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Modelling at-level allodynia after mid-thoracic contusion in the rat

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Abstract

Background: The rat mid-thoracic contusion model has been used to study at-level tactile allodynia, a common type of pain that develops after spinal cord injury (SCI). An important advantage of this model is that not all animals develop hypersensitivity. Therefore, it can be used to examine mechanisms that are strictly related to the development of pain-like behaviour separately from mechanisms related to the injury itself. However, how to separate animals that develop hypersensitivity from those that do not is unclear.

Methods: The aims of the current study were to identify where hypersensitivity and spasticity develop and use this information to identify metrics to separate animals that develop hypersensitivity from those that do not to study differences in their behaviour. To accomplish these aims, a grid was used to localize hypersensitivity on the dorsal trunk relative to thoracic dermatomes and supraspinal responses to tactile stimulation were tallied. These supraspinal responses were used to develop a hypersensitivity score to separate animals that develop hypersensitivity, or pain-like response to nonpainful stimuli.

Results: Similar to humans, the development of hypersensitivity could occur with the development of spasticity or hyperreflexia. Moreover, the time course and prevalence of

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AUTHOR CONTRIBUTIONS

G.H.B., B.N., A.K.S. and K.A.M. wrote the manuscript, G.H.B., B.N., A.K.S. and J.R. performed all experimental work, G.H.B., B.N., A.K.S. and K.A.M. analysed the data, G.H.B, B.N., M.R.D, J.R.B. and K.A.M. were involved in experimental design. All authors contributed to the discussion and review of the results and the manuscript.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

hypersensitivity phenotypes (at-, above-, or below level) produced by this model were similar to that observed in humans with SCI.

Conclusion: However, the amount of spared spinal matter in the cord did not explain the development of hypersensitivity, as previously reported. This approach can be used to study the mechanisms underlying the development of hypersensitivity separately from mechanisms related to injury alone.

1 | BACKGROUND

After a spinal cord injury (SCI), over 50% of individuals develop chronic neuropathic pain (CNP; Burke et al., 2017) described as ‘severe or excruciating’ in nearly half of all patients that experience it (Siddall et al., 2003). Unfortunately, CNP remains largely refractory to treatment and may be accompanied by comorbidities such as depression (Cairns et al., 1996), further reducing the quality of life. A greater understanding of the mechanisms underlying CNP is essential to the development of more effective treatments (Cohen & Mao, 2014). However, before exploring these underlying mechanisms, it is important that animal models used in these investigations include the relevant control group that undergoes an SCI but does not develop hypersensitivity, defined by pain-like behaviours. Moreover, these models should produce a similar prevalence and time course of hypersensitivity above-, at- and below- the spinal level of the SCI, as observed in humans (Finnerup et al., 2014).

The mid-thoracic spinal cord contusion model in the rat has been used to study CNP due to its clinical relevance and ease of implementation (Carter et al., 2016; Metz et al., 2000; Sharif-Alhoseini et al., 2017). An advantage of this model is that not all animals display pain-like behaviours in response to non-noxious tactile stimulation and, therefore, this model could be used to study differences in underlying mechanism specifically related to pain separately from mechanisms related to the injury itself (Crown et al., 2006; Nesic et al., 2005). Moreover, the development of allodynia can be modelled at multiple levels relative to the site of injury: above- (forepaws), at- (trunk) and below- (hindpaws) level allodynia (Hulsebosch et al., 2000; Lindsey et al., 2000), yet, differences between these subgroups of animals are less clear.

Thus, the primary aim of this study was to develop a rat model of at-level tactile allodynia by refining the assessment to separate animals that display pain-like behaviours from those that do not. The secondary aim was to describe the prevalence and onset of the different hypersensitivity phenotypes (above-, at- and below-level) to examine the time course of development and to determine if spared grey or white matter could be used to predict these phenotypes.

To accomplish these aims, a grid drawn on the dorsal trunk was mapped to thoracic dermatomes and the distribution of supraspinal responses to tactile the stimulation of the trunk, forepaws and hindpaws was studied. Trunk tactile hypersensitivity was located just rostral to the level of the lesion and audible vocalisations and/or avoidance behaviours were the most informative responses to identify animals that developed hypersensitivity. Above- and below-level hypersensitivity were also identified using supraspinal responses. At-level hypersensitivity was the most prevalent phenotype, developing early, while above- and

below-level tactile hypersensitivity were less common and developed later. Finally, spared matter within the cord did not correlate with behavioural measures of hypersensitivity. Given similarities to the human prevalence of CNP, including the distribution of phenotypes and time course of development, as well as the availability of a control group of SCI animals that do not develop hypersensitivity suggests this is a good model to study the mechanisms underlying the development of pain.

2 | METHODS

2.1 | Subjects

One hundred and fifty-nine adult, female Sprague Dawley rats (225–250 g; Envigo) were used in this study. One hundred and thirty-eight rats received a moderate mid-thoracic spinal cord contusion, six received a laminectomy and 15 non-injured animals were used to identify the location of thoracic dermatomes. Of the contused animals, nine died during SCI surgery due to complications and three were removed from the study due to improper SCIs, defined as BBB scores greater than 20 one week after SCI. Of the remaining contused animals, nine were used to assess locations on the trunk that, when stimulated, produced a pain-like behavioural response. These nine animals along with an additional 37 used to quantify the prevalence of evoked supraspinal responses to tactile stimuli on the trunk. A subset of these 46 animals and 79 additional animals, were used to assess the impact of mid-thoracic spinal contusion on the development of trunk, forepaw and hindpaw hypersensitivity ($N = 117$). Finally, to determine if the extent of damage in the cord was related to the development of hypersensitivity, the amount of spared white matter was correlated to the number of supraspinal responses in 11 of these animals.

All animals were maintained on a 12/12 hr light-dark cycle with food and water ad libitum. All experimental procedures were approved by the Drexel University and the University of California, Davis Institutional Animal Care and Use Committees (IACUC).

2.2 | Standardized grid

To identify the location of thoracic dermatomes and thereby localize hypersensitivity on the dorsal trunk relative to the location of the injury, a standardized grid was drawn on the dorsal trunk in a subset of animals, while animals were anaesthetized with 2% isoflurane at least 24 hr before testing. Each animal's dorsum was shaved. To define the length of the trunk, the midpoint along a virtual line connecting the intertragic notches of the ears was connected to a point at the base of the tail and this distance was divided into 16 equally spaced grid rows. Next, four equally spaced grid columns were defined on each side of the vertebral column, from the midline to the lateral aspect of the dorsal trunk parallel to the knee for a total of eight columns (Figure 1a). These columns and rows were drawn on the animal's skin to define the large trunk grid consisting of 128 grid squares, each approximately 1 cm^2 due to the similar size of all animals. For additional behavioural testing of trunk hypersensitivity in a larger group of animals, a smaller trunk grid with similar spacing localized to the region of the trunk most likely to develop hypersensitivity (see Section 3) was used, which consisted of 40 grid squares, each approximately 1 cm^2 (Figure 1a).

2.3 | Thoracic dermatome map

To identify which dermatomes were likely to be associated with trunk hypersensitivity after mid-thoracic SCI, thoracic dermatomes were identified in relation to the large trunk grid (Figure 1b). Naïve uninjured animals were anaesthetized with urethane (1.5 g/kg) via IP injection and maintained at a Stage III-3 anaesthetic state (Friedberg et al., 1999). The skin and musculature overlying thoracic vertebrae T1-T13 were retracted carefully to avoid damage to any spinal nerves. The spinous processes, lamina and transverse processes of selected vertebrae were removed unilaterally to expose the dorsal root ganglions (DRG). The spinal column was stabilized by attaching locking forceps to the transverse process immediately rostral and caudal to the selected vertebrae. A single high-impedance (4–10 MΩ) tungsten microelectrode (FHC Inc.) was attached to a stereotaxic manipulator and positioned to a single DRG. A ground wire was placed in contact with the body cavity. The electrode was slowly inserted into the DRG as the neural signal was amplified (100X), bandpass filtered, (150–8000 Hz), digitized (40 kHz; Plexon Inc., Dallas, TX) and monitored both on an oscilloscope and through audio speakers. Once a single unit was isolated, the advancement of the electrode was paused and light tactile stimulation was applied to the animal's skin. By identifying the grid locations which when given light tactile stimulation resulted in the cell increasing its firing rate, the cell's receptive field was determined relative to the trunk grid. The electrode was then advanced and the process was repeated until another cell was identified or the electrode punctured through the DRG. Each DRG was sampled at least three times in different locations of the ganglion.

2.4 | Spinal cord contusion

Animals were anaesthetized with ketamine (63 mg/kg), xylazine (5 mg/kg) and acepromazine (0.05 mg/kg) via IP injection. Animals were considered sufficiently anaesthetized with the absence of a toe pinch reflex. The skin and musculature overlying the spinal column was retracted from spinal levels T4 to T12 and a laminectomy was performed at vertebral level T10. The spinal cord was stabilized by securing locking forceps to the transverse processes of T9 and T11. SCI rats received a moderate contusion injury at vertebral level T10 using the Infinite Horizon impactor device (Precision Systems and Instrumentation, LLC) with 150 kdynes of force and a 1 s dwell time. The musculature was then sutured in layers and the skin was closed with wound clips. Laminectomy controls underwent the same procedure, except the spinal cord was not impacted. Animals were post-operatively hydrated with saline (7 ml), prophylactically administered an antibiotic (enrofloxacin, 5 mg/kg) and allowed to recover on a heated water pad. Animals were administered fluids and antibiotics once daily for seven days and bladders were manually expressed twice daily until they regained autonomic bladder control.

2.5 | Behavioural testing

Because the assessment of pain is subjective, it is difficult to objectively assess pain in others. Rodent models have been extensively studied and the display of supraspinal responses is generally accepted as a pain-like response (Bedi et al., 2010; Christensen & Hulsebosch, 1997; Crown et al., 2006; Lindsey et al., 2000). In keeping with the nomenclature of IASP and the recommendations of Hansson & Bouhassira (Hansson &

Bouhassira, 2015), supraspinal responses that were likely indicative of pain are referred to as hypersensitive in order to separate these responses from hyperreflexia or spasticity that are unlikely to be associated with pain. As such, here we refer to responses in which the animal displayed pain-like behaviours as hypersensitive and perform hypersensitivity testing for pain-like behaviours at (trunk), above (forepaws) and below (hindpaws) the level of the injury.

Prior to locomotor assessment and hypersensitivity testing, animals were handled and habituated to the behavioural testing environments. This consisted of placing each animal in the open field (locomotor testing environment; 15 min) and the von Frey testing cage (forepaw and hindpaw hypersensitivity testing environment; 30 min), as well as cradling each animal in the testers' forearm (trunk hypersensitivity testing environment; 30 min), once a day for 3 days.

After habituation, behavioural testing was conducted on all animals pre-operatively to establish baseline measures. Post-SCI behavioural testing began one week after the injury and animals were tested once a week for a total of 5 weeks post-SCI (Figure 1c).

Locomotor functional recovery was measured using the Basso, Beattie and Bresnahan (BBB) locomotor rating scale (Basso et al., 1995). Animals were placed in an open field (76.20 × 91.44 cm) and were observed by two trained experimenters blinded to the animals' experimental condition for 4 min. Each hindlimb was assessed for the presence of joint movements, weight support, quality of stepping, forelimb-hindlimb coordination, paw placement and toe clearance, and these observations were converted into a BBB score for each hindlimb. Scores on this scale range from 0 to 21, where a score of 0 represents a complete paralysis of the hindlimbs, while a score of 21 represents the locomotor function of an uninjured rat.

One day prior to the trunk hypersensitivity test, animals were briefly placed under isoflurane anaesthesia, the dorsal trunk was shaved and the trunk grid was drawn on the skin. During the trunk testing session, the experimenter draped an absorbent pad across their forearm and placed the animal on the pad, unrestricted such that it was free to walk back and forth across the experimenter's forearm. The animal supported its own weight on all four limbs for the entirety of the testing session. A 26 g force von Frey filament (Stoelting) was applied perpendicularly to the dorsal surface of the trunk until the filament bent (Figure 1d). The filament was randomly applied to the centre of each trunk grid square (large grid - 128 squares, smaller grid - 40 squares) until the entire grid was stimulated, with an interstimulus interval of at least 10 s. This process was repeated a total of five times.

To model allodynia, a 26 g force was selected because it has been documented to be a normally non-noxious tactile stimulus for similarly sized animals (Hulsebosch et al., 2000). Moreover, observable aversive supraspinal responses that are likely indicative of pain were used to separate hypersensitive responses from spastic responses. These responses included audible vocalisations, biting at the filament, licking the point of stimulation, looking at the filament and avoidance behaviour in direct response to the stimulus. Responses were considered avoids when animals made coordinated movements away from and in response to

the stimulus using both forelimbs and, after SCI recovery, both hindlimbs. Animals would often move from one end of the experimenter's forearm to the other to avoid the area of the testing environment where they were last stimulated. The stimulated trunk grid location that elicited the response and the type of response was documented. Animals generally never evoked more than one type of response upon a single stimulation. In addition to evoking supraspinal responses, stimulation of the trunk also evoked hindlimb movements without supraspinal responses that were unrelated to voluntary movements (Baastrup et al., 2010). These responses were considered spastic and the stimulus location that elicited them was noted.

For forepaw and hindpaw hypersensitivity testing, standard methods were used (Ängeby Möller et al., 1998). Briefly, animals were placed in a Plexiglass chamber (10.16 × 25.40 × 10.16 cm cage) with a wire mesh bottom and were allowed to acclimate to the environment for at least 20 min before testing began. An acclimated animal displayed little to no movement or exploratory behaviour. For each paw, an electronic von Frey filament (Ugo Basile) with a stiff metal tip was slowly applied to the plantar surface of the paw between the paw pads at a constant rate as the device measured the force which was being applied. Continuing our efforts to model allodynia, the force at which the animal quickly withdrew its paw was recorded as well as any supraspinal responses made during the trial that would suggest the animal was experiencing pain (Figure 1e) and could be used to separate hypersensitive responses that are likely painful from hyperreflexive responses. Five stimulations were applied to each paw per session with an interstimulus interval of at least 30 s. To ensure accurate withdrawal thresholds, testing was only carried out if animals had the ability to bear weight on all limbs.

2.6 | Histology

Histological verification of the spinal cord lesion was conducted on the subset of animals. At the conclusion of behavioural testing five weeks post-SCI, animals were transcardially perfused with cold saline followed by 4% paraformaldehyde (pH 7.4). During spinal cord tissue removal, the vertebral level of the lesion site was confirmed. Tissue was post-fixed in 4% paraformaldehyde for 24 hr and placed in 30% sucrose until the tissue sank to the bottom of the specimen container, indicating that the tissue had been cryoprotected. A 14 mm section of the spinal cord surrounding the lesion site was dissected and frozen in Shandon M1 embedding matrix (Thermo Fisher Scientific). 25 μm coronal sections of cord were sliced using a freezing microtome and every 20th section was mounted onto charged slides (Thermo Fisher Scientific premium frosted microscope slides) to evaluate sections 500 μm apart. Sections were air dried overnight. To stain, the slides were dehydrated in increasing concentrations of ethanol baths (75%, 95%, 100%) for 3–6 min each, cleared using CitriSolv (Decon Labs Inc.) for 20 min, rehydrated in decreasing concentrations of ethanol baths (100%, 95%, 75%) for 3–6 min each and were then rinsed with distilled water. The slides were stained for myelin using a Cyanine R/$FeCl_3$ solution for 10 min. Slides were rinsed and placed in differentiation solution for 1 min using 1% aqueous NH_4OH. After additional rinsing, slides were stained for Nissl in a Cresyl Violet solution for 20 min, rinsed and dehydrated once again using ethanol. Slides were coverslipped using Vectashield

mounting medium (Vector Laboratories) and digital images were taken of each section 24 hr later.

2.7 | Data analysis

2.7.1 | Dermatome map analysis—For each animal, a thoracic dermatome was identified as the union of all trunk grid locations that, when stimulated, modulated the firing rate of any cell recorded from a single DRG. Dermatome width, defined as the rostrocaudal extent of each dermatome measured in trunk rows, was calculated for each DRG sampled. Additionally, the central position of each dermatome was calculated, defined as the point on the trunk grid at the centre of each dermatome's width. Dermatome widths and central positions were then averaged across all animals and averaged thoracic dermatomes were defined by taking the average dermatome width centred on the average dermatome centre position.

2.7.2 | Behavioural assessment—The frequency, type and location of supraspinal responses to tactile the stimulation of the trunk grid were noted and used to refine trunk hypersensitivity assessment. To assess the rostrocaudal extent of hypersensitivity, the number of evoked supraspinal responses within each grid row for each animal was tallied, separately for each week. The percentage of supraspinal responses at week 5 was used to define a hypersensitivity score to separate animals that developed hypersensitivity from those that did not (see Section 3 for more details). To assess the development of spasticity, the frequency of spastic responses across each grid row was calculated at week 5.

The development of forepaw and hindpaw hypersensitivity was evaluated at each week post-SCI. The median force of the five trials performed on each paw during a testing session was considered to be the withdrawal threshold for that paw in that testing session. There was not a significant difference in baseline withdrawal threshold between the left and right forepaws [$t(47) = 1.42$, $p = .16$], or hindpaws [$t(45) = 0.13$, $p = .89$], so paw withdrawal thresholds were averaged between the left and right paws. Withdraw thresholds were then normalized to the animal's baseline score. An animal was considered to have tactile hypersensitivity in the forepaws or hindpaws if the withdrawal threshold was reduced by at least 50% compared to their baseline withdrawal threshold and the animal exhibited a supraspinal response during stimulation (Detloff et al., 2013) at week 5. If an animal had a >=50% decrease in withdrawal threshold at week 5 compared to baseline, but did not exhibit supraspinal responses during stimulation, it was considered to have hyperreflexia, but not hypersensitivity.

Finally, to assess locomotor recovery, the BBB scores from the left and right hindlimb of each animal were averaged together such that each animal had a single BBB score for each week.

2.7.3 | Histological analysis—To determine if there was an association between spared matter around the lesion site and the presence of hypersensitivity, the amount of spared white and grey matter in each section was calculated using Image J Software (NIH; Schneider et al., 2012). Tissue was considered spared if staining was uniform and it was absent of extensive cellular debris or vacuoles. All measured sections were then normalized

to the section with the largest amount of total spared tissue and converted to a percentage of spared tissue. The section with the least amount of total spared tissue was considered the lesion epicentre.

Because asymmetry in the ventrolateral funiculi (VLF) has been suggested to occur more often in animals that develop hypersensitivity compared to those that do not (Hall et al., 2010), the relationship between asymmetries in the amount of spared white/grey matter and the number of supraspinal responses was assessed using a similar approach. Briefly, the cord was divided into quadrants by drawing a vertical line and a horizontal line through the central canal. To isolate the VLF from the ventromedial funiculus, the lower quadrants were then further divided by a line drawn from the tip of each ventral horn to the edge of the section (Figure 6a). As in Hall et al., 2010, if the ventral horns were damaged to such an extent that they could not be identified, their medial border was estimated by drawing a line from the central canal to the ventral edge of the section at a 30° angle, which is approximately the angle of the line drawn on a naïve cord (Figure 6b).

2.8 | Statistical analysis

Analysis of supraspinal responses to trunk tactile stimulation at week 5 was used to refine our model of trunk allodynia. The distribution of the number of supraspinal and spastic responses per row of the large grid was used to assess the location of trunk hypersensitivity and define a smaller grid for hypersensitivity testing. The distribution of the different types of supraspinal responses across the smaller grid was used to further develop a method to separate animals with at-level hypersensitivity from those that did not develop at-level hypersensitivity (see Section 3). Chi-square test was used to assess the importance of supraspinal responses to distinguish hypersensitivity from hyperreflexia in response to paw stimulation.

Using our operational definition of hypersensitivity, differences in behavioural measures between animals that developed hypersensitivity compared to those that did not were compared over time using a repeated measures restricted maximum likelihood estimation linear mixed model with a Greenhouse-Geisser correction. Where appropriate, post hoc Sidak pairwise comparisons were performed at a significance level of .05. This procedure was implemented to prevent list-wise deletion due to missing data. All statistical analyses were conducted using GraphPad Prism 8.0.2 for Windows (GraphPad Software).

To assess the effect of the amount of spared white and grey matter on the development of trunk hypersensitivity, the percentages of spared white and grey matter were separately averaged across animals for the sections located at the same distance from the lesion epicentre. Spared tissue across the lesion site in animals with at-level hypersensitivity was compared to that of animals with no hypersensitivity anywhere. The percent of total spared white or grey matter along the entire lesion site between groups was compared using the same repeated measures statistical test as that used for differences in behavioural measures. Finally, to evaluate if asymmetry of spared white matter in the VLF near the lesion epicentre could account for the development of hypersensitivity, the percent difference of spared matter between the VLFs of the right and left side of the cord were compared and correlated to the number of supraspinal responses per animal using Pearson correlation tests.

3 | RESULTS

3.1 | Localisation of trunk hypersensitivity and spastic responses

The full trunk grid was used to localize and subsequently quantify, responses evoked by innocuous tactile stimulation. Of the 10 animals that were tested for trunk hypersensitivity using the full trunk grid, seven animals exhibited supraspinal responses elicited primarily from the stimulation of rostrocaudal grid rows 5–9. Electrophysiology determined that these grid rows correspond to spinal dermatome levels T4–T10 and include part of dermatome T11 (Figure 2a,b). In fact, all animals that exhibited supraspinal responses responded to stimuli within the T4-T11 dermatomes. A subset of these animals had larger areas that elicited hypersensitive responses, predominately avoids, expanding into upper thoracic and cervical dermatomes. Very few supraspinal responses were elicited below the level of the lesion. Therefore, a moderate T10 spinal cord contusion consistently produced hypersensitive responses at and immediately rostral to, the site of the lesion, with the majority of the responses defining a hypersensitive region up to six dermatomal levels above the lesion site.

Spastic or rapid extension of the hindlimbs in response to trunk stimulation was also observed. Tactile stimulation to trunk grid rows 11–16 elicited these spastic responses, which correspond to dermatome levels T12 and below, extending into lumbar dermatomes (Figure 2c,d). Responses were mainly bilateral and were in response to stimulation across the mediolateral extent of the trunk dorsum. These spastic responses were not accompanied by supraspinal responses. While grid locations that produced spastic responses were rarely co-localized (occurring in only one animal), spastic and supraspinal responses did occur in the same animal: of the 10 animals tested with the full grid, two had supraspinal responses only, three had spastic responses only and five had both supraspinal and spastic responses. Because the majority of supraspinal responses occurred mainly rostral to the lesion site (T4–T11) and were generally not co-localized with spastic responses (below T11), it was determined that a smaller grid could be used to identify hypersensitive responses.

This smaller grid was used on a larger sample of animals to determine the best behavioural markers of trunk hypersensitivity (Table 1). As expected, sham SCI animals elicited few supraspinal responses to non-noxious tactile stimulation. At week 5, the most common supraspinal response in SCI animals was vocalisation, comprising 52.71% of all responses, followed by avoiding, which comprised 28.93% of responses. To localize these responses, the stimulus location was mapped to the grid (Figure 3a). Vocalissations were located across the entire grid, which encompassed dermatomes T4-T11, while avoids were concentrated more medial and anterior. Because lick, look and bite responses occurred relatively infrequently (9.11%, 7.79% and 1.45%, respectively), vocalisation and avoid responses were sufficient to discriminate animals that developed hypersensitivity from those that did. Therefore, a hypersensitivity score was defined that represented the prevalence of pain-like behaviours evoked by non-noxious tactile stimulation of the trunk. The trunk hypersensitivity score was defined as the percentage of vocalisations and avoids elicited in response to the stimulation of the small grid ($N = 200$ total stimuli given within one testing session).

To identify a threshold that could separate animals that develop tactile hypersensitivity from those that do not, the distribution of hypersensitivity scores in the smaller grid across all animals was evaluated (Figure 3b). The majority of animals had a hypersensitivity score of zero, five animals had a hypersensitivity score less than or equal to three and 11 animals had a hypersensitivity score greater than or equal to five ($\mu = 26.95 \pm 17.71\%$). The average raw number of vocalize + avoid responses per animal was 53.91 ± 35.41 occurring in an average of 20.91 ± 7.80 grid locations. For those animals with a hypersensitivity score of at least five, only two had a hypersensitivity score less than 10 and over half had a hypersensitivity score greater than 25 (Figure 3b). Because animals that did not develop tactile hypersensitivity were highly unlikely to produce a supraspinal response to a non-noxious stimulus, a threshold of five for the hypersensitivity score can differentiate spinal injured animals that develop hypersensitivity from those that do not.

To test if restricting the hypersensitivity score to only include vocalisation and avoid responses misrepresented the likelihood of identifying cases of hypersensitivity, we compared our threshold hypersensitivity score using only vocalisations and avoids to a threshold using all supraspinal responses (i.e. vocalize, bite, lick, look, avoid). We found that the same 11 animals would have been considered to have hypersensitivity. Therefore, not using bites, licks, or looks did not affect whether an animal was considered to develop hypersensitivity. Moreover, the percentage of all other evoked supraspinal responses (i.e. look, lick, bite) for animals with trunk hypersensitivity was low ($\mu = 5.72 \pm 6.61$), suggesting that these supraspinal responses may not be the best indicator of hypersensitivity and that using only vocalisations and avoids is sufficient to assess tactile hypersensitivity in the trunk. Therefore, a hypersensitivity score using only vocalisation and avoidance behaviours was used to distinguish animals that developed trunk hypersensitivity from those that did not.

The same set of contused animals was also tested for the presence of spasticity of the hindlimbs in response to trunk stimulation at week 5. In addition to testing within the small grid, animals were stimulated a total of 20 times on each side of the dorsal trunk between the caudal border of the small grid and the tail in random locations. Almost half of the animals (21 of 46) had at least one spastic response. Of the 541 total spastic responses detected, only five responses from two animals were elicited from within the small grid, whereas all other responses were elicited from between the caudal border of the small grid and the tail. Over half of the animals with hypersensitivity (6 of 11) also had spastic responses, whereas only 28.57% (6 of 21) of animals with spastic responses also had trunk hypersensitivity. Supraspinal and spastic responses were never elicited from the same trunk location in the same animal. Therefore, the trunk locations which elicit spasticity of the hindlimbs upon stimulation arise caudal to the region of trunk hypersensitivity development and the development of one does not require nor exclude the development of the other.

3.2 | Animals unlikely to have tactile hypersensitivity at multiple levels

To better understand the development of tactile hypersensitivity in this model, a larger group of animals was tested for trunk hypersensitivity as well as forepaw and hindpaw hypersensitivity. Each animal was classified into one of the eight hypersensitivity phenotype

groups depending on the levels where hypersensitivity developed (Figure 4). An animal was more likely to develop hypersensitivity at only one level (27.35% of animals: 18.80% trunk alone, 5.13% forepaw alone, 3.42% hindpaw alone) than at multiple levels (6.83% of animals: 2.56% forepaw + hindpaw hypersensitivity, 1.71% trunk + forepaw hypersensitivity, 2.56% trunk + hindpaw, 0% trunk + forepaw + hindpaw hypersensitivity). Over half of animals (65.90%) did not display any supraspinal responses to forepaw, hindpaw or trunk tactile stimulation, suggesting that they did not develop tactile hypersensitivity. In summary, 23.07% of animals developed trunk, 9.40% developed forepaw and 8.54% developed hindpaw hypersensitivity with 34.18% developing hypersensitivity in at least one region.

During forepaw or hindpaw tactile stimulation, 17.95% displayed hyperreflexia (i.e. a 50% or greater reduction in forepaw and/or hindpaw withdrawal threshold) without a supraspinal response. In fact, the overall proportion of animals that developed hyperreflexia in the hindpaws (13.59%) was greater than the proportion of animals that developed hindpaw hypersensitivity (3.54%; X^2 (1, n = 117) = 6.02, p = .01) with no difference between the overall number of animals that developed forepaw hyperreflexia (9.34%) and the number that developed forepaw hypersensitivity (5.41%; X^2(1, n = 117) = 1.07, p = .30).

3.3 | Trunk hypersensitivity develops early post injury

To assess the effect of developing hypersensitivity on locomotor recovery, the BBB scores of animals determined to have hypersensitivity at any level (forepaw, trunk and/or hindpaw; n = 40) were compared to animals that did not develop hypersensitivity anywhere (n = 38). As expected, BBB scores decreased immediately after SCI, but then increased with each successive week post-SCI for both groups (main effect of week: F(2.61, 194.70) = 239.4, p < .001; main effect of group: F(1, 76) = 0.44, p = .88; interaction: F(5, 373) = 1.06, p = .46, (Figure 5a). This suggests that there is no relationship between locomotor recovery and hypersensitivity development.

To assess the development of the different hypersensitivity phenotypes over time, the trunk hypersensitivity score and the hindpaw and forepaw withdrawal thresholds were compared separately across weeks between animals that developed hypersensitivity at each level and those that did not develop hypersensitivity at any level (n = 38). The trunk hypersensitivity score for animals that developed trunk hypersensitivity (n = 29) significantly increased over time, but not the score for animals that did not develop hypersensitivity [main effect of week: F(3.39, 213.90) = 17.24, p < .001; main effect of group: F(1, 65) = 48.70, p < .001; interaction: F(5, 315) = 16.09, p < .001. Importantly, the hypersensitivity scores differed between groups starting in week 1 and continued through week 5 with the difference becoming greater with each successive week (Figure 5b). Similarly for forepaw hypersensitivity (n = 10), withdrawal thresholds for animals that developed hypersensitivity decreased over time, while withdrawal thresholds for animals that did not develop hypersensitivity remained stable [main effect of week: F(3.76, 166.90) = 4.05, p < .01; main effect of group: F(1, 46) = 6.50, p < .05; interaction: F(5, 222) = 6.33, p < .001]. However, differences between groups were apparent only at weeks 2 and 5 (Figure 5c). For hindpaw hypersensitivity (n = 8), there were again differences between groups [main effect of week:

$F(3.66, 156.60) = 7.77$, $p < .001$; main effect of group: $F(1, 44) = 11.58$, $p < .005$; interaction: $F(5, 214) = 3.34$, $p < .01$]. Withdrawal thresholds for animals that developed hindpaw hypersensitivity decreased with time, becoming significantly different from those that did not develop hypersensitivity at weeks 4 and 5 (Figure 5d). Taken together, these data suggest that trunk hypersensitivity emerges early, while forepaw and hindpaw hypersensitivity emerge at later time points after SCI. This is consistent with the development of chronic neuropathic pain in human SCI patients where below-level pain was found to have a later onset than at-level pain (Finnerup et al., 2014).

3.4 | Trunk hypersensitivity is not related to differences in lesion

Residual function of spared spinal tissue such as GABAergic inhibitory cells in the dorsal horn (Gwak & Hulsebosch, 2011) or the spinothalamic tract (Wasner et al., 2008) is likely to affect hypersensitivity development. To understand if differences in spared tissue (left vs. right) were associated with trunk hypersensitivity, the amount of spared grey and white matter across the lesion site was compared in a subset of animals with trunk hypersensitivity ($n = 7$) and a subset of animals with no hypersensitivity anywhere ($n = 5$). While thoracic sections of spinal cord distal to the lesion site (~7 mm) appeared completely undamaged (Figure 6a), sections from the lesion epicentre generally suffered extensive damage, with a complete absence of grey matter and a small amount of spared white matter usually found along the ventral periphery of the cord (Figure 6b). However, along the entire lesion site, there were no differences between the percent of total spared white or grey matter between hypersensitivity groups [white matter main effect of group: $F(1, 9) = 0.50$, $p = .50$; main effect of distance from the epicentre: $F(27, 186) = 24.94$, $p < .0001$; interaction: $F(27, 186) = 0.73$, $p = .83$; (Figure 6c); grey matter main effect of group: $F(1, 9) = 1.291$, $p = .96$; main effect of distance from the epicentre: $F(2.76, 17.71) = 29.32$, $p < .001$; interaction: $F(28, 180) = 0.58$, $p = .96$, (Figure 6d). This suggests that total sparing is unlikely to predict the development of hypersensitivity. Because the STT lies within the VLF, sparing of this funiculus was studied in more detail. However, we found no correlation between trunk hypersensitivity score and spared white matter in the VLF at the epicentre [$r(9) = .06$, $p = .89$ (data not shown)]; nor 1 mm rostral [$r(9) = .03$, $p = .92$] (Figure 6e). Nor was there any correlation between trunk hypersensitivity score and asymmetry of the funiculi at the lesion epicentre [$r(9) = -.25$, $p = .46$ (data not shown)] nor 1 mm rostral [$r(9) = .17$, $p = .61$] (Figure 6f). These results suggest that the amount of spared tissue at the lesion site does not explain the development of trunk hypersensitivity.

4 | CONCLUSIONS

To provide more insight into the development of allodynia, a rodent model of at-level allodynia was refined to include measures of supraspinal responses that are likely to be indicative of pain as opposed to spastic or hyperreflexive responses that are not. Pain-like behaviours such as vocalisations and avoids were sufficient to separate animals that developed at-level hypersensitivity from those that did not. Hypersensitivity was found to occur at and immediately rostral to the lesion and in distinct dermatomes from spastic responses. Of the animals that developed hypersensitivity, the majority developed it at-level, which developed earlier than below- or above-level hypersensitivity. These findings are

similar to the prevalence and time course of pain phenotypes observed in humans (Finnerup et al., 2014). Moreover, more than half of all injured animals showed no hypersensitivity at any level which ensures an important control group in studies of CNP to account for any physiological changes due to the injury itself (Burke et al., 2017; Finnerup et al., 2014).

Though a variety of methods have been used to model allodynia in response to SCI in rodents, this study was primarily focused on modelling at-level allodynia evoked by a tactile stimulus. At-level hypersensitivity is most often assessed by providing tactile stimuli to the trunk and quantifying painful responses (Baastrup et al., 2010; Crown et al., 2005, 2006, 2008; Hall et al., 2010; Hubscher & Johnson, 1999; Hulsebosch et al., 2000; Lindsey et al., 2000). Other stimulus modalities including cold (Lindsey et al., 2000; Yoon et al., 2004) and heat (Carlton et al., 2009; Putatunda et al., 2014) stimuli have also been used. A more comprehensive understanding of hypersensitivity phenotypes could be obtained by combining the current testing method with other stimulus modalities (Deuis et al., 2017).

In addition to stimulus-evoked pain, spontaneous pain is a common occurrence in humans after SCI. In humans, self-reporting of spontaneous pain may be possible (Ness et al., 1998); however, measuring spontaneous pain in animal models has been challenging. Spontaneous pain has been assessed in rodents through the use of operant and classical conditioning (Chhaya et al., 2019; Harte et al., 2016; Labuda & Fuchs, 2000; Sufka, 1994; Yang et al., 2014) or assessment of exploratory behaviour (Mills et al., 2001). However, these measures introduce confounding factors (e.g. motivation) that have yet to be resolved and may therefore interfere with pain assessment.

In the current study, we limited our investigation to female rats. However, it is important to note that there are likely differences in the development of chronic pain between the sexes and differences have been found between the sexes in rat models. For example, male rats have higher rates of hypersensitivity (Crown et al., 2006, 2008; Hubscher et al., 2010) and both hormones (Hubscher et al., 2010) and rat breed (Dominguez et al., 2012) were shown to impact prevalence. Considering that prevalence of SCI in the clinical population is higher in males, further study of these differences would be of great benefit to the clinical population.

Like humans, animals with an SCI do not consistently develop pain-like behaviours. It is of high clinical and scientific value to understand why this is. In the periphery, increased spontaneous activity of primary afferents has been shown to be correlated with increased pain-like behaviour after SCI (Bedi et al., 2010). In the spinal cord, over-activation of astrocytes (Nesic et al., 2005), microglia (Detloff et al., 2008) and upregulation of mitogen-activated protein kinases (Crown et al., 2006; Detloff et al., 2008) have been seen in SCI animals with at- and below-level hypersensitivity. This may cause chronic inflammation and sensitisation in the central nervous system, leading to a neuropathic state. Supraspinally, modified sodium channels in the thalamus (Hains et al., 2003, 2005), increased cortical plasticity (Wrigley et al., 2009) and anatomical changes in pain-related brain regions (Gustin et al., 2010; Jutzeler et al., 2016) have been associated with below-level hypersensitivity after SCI, which may result in the aberrant central processing of normally non-noxious

stimuli. Taken together, it is likely that CNP develops due to changes along the entire neural axis.

To refine the assessment of at-level hypersensitivity, we determined that pain-like behaviours were localized predominately at and just rostral to the site of the lesion (dermatomes T4-T11), consistent with the girdle region as previously reported (Baastrup et al., 2010; Hulsebosch et al., 2000; Lindsey et al., 2000). Interestingly, at-level pain in humans has been clinically defined as pain spanning one dermatome above and three below the level of the SCI (Bryce et al., 2012). The relevance of this difference in the location of at-level hypersensitivity between the rat model and the human is unclear, but could be due to differences in overall body size or bipedal versus quadrupedal stance.

Furthermore, it was evident that at-level hypersensitivity could be confidently detected by quantifying only evoked vocalisation and avoidance behaviours, consistent with earlier studies (Baastrup et al., 2010; Christensen & Hulsebosch, 1997; Hulsebosch et al., 2000; Lindsey et al., 2000; M'Dahoma et al., 2014). While it is possible that animals may have vocalized at frequencies inaudible to the experimenters (Knutson et al., 2002), ultrasonic vocalisations have not been shown to be a superior indicator of hypersensitivity than audible vocalisations (Williams et al., 2008).

The use of a grid system to localize at-level hypersensitivity adds several benefits. Behaviourally, it provides consistency both within and across animals, important for studies that look at hypersensitivity development over time. It can also be adapted for hypersensitivity testing in other injury models or stimulus modalities, allowing for comparisons across models and methods. Additionally, it can be used to study the relationship between the localisation of behavioural hypersensitivity and neuronal hyperexcitability in the form of changing receptive fields, providing greater insight into the mechanisms underlying the development of hyperexcitability. Thus, we conclude that a standardized grid should be used to uniformly assess the trunk region for hypersensitivity testing.

We report that trunk stimulation post-SCI can evoke spasticity of the hindlimbs without supraspinal responses. An SCI model that produces both hypersensitivity and spasticity is clinically relevant due to the prevalence of spasticity in SCI patients with CNP (Andresen et al., 2016). Moreover, because the stimulation locations which evoke hypersensitivity and spasticity are not co-localized, it is likely that they have different underlying mechanisms, but additional studies would be needed to investigate this.

In assessing hypersensitivity in the paws, our results further support the necessity of the detection of supraspinal responses. We found that over half of the animals that developed hyperreflexia did not exhibit any supraspinal responses to paw stimulation. Consistent with others (Baastrup et al., 2010; Van Gorp et al., 2014), these results suggest that not all animals that develop hyperreflexia develop hypersensitivity and therefore the prevalence of hypersensitivity is likely overestimated in studies that define hypersensitivity based on a withdrawal threshold criterion alone. Misclassifying animals into hypersensitivity

experimental groups could have substantial effects on the interpretation of results and the study of CNP in general.

While the incidence of hindpaw hypersensitivity in this study was relatively low compared to others, many studies only use animals that develop pain-like symptoms without reporting the number of animals which do not. In studies that do, incidences are highly variable. Differences in the SCI, varying definitions of how animals are classified into groups and methods for testing withdraw threshold may affect this. Additionally, the low incidence of above-level hypersensitivity was not unexpected, as above-level hypersensitivity may not be due to the SCI itself (Cruz-Almeida et al., 2009), but rather associated with peripheral sensitisation in response to secondary injury after SCI (Hulsebosch et al., 2009; Widerström-Noga, 2017).

Of the animals that developed hypersensitivity, the majority developed it at-level, which developed earlier than below- or above-level, suggesting that different mechanisms may be involved in the development of hypersensitivity in different regions (Hulsebosch et al., 2009). The fact that at-level hypersensitivity developed earlier than below- or above-level is consistent with clinical studies (Finnerup et al., 2014). Moreover, the prevalence of at-level sensitivity is similar to the prevalence of at-level pain observed in a meta-analysis of humans (19%; Burke et al., 2017). However, this meta-analysis also found a higher prevalence of below-level pain (27%) than observed in this study which could be due to the inclusion of spontaneous, itch and thermal pain as well as tactile. Therefore, though more work needs to be performed, the model presented here reproduces some of the important characteristics of at-level pain experienced by humans after SCI.

In summary, we demonstrated that at-level tactile allodynia could be modelled in the rat after mid-thoracic contusion by calculating a hypersensitivity score derived from the percentage of vocalisation and avoidance behaviours in response to the tactile stimulation of the T4–T11 region of the trunk. This model produces a similar distribution and time-course of hypersensitivity phenotypes as observed in human SCI. This at-level hypersensitivity often occurred without hypersensitivity at other levels and was distinguishable from spasticity and hyperreflexia. These characteristics are important for the study of the pathophysiology associated with unique hypersensitivity phenotypes and the development of effective treatments.

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Significance

To model at-level tactile allodynia, rat hypersensitivity was assessed by carefully observing supraspinal responses that are indicative of pain-like behaviours after mid-thoracic SCI. By quantifying these supraspinal responses, a hypersensitivity score was developed that models allodynia and was used to separate animals that develop at-level hypersensitivity from those that did not. Hypersensitivity that included supraspinal responses was localized to thoracic dermatomes T4-T11 and could be identified by considering only audible vocalisation and avoidance behaviours. Like human allodynia, at-level hypersensitivity often occurred without hypersensitivity at other levels, was distinguishable from spasticity and hyperreflexia, and developed early after SCI, suggesting that this model could be used to study mechanisms underlying at-level allodynia.

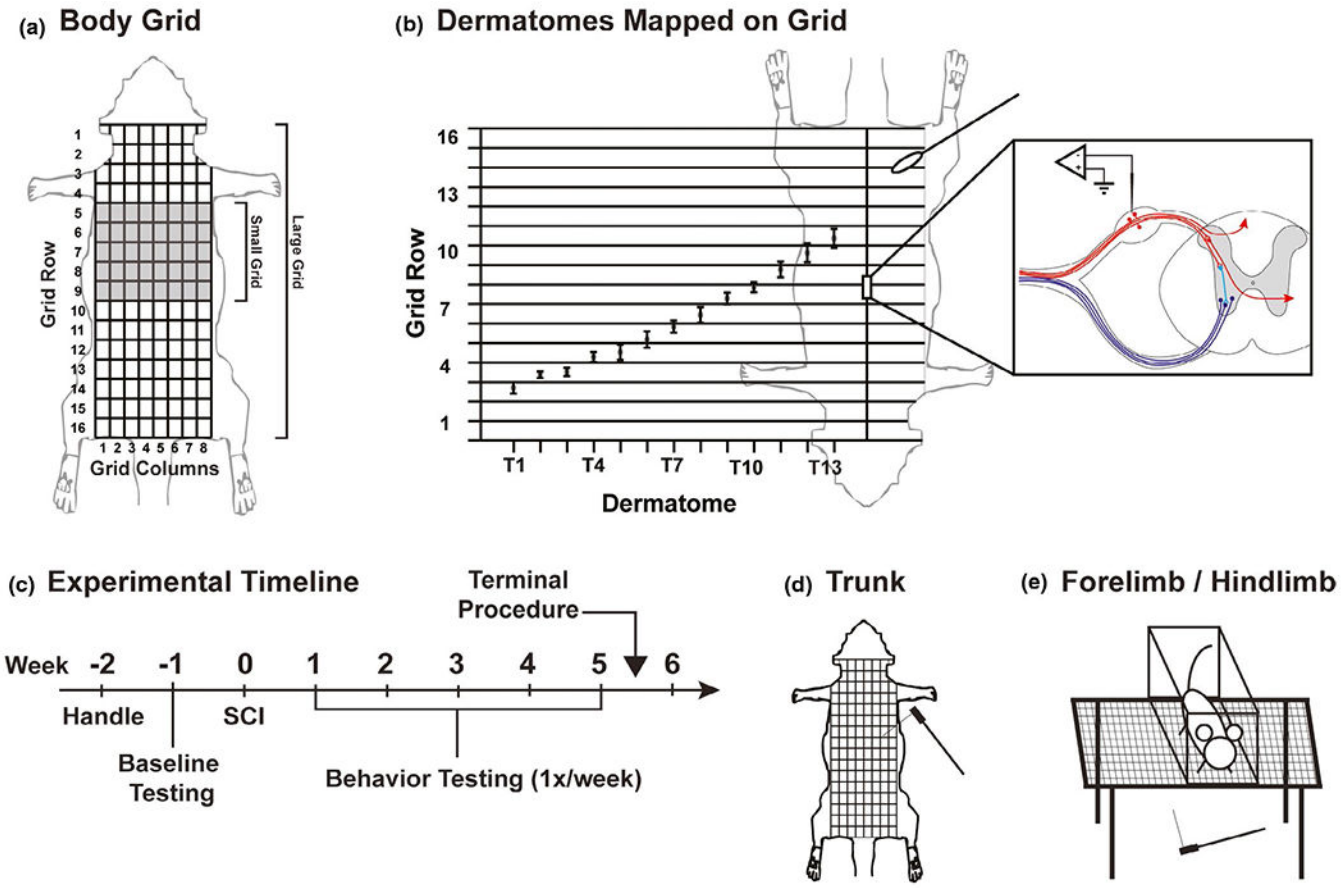

FIGURE 1.

Experimental methods and timeline. (a) A large body grid was used to identify locations that when stimulated evoked a painful response, while a small body grid was used to identify at-level hypersensitivity in a larger group of animals. (b) Grid locations were mapped to thoracic dermatomes by recording from multiple cells within each thoracic DRG and mapping their receptive fields relative to the grid. (c) Experimental timeline: Animals were handled and acclimated to each testing environment. Behavioural testing was performed once pre-SCI (baseline) and once a week for 5 weeks post-SCI, beginning one week after the injury. All terminal procedures were performed after collecting week 5 behavioural data. (d) A 26 g von Frey monofilament was used on the dorsum of the trunk to detect and quantify trunk tactile hypersensitivity. (e) An electronic von Frey anaesthesiometer was used to detect and quantify forepaw and hindpaw tactile hypersensitivity

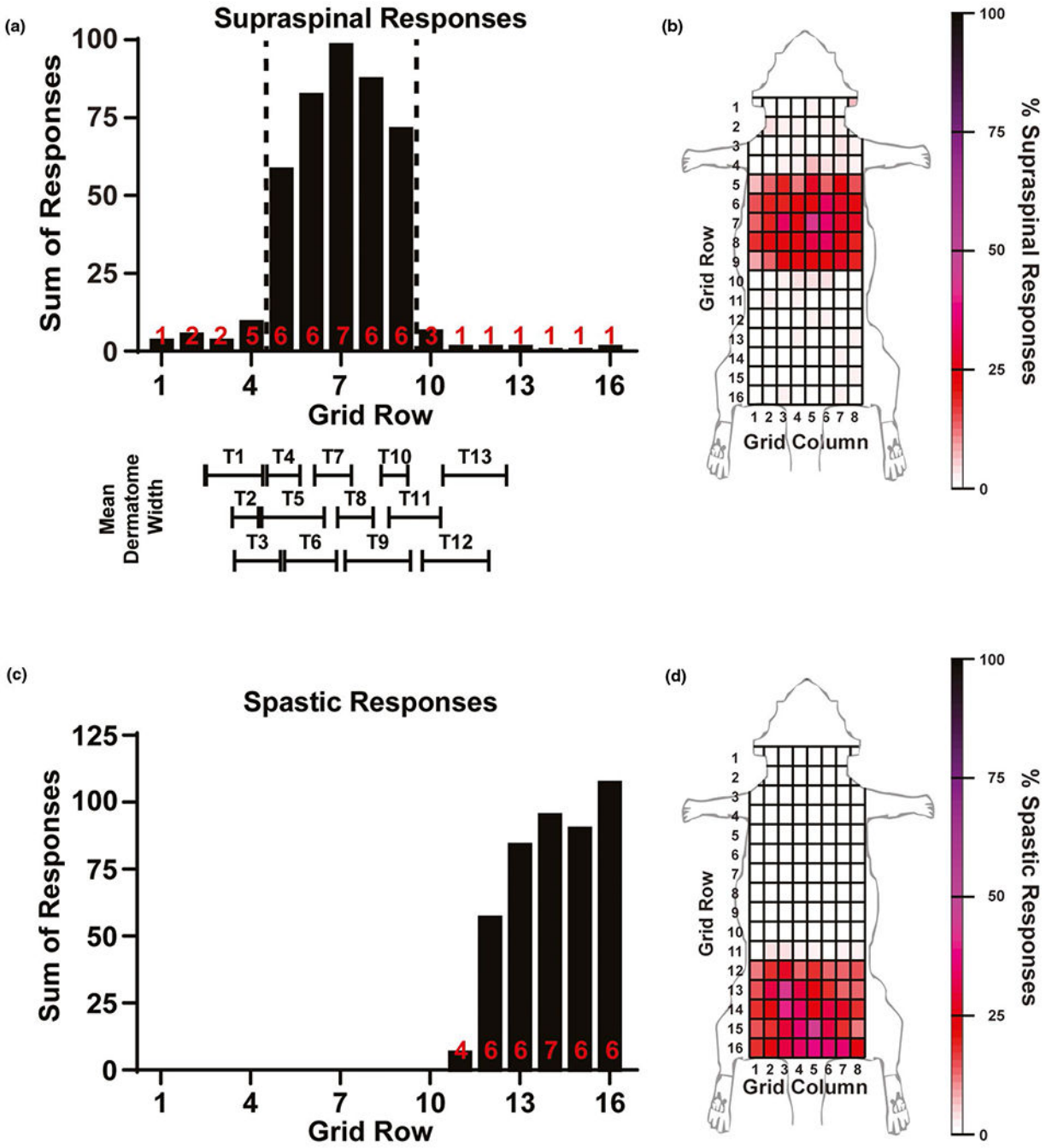

FIGURE 2.

Localisation of hypersensitivity and spasticity. (a) Distribution of supraspinal responses to the tactile stimulation of the dorsal trunk of the rat by grid row. The number of animals from which supraspinal responses were detected in each grid row is indicated in red on the histogram. A mapping of the grid rows to the thoracic dermatomes is shown below. Note that there is considerable overlap between adjacent dermatomes. (b) Heat maps displaying the locations and percent of possible evoked responses for each supraspinal response. In each grid square, 100% = 50 responses or five stimulations per square × 10 animals). (c)

Distribution of spastic responses to the tactile stimulation of the dorsum of the rat. (d) Heat map displaying the locations and percent of possible evoked spastic responses

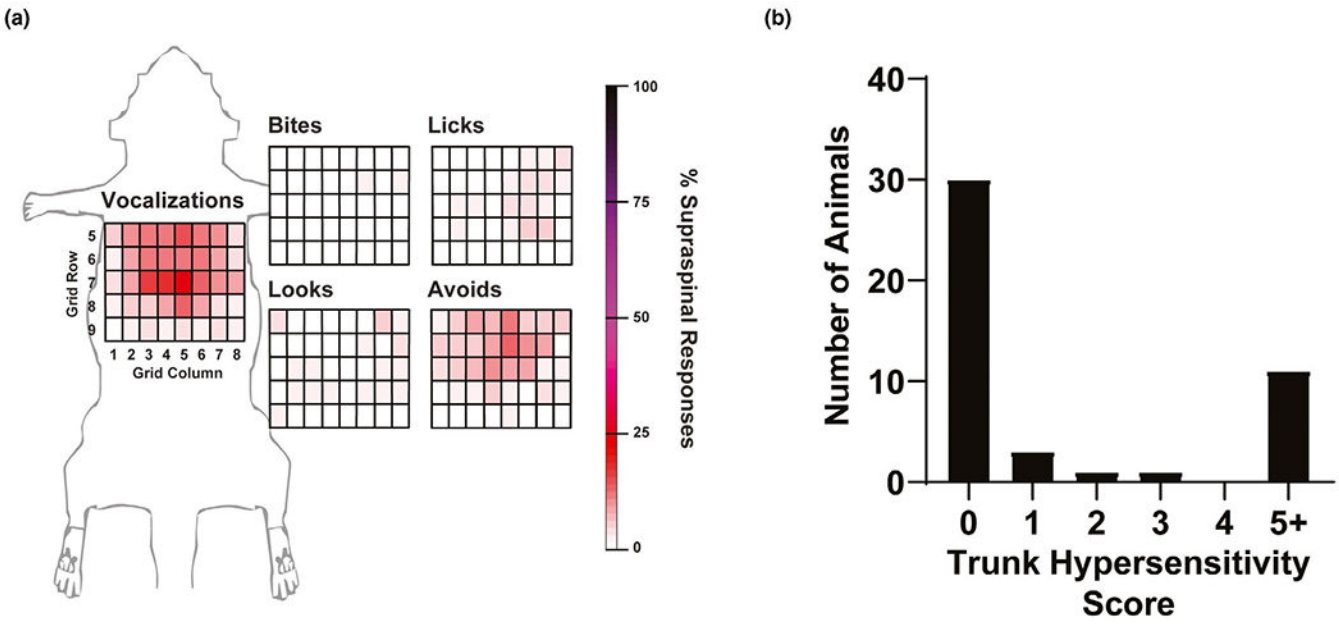

FIGURE 3.

Characterisation of at-level hypersensitivity. (a) Heat map displaying percentage of each type of supraspinal response within each small grid location. (b) Distribution of hypersensitivity scores. The hypersensitivity score is defined as the sum of vocalisation and avoid responses divided by the total number of stimulations (200). From this distribution, a score >=5 was used to distinguished animals with at-level hypersensitivity from animals without at-level hypersensitivity

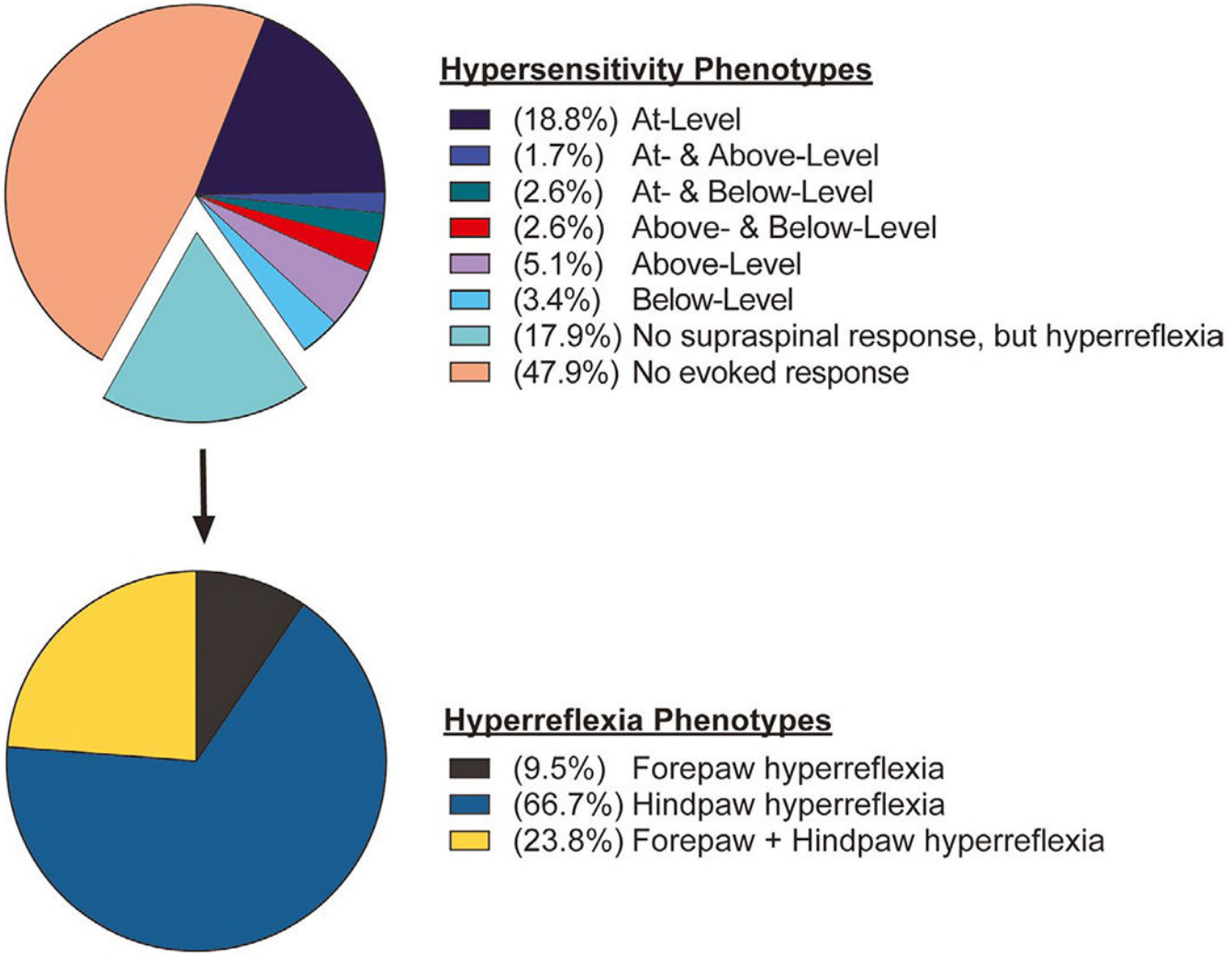

FIGURE 4.

Frequency of hypersensitivity phenotypes. Hypersensitivity phenotypes were determined for each animal at week 5 and were assigned based on whether the animal had at-, above-, below-level hypersensitivity, or some combinations of the three. For animals that had no supraspinal responses but demonstrated hyperreflexia in the limbs, the frequency of hyperreflexia for forepaw, hindpaw, or both is broken out in the second pie chart

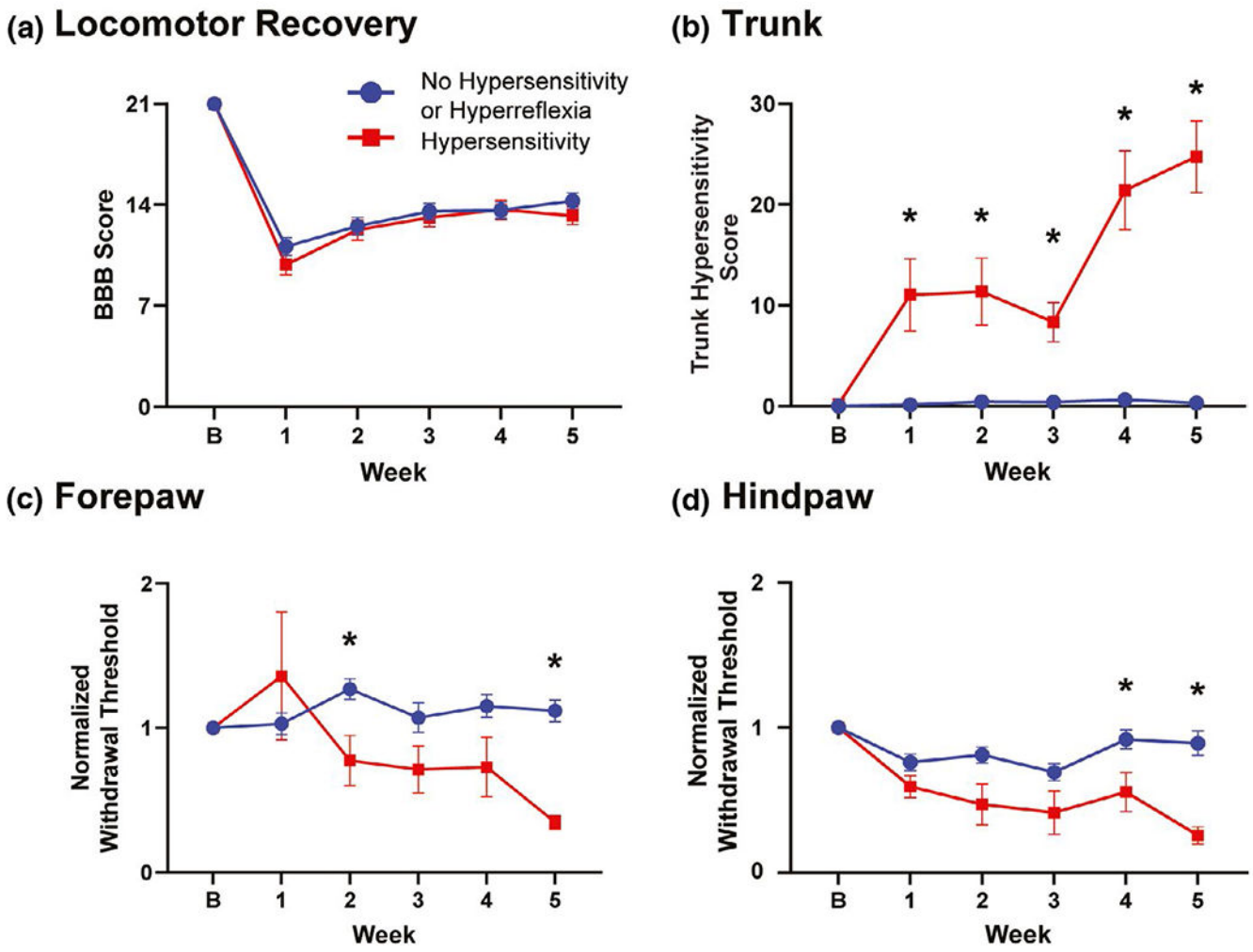

FIGURE 5.

Temporal development of hypersensitivity. Changes in behaviour were compared across weeks for animals the developed hypersensitivity and those that did not. (a) Locomotor ability (BBB score) between animals that developed hypersensitivity at any level ($n = 40$) was compared to that of animals that did not develop hypersensitivity anywhere ($n = 38$). (b) Hypersensitivity scores were compared between animals that developed at-level hypersensitivity ($n = 29$) and animals that did not develop hypersensitivity anywhere. (c) & (d) Withdrawal thresholds for forepaws and hindpaws of animals that developed forepaw ($n = 10$) or hindpaw ($n = 8$) hypersensitivity, respectively, were compared to the animals that did not develop hypersensitivity anywhere. For all plots (a–d) the no hypersensitivity group was the same group of animals that did not develop hypersensitivity anywhere ($n = 38$). Animals were classified into hypersensitivity phenotypes at week 5. B = baseline before SCI, numbers indicate weeks post SCI. *$p < .05$

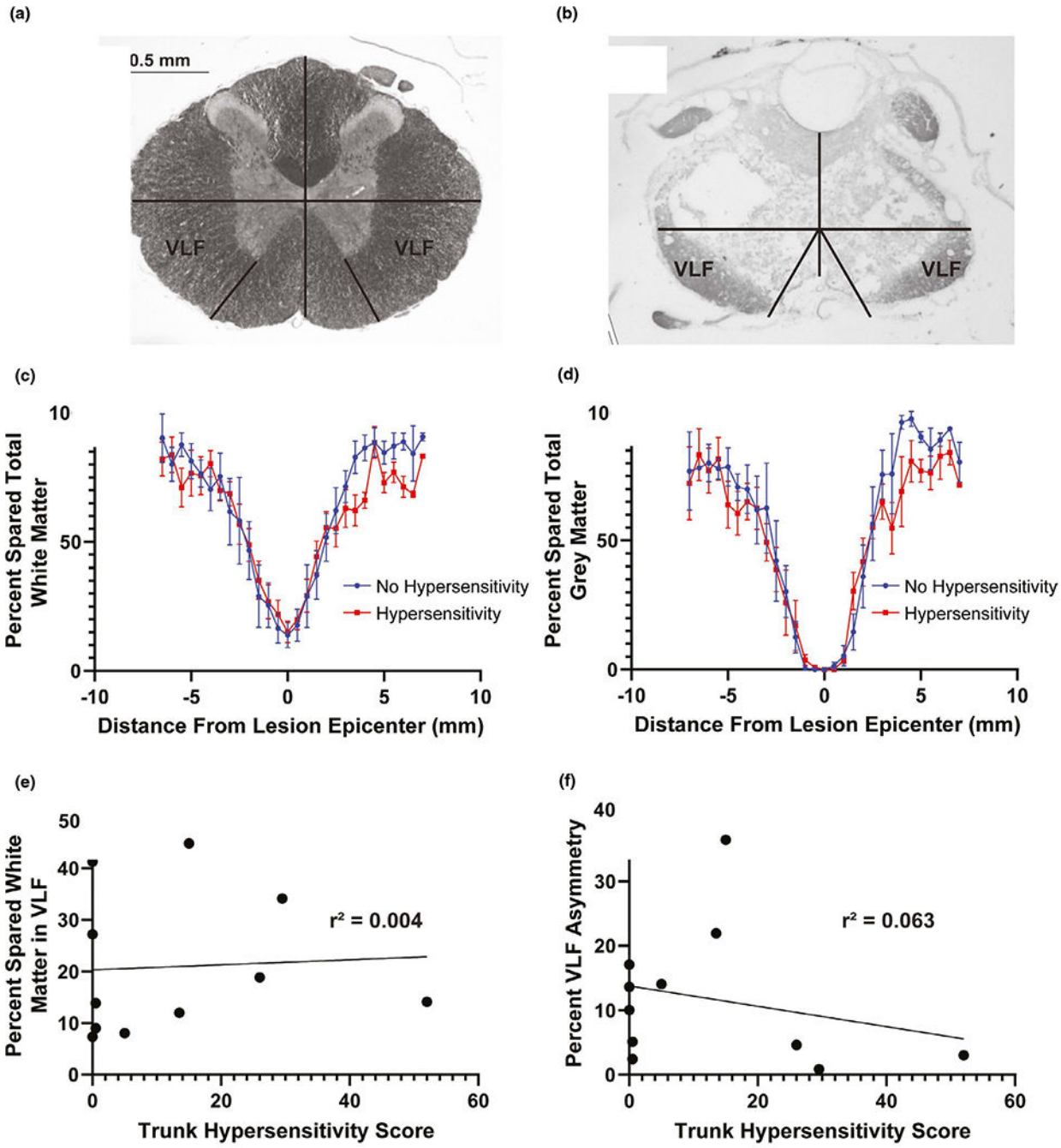

FIGURE 6.

Assessment of spared white and grey matter. (a) An example of an undamaged histological section distal from the lesion site noting the area estimated to be the ventrolateral funiculi (VLF). (b) If the section had little spared matter, the locations of the ventrolateral funiculi were estimated (see Section 2). (c, d) The amount of spared white or grey matter across the lesion site of animals that developed trunk hypersensitivity was compared to animals that did not develop hypersensitivity and no differences were found. (e, f) No correlation was found between the amount of spared white matter in the VLF, or the degree of the asymmetrical

sparing of the VLF at the lesion epicentre (data not shown) or 1 mm rostral to the lesion and the trunk pain score

TABLE 1

Incidence of supraspinal responses to tactile stimulations of the trunk

	Baseline		Week 1		Week 2		Week 3		Week 4		Week 5		
Response	**Sham**	**SCI**	**Sham**	**SCI**	**Sham**	**SCI**	**Sham**	**SCI**	**Sham**	**SCI**	**Sham**	**SCI**	**%**
Vocalize	2	4	2	55	0	21	4	164	0	137	2	399	52.7
Avoid	0	9	1	62	0	47	0	149	0	204	0	219	28.9
Look	0	19	3	36	0	30	7	39	2	27	0	59	7.8
Lick	0	6	0	0	0	2	0	49	0	82	0	69	9.1
Bite	0	3	0	4	0	0	0	0	0	3	0	11	1.5

Note: Sham = laminectomy animals ($n = 6$) and SCI = contused animals ($n = 47$). % indicates the percentage of all supraspinal responses that are comprised of that type of response, at week 5, in contused animals. Contused animals primarily vocalize and elicit avoidance behaviour in response to tactile stimulation of the trunk. The incidence of their evoked supraspinal responses increases over time. In contrast, laminectomy animals elicited few supraspinal responses and the incidence of their responses did not change over time.