Temporal Kinetics of Microgliosis in the Spinal Dorsal Horn after Peripheral Nerve Injury in Rodents

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Neuropathic pain, a highly debilitating chronic pain following nerve damage, is a reflection of the aberrant functioning of a pathologically altered nervous system. Previous studies have implicated activated microglia in the spinal dorsal horn (SDH) as key cellular intermediaries in neuropathic pain. Microgliosis is among the dramatic cellular alterations that occur in the SDH in models of neuropathic pain established by peripheral nerve injury (PNI), but detailed characterization of SDH microgliosis has yet to be realized. In the present study, we performed a short-pulse labeling of proliferating cells with ethynyldeoxyuridine (EdU), a marker of the cell cycle S-phase, and found that EdU+ microglia in the SDH were rarely observed 32h after PNI, but rapidly increased to the peak level at 40h post-PNI. Numerous EdU+ microglia persisted for the next 20h (60h post-PNI) and decreased to the baseline on day 7. These results demonstrate a narrow time window for rapidly inducing a proliferation burst of SDH microglia after PNI, and these temporally restricted kinetics of microglial proliferation may help identify the molecule(s) that causes microglial activation in the SDH, which is crucial for understanding and managing neuropathic pain.

Key words: proliferation; microgliosis; spinal dorsal horn; neuropathic pain; peripheral nerve injury

Neuropathic pain is a debilitating chronic pain state caused by damage to the nervous system incited by cancer, diabetes, chemotherapy and trauma. One of the hallmark symptoms is mechanical allodynia (non-nociceptive mechanical stimuli elicit pain), and neuropathic pain is often resistant to currently available therapies. Injury to peripheral nerves of rodents, which are frequently used as models of neuropathic pain, produces mechanical pain hypersensitivity and alterations at molecular and cellular levels that result in multiple forms of neuronal plasticity and structural reorganization, not only in the affected sensory ganglion cell body, but also in the spinal dorsal horn (SDH). The peripheral nerve injury (PNI)-induced alterations in the SDH are observed not only in neurons, but also in glial cells, especially microglia, which are known as resident immune cells in the central nervous system (CNS). Following PNI, SDH microglia become activated through a progressive series of changes in their morphology, expression of a variety of genes and cell number.

One of the most prominent features of microglial activation is an increase in the number of microglia (called microgliosis). Since PNI-induced microgliosis in the SDH was recorded in 1970s and the rodent models of neuropathic pain were established in 1990s, studies have investigated the mechanism for microgliosis in the SDH after PNI. Two possible mechanisms (proliferation of resident microglia and infiltration of bone marrow-derived monocytes) have been considered, but it is now thought that local expansion of resident microglia by proliferation is the primary cellular basis for SDH microgliosis after PNI. However, in stark contrast to the characterization of temporal and spatial changes in the immunostaining levels of microglial markers (CD11b or ionized calcium-binding adapter molecule 1 (Iba1)) in the SDH that have so far been extensively studied, the temporal kinetics of microglial proliferation after PNI have yet to be fully characterized. While recent studies reported an involvement of colony-stimulating factor 1 (CSF1) expressed in injured dorsal root ganglion (DRG) neurons in the PNI-induced proliferation, the expression of CSF1 in DRG neurons (and also its receptor CSF1R in microglia) is upregulated for a few weeks after PNI when microglial proliferation has already terminated. Thus, determining the kinetics of microglial proliferation after PNI would provide an important clue to identify a molecule(s) for the PNI-induced proliferation of SDH microglia. Because a recent study has demonstrated that interrupting spinal microgliosis suppresses PNI-induced mechanical pain hypersensitivity and provided evidence that it is a crucial step in neuropathic pain, the kinetics of microglial proliferation after PNI would also provide valuable information for developing a new therapeutic strategy.

In the present study, we fully characterized the kinetics of microglial proliferation in the SDH after PNI and identified for the first time an unexpected narrow time window to rapidly induce a proliferation burst of SDH microglia after PNI.

MATERIALS AND METHODS

Animals Male C57BL/6 mice (Clea, Japan) and male Wistar rats (Japan SLC, Japan) were used. All mice and rats were aged 8–12 weeks at the start of each experiment, and were housed at 22±1°C with a 12-h light–dark cycle. All animals were fed food and water ad libitum. All animal experiments were conducted according to relevant national and international guidelines contained in the ‘Act on Welfare and Management of Animals’ (Ministry of Environment of Japan)

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Peripheral Nerve Injury  We used the spinal nerve injury model as described previously. Under isoflurane [2% (v/v)] anesthesia, a small incision at L3–S1 was made. Paraspinous muscle and fat were removed from the L5 (mice) and L6 (rats) traverse process, and the part of this traverse process was removed to expose the parallel-lying L3/4 (mice) and L4/5 (rats) spinal nerves. The L4 nerve was carefully isolated and cut (mice). In rats, isolated L5 nerve was tightly ligated by a 5-0 silk suture and cut just distal to the ligature. The wound and the surrounding skin were sutured with 5-0 silk and closed with staples. As a control in mice, sham operation was performed by exposing and drilling the root of the transverse process of the lumbar vertebra without removing the process to keep the L4 spinal nerve intact.

Immunohistochemistry  Mice and rats were deeply anesthetized by intraperitoneal (i.p.) injection of pentobarbital and perfused transcardially with phosphate-buffered saline (PBS; composition in mM: NaCl 137, KCl 2.7, KH₂PO₄ 1.5, NaH₂PO₄ 8.1; pH 7.4), followed by ice-cold 4% paraformaldehyde/PBS. The L4 (mice) or L5 (rats) segments of the spinal cord were removed, postfixed in the same fixative for 3 h at 4 °C, and placed in 30% sucrose solution for 24 h at 4°C. Transverse L4 spinal cord sections (30 µm) were incubated in blocking solution (3% normal goat serum) for 2 h at room temperature and then incubated for 48 h at 4°C with primary antibodies: rabbit polyclonal anti-Iba1 (1:2000, Wako, Japan), rabbit monoclonal anti-PU.1 (1:1000, Cell Signaling, U.S.A.), and rat monoclonal anti-glial fibrillary acidic protein (GFAP; 1:2000, Invitrogen, U.S.A.). Following incubation, tissue sections were washed and incubated for 3 h at room temperature with secondary antibodies conjugated to Alexa Fluor 546, Alexa Fluor 488 (1:1000, Molecular Probes, U.S.A.) or CF405 (1:1000, Bio- tium, U.S.A.). The tissue sections were washed, slide-mounted

Fig. 1. The Longitudinal Time–Course of Microgliosis after PNI in Mice
(A) Immunolabeling of PU.1 in the SDH before and after PNI. (B) The time course of changes in the density of microglial cells in the SDH ipsilateral and contralateral to PNI (n=3 mice; ****p<0.0001). Scale bar: 200µm (A). (Color figure can be accessed in the online version.)

Fig. 2. Microglial Proliferation in Early and Late Phases after PNI in Mice
(A) Schematic timeline for intraperitoneal injection of EdU (50 mg/kg) and fixation. (B) The numbers of EdU⁺ cells, EdU⁺ Iba1⁺ cells, EdU⁺ GFAP⁺ cells, and EdU⁺ Iba1⁻ GFAP⁻ cells in the SDH sections from mice treated EdU during the early or late phase after PNI (n=5 mice; ****p<0.0001). (Color figure can be accessed in the online version.)
and subsequently coverslipped with Vectashield hardmount (Vector Laboratories, U.S.A.). To visualize proliferating cells in mice, ethynyldeoxyuridine (EdU; 50 mg/kg; Invitrogen) was injected i.p. to mice. EdU is a thymidine analog that is incorporated into newly synthesized DNA of the cells undergoing S-phase (DNA synthesis phase) of the cell cycle. For the repeated injection, mice were injected with EdU once a day for 4 d (days 0 to 3 or days 4 to 7 after PNI) and were fixed at day 7. For a short-pulse labeling of proliferating cells, mice were injected with a single dose of EdU (50 mg/kg) 2 h prior to fixation at 0, 24, 32, 40, 48, 60, 72, 96, 168, and 336 h after PNI. Spinal sections immunolabeled by Iba1 were used for EdU staining using Click-iT EdU (Invitrogen) according to manufacturer’s protocol. For experiments using rats, bromodeoxyuridine (BrdU, another thymidine analog that is also incorporated into DNA of the cells undergoing S-phase; 75 mg/kg, Sigma) was injected i.p. into rats 2 h prior to fixation at 0, 24, 28, 32, 36, 48, 60, 72, 168, and 336 h after PNI.

Fig. 3. Detailed Kinetics of Microglial Proliferation in the SDH after PNI in Mice
(A) The representative images of EdU and Iba1 immunofluorescence in the SDH before and after PNI. (B) The time-course of EdU+ Iba1+ or EdU+ Iba1− cells quantification in the SDH ipsilateral and contralateral to PNI (n=3 mice; ****p<0.0001 vs. EdU+ Iba1−; *p<0.05, ****p<0.0001 vs. contralateral side at the corresponding time point). Scale bar: 200 µm (A).
Fixed spinal sections (30 µm) were incubated with 2 N HCl to denature DNA followed by incubation with 0.1 M boric acid. After a rinse with PBS, sections were incubated with the primary antibody for BrdU (anti-BrdU, 1:2000, Chemicon) and the marker of microglia Iba1 (1:2000, Wako).

The images were captured by using a confocal laser scanning microscope (LSM700, Carl Zeiss, Oberkochen, Germany) and 3–5 sections from the L4 and L5 spinal cord segments of each mouse and rat, respectively, were randomly selected and analyzed using ImageJ software. For quantification of the number of microglial PU.1+ nucleus in the SDH, images were converted to binarized images by setting a threshold for fluorescence intensity (10000:2 times higher than that for background levels). Background levels were obtained from an area in the dorsal horn of the same section where immunoreactive cells are not contained. The numbers of particles were counted using the Analyze Particles function. The region of interest (ROI) was made by drawing the boundary between gray and white matter of the SDH based on the differential interference contrast (DIC) image. The number of Iba1+ microglia or GFAP+ astrocytes with clear cell bodies and EdU+ and BrdU+ nuclei with an S/N ratio of 2.0 or more were counted. For morphological change analysis, single microglia images with 40–60 stacks were obtained (7 cells from 6–7 slices from 3 mice). The topological skeletonized images of each microglia were made by tracing microglial processes using the Simple Neurite Tracer software program plug-in. To count branch number and total length of microglial processes, skeletonized images were analyzed by using the Analyze Skeleton function. For quantification of the morphological complexity, Sholl Analysis was performed on Z-projection images of skeletonized microglial processes. The number of processes that intersected the concentric circles that were spaced 5 µm apart originating from center of the soma were counted.

Fig. 4. Proliferation Kinetics of SDH Microglia after PNI in Rats

The time-course of BrdU+ cells quantification in the SDH ipsilateral and contralateral to PNI (n=3–4 rats; *p<0.05, ****p<0.0001). Inset: the percentage of BrdU+ Iba1+ cells per total BrdU+ cells in the ipsilateral SDH at 32, 36, 48 and 60 h after PNI.

Fig. 5. Morphological Alterations of SDH Microglia after PNI in Mice

(A) Upper: Z-Projection images of microglia in the SDH before and after PNI. Lower: The topological skeletonized images from stacked images of a single microglial cell. (B, C) The number of branches (B) and total length (C) of microglial processes (n=7 cells from 3 mice; *p<0.05, **p<0.01, ***p<0.001). (D) Sholl analysis plot of microglia in the SDH (n=7 cells from 3 mice; *p<0.05, ****p<0.0001). Scale bar: 20 µm (A). (Color figure can be accessed in the online version.)
Statistical Analysis  All data are expressed as the mean±standard error of the mean (S.E.M.) and were analyzed by the two-way ANOVA with post hoc Tukey’s test (Figs. 1B, 2B, 3B, 4). Student’s t test (Figs. 5B, C), or two-way repeated measures ANOVA with post hoc Tukey’s test (Fig. 5D). Statistical analysis was performed using GraphPad Prism 7 software. Values were considered significantly different at p<0.05.

RESULTS

To longitudinally analyze the number of microglia in the SDH after PNI in mice, we immunolabeled the nucleus of individual microglial cells by using PU.1, a microglia-specific transcription factor in the CNS21,22) (Fig. 1A). By counting the number of PU.1-immunoreactive signals in the SDH, we found that PU.1+ SDH microglia were markedly increased at day 3 post-PNI, peaked at day 7 and gradually decreased (Fig. 1B). However, compared with that of the contralateral side, a significantly higher number of microglia in the ipsilateral SDH was observed even at day 56.

Because the peak of PNI-induced increase in SDH microglia was observed at day 7 post-PNI, proliferation of SDH microglia could occur within the first 7 d. To examine its temporal pattern, we visualized proliferating cells in the SDH by using EdU, a marker of the S-phase of the cell cycle. We divided 7 d into two phases (the first 3 d as early phase, and the next 4 d as late phase), administered EdU to mice once a day during each phase, and fixed at day 7 (Fig. 2A). In the SDH of mice with EdU injected during the early phase, there were many strong EdU+ cells in the SDH ipsilateral to the PNI, compared with that in the contralateral SDH (Fig. 2B). Triple immunolabeling with Iba1 and GFAP (markers of microglia and astrocytes, respectively) confirmed that almost all EdU+ cells in the SDH were microglia and a small portion of remaining EdU+ cells was GFAP+ astrocytes (Fig. 2B). By contrast, in the SDH of mice with EdU during the late phase, the number of EdU+ microglia was much smaller. These results indicate that a wave of microglial proliferation occurs during the first 3 d after PNI.

To determine the detailed temporal kinetics of the proliferation, we injected a single dose of EdU into mice at 8-h intervals from 22 h after PNI and, 2 h later, fixed the EdU-injected mice. There were few EdU+ cells in the SDH in mice fixed at 32 h after PNI, but the number of EdU+ cells rapidly increased in mice fixed at 40 h (Fig. 3A). The peak was also observed at this time point (40 h), and numerous EdU+ cells persisted for the next 20 h (60 h after PNI). EdU+ cell number declined from 72 h post-PNI and returned to the control level over the next 4 d (7 d after PNI). No significant change in the number of EdU+ microglia was observed at 7 and 14 d after PNI (Figs. 3A, B). The number of EdU+ microglia was not altered in the contralateral SDH (Figs. 3A, B), and there were few EdU+ Iba1− cells at all time-points tested (Fig. 3B). These results indicate that PNI-induced microgliosis is derived by a single wave of proliferation starting from 40 h post-PNI.

To exclude the possibility that this characteristic proliferation kinetics of SDH microglia after PNI is dependent on our experimental condition including regents and animal species, we also performed a similar short-pulse labeling by BrdU, another proliferating marker, in rats. The number of BrdU+ microglia was rapidly increased from 32 h post-PNI, peaked at 36 h, and persisted for at least the next 12 h (48 h post-PNI). The proliferating cells declined and returned to the control level over the next 4 d (7 d after PNI). Double-immunolabeling with Iba1 showed that almost all BrdU+ cells during 32–60 h post-PNI were microglia. In the contralateral side of the SDH, there were few BrdU+ microglia in all time point tested (Fig. 4). These results suggest that rapid proliferation burst of SDH microglia may be commonly induced following PNI.

From our previous report23) which longitudinally measured mechanical pain hypersensitivity after PNI, the observed PNI-induced proliferation burst could occur during the initiation phase of pain. Nevertheless, the paw withdrawal threshold had already decreased at day 1 post-PNI23) when microglial proliferation had not yet started. To further characterize microglial changes during the first 24 h after PNI, we performed individual cell-by-cell morphological analysis of SDH microglia (Fig. 5A). At 24 h post-PNI, the number of branches of microglial processes was decreased (Fig. 5B), and the length of processes was shortened compared with those in sham-operated mice (Fig. 5C). These changes were also observed at 12 h after PNI (Figs. 5A–C). Sholl analysis, a method to measure the process complexity of microglia,24) showed that PNI simplified microglial processes within the first 12 h (Fig. 5D). Therefore, these results suggest that SDH microglia might respond to PNI at a relatively early time point after the injury.

DISCUSSION

In this study, we examined the full temporal kinetics of proliferation of SDH microglia after PNI, which had remained to be determined, although the temporal pattern of the staining levels of microglial markers (such as CD11b and Iba1) has been extensively studied.4) The time course of the PNI-induced SDH microgliosis in mice was revealed to be that microglia number increased from day 3, peaked at 1 week and declined later, which is almost similar to the temporal pattern of microgliosis reported in previous studies.11,22,26) Under such conditions, we clearly showed that a proliferation burst of SDH microglia occurred during the early phase (the first 3 d after PNI) but not during the later phase (at least until 2 weeks post-PNI). The early phase proliferation is consistent with several previous papers.11,13) but the later phase is controversial. It has previously been described that newly generated SDH microglia in response to PNI in rats continued to divide for at least 14 d after the injury.27) Conversely, in a recent report using transgenic mice that microglia are genetically labeled by fluorescent proteins, SDH microglia in the late phase after PNI have been shown to be negative to Ki-67,19) a marker of all stages of the cell cycle except the resting one. Although the difference in the late phase could be due to the animal species used in these studies (rat vs. mouse), we also demonstrated a robust and rapid proliferation of SDH microglia with similar kinetics after PNI in rats. Thus, it seems that PNI induces a temporally restricted proliferation of SDH microglia that occurs during the 3 d after the injury.

The most notable finding in this study is the determination of the detailed temporal kinetics of microglial proliferation. Based on microglial proliferation during the early phase, we performed a short-pulse labeling of proliferating cells by using EdU and demonstrated for the first time an unexpected rapid induction of proliferation of SDH microglia after PNI.
during a short time period (32–40h after PNI). In rats, we also demonstrated almost identical temporal dynamics of microglial proliferation by PNI. Thus, we identified a starting time point for inducing the rapid proliferation burst of SDH microglia after PNI. The mechanism underlying such a temporally restricted robust proliferation of microglia remains to be determined, but one candidate could be CSF1 derived from injured DRG neurons.\textsuperscript{13,14} Indeed, CSF1 mRNA is induced day 1 in DRG neurons after PNI, which seems to temporally coincide with proliferation of SDH microglia. Furthermore, a conditional knockout of CSF1 in DRG neurons suppressed the PNI-induced microglial proliferation.\textsuperscript{13} Conversely, intrathecal administration of CSF1 to normal mice induces proliferation of microglia.\textsuperscript{13,14} These data suggest a necessity and sufficiency of CSF1 for microglial proliferation. However, it should be noted that upregulation of CSF1 in DRG neurons and its receptor CSF1R expressed in microglia are not only observed at day 1 after PNI but also persist for a few weeks later\textsuperscript{13,14} when microglial proliferation has already terminated as shown in this study and others.\textsuperscript{1,13,15} Thus, CSF1 critically contributes to the PNI-induced microglial proliferation, but it might not be the sole factor. Therefore, our present findings provide an important clue for future investigations to identify the molecule(s) that induces the temporally restricted dramatic proliferation burst of SDH microglia after PNI.

Correlating with behavioral pain hypersensitivity after PNI,\textsuperscript{23} the duration of microglial proliferation corresponded to an initiation phase of neuropathic pain. Pain hypersensitivity was already initiated on day 1 when microglia had not proliferated. However, SDH microglia seemed to have already responded, because morphology of microglia changed during the first 24h: the processes of SDH microglia were retracted, and their complexity became simplified. A recent study has reported that long-term exposure (1 h) of rats to the volatile anesthetic isoflurane shortens microglial processes,\textsuperscript{13} but under our experimental condition in which mice were anesthetized by isoflurane for a short period (approximately 15 min), the process morphology of SDH microglia was indistinguishable between sham-operated mice and naïve mice with or without anesthesia, respectively. Thus, it seems that microglia could receive a signal(s) presumably from DRG neurons at least 12 h after PNI and change their phenotype from normal surveillance to reactive. Whether the alteration in the process morphology of SDH microglia contributes to subsequent proliferation and neuropathic pain induction remains unknown.\textsuperscript{13} Given that PNI-induced pain hypersensitivity is blunted in mice lacking a molecule (P2Y12 receptor or transmembrane protein 16F) that regulates the shape of microglial processes,\textsuperscript{29,30} it is possible that the first morphological change in SDH microglia by PNI may also have a role in subsequent microglial activation and neuropathic pain.

After proliferation, microglial number dramatically increased: it became approximately 4-fold higher. This implies that microglia could proliferate multiple times during the first 3 d. Alternatively, it is also possible that microgliosis could be due to migration of SDH microglia from neighboring spinal segments (e.g., rostral L3 and caudal L5 segments) because microglia show chemotaxis.\textsuperscript{31,32} However, infiltration of circulating monocytes in the SDH,\textsuperscript{12} which is reported to express Iba1, does not contribute to the PNI-induced SDH microgliosis, since recent studies using bone marrow chimeric mice subjected to mild irradiation, parabiosis mice and double-transgenic mice (enable distinct visualization of resident microglia and circulating monocytes) demonstrated no evidence for the involvement of circulating monocytes.\textsuperscript{4,10,11}

Numerous microglia induced by PNI gradually returned to pre-PNI levels, the time–course of which is similar to a recovery of PNI-induced pain hypersensitivity.\textsuperscript{23} Interestingly, SDH microglia still retained a significantly high cell number even about 2 months after the injury when pain hypersensitivity was fully recovered. The role of microglia in this stage remains unknown, but cholinergic immunoprecipitation (ChIP)-sequencing analysis has revealed that several putative latent enhancer regions in close proximity to transcriptionally-regulated microglial genes are maintained one month after PNI,\textsuperscript{33} raising the possibility that spinal microglia may retain a memory of past activation by PNI at a molecular level. How this genetic ‘memory’ in activated microglia affects their function and neuropathic pain remains unknown, but will be important in future research because a recent study in the facial motor nucleus found that some microglia proliferated by PNI persist for approximately two months post-PNI.\textsuperscript{34}

In summary, our longitudinal and detailed analyses of SDH microgliosis after PNI further characterized a series of their activation. In particular, by using short-pulse labeling of proliferating cells, this study identified for the first time a narrow time window to induce a proliferation burst of SDH microglia after PNI. Because microglial proliferation is a crucial step for generating reactive microglia and neuropathic pain, our findings provide an important clue for both the future identification of molecules that govern the temporally restricted dramatic proliferation burst of SDH microglia after PNI and the development of strategies for controlling microglial activation and neuropathic pain.

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Conflict of Interest The authors declare no conflict of interest.

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