic symptoms after SCI.

Key words: autophagia; autotomy; excessive grooming; overgrooming; sensory testing

Introduction

ABERRANT SENSATIONS, including neuropathic pain, dysesthesias, and paresthesias, develop in a large proportion of individuals with spinal cord injury (SCI) (Siddall et al., 2001; Störmer et al., 1997). These chronic, often intense, sensory symptoms may interfere with daily function and quality of life to a greater degree than even motor impairments (Jensen et al., 2005; Westgren et al., 1998). Neuropathic pain and dysesthesias, defined as abnormal and unpleasant sensations, can arise above, at, or below the level of the lesion, and can be elicited by sensory stimuli (evoked) or occur spontaneously, in the absence of external sensory input (Eide et al., 1996; Siddall et al., 2002). While animal models are crucial for the understanding of mechanisms underlying abnormal sensation and the future development of effective treatments, evaluating sensation in rodents is challenging. Most animal models of neuropathic pain focus on hyper-

responsiveness to mechanical or thermal stimuli as an indication of evoked pain (Christensen et al., 1996; Hutchinson et al., 2004; Kloos et al., 2005; Lindsey et al., 2000; Takasaki et al., 2005). However, these commonly used outcomes address the occurrence of spontaneous pain or dysesthesia indirectly at best (Mogil et al., 2004).

One possible indication of spontaneous pain or dysesthesia in animal models is self-directed overgrooming (OG) or “excessive grooming” behavior, including scratching, licking, and/or biting (Brewer et al., 2008; Kerr et al., 2007; Yeziarski et al., 1998; Zhang et al., 2001; reviewed in Kauppila, 1998). Overgrooming after SCI has been described thoroughly in lesion models induced by focal excitotoxicity (Yeziarski et al., 1998). While OG has also been noted as an occasional consequence of spinal cord transection or contusion injuries (Hook et al., 2009; Zhang et al., 2001), most recently in studies using mice (Aguilar and Steward, 2010; Kerr et al., 2007), the instances are typically sporadic and frequently described

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anecdotally, with subjects that display the behaviors often removed from further analysis. The causes of the occasional clusters of affected animals are not understood. In previous studies, we examined responses of mice to mechanical and nociceptive stimulation on the dorsal trunk and hind paws. We noted an unusually high incidence of OG, particularly in groups of mice for which a large body of pre-injury baseline data had been gathered. Efforts to eliminate OG by reducing baseline testing prior to injury and lengthening the time between baseline testing and subsequent SCI seemed to reduce the incidence of OG. Based on these observations, we hypothesized that repeated sensory stimulation prior to injury contributed to increased incidence of OG in mice. To address this, mice were acclimated to a testing environment and then subjected to nociceptive and mechanical stimulation, mechanical stimulation only, the testing situation without stimulation, or no treatment. Three days after the stimulation ended, the mice received a mid-thoracic moderate contusion injury or laminectomy only. They were observed for 14 days after surgery by an investigator with no knowledge of the pre-injury paradigm. Mice that received sensory stimulation rostral and caudal to the future sight of a contusive SCI had increased incidence of OG just below the level of injury. We found no difference in spinal cord lesion size between overgroomers and non-overgroomers, and no effect of pre-injury stimulation paradigm on lesion length. These findings indicate that sensory stimulation before injury can exacerbate OG after SCI and suggest that the combination of prior afferent activity and the pathophysiology of trauma contribute to the induction of this manifestation of spontaneous dysesthesia or pain.

Methods

Animals and surgery

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 47 adult, female C57BL/6 mice (8–12 weeks of age at the beginning of the experiments) were obtained from The Jackson Laboratory (Bar Harbor, ME) and used for this study. Mice were singly housed and were maintained on a 12-hour light/dark cycle with food and water (pH 6.5) *ad libitum* for the duration of the study. A total of 38 mice received a moderate (0.5 mm displacement) contusion injury to the mid-thoracic (T9) spinal cord with the OSU electromagnetic spinal cord injury device (ESCID) (Jakeman et al., 2000, 2009; Ma et al., 2001). The remaining nine mice served as laminectomy controls. All injury and laminectomy mice were anesthetized intraperitoneally with ketamine (80 mg/kg) and xylazine (10 mg/kg) and given a T9 vertebral level laminectomy. After the injury or laminectomy, incisions were closed and mice were allowed to recover in a warmed cage overnight. Post-operative care included saline injections (2 cc/day s.q.) and antibiotics (5 mg/kg gentocin, s.q.) for 5 days following surgery, and bladder expression twice a day for the duration of the study (Hoschouer et al., 2008).

Sensory stimulation and behavioral observations

Prior to injury and sensory stimulation, mice were acclimated for 15 min on 3 separate days to the two testing

apparatus, including an open field pool (Basso et al., 2006) and a small plastic box (6.5×8.6×3.4 cm) that would be used as the sensory stimulation environment. Beginning at 1 week prior to surgery, the mice were randomly assigned to one of four defined stimulation paradigms for 4 days. Paradigms included nociceptive and mild mechanical stimulation, mild mechanical stimulation alone, sham stimulation, and no stimulation. Mechanical and nociceptive stimulation was performed by applying nociceptive or mechanical probes (as described below) to the trunk of the mouse at 1 cm to the right of midline, rostral to the future T9 injury site (in line with the axilla of the mouse) and about 1 cm caudal to the T9 (vertebral) injury level (Fig. 1A). The small plastic box used to contain the mice for sensory stimulation is depicted in Figure 1B. The dorsal trunk of all mice in all groups was shaved at least 1 day prior to the first day of stimulation to expose the sites and minimize variations due to manipulation of the fur.

Nociceptive stimulation was administered using a standard household straight pin available at any department store. The pin was positioned perpendicular to the surface of the skin at each of the testing sites. Pressure was applied so that the skin dimpled, but the pin did not penetrate or damage the skin (Rigaud et al., 2008). Each animal received 10 pin touches per site per day, with 30 sec to 1 min between sequential touches at the same site.

Mechanical stimulation was applied using calibrated Touch Test filaments (von Frey, Semmes-Weinstein monofilaments; Stoelting, Wood Dale, IL) (Hoschouer et al., 2008; Mogil et al., 1999) with two different paradigms applied on alternate days. On the first and third days of stimulation, mice received 10 stimuli at each site with a 0.04 g force. On the second and fourth days of stimulation, mice received 15 stimuli starting at 0.4 g and following the pattern of the “up down method” (Chaplan et al., 1994; Dixon, 1980) used to establish a sensory threshold. Stimuli applied with the up-down method ranged from 0.008 to 0.4 g. Mice receiving sham stimulation were also shaved and placed in the small plastic boxes for the same duration and number of sessions, but

FIG. 1. Sensory stimulation paradigm and overgrooming lesion. (A) Schematic of the dorsal aspect of a mouse and the two sites used for mechanical and nociceptive sensory stimulation. The black vertical line represents midline, the horizontal gray line represents the T9 vertebral injury level, and the small circles represent the stimulation sites. Scale bar, 1 cm. (B) Sensory stimulation environment. Mice were enclosed in small plastic containers with a mesh-snapping lid to provide access to the dorsal trunk. Scale bar, 3 cm. (C) An example of a skin lesion caused by overgrooming (circle). The collar used to prevent further self-inflicted tissue damage can also be seen. Scale bar, 1.5 cm.

received no stimulation. A fourth, control, group was shaved but remained in their home cages, except during open field locomotion acclimation and testing, and received no stimulation.

Three days elapsed between the last day of pre-injury stimulation and injury because this was the interval between baseline testing and injury in a prior study where overgrooming was observed at an unexpectedly high rate. After injury or laminectomy, all mice were returned to their home cages and were singly housed to ensure that cagemates could not contribute to the observed hair removal and biting. Housing was distributed so that animals in different groups were adjacent to each other, to minimize any overestimation of treatment effects because of visual communication (Langford et al., 2006). Mice were observed twice daily for signs of OG (patches of hair removal and/or skin lesions) by an observer unaware of treatment group. Any distinct sites of hair removal or lesions were measured with a digital caliper and recorded on anatomical surface maps. Areas larger than 2 mm in the longest dimension were defined as OG sites. OG sites with evidence of skin penetration were defined as bites. Mice that met the criteria for OG were fitted on the day of identification with a collar designed to inhibit access to the wound/bald patch to prevent excessive skin damage. The collars were constructed from Plast-o-fit (Sammons Preston, Bolingbrook, IL) and Vetwrap (3M, St. Paul, MN) and allowed normal function (eating, drinking, and locomotion) but limited accessibility of the lower back and haunch where OG generally occurred (Fig. 1C). The collars were left on for the duration of the study and removed for behavioral testing. No sensory testing was performed on any of the mice after injury. The dates of onset and final distribution of OG sites were recorded for each animal, and the incidence of OG was recorded as the total number of mice classified with OG divided by the total number of mice in the treatment group. Mice that were fitted with collars did not continue to OG and the lesions did not continue to expand. Thus, the lesion size beyond 2 mm was not used as an outcome measure.

Open field locomotion was assessed using the Basso Mouse Locomotor Scale (BMS), 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 injury in mice (Basso et al., 2006). Testing sessions were conducted by a team of two trained investigators prior to injury and then at 1, 7, and 14 days post-injury (dpi). BMS scores were calculated for left and right hind limbs and averaged to obtain a single value per mouse per test.

Histological analysis

Fourteen days after SCI, mice were deeply anesthetized with a lethal dose of ketamine (120 mg/kg) and xylazine (15 mg/kg), and were transcardially perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde. The spinal cords were removed and post-fixed for 2 h. After an overnight rinse in phosphate buffer and cryoprotection for 3 days in 30% sucrose, they were frozen in optimal cutting temperature (OCT) compound (Sakura Finetek USA) in blocks from 2 mm rostral to the injury epicenter to 4 mm caudal (6 mm total). Each block was cut on a cryostat in 10 μ m transverse sections, mounted on slides in 10 alternating sets, and stored at -20°C until they were stained (Ma et al., 2001).

Spinal cords from overgrooming and non-overgrooming mice were blocked together and subsequently mounted and stained on the same slides. One set of sections spaced 100 μ m apart and spanning the entire block was stained with Eriochrome Cyanine (EC) to define the extent of damage based on the distribution of myelin (Jakeman et al., 2006). 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, 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). The rostral and caudal extents of each lesion were determined by inspection, and lesion length was calculated by multiplying the number of sections containing lesioned tissue by the distance between each section (100 μ m). To determine if there were differences in the extent of gray and/or white matter damage across the length of the lesion, seven sections spaced 300 μ m apart starting at 900 μ m rostral to the lesion epicenter, was captured for each specimen using the MCID system and Sony 970 color CCD camera and stored as .tif files. The image maps were printed and the area measurements of total, lesion, gray matter, and white matter tissue sparing were each quantified separately. An unbiased estimate of the area and volume of spared tissue was calculated with the Cavalieri method by randomly orienting a series of equally spaced points (0.122 mm apart) over each section diagram, with each point representing 0.0149 mm² (Howard and Reed, 1998). All lesion analysis was done with coded sections and by an investigator unaware of treatment or outcome groups.

Statistical analysis

Graphing and analyses were performed with Graphpad Prism 4.01 (Graphpad Software, San Diego, CA). Fisher's exact test was used for 2 $\times$ 2 contingency tables. Chi squared (χ^2) analyses with contingency tables larger than 2 $\times$ 2 included a trends analysis, and multiple comparisons were corrected for with a Bonferroni post-test. Comparisons of latency to onset of grooming and histological outcomes were made using one-way ANOVA and post-hoc analysis with Bonferroni tests. A Kaplan-Meier survival curve was plotted to compare the incidence and onset of grooming between groups, with a log-rank test for trends to compare the curves. One-way ANOVA with repeated measures was used for analysis of locomotor (BMS) scores over time and region tissue sparing across sections. In all experiments, differences were considered significant at $p < 0.05$.

Results

To test the hypothesis that pre-injury sensory stimulation of the trunk increased OG after injury, we used the same sensory testing paradigms used previously to subject mice to mechanical and pin prick stimulation before injury. In the days following injury or laminectomy, individual mice began to exhibit evidence of OG. Overgrooming was located primarily on the dorsal trunk near, but not limited to, the sites of pre-injury stimulation as shown on the composite maps (Fig. 2A). While stimulation was limited to the right side of the trunk,

FIG. 2. Distribution and incidence of overgrooming. (A) Composite drawings of areas of overgrooming noted from all mice enrolled in the study. Gray areas represent regions of hair removal, and black areas represent sites of skin damage. (B) Frequency distribution of the day of onset of grooming by group. No mice showed signs of overgrooming before injury. There was no difference in average latency to overgrooming onset between groups (one-way ANOVA, $p = 0.5$). Mean latency to overgrooming for animals that overgroomed was 6.1 ± 0.92 days post-injury. (C) A Kaplan-Meier survival curve comparing the onset and incidence of OG in each group. The group that received pin prick and mechanical stimulation before injury (PP,VFH-Inj) began OG earliest, followed by the mechanical stimulation and injury group (VFH-Inj). In contrast, groups without stimulation had fewer overgroomers that began later. The curves are significantly different as defined by log-ranks analysis ($p = 0.0002$).

OG sites extended bilaterally. OG sites were predominantly below the level of the lesion. Sites of OG included hair removal (gray shading) and/or bite wounds that penetrated the epidermis (black dots). All bite marks were located below the lesion on the dorsal trunk. One mouse had hair removal

on the dorsal trunk above the lesion level and three animals had hair removal on the ventral trunk. However, in no cases was evidence of OG observed on the forelimbs or hind feet.

Despite the consistent time course of acclimation, stimulation, and injury or laminectomy in all mice, the latency to initiation of OG was highly variable. Evidence of OG began as early as 1 dpi to as late as 13–14 dpi, with new occurrences spread throughout the 2-week survival period. Mean latency to onset of OG for all animals that overgroomed was 6.1 ± 0.92 days after injury. The type of pre-injury stimulation did not affect the average latency to the first evidence of OG (one-way ANOVA, $p = 0.5$, Fig. 2B). However, a survival curve demonstrates that mice with the most intense sensory stimulation before injury (pin prick + mechanical stimulation) began OG earliest, followed by mice with mechanical stimulation alone. New occurrences of OG continued in these groups throughout the study, producing a steeper curve than groups without stimulation or injury (Fig. 2C). The curves were significantly different by log-ranks test ($p = 0.0002$).

The incidence of OG was significantly different across pre-injury treatment groups (χ^2 test, $p < 0.001$, Fig. 3A). A trends analysis showed that the more extensive stimulation paradigms led to greater incidence of overgrooming than control conditions. A total of 80% (8 out of 10) of the mice subjected to nociceptive plus mechanical stimulation prior to injury and 78% (7 out of 9) of the mice subjected to mechanical stimulation alone overgroomed. In contrast, 40% (4 out of 10) of the mice with sham stimulation, 22% (2 out of 9) of the mice with no stimulation, and 11% (1 out of 9) of the laminectomy mice exhibited OG. Bonferroni post-hoc analysis revealed significant differences between the pin prick plus von Frey stimulation and the laminectomy group and von Frey stimulation alone and the laminectomy group ($p < 0.01$ and $p < 0.05$ vs. pooled laminectomy groups, respectively). Surprisingly, we noted that the group subjected to sham stimulation prior to injury (housed in the stimulation box for the same length of time as treated subjects) developed an intermediate incidence of OG, indicating some contribution of exposure to the testing environment to OG.

To test the hypothesis that the combination of stimulation and injury specifically enhanced the incidence of OG over either condition alone, the incidence of OG in mice that received mechanical and/or nociceptive stimulation prior to injury were combined, and control injured and laminectomy groups were collapsed to form a group that received either stimulation or injury, but not both (Fig. 3B). The difference between the two groups was clearly significant ($p = 0.0002$, χ^2 test), showing that the pre-injury stimulation reliably increases the incidence of OG.

Locomotor testing was performed to determine if sensory stimulation prior to injury might alter the time course or extent of functional motor recovery. A slight drop in average BMS scores was observed on day 1 post-laminectomy. This was due to decreased trunk stability in four of the nine mice, a finding that is not uncommon following incision and laminectomy surgery in this species. Only one of these four mice went on to develop OG at 3 days post-laminectomy.

All injured mice showed a profound decrease in locomotor capability after injury compared to laminectomy controls ($p < 0.0001$ main effects of group, time, and interaction, two-way ANOVA with repeated measures over time, Fig. 3C; if the laminectomy group is removed, there are no effects of

FIG. 3. Incidence of overgrooming due to sensory stimulus. (A) Percentage of mice that overgroomed grouped by pre-injury stimulus paradigm. PP,VFH-inj mice (8/10 mice overgroomed) had pin prick and mechanical (von Frey hair) stimulation, followed by a T9 contusion injury. VFH-inj (7/9 overgroomed) mice had mechanical von Frey hair stimulation followed by SCI. Box-inj (4/10 overgroomed) mice had no sensory stimulation, but were placed in the stimulation boxes followed by SCI. Ctl-inj mice (2/10 overgroomed) remained in their home cages and then received the SCI. The Lam group is collapsed from uninjured mice that were subjected to pin prick and mechanical stimulation ($n = 2$), mechanical stimulation ($n = 4$), or were placed in the boxes with no stimulation ($n = 3$). (1/9 overgroomed; See Table 1). Incidence of overgrooming was significantly different by group (χ^2 test, $p < 0.001$), with differences between the PP,VFH-Inj group and the Lam group and the VFH-Inj and Lam group (Bonferroni; $**p < 0.01$ and $*p < 0.05$, respectively). A trends analysis showed that increased stimulation led to increased incidence of overgrooming. (B) The combination of pre-injury stimulation and SCI increases the incidence of overgrooming. ($p = 0.0002$, χ^2 test). (C) Pre-injury stimulus had no effect on locomotor recovery after injury. There was a significant difference between the laminectomy and SCI groups (main effects of time, group, and interaction $p < 0.0001$, two-way ANOVA), but no difference between locomotor performance of injured mice by sensory stimulation. Asterisks represent post hoc differences between the laminectomy group and all other groups, $***p < 0.001$, Bonferroni post hoc. Vertical dashed line represents the day of injury.

group or interaction effects). All mice recovered over the 2 weeks of the study from 0.91 ± 0.12 at 1 day post-injury to 5.1 ± 0.12 at 14 days post-injury. There were no differences in locomotor performance at any time point between injury groups subjected to stimulation or not.

To test the hypothesis that the incidence of OG behavior was dependent on the total size or extent of the lesion (Gorman et al., 2001), measures of lesion length and proportional white matter sparing at the epicenter were determined from sections stained with Eriochrome cyanine (Fig. 4A). There was no difference in either of these measures between mice that overgroomed and those that did not (Fig. 4B, C). Then, to determine whether the sensory stimulation paradigm affected the final lesion size, the same methods were applied to compare lesion length and cross-sectional sparing between mice across the four stimulation groups, and no significant differences were found (Fig. 4D). Because the incidence of overgrooming can be associated specifically with damage to gray matter, especially in the dorsal horn, the area of the lesion core, spared gray matter and spared white matter were determined in equally spaced sections from $900 \mu\text{m}$ rostral to $900 \mu\text{m}$ caudal to the lesion epicenter. There were no differences in any of these measures between the lesion site of animals that overgroomed and those that did not (Fig. 4E), and no differences in the total volume of these regions (not shown).

Discussion

A large proportion of individuals with SCI suffer from neuropathic pain and other dysesthesias. Although spontaneous pain and dysesthesias are more common than evoked pain in individuals suffering from neuropathic sensory symptoms (Backonja et al., 2004), investigations of the causes and underlying mechanisms of spontaneous pain and dysesthesias in SCI animal models are limited (Mogil et al., 2004). In this experiment, we investigated prior observations of a high incidence of overgrooming behavior after SCI in mice. We found that sensory stimulation on the trunk prior to SCI increases the incidence of OG after injury. Notably, the combination of sensory stimuli and contusion injury administered after the stimulation increased the occurrence of OG and self-injurious biting, especially in insensate trunk regions below the level of injury. Further investigation using this model could elucidate important mechanisms of clinical post-SCI spontaneous neuropathic pain and dysesthesia, and may offer a pre-emptive target for treatment.

Overgrooming behaviors are not commonly reported in the context of traumatic SCI. OG is often unpredictable and interferes with research outcomes (Zhang et al., 2001). Excessive self-directed licking and biting are generally interpreted as abnormal sensation: most commonly, pain, but more recently interpreted as symptoms of dysesthesia, paresthesia, or itch (Abraham et al., 2004; Fairbanks et al., 2000; Gorman et al., 2001; Hendricks et al., 2006; Yeziarski et al., 1998, 2004, 2005; Yu et al., 2003; Wang et al., 2003; reviewed in Eaton, 2003). Because excessive OG can cause significant damage to the skin and overall health of the animal, animals are usually removed from the study for ethical and scientific reasons, especially if the behavior is not successfully managed with ointments, wraps, or other treatments (Karas et al., 2008; Zhang et al., 2001).

TABLE 1. SUMMARY OF EXPERIMENTAL GROUPS

<i>Stimulation paradigm</i>	<i>Group</i>	<i>Injury</i>	<i>No.</i>	<i>OG (n)</i>	<i>DPO onset</i>
Mechanical and pin prick stimulation	PP,VFH	Inj	10	8	4.8
Mechanical stimulation	VFH	Inj	9	7	7.3
Sham stimulation (boxes only)	Box	Inj	10	4	7.2
None (remained in home cages)	Ctl	Inj	9	2	7
Mechanical and pin-prick stimulation	PP,VFH	Lam	2 ^a	0	NA
Mechanical stimulation	VFH	Lam	4 ^a	1	2
Sham stimulation (boxes only)	Box	Lam	3 ^a	0	NA

Distribution and numbers of animals in the stimulation groups. No., total animals assigned to each condition; OG(n), no. of animals that exhibited overgrooming resulting in hair loss or skin wound ≥ 2 mm in longest diameter; DPO onset, average of first day of overgrooming in those animals that demonstrated the behaviors for each group. Total number of injured mice that overgroomed = 21; total number of injured mice that did not overgroom = 17.

^aAnimals that received laminectomy only with no injury were pooled for group comparisons and statistical analyses.

The few studies reporting OG after trauma-induced SCI do so anecdotally, often as explanations for ending a study early or removing a subject from further analysis (Kerr et al., 2007). In some cases of severe SCI in mice or rats, biting or autophagy of the tail or hindlimbs has been documented as a complication of injury (Zhang et al., 2005). Two recent studies have examined the incidence of overgrooming-type behaviors in mice after spinal cord injury. In the first, (Kerr et al., 2007), dysethesia was identified as “caudally-directed nociceptive behavior” and was found prominently in Balb/c mice following contusion injury. Notably, these mice underwent a full battery of sensory baseline testing prior to injury, including pre-injury stimulation of the ventral trunk, corresponding to the site where the overgrooming was documented. The second study did not include pre-injury sensory testing; instead, mice were subjected to extensive forelimb grip training prior to a cervical contusion injury. After the injury, a large proportion of these mice exhibited overgrooming and/or extensive hair loss in the forepaw and neck region (Aguilar and Steward, 2010). Together with the present experiment, these findings strongly indicate that the extensive baseline sensory testing or training can contribute directly to the incidence of OG in mice.

Self-directed OG has been studied as a reproducible behavioral response in the context of excitotoxic lesions applied by microinjection in the dorsal horn of rats. This excitotoxicity model of SCI has been used for over a decade as a model of spontaneous central pain similar to that seen clinically in humans (Abraham et al., 2004; Fairbanks et al., 2000; Gorman et al., 2001; Hendricks et al., 2006; Yeziarski et al., 1998, 2004, 2005; Yu et al., 2003). The OG and biting lesions observed in the present study closely resemble the lesions on the trunk

seen following these excitotoxic injuries. Because the location and extent of these excitotoxic lesions, particularly in the dorsal gray matter correlates with excessive grooming, we hypothesized that the incidence of overgrooming would correlate with the size of the contusion injury lesion, especially as it extends caudally to the spinal segments below the lesion epicenter. Despite a wide range of incidence of OG in this study, the lesion length, epicenter damage, and extent of locomotor recovery were very consistent, as is expected with our injury model. In order to determine if gray or white matter damage were predictive of the OG behavior, we estimated the volume of spared gray and white matter across a 2100 micron block of tissue centered on the lesion site. Surprisingly, there were no differences in gray or white matter sparing at any level along the rostro-caudal extent of the lesion block and no differences in the distribution of dorsal horn damage between the mice that overgroomed and those that did not. These findings indicate that the interaction between pre-injury stimulation and injury that leads to overgrooming behaviors are likely to be caused by physiological activation and not directly due to toxicity or specific damage to the dorsal horn or spinal cord gray matter or white matter regions.

A second explanation for the site and cause of OG in this study is that the pre-injury sensory stimuli induced a peripheral irritation (Brewer et al., 2008) that was exacerbated by SCI. Indeed, the proportion of subjects that exhibited OG increased with the intensity of the pre-injury stimulation. Self-directed licking and biting have been studied extensively in peripheral nerve injury models, including a neuroma model induced by ligation of a nerve (Wall et al., 1979) and the chronic constriction injury (CCI) model, which induces peripheral nerve inflammation (Bennett et al., 1988). While not

FIG. 4. Lesion size is not associated with overgrooming. (A) Images of intact white matter at the injury epicenter from overgrooming and non-overgrooming mice. Sections are stained for Eriochrome cyanine, which labels myelin. Scale bars are 200 μ m. (B) There was no difference in the proportional area of white matter sparing at the injury epicenter between mice that did ($n = 21$) and did not ($n = 17$) exhibit overgrooming. (C) Total lesion length did not differ between overgroomers and those that did not overgroom. (D) There was also no effect of stimulation paradigm on the length of the lesion or proportional area of white matter sparing at the epicenter. (E) The area of the lesion core, spared gray matter, spared white matter, and total section area was estimated at 300 μ m intervals across the lesion block using an unbiased estimator method. -900, -600, -300 represent the distance in microns of sections from rostral to the epicenter; +300, +600, and +900 represent distances in microns caudal to the epicenter section. All section analyses were evaluated by two-way ANOVA with repeated measures, with significant effects of section position and matching, but no differences due to grooming outcome or interaction effects.

A Epicenter White Matter Staining
Overgroomer Non-Overgroomer

B Epicenter SWM by Outcome

C Lesion Length by Outcome

D Lesion Length by Stimulation Group

E Section Analysis of Lesion and Spared Tissues

undebated, autotomy or self-mutilation of the feet and toes in these models is generally interpreted to represent a form of spontaneous neuropathic pain (Kauppila, 1998; Minert et al., 2007; Wang et al., 2003). In peripheral nerve injury models, tissue damage or pain-related peptides administered prior to the nerve injury dramatically increase the incidence of overgrooming (Asada et al., 1996; Katz et al., 1991; Saade et al., 1993). Autotomy in these models has been shown to be caused by spontaneous afferent activity induced by the nerve injury (Asada et al., 1990; Xie et al., 2005), which would likely be enhanced by increased sensory input, resulting in a higher incidence of self-directed autotomy or OG. Interestingly, autotomy occurs in the neuroma (complete peripheral nerve transection) model, where transmission of pain that would be caused by the self-directed behavior is eliminated (Minert et al., 2007). Similarly, OG in the present study occurs in areas denervated by the SCI (Hoschouer et al., 2009).

If the mechanism of induction of self-directed behavior is similar between peripheral nerve models and our SCI model, we would expect the high incidence of OG to be due to a combination of repeated afferent stimulation, followed by an afferent barrage or cytokine release within the injured spinal cord as a result of the contusion injury. These signals could cause long-lasting plastic changes, including sensitization and spontaneous ectopic firing in relay centers and targets in the spinal cord and brain (Drdla et al., 2008; Rosso et al., 2003; Wang et al., 2008; Willis, 2002), resulting in perceived pain or dysesthesia and OG behavior (Hoheisel et al., 2003; Zhang et al., 2005). Interestingly, since this study was completed, we have continued to study responses to sensory stimuli in mice with contusion SCI and have found that if the time between baseline testing and injury is extended to 2 weeks, <10% of the mice show signs of overgrooming after injury.

Sensory stimulation completed 3 days before injury increases the occurrence of behavior indicative of spontaneous abnormal sensation, and the combination of stimulation and injury is sufficient to cause OG in most cases. However, the paradigm that we have employed was not always sufficient to cause OG, as indicated by the few animals in the stimulated groups that did not overgroom. This finding highlights the highly variable incidence of spontaneous pain and dysesthesias in the clinical SCI population (Defrin et al., 2001; Siddall et al., 1999, 2001, 2003; Störmer et al., 1997) and the plethora of mechanisms that converge to result in neuropathic pain (Campbell et al., 2006). Surprisingly, however, we also observed a few mice in the laminectomy control groups that exhibited OG behaviors and an increased incidence of OG in mice that were acclimated to the testing boxes but received no stimulation. This incidence of OG is not common in most of our studies of SCI, so we suspect that additional influences were produced in this study. One possibility is that the confining testing boxes may have induced systemic stress, which has been shown to have an effect on pain (Alexander et al., 2009; Ashkinazi et al., 1999). Given that stress has been indicated to have effects on pain thresholds via NMDA receptor activation (Alexander et al., 2009; Wang et al., 2005), confinement could exacerbate injury-induced neuronal hyperexcitability. However, this still does not clearly explain the restriction of these sites to the trunk dermatomes just caudal to the laminectomy or injury site. A second possibility is that shaving the fur of the mice to allow sensory stimulation or in sham animals was suffi-

cient to irritate the skin afferents. Additional studies could be done to test that directly. Finally, another explanation for the incidence of these observations and the anecdotal accounts that OG occurs in groups of mice is that mouse behaviors are highly influenced by group activity. A recent study has shown that mice observing other mice that are in pain will develop pain-like behaviors independent of physiological stimuli (Langford et al., 2006). The transference of pain-like behaviors was found to be dependent upon visual observation rather than auditory or olfactory cues, as it could be blocked by placing an opaque barrier between the mice. Based on this possibility, we recommend that mice that exhibit OG activity in SCI studies be isolated physically from other mice, to ensure that there is no exacerbation by cagemates, and a visual barrier be positioned between the cages so they do not influence mimicking or empathetic behaviors from the other mice in the colony.

Reliable and thorough assays of sensation are essential, both for the end objective of improving sensation and to avoid exacerbating or causing neuropathic pain while striving to improve motor function (Hofstetter et al., 2005). Sensory testing on the trunk is rapid, efficient, and relevant (Hoschouer et al., 2009). However, we show here that extensive sensory testing prior to mid-thoracic contusion injury increases overgrooming, indicative of aberrant sensation. We propose that care should be exercised to minimize stress and sensory stimulation immediately prior to injury to avoid altering the very systems that are the target for assessment (Alexander et al., 2009; Ashkinazi et al., 1999; Wang et al., 2005).

Despite the vast insensitivity of dermatomes below the site of injury in the clinical population, correlates of self-directed autophagy are extremely rare in humans, a fact that is logical considering the sociological context of these behaviors. The occasional case reports of autophagy after SCI implicate psychological history and/or prior mutilation behaviors as important predicated factors (Couts and Gleason, 2006), although physiological mechanisms associated with adhesions may also contribute (Tubbs and Oakes, 2005). However, this study may also offer some explanation for the idiopathic nature of a wider range of spontaneous SCI-induced neuropathic pain or dysesthesias. The incidence of neuropathic pain in humans after SCI varies widely, with 65% commonly cited as an average occurrence (Siddall et al., 2001; Störmer et al., 1997). These symptoms are commonly localized to the level of dorsal root or dorsal horn damage, but they also occur in regions below the injury level that are insensate. While many mechanisms have been elucidated through animal models, no explanation has been provided for the irregular occurrence of pain in humans with seemingly similar injuries. The results of the present study suggests that trauma or sensory fiber barrage occurring due to injuries sustained prior to or concurrent with the SCI may combine with the pathological sequelae of SCI to provide one possible mechanism for the development of spontaneous pain and dysesthesia. Future work could be directed at determining if a subpopulation of patients that exhibit chronic at-level and below level pains following SCI has experienced any form of excessive stimulation prior to their injuries. In addition, future animal studies are implicated in order to establish whether concurrent sensory barrage or sensory activation immediately after SCI also induces dysesthesias and confounding OG behaviors.

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Author Disclosure Statement

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