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Lack of correlation between spinal microgliosis and long-term development of tactile hypersensitivity in two different sciatic nerve crush injury

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Abstract

Microglia activation following peripheral nerve injury has been shown to contribute to central sensitization of the spinal cord for the development of neuropathic pain. In a recent study, we reported that the amount of nerve damage does not necessarily correlate with chronic pain development. Here we compared the response of spinal microglia, using immunohistochemistry as a surrogate of microglial activation, in mice with two different types of crush injury of the sciatic nerve. We confirmed that incomplete crush of the sciatic nerve (partial crush injury, PCI) resulted in tactile hypersensitivity after the recovery of sensory function (15 days after surgery), whereas the hypersensitivity was not observed after the complete crush (full crush injury, FCI). We observed that immunoreactivity for Iba-1, a microglial marker, was greater in the ipsilateral dorsal horn of lumbar (L4) spinal cord of mice 2 days after FCI compared to PCI, positively correlating with the intensity of crush injury. Ipsilateral Iba-1 reactivity was comparable between injuries at 7 days with a significant increase compared to the contralateral side. By day 15 after injury, ipsilateral Iba-1 immunoreactivity was much reduced compared to day 7 and was not different between the groups. Our results suggest that the magnitude of the early microgliosis is dependent on injury severity, but does not necessarily correlate with the long-term development of chronic pain-like hypersensitivity after peripheral nerve injury.

Keywords

Chronic pain, microglia, neuropathic pain, partial crush injury

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Chronic pain is a common maladaptive response after peripheral nerve injury. Activation of microglia in the dorsal horn of spinal cord is now established as a significant mechanism underlying central sensitization for the development of chronic pain.[1–5] It is widely considered that microglia activation after nerve damage is responsible for neuropathic pain development.[6–9] In our recent study, we found that the degree of nerve damage does not necessarily correlate with chronic pain development: long-term tactile hypersensitivity was induced by an incomplete partial crush injury (PCI), but not by complete full crush injury (FCI) of the sciatic nerve in adult mice.[10] Here we asked whether the activation of spinal microglia might underlie the difference in pain-like behavior between the two crush injury conditions (PCI versus FCI). We examined the time course of spinal microgliosis in the two crush models using immunohistochemistry.

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Male C57BL/6 mice aged over 6 weeks were used for this study. Experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Seoul National University. Full and partial crush injury were performed as previously described.[10] Surgeries were performed in right side of the sciatic nerve under isoflurane anesthesia (2% induction in 100% oxygen maintained at 1.5% using a face mask). The depth of anesthesia was constantly assessed by monitoring the breathing rate and adjusted during surgery accordingly. The sciatic nerve was crushed for 15 s using an ultra-fine hemostat (Cat no. 13020–12, Fine Science Tools). For partial crush in the sciatic nerve, the hemostat was fitted with a custom spacer created from two layers of aluminum foil (15 μm thick) to create 30 μm gap when fully closed. Full sciatic nerve crush was performed in the same way except for closing the hemostat on the third locking position without a spacer. The wound was closed in a suture of the overlying muscle facia and then closed with a skin clip. Aseptic technique was maintained throughout. Mice were recovered in a warm, darkened cage and monitored until regaining conscious and locomotion.

Pinprick sensory recovery testing was performed as previously described.[10,11] Briefly, the lateral side of the affected hind paw was separated into five regions from the toe to the heel and stimulated with a stainless-steel Austerlitz insect pin (Size 000, FST, Germany). A sensory response was confirmed by rapid lifting or flinching of the paw. The number of responses to two consecutive pin applications to the skin was recorded per region providing a score out of 10 (100% response equals to score 10). The pinprick analysis was conducted every two days up to 15 days post-injury (dpi). Mechanical sensitivity was assessed in both ipsilateral and contralateral hind paws at dpi 15, when sensory response to pin prick stimuli is fully recovered in both groups, using a series of von Frey filaments. The 50% withdrawal threshold was determined using the 'up-down' method.[12,13] All behavioral testing were performed by an investigator who was blind to the injury group.

In all mice used for immunohistochemical analysis, we performed pinprick tests on dpi 1 to confirm the surgery as described above. For dpi 15 samples for IHC analysis, we measured the tactile hypersensitivity with von Frey test before the sampling. The mice were then perfused with PBS and then fixed with 4% paraformaldehyde transcardially after terminal anesthesia with pentobarbital (>150mg/kg). L3-5 spinal segments were isolated,[14] and then protected in 30% sucrose solution for frozen tissue section. Spinal cords were sliced into 30 μm sections and every third sections were collected for the staining steps using free-floating methods, as previously described.[15–17] For the staining procedure, the spinal cord sections were washed with PBS several times and pre-incubated with 5% normal donkey serum in PBS containing 0.3% Triton-x (PBST) for 1 h before the antibody application. Primary antibody against ionized calcium binding adaptor molecule-1 (Iba-1) (1:1,000; Cat no. 019–19741, Wako) was diluted in 1% normal donkey serum and incubated overnight at 4 °C. Donkey anti-rabbit-Cy3 (1:200; Cat no. 711–165-152, Jackson laboratory) secondary antibody was diluted in 1% normal donkey serum and incubated for 1 h at room temperature (RT). The stained sections were then mounted on a slide glass and covered with coverslips after applying anti-fade mounting medium (Cat no. H-1000, Vector) for image analyses. After visual assessment, three sections with the brightest fluorescence that have L4 spinal anatomy were selected for further analyses.[18,19] Fluorescence images (512×1024) were acquired on a laser-scanning confocal microscope (LSM 700 Zeiss) with Zen 2010 software (v8.1 SP1, Zeiss). Five z-stack images (2 μm interval) were merged as a maximum projection for the analysis. Ipsilateral and contralateral spinal dorsal horn areas were selected from each image by referring to the accompanying phase image and the mean Iba-1 fluorescence intensity was analyzed using ImageJ software. Both image acquisition and the analysis were performed by an investigator who was blind to the injury group. The mean fluorescence intensity from three sections was calculated for each animal. Individual data points represent a single animal and data are presented as mean +/− standard error of the mean. Two-group analyses were performed by Student's t-test; behavioral time course data were analyzed by either two-way or one-way ANOVA; spinal Iba1 immunoreactivity intensities were compared by one-way ANOVA using Prism 5 (GraphPad).

We measured pinprick responses in the affected hind paw after FCI and PCI to test sensory function every two days (Figure 1(a)). One day after crush injury, the pinprick response score was zero in FCI, whereas sensory function remained partially intact in the PCI group due to the incomplete crush injury in the sciatic nerve. The pinprick response began to recover from 7 days post-injury (dpi) and reached full recovery of pinprick response at dpi 15 in FCI. The PCI group also took 15 days for full recovery. This observation confirms our previous report.[10] We next measured withdrawal thresholds to tactile stimuli applied to the hind paws at dpi 15, when pinprick sensory recovery is complete (Figure 1(a)). Compared to FCI, the PCI group displayed greater tactile hypersensitivity in the ipsilateral hind paw (dpi 15, Full ipsi × Partial ipsi, $P = 0.0001$) (Figure 1(b)) which is consistent with our previous report.[10] Also, when compared to the baseline threshold, the PCI group developed tactile hypersensitivity in the ipsilateral hind paw at dpi 15 whereas FCI did not (Figure 1(b)). Both groups showed a significant decrease

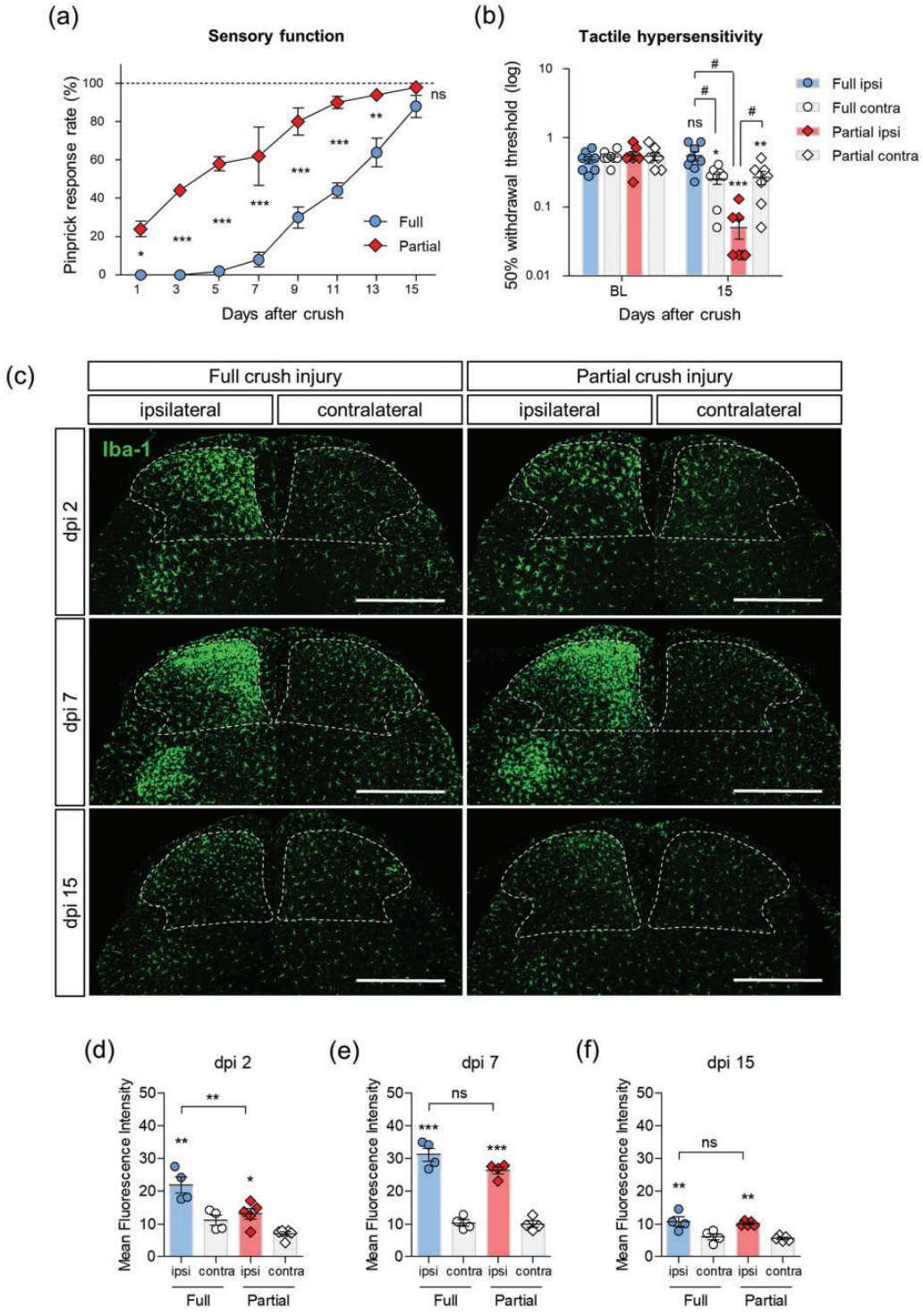

Figure 1. (a) Pinprick response score measured every 2 days after crush injury. Repeated measures of Two-way ANOVA: Effect of surgery, $F(1, 56) = 183.95$, P value < 0.0001 $n = 5$ mice per group. Bonferroni post-test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ ($t = 3.054 \sim 7.126$). ns; $P > 0.05$ ($t = 1.23$). Data are presented as means ± SEM. (b) 50% paw withdrawal threshold measured before the surgery and on dpi 15 in FCI and PCI groups in both hind paws. Repeated measures of One-way ANOVA between the time points. Tukey's multiple comparison test, ns > 0.05; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Paired Student's t-test (Full ipsi x contra: $P = 0.0254$; Partial ipsi x contra: $P = 0.0127$) and unpaired Student's t-test (Full ipsi x Partial ipsi: $P = 0.0001$), $^{\#}P < 0.05$. Data are presented as means ± SEM. Each data point represents a single animal. FCI $n = 8$, PCI $n = 7$. (c) Immunofluorescence of Iba-1 in spinal dorsal horn (L4) after FCI and PCI. White dashed lines indicate regions of interest for image analyses. Scale bars, 500 μm. Quantitative analyses of Iba-1 immunofluorescence intensity within the ipsi- and contralateral dorsal horn at (d) dpi 2, (e) dpi 7 and (f) dpi 15. One-way ANOVA. Tukey's multiple comparison test, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Data are presented as means ± SEM. A dot represents an animal. $n = 4 \sim 5$ each.

in the mechanical threshold in the contralateral side after injury compared to baseline, which was not different between two groups (dpi 15, Full contra x Partial contra, $P = 0.8832$) (Figure 1(b)).

We next assessed the fluorescence intensity of immunoreactivity against Iba-1 in the dorsal horn of L4 lumbar spinal cord as a surrogate of microglial activation at three time points after injury in the two models. These time points were chosen according to the behavior phenotypes: dpi 2 (early time), dpi 7 (end of the axonal degeneration) and dpi 15 (full recovery of sensory function, development of tactile hypersensitivity) (Figure 1 (c)). The expression of Iba-1 in the ipsilateral dorsal horn was significantly upregulated 2 days after FCI and PCI when compared to contralateral dorsal horn; however, Iba-1 reactivity in the FCI group was significantly greater than the PCI group (Figure 1(d)). The Iba-1 immunoreactivity was the most prominent at dpi 7 and was comparable in both groups with a significant increase in intensity compared to the contralateral side (Figure 1(e)). At dpi 15, Iba-1 immunoreactivity was reduced, although still significantly higher than the contralateral side in both groups (Figure 1(f)).

Our results show that initial microglial reactivity in the spinal cord is more prominent after more severe nerve damage (FCI) compared to an incomplete crush injury (PCI), despite the fact that the PCI group developed greater mechanical sensitivity - a surrogate of neuropathic pain in mice. Although some studies have reported graded nerve constriction injury influences the extent of spinal glial reactivity,[20,21] and numerous studies support that microglia function is required for pain development,[22–24] the correlation of the magnitude of spinal microglial reactivity with pain behavior is less clear.[21,25,26] Our observations from two crush models add to growing evidence that early and predominant microgliosis is neither necessary nor sufficient for neuropathic pain-like behavior in mice.[5] Nevertheless, the role of microglial function in the development of neuropathic pain after PCI requires further investigation.

Spinal microgliosis after peripheral nerve injury is considered a transient event, which increases in few days after the injury and the maximal activity is observed within a week in various animal models of neuropathic pain.[5,27,28] The peripheral nerve injury models that are commonly used for studying chronic pain in rodents typically develop pain within a week after nerve injury.[29–31] As a consequence, the linkage between chronic pain and spinal microglia activation has been studied at relatively early time points (within 2 weeks post-injury).[6,32,33] The partial crush injury, representing a novel animal model of neuropathic pain, showed maximal microgliosis at dpi 7, which significantly decreased at dpi 15. The observation is consistent with other previous peripheral nerve injury (PNI) models.[27,34] However, unlike other PNI models with pain, the PCI model develops pain after the sensory function is recovered (dpi 15). This may imply that the spinal microgliosis is rather injury-dependent than being associated with long-term development of neuropathic pain.

From our immunohistochemical images (Figure 1(c)), we consistently observed the Iba-1 reactivity in the ventral horn where the cell bodies of motor neurons exist, and the pattern of the microgliosis in the ventral horn was similar to that of the dorsal horn. Given that the sciatic nerve contains sensory afferents and motor efferents, our results may also suggest the response of microglial reactivity is an injury-dependent event, irrespective of nerve types. However, as it has been shown that dysfunctions of motor neurons elicit pain behavior,[35,36] further investigation is required whether microglial activity in the ventral horn is indeed involved in the development of neuropathic pain.

Microglial activity is considered as a key mediator of sex-dimorphism in pain,[37] and the activation of spinal microglia after nerve injury plays a limited role in female mice, unlike in the male mice.[38–41] Interestingly, recent studies have reported that Iba-1 immunoreactivity is not different between male and female mice after peripheral nerve injury,[5,39,40] suggeting the limited involvement of microgliosis in the generation of neuropathic pain. Although it remains to be tested in the crush models, our finding seems to be in line with these reports.

It has been reported that the activation of spinal microglia not only modulates neuronal synapses but also augments astrocyte activity.[7,42–44] Whether the subsequent activation of astrocytes after microglia activation is also injury-dependent or not is worthy of further investigation. Comparing the time-dependent changes of astrocyte activation in the spinal cord between the FCI and PCI groups may provide clues as to whether astrocytes are functionally relevant to pain independently of microglial reactivity.

Our results suggest that while spinal microgliosis is injury severity-dependent, it does not necessarily correlate with long-term development of neuropathic pain. A small number of clinical studies have tested compounds that interfere with glial function for neuropathic pain,[45] however microglial modulatory agents are yet to show robust or consistent results in trials.[46–49] Greater understanding of the time-dependence of microglial contributions to persistent pain may improve the translation of pre-clinical animal studies on microglia as targets in chronic pain.[50] The ability to induce contrasting neuropathic pain phenotypes despite a similar nerve injury makes the PCI model useful in the optimization of drug administration relative to the injury and for further validation of the molecular mechanisms of microglia activity in chronic pain.

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Author Contributions

HWK and SBO designed the experiment and wrote the manuscript. HWK and CHW have performed the experiments and analysis.

Declaration of Conflicting Interests

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