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Contribution of mechanoreceptors to spinal cord injury–induced mechanical allodynia

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Abstract

Evidence from previous studies supports the concept that spinal cord injury (SCI)-induced neuropathic pain (NP) has its neural roots in the peripheral nervous system. There is uncertainty about how and to which degree mechanoreceptors contribute. Sensorimotor activation-based interventions (eg, treadmill training) have been shown to reduce NP after experimental SCI, suggesting transmission of pain-alleviating signals through mechanoreceptors. The aim of the present study was to understand the contribution of mechanoreceptors with respect to mechanical allodynia in a moderate mouse contusion SCI model. After genetic ablation of tropomyosin receptor kinase B expressing mechanoreceptors before SCI, mechanical allodynia was reduced. The identical genetic ablation after SCI did not yield any change in pain behavior. Peptidergic nociceptor sprouting into lamina III/IV below injury level as a consequence of SCI was not altered by either mechanoreceptor ablation. However, skin-nerve preparations of contusion SCI mice 7 days after injury yielded hyperexcitability in nociceptors, not in mechanoreceptors, which makes a substantial direct contribution of mechanoreceptors to NP maintenance unlikely. Complementing animal data, quantitative sensory testing in human SCI subjects indicated reduced mechanical pain thresholds, whereas the mechanical detection threshold was not altered. Taken together, early mechanoreceptor ablation modulates pain behavior, most likely through indirect mechanisms. Hyperexcitable nociceptors seem to be the main drivers of SCI-induced NP. Future studies need to focus on injury-derived factors triggering early-onset nociceptor hyperexcitability, which could serve as targets for more effective therapeutic interventions.

Keywords: Spinal cord injury, Dorsal root ganglion, Dorsal horn, Below level, Neuropathic pain, TrkB, Aβ low-threshold mechanoreceptors, Nociceptors, Mechanical allodynia, Hypersensitivity, Hyperexcitability

1. Introduction

Neuropathic pain (NP) represents a frequent concomitant condition in individuals suffering from spinal cord injury (SCI). Available treatments are ineffective and frequently associated with significant side effects. Identification of mechanisms underlying SCI-associated NP and subsequent development of targeted interventions represent important aims to ultimately help improve the quality of life of SCI survivors.

Over the years, several neuroanatomical regions have been identified as potential sites of NP initiation and transmission. There is the injury site characterized by an unpredictable degree of spinal cord damage that varies with respect to preserved ascending sensory pathways—the spinothalamic and the lemniscal tracts. Recently, secondary alterations in the peripheral nervous system (PNS) below injury level have come more into focus. We wondered whether hyperexcitable nociceptors are no longer properly controlled by appropriate mechanoreceptor input because of SCI-induced paraparesis or tetraparesis with resulting inability to stand or walk, thus depleting physiological sensory input from the periphery into the dorsal horn. Accordingly, a number of studies were conducted, which showed that sensorimotor activation through treadmill training in rodents with moderate thoracic contusion reduced pain behavior, associated with reversal of structural rearrangements below injury level in the dorsal horn, namely reduction of peptidergic nociceptor sprouting into laminae III to IV.[34,41,44]

If mechanoreceptors transmit sensory input provided by sensorimotor activation (eg, treadmill training) into the dorsal horn, they should keep by these means, based on the gate control theory of pain, nociceptor firing in check.[1,13,30] Specific genetic ablation of tropomyosin receptor kinase B (TrkB) expressing low-threshold mechanoreceptors (LTMR) in mice undergoing SCI would allow us to address this hypothesis.

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Electrophysiological characterization provides another window into the relevance of mechanoreceptors in NP induction/modulation. Hyperexcitability has previously been demonstrated in nociceptors after SCI.[46] However, these findings were primarily based on dorsal root ganglia (DRG) cultures taken from SCI animals. While planning this study, such analyses have not been reported yet in ex vivo skin-nerve preparations from below injury level, considered to be a better approximation towards pathophysiological events in the living animal compared with cultures.

The relevance and comparability of animal SCI models in respect to the situation in human SCI subjects is very important. Of course, identical analyses are not applicable to human subjects for obvious reasons. However, quantitative sensory testing (QST), which stands for a battery of noninvasive psychophysical measures, allows assessment of the somatosensory system and—more specifically—hypersensitivity towards pain and mechanical stimuli,[45] thus matching preclinical assessments.

Our main aim was to understand the contribution of components of the PNS, in particular mechanoreceptors, to induce and/or maintain SCI-mechanical allodynia after genetic ablation of mechanoreceptors and post-SCI in skin-nerve preparation electrophysiological examination. Findings were compared with noninvasive psychophysical measures (QST) reflecting the state of pain/touch hypersensitivity in SCI subjects up to 1 year after injury.

2. Materials and methods

2.1. Animal subjects and experimental groups

Adult female C57BL/6J mice (8-12 weeks old at experimental initiation; wild type; JANVIER LABS) of varying genotypes subdivided into different experiments weighing between 20 and 25 g were used in this project. Animals were housed in groups of 4 to 5 mice per cage on a 12/12-hour light/dark cycle with access to water and food ad libitum. To ensure optimal conditions for housing and behavioral testing, the facility was controlled for temperature (22˚C ± 1˚C) and humidity (45%-65%) on a daily basis. All experiments were planned and conducted according to the PREPARE and ARRIVE guidelines.[36,42]

For experimental purposes and to ensure that the same investigator can perform behavioral testing, animals were divided in different cohorts for each experiment. Data from the different cohorts were combined in the end for statistical analysis.

2.2. Spinal cord injury

All contusion surgeries were conducted with the Infinite Horizon Impact Device (IH-0400 Impactor; Precision Systems & Instrumentation, Lexington, KY) adapted for the mouse spinal cord using a standard mouse steel-tipped impactor with a diameter of 1.3 mm.[34,41] Before surgery, mice were deeply anesthetized with an intraperitoneal (i.p.) injection using a cocktail (2.5 mL/kg) containing ketamine (31.25 mg/kg), xylazine (1.58 mg/kg), and acepromazine (0.31 mg/kg). T9 laminectomy (corresponding to the T11 spinal level) was performed on mice of the injury group followed by a moderate contusion with a force of 50 kDyn. After contusion, the paravertebral muscle layers were sutured (4/0 terylene) and the skin incision was stapled. In sham mice, skin incisions and separation of paravertebral muscles to isolate the T9 vertebra was performed followed by suturing and stapling of the skin. Postsurgical care included analgesic medication, volume substitution, manual bladder voiding twice per day, and prophylactic antibiotic treatment to prevent bladder infection for 10 to 14 days. Because of the difficulty of emptying male bladders, infections, and post-SCI fighting, only female mice were used in the studies presented here.

All mouse experiments were conducted in accordance with the European Communities Council Directive (Directive 2010/63/EU amended by Regulation (EU) 2019/1010 and institutional guidelines) and approved by the local governing body (Regierungspräsidium Karlsruhe, Abteilung 3—Landwirtschaft, Ländlicher Raum, Veterinär-und Lebensmittelwesen', Germany (approval number: G-196/15)).

2.3. Transgenic animals

To investigate the contribution of low-threshold mechanosensory neuronal subpopulations to SCI-induced below-level NP, heterozygous $TrkB^{CreERT2}$::$Avil^{iDTR}$ (TrkB inducible diphtheria toxin receptor (iDTR))[12,22] in which mechanosensory neurons can be specifically ablated have been used. To generate heterozygous $TrkB^{CreERT2}$::$Avil^{iDTR}$ ($TrkB^{iDTR}$) animals, C57BL/6-Tg(TrkB-CreERT2)^{1Phep}/Embph ($TrkB^{CreERT2}$) mice were crossed to C57BL/6-Tg(Avil-$^{tm1\text{-}DTR}$)^{1Phep}/Embph ($Avil^{iDTR}$) mice.

Administration of tamoxifen (75 mg/kg i.p. for 5 consecutive days) into $TrkB^{iDTR}$ mice specifically renders Avil-expressing $TrkB^{+}$ sensory neurons susceptible to ablation by diphtheria toxin (DTX). Adult female $TrkB^{iDTR}$ (n = 19) mice were injected i.p. with 40 μg/kg DTX (Sigma, D0564) with a second dose after 72 hours. In $TrkB^{iDTR}$ animals, DTX was administered 14 days after the last tamoxifen injection unless described otherwise. Control animals were injected i.p. with 100 μL saline twice in the same time frame. $TrkB^{iDTR}$ animals were used to investigate the effect of ablation after SCI when mechanical allodynia has developed (SCI n = 17; +Tam/+DTX n = 9; +Tam/+saline n = 6) or on development of mechanical allodynia (before SCI n = 18; +Tam/+DTX n = 10; +Tam/+saline n = 8).

2.4. Behavioral testing

Mice were habituated to the individual testing set-up for 2 to 3 days for at least 1.5 hours per day before behavioral testing and for 30 to 60 minutes in each set-up on testing days. All behavioral testing was performed in awake, unrestrained mice by the same investigator blinded to group identity.

2.4.1. Basso mouse scale

Recovery of motor function was assessed using the Basso Mouse Scale (BMS) for locomotion as described before.[34,41] The motor function was scored 1 day post-injury to confirm an adequate injury and weekly starting 7 days post-injury. Each hind paw was scored individually and averaged to get a single score for each animal per testing day. A score of 0 reflects complete hind limb paralysis without ankle movement, whereas a score of 9 indicates normal locomotion. The BMS was used to evaluate sensorimotor function to ensure proper recovery of motor function for sensory testing, which requires the animals to show weight support (BMS ≥ 3).

2.4.2. Mechanical sensitivity testing

To evaluate changes in mechanical sensitivity after injury and ablation of specific sensory neuronal subpopulations, von Frey hair filaments with ascending diameter and force (0.04, 0.07, 0.16, 0.4, 0.6, and 1.4 g; Touch Test Sensory Evaluators; North

Coast Medical, Gilroy, CA) were applied to the hind paws of mice as described before.[34,41]

Starting with the smallest filament (0.04 g), the plantar surface of each hind paw was stimulated as previously described, resulting in 10 stimuli per filament per mouse.[34,41] To avoid stimulus-induced sensitization of the hind paw, up to 12 animals were tested in parallel and hind paws were stimulated in an alternating manner. Mechanical sensitivity towards each filament is defined as paw withdrawal frequency (number of positive responses divided by the number of stimuli) and expressed as the average for both hind paws. Each animal was tested on 2 consecutive days before surgery and the mean was defined as the preoperative baseline response rate.

To investigate nocifensive response duration (removal, licking, and shaking) of a subcohort of wild-type mice (SCI n = 4, sham n = 4), video recording of the response towards the 0.16- and 1.4-g von Frey filament was performed preoperatively and 7 days post-injury (iPhone XR, 1080p HD, 240 fps, slow-motion function, 4.16 milliseconds/frame). Response duration was defined as the time until the hind paw was placed down again.

2.4.3. Place escape/avoidance paradigm

The place escape/avoidance paradigm (PEAP) is based on the assumption that animals escape and/or avoid an aversive mechanical stimulus. Animals have the active choice between a naturally preferred dark environment or pain relief in a more aversive light environment.[3] Here, it was used to address the aversive quality of pain and thereby supraspinal processing of nociception as previously described.[41] In short, animals were placed in the middle of the chamber, and the time spent on the dark side of the chamber was measured. An initial 10-minute exploration phase (baseline) without any stimulus was followed by a 15-minute testing phase (3× 5-minute blocks) in which the hind paws were stimulated using a 0.16-g von Frey filament every 15 seconds when the mouse was present on the dark side of the chamber. The left and right hind paws were stimulated in an alternating manner.

2.4.4. Thermal sensitivity testing

Thermal sensitivity was assessed according to Hargreaves' method using the plantar test (Hargreaves Apparatus; Ugo Basile) as previously described.[34,41] In short, an infrared laser beam (190 ± 1 mW/cm^2) was presented to the plantar surface of the hind paws and the time (sec) until withdrawal was recorded, resulting in the response latency to the heat stimulus.[24] To avoid tissue damage, a 15-second cut-off time, as well as a delay of at least 1 minute between 2 trials for the same paw and mice, was introduced. In each animal, the mean of 4 trials for the left and right hind paws was used to express thermal sensitivity, and testing was always performed after mechanical sensitivity was assessed. Similar to von Frey testing, the mean latency of 2 consecutive testing days before surgery or any other intervention determined the baseline of the animals (preoperatively; preintervention).

2.5. Tissue processing and morphological analysis

All animals were euthanized with an overdose of anesthesia cocktail (ketamine, xylazine, and acepromazine) and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer.[34] The spinal column was dissected, postfixed in 4% PFA for 1 hour at room temperature, and briefly rinsed with ddH_2O followed by a 5-minute washing in 0.1 M phosphate buffer before the tissue was cryoprotected in 30% sucrose in 0.1 M phosphate buffer at 4°C. The lesion site (T11; if applicable), the L4 to L6 spinal cord, as well as L4 to L6 DRG were identified using anatomical landmarks,[25] embedded in Tissue-Tek O.C.T compound (Sakura Finetek, Staufen, Germany) followed by serial sectioning (spinal cord, 16 and 25 μm coronal; DRG, 10 μm) directly on to slides as previously described.[34,41]

2.5.1. Eriochrome Cyanine staining

The lesion size of injured animals was analyzed by quantifying the spared tissue at the lesion epicenter. Two consecutive series were stained for myelin using Eriochrome Cyanine (EC) as previously described.[34,41] Tissue sparing at the lesion epicenter (smallest area of spared white matter) was quantified by outlining areas with light and dark blue staining and expressed as the percent of cross-sectional area calculated by dividing the white matter area by the total cross-sectional area. Imaging and analysis was performed blinded to group identity using ImageJ.

2.5.2. Immunohistochemistry

Immunohistochemical labeling was performed on slides with serial sections of the lumbar spinal cord (L4-L6) and the corresponding L4 to L6 DRG. Slides were dried for 30 minutes at room temperature, encircled with a liquid blocker, and rinsed in 0.1 M Tris-buffered saline (TBS). Sections were blocked and permeabilized by incubating in TBS/0.25% Triton TX-100/5% donkey serum followed by incubation with primary antibodies diluted in TBS/0.25% Triton TX-100/1% donkey serum overnight at 4°C. On day 2, sections were incubated with secondary antibodies and 4′,6-diamidino-2-phenylindole (DAPI), rinsed, dried, and cover-slipped with Fluoromount G (Southern Biotechnology Associates, Birmingham, AL).[41] For each animal, images of the left and right dorsal horn of 3 labeled sections with an intersection distance ranging from 700 to 1400 μm were taken at ×20 magnification with the same resolution, lens aperture, and exposure time using a XC30 camera mounted on an Olympus BX53 microscope with epifluorescence illumination and appropriate filter cubes. Lumbar DRG (L4-L6) were imaged bilaterally from each animal at ×10 magnification using the above-described set-up, and images from 3 sections per DRG (140 μm apart) were analyzed.

The following primary antibodies were used: rabbit anti-calcitonin gene-related peptide (CGRP) (Immunostar, Hudson, WI, 24112; 1:200), rabbit anti-protein kinase Cγ (PKCγ [C19]; Santa Cruz Biotechnology, Dallas, TX, SC-211; 1:200), guinea pig anti-NeuN (Millipore, Darmstadt, Germany, ABN90; 1:2000) and goat anti-human HB-EGF (R&D Systems, Minneapolis, MN, AF-259-NA; 1:40). Secondary antibodies were Alexa Fluor 488 and Alexa Fluor 594 donkey anti-rabbit and donkey anti-goat (all Life Technologies, Carlsbad, CA; 1:300), Alexa Fluor 594–conjugated streptavidin (Jackson ImmunoResearch Ely, United Kingdom; 1:300), and Alexa Fluor 488 donkey anti-guinea pig (Jackson ImmunoResearch; 1:300).

2.5.3. Quantification of labeling density in the spinal cord

The termination pattern of peptidergic fiber and nonpeptidergic fiber was analyzed in lamina III to IV of the dorsal horn as previously described.[41] In short, an analysis box was placed in the center of the dorsal horn adjoining the ventral border of lamina Iii,

which was identified by PKCγ labeling. The dorsoventral extent of lamina IIi was used as a reference for the dimensions of the region of interest (ROI) analysis box. Images were processed by setting a labeling threshold minimizing background and accurately reflecting CGRP- and Tomato-positive fiber labeling. Labeling density was expressed as percentage of positive labeling within the analysis box, and the values for 3 sections per animal were averaged. Quantification of labeling density was performed blinded to group identity using ImageJ.

2.5.4. Quantification of ablated DRG neurons

In $TrkB^{iDTR}$ L4 to L6 DRG quantification of HB EGF^+ in $NeuN^+$ neurons was used to evaluate the proportion of neurons expressing diphtheria toxin receptor (DTR) ($DTR^+/NeuN^+$).

2.6. Ex vivo skin nerve recordings

Skin-nerve recordings were performed on 10- to 12-week-old SCI or Sham mice killed with CO_2 followed by cervical dislocation. The hind paw skin was dissected free together with the sural nerve and was placed in an organ bath chamber that was perfused with 32°C-warm synthetic interstitial fluid (SIF) consisting of 108 mM NaCl, 3.5 mM KCl, 0.7 mM $MgSO_4$, 26 mM $NaHCO_3$, 1.7 mM NaH_2PO_4, 1.5 mM $CaCl_2$, 9.5 mM sodium gluconate, 5.5 mM glucose, and 7.5 mM sucrose at a pH of 7.4. The sural nerve was led through a small hole into the adjacent mineral oil filled recording chamber. The nerve was teased into thin bundles that were laid on a silver wire recording electrode connected to a differential amplifier (Digitimer, modules NL104, NL125/NL126) and nerve fiber activity was recorded with the Powerlab 2 4SP system and Labchart 7.1 software (AD Instruments, Sydney, Australia). The receptive fields of single nerve fibers were located by manually probing the skin using von Frey hair filaments. To distinguish between Aβ fibers, Aδ fibers, and C fibers, action potentials were evoked by electrical stimulation of the receptive fields, and the axonal conduction velocities were calculated by dividing the distance between the stimulation electrode and the recording electrode by the delay between the onset of electrical stimulation and the arrival of the action potential at the recording electrode. Nerve fibers with a conduction velocity >10 m/second were classified as Aβ fibers, fibers with a CV between 1 and 10 m/second as Aδ fibers, and fibers with a CV <1 m/second as C fibers. To further distinguish between nociceptors and mechanoreceptors, the mechanical activation thresholds were determined with von Frey hair filaments. Finally, to determine the action potential firing rates and adaptation patterns at different stimulus strengths, the receptive fields were stimulated with ramp-and-hold stimuli applied with a linear piezo actuator (Nanomotor; Kleindiek, Reutlingen, Germany) equipped with a force measurement system (FMS-LS; Kleindiek, Reutlingen, Germany) to measure the exact force of the applied stimulus.

2.7. Human spinal cord injury study

Included participants (n = 17) were identified and recruited from July 2016 to June 2021 by convenient sampling. All of them provided written informed consent. The human SCI study was conducted within the framework of the European Multicenter Study about Spinal Cord Injury (EMSCI) at the SCI center, Heidelberg University Hospital (ClinicalTrials.gov register-no. NCT01571531; https://emsci.org). The study protocol was approved by the local ethical review board (S-188/2003). Inclusion criteria for the EMSCI study are acute traumatic or ischemic SCI. Furthermore, the initial assessments must be performed within the first 6 weeks after injury to be eligible for inclusion. Exclusion criteria were a nontraumatic cause of SCI other than spinal cord ischemia, impaired capabilities in respect to cooperation or giving informed consent, medical history of polyneuropathy, and concomitant traumatic brain injury.[9]

Sensory function was assessed in SCI subjects by both the International Standards for Neurological Classification of SCI (ISNCSCI) and highly standardized QST.[27,38] Quantitative sensory testing represents a comprehensive protocol for assessing the integrity of the sensory system along the complete nervous system. For the classification of existing spontaneous pain problems in individuals with SCI, all participants received the clinical assessment EMSCI Pain Assessment Form (EPAF) that is in accordance with international guidelines and recommendations for the clinically documentation and assessment of pain in SCI.[48] Within EMSCI, all study participants underwent recurrent comprehensive clinical examinations in predefined time windows (up to day 40, day 70-98, day 150-186, and day 300-546) within the first year after injury.[9] For this study, only data of 3 timepoints (1, 3, and 12 months) were used. Six individuals did not complete the final timepoint.

In consideration of the methodological approach for the animal model, special focus was set on the evaluation of the mechanical detection threshold (MDT) representing a test for Aβ fibers (mechanoreceptors) and the mechanical pain threshold (MPT) to function as indicator for Aδ fibers (nociceptors).[4,21,51] Although MDT was performed via modified von Frey hair filaments (contact area 0.5 mm in diameter; Optihair2-Set, Marstock Nervetest, Germany) applying forces from 0.25 to 512 mN, starting with 8 mN, MPT was assessed by means of (weighted) pinprick examination (contact area 0.25 mm in diameter) applying forces from 8 to 512 mN, starting with 8 mN.[4]

Dedicated areas being tested as to alterations of sensory function were the dermatomes L4 (medial right shin, midway between knee and ankle) and L5 (dorsum of the foot, third metatarsal phalangeal joint). The rationale for choosing L4/L5 was based on related literature reporting a frequent localization of NP in these regions.[10,17,20,49]

Evaluation and analysis of QST data was done based on standard reference MDT and MPT values provided by 180 healthy subjects (respecting test site, sex, and age, split by decades) as provided by the German Research Network on Neuropathic Pain (DFNS) by means of a QST reference database.[32,33]

2.8. Statistical analysis

Behavioral results were analyzed by repeated-measures (RM) 2-way analysis of variance (ANOVA) to reveal overall group and timepoint differences, without assumption of sphericity (Geisser–Greenhouse corrections). Significant group differences were followed by Sidak pairwise comparisons. Significant timepoint differences between baseline vs post-SCI or baseline vs post-DTX were followed by post hoc Fisher least significant difference (FLSD) test.

For experiments in which transgenic animals have been used, the lesion size, fiber density in the lumbar spinal cord, and changes in DRG neurons (ablated neurons) were only compared between saline injected control SCI and ablated SCI group and consequently analyzed by unpaired *t* test.

Electrophysiological data were analyzed with multiple χ^2 tests and multiple Mann–Whitney tests.

All data are presented as mean ± SD, except for skin-nerve preparation data, which are presented as mean ± SEM.

Statistical analysis was done using Prism 9 software (GraphPad Software Inc, La Jolla, CA) with an alpha level of 0.05 for significance.

Quantitative sensory testing data were processed and analyzed using the software "eQUISTA" (Casquar GmbH, Bochum, Germany). Accordingly, results are expressed as Z-transformed standard values ("Z-scores"). Values greater than 1.96 and lower than −1.96, indicating ~2 SDs from healthy matched controls findings (with 95% confidence interval), meaning hypersensitivity or hyposensitivity to the respective stimulus.[38,39]

3. Results

3.1. Pre-injury ablation of tropomyosin receptor kinase B–expressing mechanosensory neurons reduces neuropathic pain behavior after mouse contusion spinal cord injury

We hypothesized that low-threshold mechanoreceptors (LTMRs), responsible for light touch, mediate beneficial sensory input provided by natural locomotor activity or in an enhanced setting such as treadmill training.[16,28,29,44] Therefore, we investigated the contribution of TrkB-expressing LTMRs (identified as Aδ-LTMRs, D-hair, and rapidly adapting Aβ mechanoreceptors, Aβ-RAMs[12]) to SCI-induced neuropathic pain behavior represented by mechanical allodynia by ablating TrkB-expressing LTMRs in $TrkB^{CreERT2}$:$Avil^{iDTR}$ ($TrkB^{DTR}$) mice.

First, to determine the contribution of TrkB-expressing sensory neurons to established signs of SCI-induced neuropathic pain, respective neurons were genetically ablated 14 days after thoracic contusion SCI using diphtheria toxin (DTX), when mechanical allodynia and thermal hyperalgesia have already developed (**Fig. 1A**). Before SCI, tamoxifen administration did not affect mechanical and thermal sensitivity (data not shown), and as expected, all SCI animals developed mechanical allodynia and thermal hyperalgesia at 7 days post-injury (**Figs. 1E–G,I**, 2-way RM ANOVA, time differences $P < 0.0001$). Genetic ablation resulted in a 97% reduction in $TrkB^+/DTR^+$ neurons (**Figs. 1B–D**; *t* test $P < 0.0001$ DTX vs saline). Despite the high ablation efficiency, no significant effect of post-SCI ablation on mechanical hypersensitivity was observed. Statistical analysis indicated no overall group differences comparing the response rates for the small-diameter von Frey hair filaments (0.04 g-0.16 g) between saline-injected control and ablated SCI animals (**Figs. 1E–G**, 2-way RM ANOVA group: 0.04 g, $P = 0.54$; 0.07 g, $P = 0.27$; and 0.16, g $P = 0.06$). In line with this observation, supraspinal processing assessed by the PEAP also showed no difference between groups in response to the 0.16-g filament (**Fig. 1H**, 2-way RM ANOVA $P = 0.23$ for group differences).

Post-SCI ablation did not affect response rates measured using larger diameter innocuous von Frey hair filaments (Suppl. Fig. 1A, available at http://links.lww.com/PAIN/B970, 0.4 g: 2-way RM ANOVA $P = 0.17$) but did have significant group differences for noxious stimuli (0.6 g and 1.4 g) with inconsistent or nonsignificant pairwise comparisons at each timepoint (Suppl. Fig. 1B-C; 2-way RM ANOVA, group differences 0.6 g, $P = 0.04$; 1.4 g, $P = 0.01$). TrkB-expressing LTMR ablation did not affect motor recovery (**Fig. 1J**). After SCI, significant thermal hypersensitivity (Hargreaves method) was observed for all SCI animals irrespective of ablation (**Fig. 1I**, 2-way RM ANOVA, baseline vs 7 dpi $P < 0.0001$, group differences $P = 0.02$, no significant Sidak pairwise comparisons). No significant differences in white matter sparing (lesion size) were observed between saline-injected controls and ablated SCI mice (Suppl. Fig. 2A, available at http://links.lww.com/PAIN/B970; *t* test $P = 0.52$). Taken together, these results suggest that TrkB-expressing sensory neurons do not play a role in already developed and stably present mechanical allodynia after SCI.

Second, to determine the role of TrkB-expressing sensory neurons in the initiation of SCI-induced neuropathic pain, neurons were ablated before SCI. This resulted in an effective reduction of >90% of DTR^+ neurons in lumbar DRG (L4-L6) (**Figs. 2B–D**; *t* test $P < 0.0001$ DTX vs saline control group). Without injury, no differences in mechanical or thermal sensitivity were observed (**Figs. 2**, Suppl. Fig. 1D-F, available at http://links.lww.com/PAIN/B970). All SCI animals of the saline-injected control group showed mechanical hypersensitivity at 7 days post-injury compared with pre-SCI values using small-diameter von Frey filaments (**Figs. 2E–G**). Interestingly, a significant group effect was observed (**Figs. 2E–G**; 2-way RM ANOVA group differences: 0.04 g, $P = 0.004$; 0.07 g, $P = 0.008$; 0.16 g, $P = 0.013$). However, TrkB-ablated SCI animals (DTX group) showed a lower response rate than the control group only at 21 days post-injury, indicating a small but significant effect of pre-injury ablation of TrkB-expressing sensory neurons on SCI-induced mechanical allodynia. In line with the lack of group differences to the 0.16-g von Frey hair filament at the end of the experiment, the PEAP indicated SCI-induced mechanical allodynia in both groups (**Fig. 2H**, 2-way RM ANOVA group differences $P = 0.49$). Pre-SCI ablation did not affect response rates measured using larger diameter (0.4-1.4 g) von Frey hair filaments (Suppl. Fig. 1D-F, 2-way RM ANOVA, group differences 0.4 g, $P = 0.11$; 0.6 g, $P = 0.11$; 1.4 g, $P = 0.13$). Both, thermal sensitivity (**Fig. 2I**, $P = 0.14$) and motor recovery (**Fig. 2J**) were not influenced. Additionally, differences in sensory behavior of saline-injected and ablated SCI animals cannot be attributed to difference in white matter sparing (lesion size) (Suppl. Fig. 2B, available at http://links.lww.com/PAIN/B970, *t* test $P = 0.69$). Taken together, only pre-injury—not post-injury—ablation of TrkB-expressing mechanosensory neurons reduces SCI-induced mechanical allodynia.

Regardless of the ablation timing (post- or pre-SCI), calcitonin gene-related peptide (CGRP) density in laminae III to IV of the lumbar dorsal horn was not reduced in respective transgenic SCI mice compared with the non-ablated SCI control group (**Figs. 3A–F**). Therefore, SCI-induced maladaptive structural changes regarding sprouting of CGRP-expressing fibers were not influenced by ablation of TrkB-expressing sensory neurons and existed while mechanical allodynia was present.

3.2. Experimental contusion spinal cord injury induces nociceptor, but not mechanoreceptor, hyperexcitability

To identify the key neuronal population (nociceptors or mechanoreceptors) driving mechanical allodynia as a correlate of NP behavior, ex vivo skin-nerve preparations were investigated.

Single-unit teased fiber recordings from ex vivo hind paw skin-nerve preparations revealed that C- and Aδ-fiber nociceptive sensory afferents displayed decreased mechanical activation thresholds 7 days post-injury (**Figs. 4A–E**). Thus, a significantly higher proportion of C-fiber nociceptors responded to forces smaller than 125 mN (**Fig. 4E**, multiple χ^2 tests). Moreover, C-fiber nociceptors fired significantly more action potentials in response to mechanical ramp-and-hold stimuli (**Fig. 4F**, multiple Mann–Whitney tests) and exhibited continuous action potential firing after removal of the stimulus (**Figs. 4G–I**, multiple Mann–Whitney tests). These afterdischarges were also observed in Aδ-fiber nociceptors (**Figs. 4G and H**). Additionally, a significantly higher proportion of Aδ-fiber nociceptors responded to

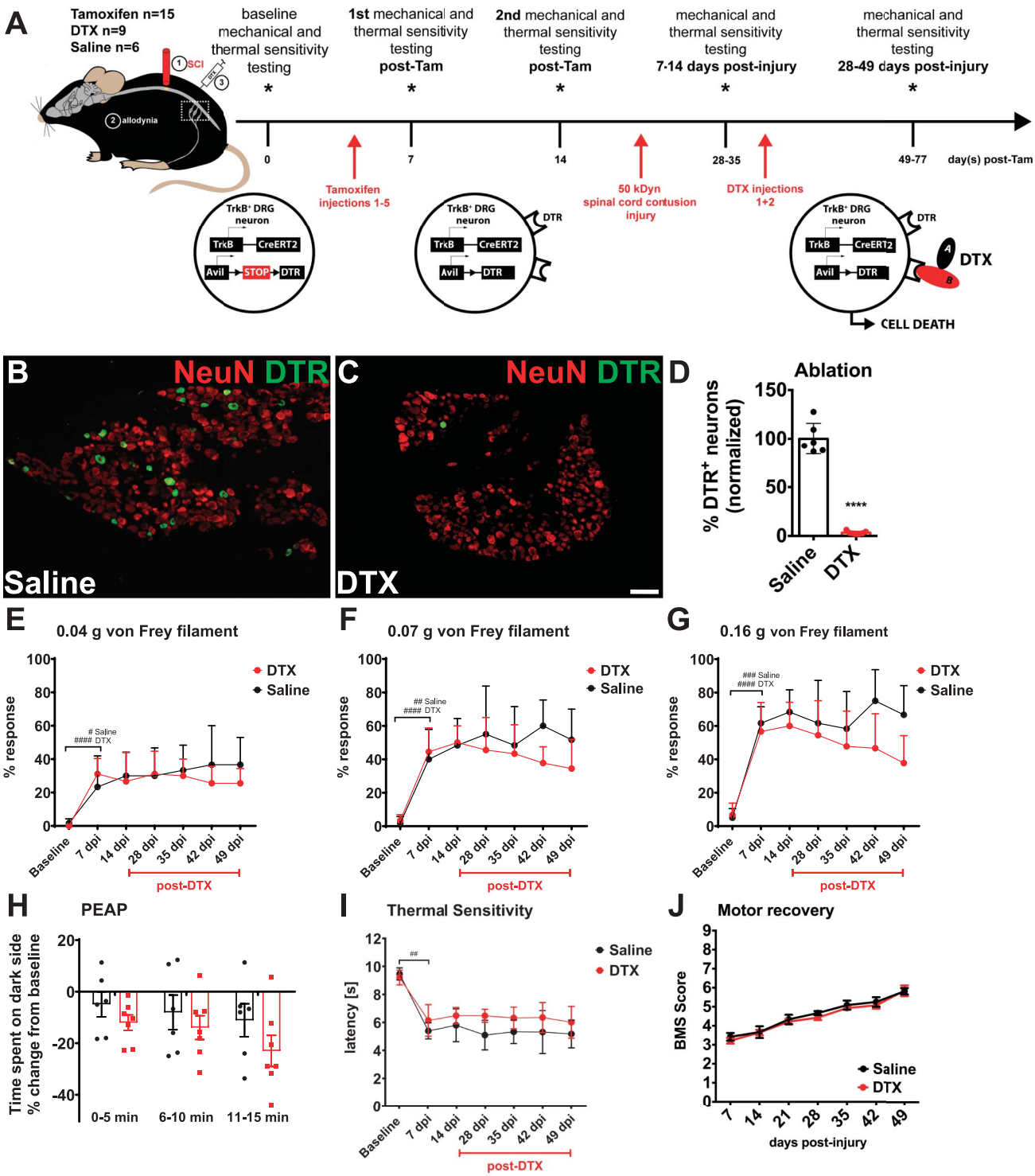

Figure 1. Post-SCI ablation of TrkB-expressing mechanoreceptors. (A) Schematic of experimental design. (B) Representative lumbar (L4-L6) DRG neurons of saline-treated control animals stained for HB-EGF shows robust expression of DTR in a subset of sensory neurons. (C) In ablated TrkB^{DTR} animals, DTR expression is almost completely absent (scale bar, 100 μm). (D) Quantification (*t* test, $P < 0.0001$; DTX, n = 7; saline n = 6, L4-L6 bilaterally). (E–G) SCI-induced mechanical hypersensitivity to small-diameter light touch eliciting von Frey hair filaments (0.04, 0.07, and 0.16 g, 2-way RM ANOVA, time differences $P < 0.0001$, baseline vs 7 dpi FLSD #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$, ####$P < 0.0001$), but DTX-mediated ablation of TrkB+ sensory neurons (28-49 dpi) did not change SCI-induced mechanical hypersensitivity. (H) The Place Escape Avoidance Paradigm (PEAP, 0.16 g) performed at the end of the study shows that both groups spent more time on the light side of the chamber (2-way RM ANOVA $P = 0.23$ for group differences). (I) Injured TrkB^{DTR} animals of both groups develop thermal hypersensitivity assessed by the Hargreaves test (2-way RM ANOVA, baseline vs 7 dpi $P < 0.0001$, FLSD ##$P < 0.01$, group differences $P = 0.02$, no significant Sidak pairwise comparisons). (J) Except for the SCI-induced motor deficits scored by the Basso Mouse Scale (BMS), no further motor deficits are observed after post-SCI ablation of TrkB^{+} sensory neurons. Mean ± SD for all graphs. 2-way RM ANOVA, 2-way eepeated-measures analysis of variance; DTR, diphtheria toxin receptor; DTX, diphtheria toxin; FLSD, Fisher least significant difference; FLSD, Fisher least significant difference; HB-EGF, heparin binding EGF-like growth factor ; SCI, spinal cord injury; TrkB, tropomyosin receptor kinase B.

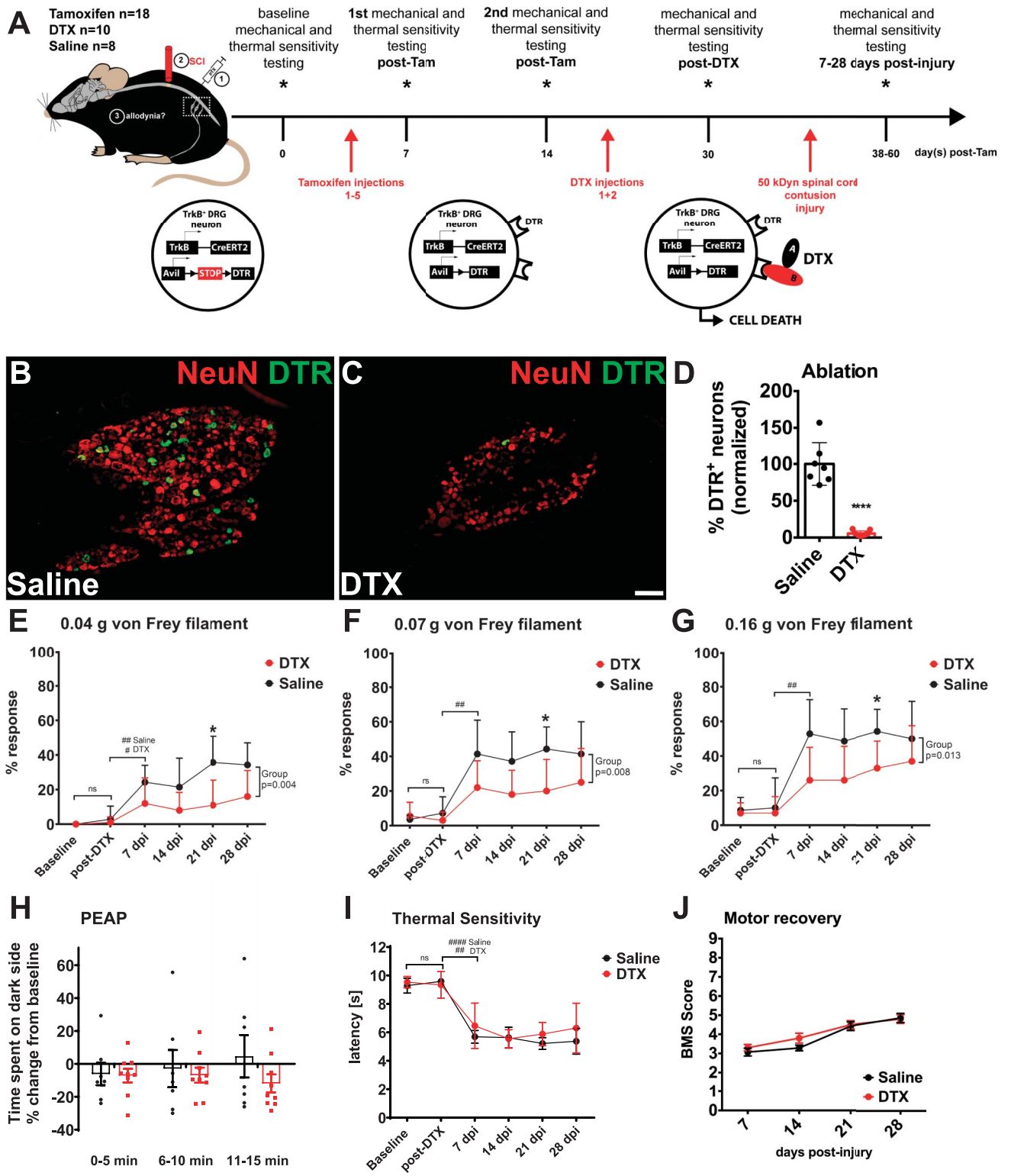

Figure 2. Pre-SCI ablation of TrkB-expressing mechanoreceptors. (A) Schematic of experimental design. (B) Saline-injected control animals show robust DTR expression in lumbar (L4-L6) DRG neurons. Compared with control animals, DTR+ neurons are almost completely ablated in DTX-injected animals (C) (scale bar, 100 μm). (D) Quantification (*t* test $P < 0.0001$; DTX, n = 9; saline n = 7, L4-L6 bilaterally). (E–G) SCI-induced mechanical hypersensitivity to small-diameter filaments (0.04, 0.07, and 0.16 g, 2-way RM ANOVA, time differences $P < 0.0001$, post-DTX vs 7 dpi FLSD #$P < 0.05$, ##$P < 0.01$, no significant (ns) difference between baseline and post-DTX) is significantly altered in ablated animals, but inconsistently (2-way RM ANOVA group differences: 0.04 g, $P = 0.004$; 0.07 g, $P = 0.008$; 0.16 g, $P = 0013$, Sidak pairwise comparisons *$P < 0.05$). The response rate towards the 0.16-g von Frey hair filament (G) of ablated animals is not significantly different from control animals anymore at the end of the experiment 28 dpi. (H) This is confirmed by supraspinal processing of nociception using the PEAP (0.16 g) where both groups equally spent more time on the light side of the box. (I) DTX treatment does not affect pre-injury or post-injury thermal sensitivity, but SCI did induce thermal sensitivity in both groups (2-way RM ANOVA, time differences $P < 0.0001$, post-DTX vs 7 dpi FLSD ##$P < 0.01$, ####$P < 0.0001$). (J) SCI induces significant motor deficits in all animals scored by the Basso Mouse Scale (BMS), and ablation of TrkB+ sensory neurons does not induce further motor deficits. Mean ± SD for all graphs. DTX, diphtheria toxin; FLSD, Fisher least significant difference; PEAP, place escape/avoidance paradigm; SCI, spinal cord injury; TrkB, tropomyosin receptor kinase B.

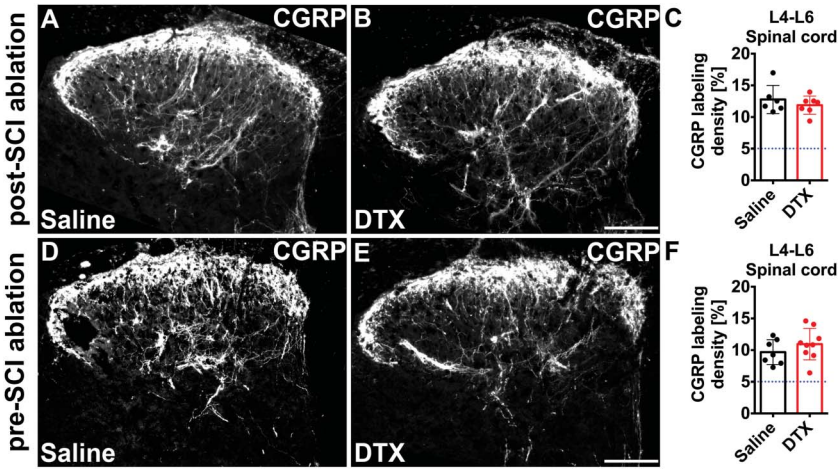

Figure 3. Peptidergic nociceptive fiber changes in the dorsal horn after TrkB-expressing mechanoreceptor ablation. Lumbar (L4-L6) spinal cord sections of injured saline control (A and D) and DTX-treated injured (B and E) $TrkB^{DTR}$ mice stained for CGRP (scale bar, 100 μm). Representative images indicate the SCI-induced increase in CGRP-labeling density in deeper laminae (III-IV) of the spinal dorsal horn in all groups. (C and F) Quantification of the CGRP-labeling density in laminae III-IV of the lumbar (L4-L6) dorsal horn shows no difference comparing the injured saline-injected control group with injured DTX-injected animals but a significant increase compared to previous sham animals (dotted line) (mean ± SD) (post-SCI; saline n = 6; DTX n = 7) (pre-SCI; saline n = 7; DTX n = 9). CGRP, Calcitonin gene-related peptide; DTX, diphtheria toxin; SCI, spinal cord injury; TrkB, tropomyosin receptor kinase B.

forces at 17.5 and 22.5 mN (**Fig. 4C**, multiple χ^2 tests); however, they did not fire more action potentials in response to mechanical ramp-and-hold stimuli (**Fig. 4D**, multiple Mann–Whitney tests). Most importantly, no significant changes in the mechanical activation thresholds or the action potential firing rates were observed after SCI in the 3 major classes of low-threshold mechanoreceptors (rapidly adapting (RA) Ab-LTMRs, slowly adapting (SA) Ab-LTMRs, and D hairs (DH) Ad-LTMRs, **Figs. 4J–M**). Therefore, a midthoracic contusion SCI induces hyperexcitability of peripheral nociceptive fibers but not mechanoreceptors of the hind paw.

Does prolonged nocifensive behavior such as removal, licking, and shaking correlate with the extension of the firing past stimulus removal? Indeed, almost a doubling of the response duration of the SCI mice to the 0.16-g filament was observed at 7 days post-injury (**Fig. 5A**, *t* test $P = 0.03$). Also the response rate for these mice was significantly increased 7 days post-injury (**Fig. 5B**, 2-way RM ANOVA, group differences, $P = 0.03$). Therefore, the continued firing observed in nociceptors appears to be behaviorally expressed by prolonged nocifensive behavior in SCI mice.

3.3. Nociceptor hypersensitivity is observed in spinal cord injury subjects up to 1 year post-injury

The best approximation towards the relevance in nociceptors vs mechanoreceptors in initiating/maintaining hyperexcitability and NP presentation, QST was used. Quantitative sensory testing was performed 1, 3, and 12 months post-injury in 17 conveniently sampled individuals with SCI varying in age (18-75 years), sex (male, n = 13; female, n = 4), cause of injury (traumatic, n = 11; ischemic, n = 5; or hemorrhagic, n = 1), initial classification according to the ASIA impairment scale (AIS D, n = 9; C, n = 3; B, n = 2; A, n = 3), and initial neurological level of injury (C2-T10, cervical = 9, thoracic = 8) (6 individuals did not complete the full study) (Suppl. Table 1, available at http://links.lww.com/PAIN/B970; **Fig. 6**). Not all SCI subjects could be tested at each timepoint and at all intended spinal segments (at 1 month, n = 14; at 3 months, n = 11; at 12 months, n = 8). The majority of sensory incomplete SCI subjects, in comparison with the healthy subject QST reference data bank, present with MPT Z-scores beyond the 1.96 SD margin (1 month: n = 10; 3 months: n = 9; 12 months: n =8; **Fig. 6**). Whereas, MDT Z-scores are mostly clustered within normal variation of 1.96 and −1.96, with some SCI subjects displaying Z-scores beyond the Z-score < −1.96 SD margin pointing towards reduced mechanosensation (5 being classified initially as exhibiting hypoesthesia at 1 month and 2 at 3 months and at 12 months in at least 1 dermatome). At 12 months, of the 8 remaining sensory incomplete individuals (AIS-B, C and D), 6 reported at-level (n = 2) or below-level (n = 4) NP.

Comparing the MDT analysis with light touch (LT) assessment based on International Standards for Neurological Classification of SCI (ISNCSCI) (**Fig. 6B**; Suppl. Table 2, available at http://links.lww.com/PAIN/B970) yields concordant results. The majority of SCI subjects rate light touch neither normal nor absent equivalent to a score of 1 (Suppl. Fig. 3, available at http://links.lww.com/PAIN/B970). Accordingly in the MDT, several of the SCI subjects display reduced light touch sensation (tested with up and down method von Frey hair filaments). Pinprick assessment according to ISNCSCI vs MPT threshold results requires a more thorough explanation. The majority of SCI subjects displayed a pinprick score of 0 (ISNCSCI), indicating the subject either feels nothing or is not able to discriminate between sharp and dull stimuli. However, in the MPT (QST), the majority are depicted as hypersensitive towards pinprick stimuli (MPT Z-score >1.96).

In summary, sensory incomplete SCI subjects are characterized by early and persistent hypersensitivity towards nociceptor-mediated stimuli, whereas such changes could not be observed in respect to mechanoreceptor-mediated stimuli.

4. Discussion

Results from the present study improve our understanding of initiation and maintenance of NP signals in traumatic SCI. Neuropathic pain ameliorating effects of mechanoreceptor ablation depend on the timing of ablation in relation to the time of injury. Only pre-SCI mechanoreceptor ablation reduces pain behavior, suggesting a potentially NP-inducing role. Replicating previously published evidence, nociceptors become hyperexcitable early after SCI.

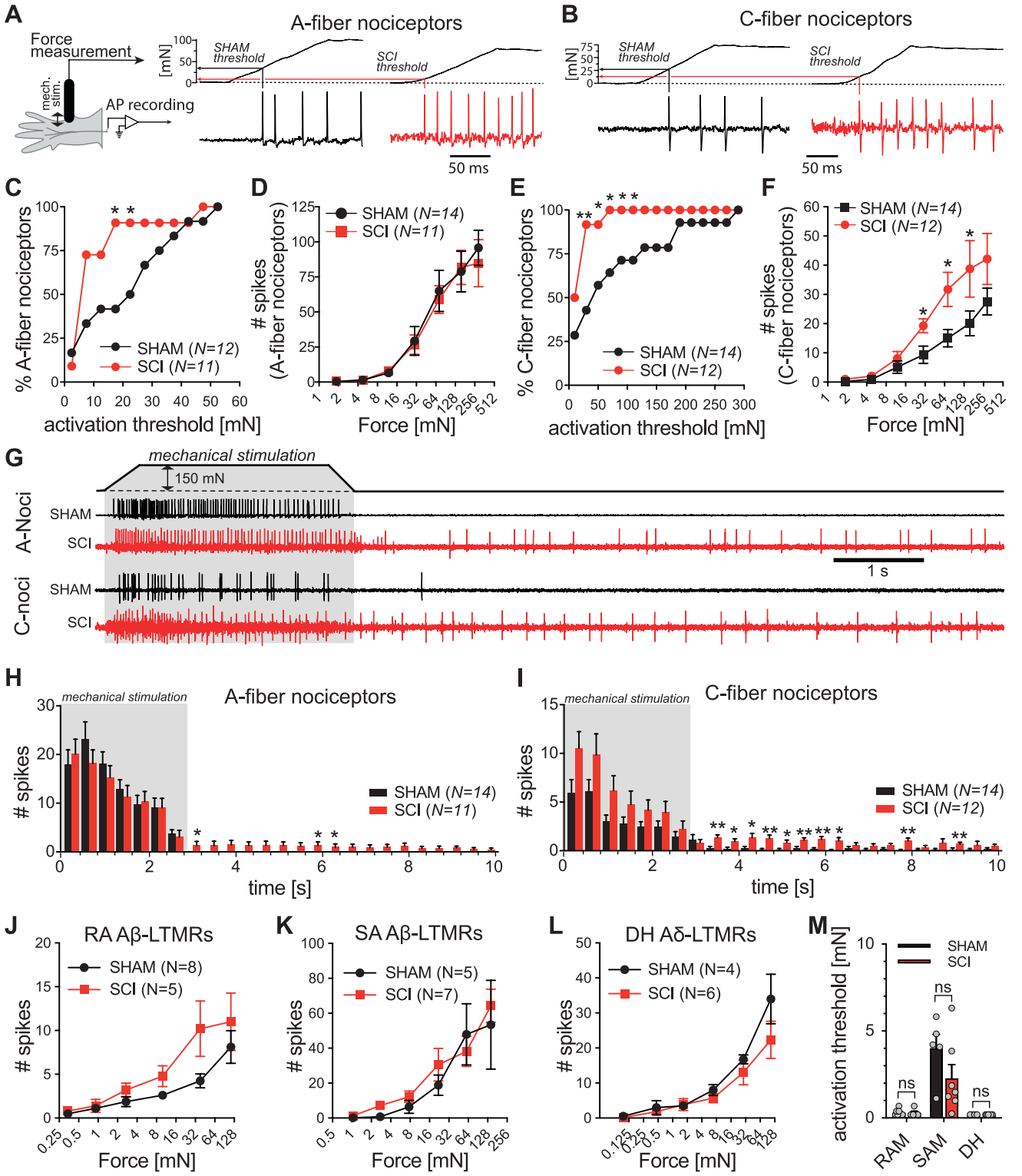

Figure 4. Mechanoreceptor and nociceptor activity after SCI. Single-unit teased fiber recordings from hind paw glabrous skin-nerve preparations show that A-fiber (A) and C-fiber nociceptors (B) have lowered activation thresholds 7 dpi T11 (50 kDyn) SCI in mice. This is quantitatively shown in (C) for A-fiber and (E) for C-fiber nociceptors responding to forces smaller than 125 mN (multiple χ^2 tests). While following SCI A-fibers (D) do not fire more frequently, C-fibers (F) significantly fire more frequently in response to mechanical ramp-and-hold stimuli. Example traces exhibit continuous firing after removal of stimulus (150 mN denoted in gray area) in the SCI group (G). This is quantitatively shown for A-fiber (H) and C-fiber (I) nociceptors (multiple Mann–Whitney tests). The firing of RA-Aβ-LTMRs (J), SA-Aβ-LTMRs (K), and DH-Aδ-LTMRs (L) was not significantly altered by SCI. Additionally, LTMR activation thresholds were not altered by SCI (M). Mean ± SEM. * $P < 0.05$, ** $P < 0.01$. DH, D hairs; LTMRs, low-threshold mechanoreceptors; RA, rapidly adapting; SA, slowly adapting; SCI, spinal cord injury.

The finding that pre-SCI ablation of mechanoreceptors—not post-SCI ablation—reduces pain behavior, points toward an NP-inducing rather than an NP-maintaining effect. This is supported by the absence of mechanoreceptor hyperexcitability in hind paw skin-nerve preparation 7 days after thoracic contusion SCI in mice. Central branches of mechanoreceptors form the lemniscal

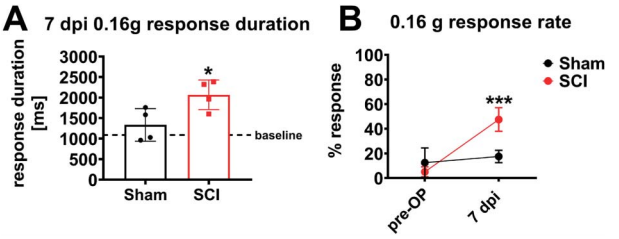

Figure 5. Prolonged nocifensive behavior after experimental SCI. (A) T11 SCI mice showed prolonged nocifensive behavior (licking, shaking, and holding) compared with Sham mice when responding to 0.16-g von Frey hair filaments 7 dpi (slow-motion video analysis, unpaired 2-tailed *t* test, $^{*}P < 0.05$). (B) The response rate of SCI rate was significantly higher than Sham mice at 7 dpi with 0.16-g von Frey hair filament (Sham n = 4, SCI n = 4, 2-way RM ANOVA, group difference $P = 0.03$, Sidak pairwise comparison $^{***}P < 0.001$). 2-way RM ANOVA, 2-way repeated-measures analysis of variance SCI, spinal cord injury.

tract, which reach from their entry point into the spinal cord below injury level all the way to and across the spinal cord injury site. There is a vast body of evidence describing molecular changes in DRG neurons caused by lemniscal tract lesions,[2,35] which not only underlie the poor regenerative capacity of these axons but may also be causally linked to nociceptor hyperexcitability. Macrophage migration inhibitory factor (MIF) released within DRG has been reported to signal nociceptor hyperexcitability after SCI.[6] Whether this a consequence of lemniscal tract injury or related to systemic signals released after SCI irrespective of specific lemniscal tract injury has yet to be determined. A previous study, which would argue against the concept of retrograde signaling along the injured lemniscal tract to trigger NP behavior, reported reduced mechanical allodynia in a rat thoracic lateral hemisection SCI model after subsequent (1 day later) rostral dorsal column (containing the lemniscal tract/central branches of mechanosensory neurons) transection.[26] Of course, animal species are different, a different SCI model and in particular a different approach to eradicate mechanosensory neuron–derived signal transmission was chosen.

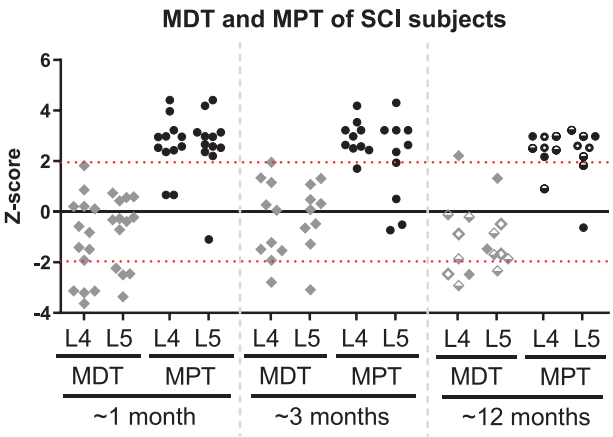

Figure 6. Quantitative sensory testing in SCI subjects. Fourteen sensory incomplete SCI individuals (and 1 initial complete SCI subject) were examined 1, 3, and 12 months post-SCI for mechanical detection threshold (MDT), reflecting Aβ mechanoreceptors, and mechanical pain threshold (MPT), reflecting Aδ nociceptors. SCI individuals were measured at the L4 (right shin) and L5 (dorsum of right foot) dermatomes and found to be consistently altered compared with healthy database matched controls in MPT but not MDT (represented by Z-scores). Red dashed lines visually mark the Z-scores at 1.96 and −1.96, the cutoff for hypersensitivity or hyposensitivity, respectively. In the 12-month timepoint, open symbols represent at-level NP, lower filled symbols represent below-level NP, and fully filled symbols represent no NP. Mean ± SD. NP, neuropathic pain; SCI, spinal cord injury.

Post-SCI mechanoreceptor ablation indicates that specific input transmitted by mechanoreceptors, such as provided by spontaneous locomotion in the cage or augmented by treadmill training, has no effect on NP behavior in SCI mice. Not only are there no major changes in respect to pain behavior compared with nonablated SCI mice, but enhanced peptidergic nociceptor sprouting in the dorsal horn post-SCI was also not altered by mechanoreceptor ablation. Nevertheless, we and others have identified a robust pain relieving effect of sensorimotor activity such as treadmill training.[11,14,34,41] Of course, other pain reducing mediators of activity/exercise have been identified, which do not rely on neural transmission through mechanoreceptors. For example, in a spinal nerve ligation model of NP, endogenous opioid content was increased in the brainstem after treadmill training, whereas such effects were reversed by opioid receptor antagonists.[43] After direct injury to the PNS—spared nerve injury (common peroneal and tibial nerve ligated and cut, sural nerve left intact)—identical transgenic mice, in which TrkB expressing mechanoreceptors were ablated, did not develop pain behavior (mechanical allodynia).[12] Of course, after experimental SCI, there is no direct mechanical injury to the peripheral branch of DRG neurons, which could account for the observed differences.

One week after moderate thoracic contusion SCI in mice, nociceptors were identified to be hyperexcitable in skin-nerve preparations (lower activation thresholds and afterdischarges). These findings are in line with studies in cultured DRG neurons[5,7,8,50] and confirm recent evidence in skin-nerve-DRG-spinal cord preparations taken 1 day post-injury in a moderate mouse contusion SCI model.[15] Moreover, the observation of afterdischarges in nociceptors represents the likely correlate of prolonged hind paw nocifensive behavior (licking, shaking, and holding) in response to lighter von Frey hair filaments in SCI contused mice 7 days post-injury and goes beyond reflexive movements, thus suggesting a supraspinal pain response.

In subjects suffering from subacute traumatic or ischemic SCI, hypersensitivity to mechanical pain stimuli was observed, which in principle is supported by preclinical findings of nociceptor hyperexcitability and dorsal horn nociceptor sprouting. Of course, in human subjects, systematic assessment options are limited, which do not allow to clearly identify neuroanatomical regions (DRG, dorsal horn), where signals for NP are initiated and maintained. The observed hypersensitivity to mechanical pain stimuli was identified in patients with SCI with and without NP. This points again towards co-factors such as the preservation of ascending sensory pathways within the spinal cord, which need to mediate signals generated by hyperexcitable DRG and augmented by dorsal horn nociceptor sprouting to induce NP. Standardized and comprehensive light touch and pinprick sensory examination in all dermatomes according to International Standards for Neurological Classification of SCI (ISNCSCI) in large cohort of traumatic and ischemic patients with SCI observed within the European Multicenter Study about Spinal Cord Injury (EMSCI) together with analysis of pain behavior and histological analysis of sensory tract preservation in a mouse contusion SCI model revealed relative preservation of the spinothalamic tract that predisposes for NP presentation (unpublished observation). This is supported by previous studies, where assessments of sensory function below injury level[19,31,47] and MRI-based structural analyses[37] also point towards preservation of the spinothalamic tract. Of course, with the tools available, it cannot be ruled out that the central mechanisms such as alterations in descending inhibition may have contributed to the observed hypersensitivity.[18,23]

The discrepancy between the results of pinprick examinations according to QST vs ISNCSCI—the majority of SCI subjects score 0 in pinprick examination according to ISNCSCI, whereas MPT Z-scores indicate hypersensitivity towards pin prick stimuli—can be attributed to the intricacies of the pinprick examination according to ISNCSCI.[40] The patient is presented a sharp (pinprick) vs a dull sensory stimulus in a given dermatome. If the subject feels nothing or is not able to discriminate between sharp and dull a score of 0 is applied. Thus, a score of 0 means not necessarily a complete anesthesia related to the applied stimulus within the ISNCSCI examination. If those individuals are exposed to different pinprick stimulus intensities (MPT) irrespective of sharp/dull discrimination capability, they apparently sense the stimulus at a lower threshold compared with healthy subjects.

Taken together, mechanoreceptors are likely involved in the initiation, not the maintenance of NP. Nociceptors are confirmed as the main drivers of NP maintenance, which is highlighted by the hyperexcitability of nociceptors, nociceptor sprouting in the dorsal horn after experimental SCI complemented by nociceptor hypersensitivity in human SCI subjects. Hyperexcitability-inducing factors, either transmitted systemically or retrogradely along injured axons (central branch of mechanoreceptors) into/towards DRG have yet to be defined. According to this understanding, the term "central NP" used to describe SCI-induced NP should be reconsidered. Of course, the initial trigger results from the central nervous system injury. However, the most likely driver of SCI-related NP sits in the peripheral nervous system showing also many similarities to pathophysiological changes observed after direct peripheral nerve injury.

Conflict of interest statement

The authors have no conflicts of interest to declare.

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Data availability statement: The data that support the findings of this study are available from the corresponding author (R.P.) upon reasonable request.

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