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Development and persistence of neuropathic pain through microglial activation and KCC2 decreasing after mouse tibial nerve injury

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Abstract (250 words)

Gamma-amino butyric acid (GABA) is an inhibitory neurotransmitter in the mature brain, but is excitatory during development and after motor nerve injury. This difference in GABAergic action depends on the intracellular chloride ion concentration ($[Cl^-]_i$), primarily regulated by potassium chloride co-transporter 2 (KCC2). To reveal precise processes of the neuropathic pain through changes in GABAergic action, we prepared tibial nerve ligation and severance models using male mice, and examined temporal relationships amongst changes in (1) the mechanical withdrawal threshold in the sural nerve area, (2) localization of the molecules involved in GABAergic transmission and its upstream signaling in the dorsal horn, and (3) histology of the tibial nerve. In the ligation model, tibial nerve degeneration disappeared by day 56, but mechanical allodynia, reduced KCC2 localization, and increased microglia density remained until day 90. Microglia density was higher in the tibial zone than the sural zone before day 21, but this result was inverted after day 28. In contrast, in the severance model, all above changes were detected until day 28, but were simultaneously and significantly recovered by day 90. These results suggested that in male mice, allodynia may be caused by reduced GABAergic synaptic inhibition, resulting from elevated $[Cl^-]_i$ after the reduction of KCC2 by activated microglia. Furthermore, our results suggested that factors from degenerating nerve terminals may diffuse into the sural zone, whereby they induced the development of allodynia in the sural nerve area, while other factors in the sural zone may mediate persistent allodynia through the same pathway.

Highlights

- Tibial nerve injury induced allodynia, KCC2 reduction, and microglial activation.
- Above changes remained after ligation but were recovered 90 days after severance.
- Microglia density was high in tibial zone until D21 but in sural zone after D28.
- Allodynia may be commonly caused by a reduction of GABAergic synaptic inhibition.
- Development and persistence of allodynia may be induced by different mechanisms.

Key words; mechanical allodynia, microglia, nerve degeneration, nerve ligation, nerve severance, potassium chloride co-transporter 2 (KCC2)

Abbreviations

ABC, avidin-biotin-peroxidase complex; BDNF, brain derived neurotrophic factor; CCL, chemokine ligand; CSF-1, colony stimulating factor-1; Cl^-, chloride ion; $[Cl^-]_i$, intracellular chloride ion concentration; D, day after operation; GABA, gamma-amino butyric acid; GAD, glutamic acid decarboxylase; Iba1, ionized calcium binding adaptor molecule1; KCC2 potassium chloride co-transporter 2; P2X4R, purinoceptor 4 receptor; PB, phosphate buffer; SEM, standard error of mean; VGAT, vesicular GABA transporter; VR1 vanilloid receptor 1.

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Declaration of interest

None.

Author contribution

The majority of experiments and construction of figure panels and tables were performed by YK. Histological studies were assisted by TY, CS-O, JK, and SK. EM analysis was assisted by NO. Statistical analysis was assisted by JK, SK, and AO. The manuscript was written by YK and CT, and checked by all authors.

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1. Introduction

In the mature central nervous system, γ-amino butyric acid (GABA) is a predominant inhibitory neurotransmitter. GABA is synthesized by glutamic acid decarboxylase (GAD), packaged into vesicles by vesicular GABA transporter (VGAT), and then released from axon terminals, and whereby it binds to post-synaptic GABA receptors. Influx of chloride ion (Cl^-) through activated $GABA_A$ receptors mediates the hyperpolarization of membrane potential. Thus, GABA negatively regulates neuronal activity (Macdonald and Olsen, 1994; Olsen and Tobin, 1990) and play important roles, such as preventing seizures (Kardos, 1999; Olsen and Avoli, 1997) and modulating anxiety (Nutt et al., 1990; Pratt, 1992). In contrast, GABA mediates depolarization of membrane potential in the immature central nervous system (Ben-Ari, 2002; Ben-Ari et al., 2007; McCarthy et al., 2002; Owens and Kriegstein, 2002; Represa and Ben-Ari, 2005) and in motor nuclei after efferent nerve injury (Nabekura et al., 2002; Toyoda et al., 2003), and thus may be involved in morphogenesis and regeneration, respectively. Differences in GABAergic action depend on the intracellular Cl^- concentration ($[Cl^-]_i$), which is regulated by the balance of two transporters, sodium potassium chloride co-transporter 1 and potassium chloride co-transporter 2 (KCC2) (Ben-Ari, 2002; Owens and Kriegstein, 2002; Payne et al., 2003). As expression of KCC2, which excludes Cl^- out of cells, frequently changes during development and after nerve injury, KCC2 may play a key role in the fine tuning of $[Cl^-]_i$, and primarily regulate the action of GABA (Hubner et al., 2001; Lee et al., 2005; Mahadevan and Woodin, 2016; Rivera et al., 2005).

Neuropathic pain, including chronic lumbar pain, is a serious problem worldwide (Bouhassira and Attal, 2016; Breivik et al., 2006; Toth et al., 2009). To establish treatments for neuropathic pain, various possible mechanisms have been discussed (Cohen and Mao, 2014; Jensen and Finnerup, 2014; Kahle et al., 2014; St John Smith, 2018; Starowicz and Przewlocka, 2012). After peripheral nerve injury, number of GABAergic terminals and neurons were decreased in the dorsal horn (Inquimbert et al., 2018; Kami et al., 2016; Lee et al., 2009; Moore et al., 2002; Scholz et al., 2005). When GABA release was halved in heterozygous VGAT knockout mice, inflammatory pain increased (Yamada et al., 2012). KCC2 expression decreased after peripheral nerve injury (Coull et al., 2003; Modol et al., 2014; Okada-Ogawa et al., 2015; Wei et al., 2013; Zhou et al., 2012). Reduction of KCC2 by micro-RNA (Zhang et al., 2017) and injection of specific KCC2 antagonist (Austin and Delpire, 2011; Keller et al., 2007) induced hypersensitivity and neuropathic pain. Peripheral nerve injury induced the proliferation and activation of microglia, which expressed purinoceptor 4 receptor (P2X4R) and released brain derived neurotrophic factor (BDNF). The activation of microglia mediated neuropathic pain (Coull et al., 2005; Gu et al., 2016; Trang et al., 2009; Trang et al., 2011; Tsuda et al., 2003). Most of above results were obtained in the male rodents, and recent studies demonstrated different mechanisms may be involved in the female (Mapplebeck et al. 2019, Sorge and Totsh 2017, Maurer et al 2016). The results of these studies suggest that in the male animals, changes in GABAergic action and their upstream signaling, namely the microglia-

BDNF-KCC2- GABA pathway, may play key roles in the neuropathic pain (Beggs and Salter, 2013; Ferrini and De Koninck, 2013; Inoue and Tsuda, 2018; Taves et al., 2013).

Nevertheless, the precise processes through changes in GABAergic action are not yet fully understood. First, after nerve injury, allodynia did not develop in the injured nerve-distributing area, but instead developed in the adjacent area (Bourquin et al., 2006; Decosterd and Woolf, 2000; Jaggi et al., 2011; Kumar et al., 2018). However, previous reports did not mention specific difference in each region. It was not clearly demonstrated whether GABAergic action changed in the adjacent neuropathic pain-related region or not. Second, temporal relationships amongst changes in the histology of tibial nerves, GABAergic action, and mechanical allodynia have not been reported, although pathological changes such as nerve degeneration and neuroma may influence GABAergic signaling and neuropathic pain (Biber et al., 2008; Chen and Devor, 1998; Guan et al., 2016; Toia et al., 2015). Third, previous studies mainly examined the time course of sensory disorder until one month after injury, and reported differences in the type of sensory disorder and their underlying mechanisms (Jain et al., 2009; Pertin et al., 2007; Shields et al., 2003; Vadakkan et al., 2005). However, as the long-term changes were not examined, the difference between development and persistence of neuropathic pain was not discussed. Thus, distinct mechanisms underlying the development and persistence of neuropathic pain should be revealed.

To address the above points, we focused on the relationship between sciatic nerve injury-induced neuropathic pain and GABAergic transmission related processes. Pain sensation in lower

limbs is conducted through the sciatic nerve (consisting of tibial, peroneal and sural nerves) and then relayed within laminae I and II of the lumbar spinal cord (Cordero-Erausquin et al., 2016; Willis and Westlund, 1997; Willis WD, 2004). In this study, we used male mice, because there is a sex dimorphism in the mechanism underlying pain hypersensitivity between male and female (Mapplebeck et al. 2019, Sorge and Totsh 2017, Maurer et al 2016), and pathway through microglia, KCC2 and GABAergic transmission, may play roles only in the male animals. To minimize invasiveness, the surgical operation was restricted to only the tibial nerve, and two mouse models of neuropathic pain were prepared. The time course of the withdrawal threshold was different between two models. One was the tibial nerve ligation model mice. Ligation models experience mechanical touch pain for a long period of time (Kim and Chung, 1992; Seltzer et al., 1990; Vadakkan et al., 2005). The other was the tibial nerve severance model mice. Severance models also exhibit allodynia after operation (Lee et al., 2000; Poppler et al., 2017), but our preliminary experiment demonstrated that the mechanical allodynia gradually recovered after two months. First, we investigated the time course of changes in mechanical withdrawal threshold in the sural nerve area (lateral part of the sole) using von Frey filament test for 90 days (Chaplan et al., 1994; Koga et al., 2004). Second, we investigated changes in the localization of various molecules involved in GABAergic transmission, including GAD, a marker of GABAergic terminals (Barker et al., 1998; Martin and Rimvall, 1993; Varju et al., 2001); VGAT, a marker of inhibitory terminals (Fujii et al., 2007; Saito et al., 2010; Wojcik et al., 2006; Yamada et al., 2012); and KCC2, which primarily regulates GABAergic action,

in laminae I and II of the dorsal horn, where GABAergic terminals are abundant but glycinergic terminals are sparse (Kosaka et al., 2012; Sunagawa et al., 2017). We individually examined these markers in sural and tibial zones, which was determined by immunohistochemistry for c-fos, one of the immediate early genes and a marker of neuronal activity (Hunt et al., 1987; Molander et al., 1992). Third, to reveal upstream signaling responsible for changes in GABAergic action, we investigated the proliferation and activation of microglia by immunohistochemistry for ionized calcium binding adaptor molecule 1 (Iba1) (Hoogland et al., 2015; Ji et al., 2013). Finally, we investigated the time course of histological change in the tibial nerve bundle, and analyzed temporal relationships amongst the changes described above in both mouse models.

2. Results

2.1 Changes in mechanical withdrawal threshold of the tibial nerve ligation model

After ligation (Fig. 1A), the threshold did not exhibit apparent changes at the intact side (Fig. 1B). In contrast, the threshold was markedly decreased to 0.4 g at day 7 on the ligated side, and continued to drop to 0.07 g until day 21. The threshold did not significantly change between day 21 and day 90 (Fig. 1B). These results suggested that mechanical allodynia developed at day 7 and was sustained for 90 days in tibial nerve ligation model mice.

2.2 Identification of tibial and sural zones in the dorsal horn of spinal cord

To identify tibial and sural zones in the dorsal horn, we examined the localization of c-fos, 3

h after the cauterization of tibial or sural nerve areas on the hind soles (Fig. 2A, B). Immunolabeling for c-fos was localized within nuclei of laminae I and II at the cauterized side of the fifth and sixth lumbar cord, but the intact side was negative (Fig. 2C–F). Nuclei positive for c-fos were observed in the medial region of the dorsal horn after cauterization of the tibial nerve area in hind soles (Fig. 2C, D), whereas they were distributed at the central part after cauterization of the sural nerve area (Fig. 2E, F). These results indicated that the tibial zone was the medial part, while the sural zone was the central part of the dorsal horn in fifth and sixth lumbar cord, consistent with a previous study (Lee et al., 2009).

2.3 Changes in localization of presynaptic terminals of GABAergic synapses (GAD and VGAT) after tibial nerve ligation

Next, we examined changes in the localization of GABAergic terminals by immunohistochemistry for GAD and VGAT. Immunolabeling of both GAD (Fig. 3A–C) and VGAT (Fig. 3D–F) exhibited as fine dots, which continued to be localized in all laminae of gray matter in the dorsal horn, as reported in previous studies (Kosaka et al., 2012; Sunagawa et al., 2017). We did not observe obvious differences in the density of their immunolabeling between ligated and intact sides, nor apparent post-surgical changes in localization within tibial (medial) or sural (central) zones determined by c-fos staining (Fig. 2C–F). Furthermore, we objectively evaluated the density of GAD- and VGAT-positive areas in laminae I and II of tibial and sural zones on both sides. The ratio between ligated and intact sides remained around 100% throughout all days after ligation in both

tibial (Fig. 3G, I) and sural zones (Fig. 3H, J). These results suggested that localization of GABAergic terminals was unaltered after tibial nerve ligation, although the withdrawal threshold was markedly decreased.

2.4 Changes in KCC2 localization

Next, we examined the localization of KCC2, which reduces $[Cl^-]_i$ and primarily regulates GABAergic action, in secondary sensory neurons in the dorsal horn. KCC2 immunolabeling was homogeneously distributed in the dorsal horn gray matter of both intact and ligated sides (Fig. 4A–I), and occupied the neuropil in tibial and sural zones of both intact (Fig. 4D, F, H) and ligated sides (Fig. 4E, G, I). Lamina I was more densely stained than laminae II and III of both sides. In both tibial and sural zones, the density of KCC2 immunolabeling of the operated side was slightly lower than the intact side at day 21 and day 90 (Fig. 4B, C, F–I). We objectively evaluated the density of KCC2 immunolabeling in laminae I and II of tibial and sural zones of both sides. The ratio of densities between ligated and intact sides was similarly changed in tibial and sural zones after tibial nerve ligation (Fig. 4J, K). The KCC2 expression was significantly decreased at day 14 in both zones, and the ratio became 77% in the sural zone and 86% in the tibial zone at day 21 (Fig. 4J, K), when the mechanical withdrawal threshold was lowest (Fig. 1B). Similar to the lack of recovery of mechanical threshold (Fig. 1B), the KCC2 ratio was not significantly rescued in either zone at day 28 or 90 (Fig. 4J, K). The ratio in the sural zone was lower than that in the tibial zone after day 14 (Fig. 4J, K).

These results indicated significantly decreased KCC2 localization in both tibial and sural zones after tibial nerve ligation, and its localization remained significantly low on the ligated side until day 90.

2.5 Changes in localization of Iba1-positive microglia

We next examined Iba1 immunohistochemistry in the dorsal horn to investigate changes in the localization of microglia, which release BDNF and reduce KCC2 expression. Many Iba1-positive cells were detected on the ligated side at day 7 (Fig. 5A), and their density continued to be higher compared with the intact side until day 90 (Fig. 5B, C). In the higher magnification view, Iba1-positive cells had many fine processes on the ligated side, and their density was higher than the intact side in the tibial zones at day 7 (Fig. 5D, E) and 21 (Fig. 5F, G), and sural zone until day 90 (Fig. 5D-I). We objectively evaluated changes in the density of Iba1-positive cells with multiple processes. In both zones, their density was rapidly increased at day 3 and day 7 (Fig. 5J, K). The rate of increase in the tibial zone was higher than that in the sural zone. After day 14, density in the tibial zone rapidly decreased, whereas that in the sural zone was sustained (Fig. 5J, K). In the sural zone, the density was not obviously changed after day 28, and continued to be significantly higher than before operation until day 90 (Fig. 5K). The density in the tibial zone was higher than the sural zone until day 14, but lower after day 21 (Fig. 5J, K). These results suggested that microglia may be rapidly proliferated and activated in both tibial and sural zones of the dorsal horn after ligation, and a significantly higher density of microglia remained until day 90 in the sural zone.

2.6 Histological changes of the tibial nerves after ligation

Next, we examined histological changes of the tibial nerve by scanning electron microscopy. Before ligation, various sizes of myelinated fibers and unmyelinated axons, contained within the Schwann cell cytoplasm, occupied the tibial nerve bundle (Fig. 6A). At day 7, numerous myelin fragments, degenerating axons, and large macrophages, containing numerous lipid vacuoles, axons, and myelin fragments, were detected in the distal part of the tibial nerve (Fig. 6B, C). Intact axons were absent, and intracellular spaces were large in the marginal region (Fig. 6B). In contrast, there were a few intact axons, and intracellular spaces were small in the central region (Fig. 6C). This result indicated that at day 7, the majority of axons were degenerating and nerve degeneration was more advanced in the marginal region than in the central region. At day 28, myelinated axons were homogeneously distributed throughout the nerve bundle of the proximal part of the tibial nerve (Fig. 6D). Pathological changes, such as neuroma detected in previous studies (Chim et al., 2013; Toia et al., 2015; Valverde Guevara et al., 2014; Yarar et al., 2015), were not observed; although, several blood vessels were expanded (Fig. 6D). In the distal part, degenerating axons and macrophages were scarce in the marginal region (Fig. 6E), whereas they were still detected in the central region (Fig. 6F). In the marginal region, many myelinated and unmyelinated axons were distributed, but the diameters of myelinated axons remained small and their myelin was thin (Fig. 6E). In the central region, unmyelinated axons were abundantly detected, but only a few myelinated axons were observed (Fig. 6F). At day 56, myelinated fibers were homogeneously distributed in the proximal part of tibial nerve bundles (Fig. 6G), and pathological changes were not detected; although, several

blood vessels were expanded (Fig. 6G). In the distal part, nerve bundles were occupied by myelinated and unmyelinated fibers (Fig. 6H, I). Diameters of myelinated axons and myelin thickness were larger in the marginal region (Fig. 6H) compared with the central region (Fig. 6I), but remained lower compared with before surgery (Fig. 6A). These results suggested that nerve degeneration may proceed more quickly in the marginal region than in the central region until day 28, and is repaired by day 56; although, the withdrawal threshold continued to be low. Present histological changes were comparable to previous studies using ligation models (Abuduhadeer, 2004; Yagasaki et al., 2013).

2.7 Changes in the tibial nerve severance model

Finally, we examined changes in the features described above in severance model mice (Fig. 7A). The allodynia test demonstrated that withdrawal threshold was also markedly decreased after severance, and was attenuated to 0.04 g at day 14 (Fig. 7B). Notably, the lowest threshold occurred one week earlier than observed in the ligation model (Fig. 1B). The threshold gradually increased after day 28, and this increase of the withdrawal threshold was statistically significant between day 14 and day 90 (Fig. 7B), suggesting that mechanical allodynia was significantly recovered in severance model mice after day 21.

Localization of presynaptic terminals were not obviously changed in the severance model (data not shown) as well as ligation model (Fig. 3). Density of KCC2 immunolabeling was slightly decreased on the severed side at day 3 (Fig. 8A, D, E) and day 14 (Fig. 8B, F, G), whereas an

apparent difference in density was not observed at day 90 (Fig. 8C, H, I). Objective analysis confirmed this result (Fig. 8J, K). The ratio between severance and intact sides decreased in both tibial and sural zones until day 14, and significantly increased back to a normal level between day 14 and day 90 (Fig. 8J, K). Indeed, there were no significant differences between day 90 and before severance. Notably, the date of lowest expression was earlier in the severance model (Fig. 8J, K) compared with the ligation model (Fig. 4J, K).

Densities of Iba1-positive cells were markedly increased in both tibial and sural zones on the severed side until day 14 (Fig. 9A, B, D–G), but this difference was not apparent at day 90 (Fig. 9H, I). Objective analysis demonstrated that in both zones, the density rapidly increased at day 3, gradually increased until day 14, and then gradually decreased after day 21 (Fig. 9J, K). Notably, density was higher in the tibial zone than in the sural zone until day 14 (Fig. 9J, K), similar to the ligation model (Fig. 5J, K). There was no significant difference between day 90 and before severance (Fig. 9J, K).

Finally, we examined histological changes in the tibial nerve after severance. At day 7, myelinated fibers disappeared from the distal part of the tibial nerve (Fig. 10A). There were many degenerating axons and macrophages, which contained axons and myelin fragments. Intracellular spaces were large (Fig. 10A), similar to the marginal region of the ligation model at day 7 (Fig. 6B). At day 28, degenerating axons and macrophages were absent, and the nerve bundle was occupied by numerous myelinated and unmyelinated axons (Fig. 10B). However, myelin around the axons

remained thinner than observed before surgery (Fig. 6A). These results suggested that degeneration was already repaired in the severance model at day 28, and regeneration (e.g. re-extending of various types of axons) was progressing. Pathological changes, such as neuroma, detected in previous studies (Chim et al., 2013; Toia et al., 2015; Valverde Guevara et al., 2014; Yarar et al., 2015) were not detected in distal (Fig. 10B) or proximal (Fig. 10C) parts of the tibial nerve.

3. Discussion

3.1 Mouse models for investigation of chronic neuropathic pain

Various animal models have been described for the investigation of neuropathic pain (Bennett, 1993; Jaggi et al., 2011; Kumar et al., 2018). Examples include spinal nerve root ligation (or constriction), sciatic nerve partial ligation, axotomy, and spared nerve injury models. All of these models involve various types of sensory disorders, such as allodynia, hyperalgesia, hyperpathia, and hypoalgesia, with symptoms occurring over the course of at least one month. Previous studies reported differences in the above symptoms in each model, and discussed their underlying mechanisms (Jain et al., 2009; Pertin et al., 2007; Shields et al., 2003; Vadakkan et al., 2005). However, they did not analyze the differences in time course of associated changes in each model, and did not mention the difference of mechanisms underlying development and persistence of allodynia. In this study, we observed differences in long-term changes in mechanical allodynia between two nerve injury models. Tibial nerve ligation model mice continued to suffer from

mechanical allodynia for three months, whereas severance model mice significantly recovered from mechanical allodynia during the second and third months after surgical induction. This result suggested that the ligation model may be better than the severance model for investigating chronic neuropathic pain and, moreover, common changes observed in both models during the first month and differences observed during the second and third months may represent key events for the development and persistence of neuropathic pain, respectively.

In the utilized ligation model, impairment of motor function was not serious because (1) the main trunk of the sciatic nerve, which distributes hamstrings, and common peroneal nerve, which distributes extensor muscles of the lower limb, were completely preserved, (2) the tibial nerves were only loosely ligated, and (3) the tibial nerves were not transected after ligation unlike the spared nerve injury model (Decosterd and Woolf, 2000; Pertin et al., 2007; Shields et al., 2003). Furthermore, motor disturbances of flexor muscles in the lower limb may recover for extended periods of time, as regenerating thick myelinated axons were markedly increased in number during the second and third post-surgical months. Considering these results, the present tibial nerve ligation model may be a novel and useful model for investigating persistent neuropathic pain.

3.2 Microglia-KCC2-GABA-allodynia pathway in the sural zone

In this study, we precisely analyzed the time course of changes, such as mechanical allodynia, GABAergic synapses, KCC2 localization, and microglia, in both tibial and sural zones of

the male mouse spinal cord. We observed a close relationship amongst the above features in the sural zone, where allodynia was relayed.

First, we did not find any statistically significant changes in the localization of molecules in presynaptic terminals of GABAergic synapses, consistent with a previous study (Polgar and Todd, 2008). Furthermore, we examined changes in the localization of postsynaptic elements of GABAergic synapses on secondary sensory neurons by immunohistochemistry for gephyrin, a $GABA_A$ receptor scaffolding protein (Fritschy et al., 2008; Kneussel and Betz, 2000; Tretter et al., 2008; Yu et al., 2007), but significant changes were not detected (Data not shown). These results suggested that the structure and number of GABAergic synapses on secondary sensory neurons may not be changed after the present operation. However, some previous studies reported decreased intensity of GAD-immunolabeling and numbers of GABAergic neurons in the dorsal horn after nerve injury (Inquimbert et al., 2018; Kami et al., 2016; Lee et al., 2009; Moore et al., 2002; Scholz et al., 2005). The underlying reason for this discrepant result was unclear, but it may suggest that (as described in the previous section) the present surgical operation was not invasive enough to alter the presynaptic elements of GABAergic synapses compared with previous studies (Inquimbert et al., 2018; Kami et al., 2016; Lee et al., 2009; Polgar and Todd, 2008; Scholz et al., 2005).

Second, we observed significantly reduced KCC2 localization after nerve injury in both models, consistent with previous reports (Coull et al., 2003; Modol et al., 2014; Okada-Ogawa et al., 2015; Wei et al., 2013; Zhou et al., 2012). However, the results of the present study revealed that

KCC2 localization decreased in both tibial and sural zones, and remained low while the model mice were suffering from allodynia. The ratio of KCC2 localization between operated and intact sides in the sural zone was significantly reduced at day 7, when the mechanical threshold was decreased to less than 50% in both models. Notably, values for both minimum withdrawal threshold and KCC2 ratio in the sural zone occurred earlier in the severance model compared with the ligation model. In the ligation model, KCC2 localization remained significantly reduced for 90 days, while the mice were experiencing allodynia. In contrast, KCC2 localization gradually and significantly increased (recovered) in tibial nerve severance model mice, whose mechanical allodynia gradually recovered. These results indicated that withdrawal threshold and KCC2 level were similarly altered, suggesting that KCC2 reduction in the sural zone may mediate allodynia in the sural nerve area. Previous studies also demonstrated markedly decreased KCC2 expression in motor neurons whose axons were transected (Kim et al., 2018; Nabekura et al., 2002; Tatetsu et al., 2012; Toyoda et al., 2003). In the present study, KCC2 was decreased in secondary sensory neurons in the tibial zone, where central terminals of the injured tibial nerves were distributed. Similarly, deprivation of auditory information by destruction of the cochlear organs (Kakazu et al., 1999) or constriction of trigeminal nerves (Wei et al., 2013) also reduced KCC2 expression in secondary sensory neurons of the cochlear nucleus and spinal trigeminal nucleus, respectively. These results suggested that decreased KCC2 expression may be a common feature after the injury of both efferent motor and afferent sensory fibers. After motor nerve injury, GABAergic action did shift from inhibition to excitation, as KCC2 mRNA almost

disappeared from motor neurons (Nabekura et al., 2002; Toyoda et al., 2003), and KCC2 localization was markedly reduced to less than 50% (Kim et al., 2018; Tatetsu et al., 2012). In contrast, KCC2 expression was approximately 80% of pre-surgical levels after sensory nerve injury, consistent with previous studies (Wei et al., 2013; Zhou et al., 2012). These results suggested that after tibial nerve injury, KCC2 localization was reduced in the sural zone, whereby $[Cl^-]_i$ may be decreased in sensory neurons, GABA may not induce depolarization but mediate weak hyperpolarization as previously reviewed (Kaila et al., 2014; Price et al., 2009), and mechanical threshold may be decreased.

Third, the number of microglia was markedly increased in the dorsal horn of both models, as previously reported (Gu et al., 2016; Tashima et al., 2016; Trang et al., 2011). Results of the present study revealed, for the first time, significant increases in microglial density in both tibial and sural zones. Moreover, when microglia significantly increased in number, KCC2 localization was significantly reduced and the mechanical threshold was markedly decreased in the respective zone and area, and vice versa. In both sural and tibial areas and zones, all events including allodynia, KCC2 reduction, and increasing of microglia, occurred earlier in the severance model compared with the ligation model. Collectively, these results suggested a close relationship among increasing microglial density and reduced KCC2 in the sural zone, and allodynia in the sural nerve area; moreover, they confirmed the involvement of a microglia-KCC2-GABA-allodynia pathway (Beggs and Salter, 2013; Ferrini and De Koninck, 2013; Inoue and Tsuda, 2018; Taves et al., 2013) in allodynia-related areas, but not in the region of nerve injury.

3.3 Upstream factors mediating development and persistence of neuropathic pain

By precisely examining various changes in both tibial and sural zones, we found that upstream factors driving the microglia-KCC2-GABA-allodynia pathway were different between development and persistence of neuropathic pain.

In both mouse models, microglia density rapidly increased in the tibial zone until day 7, whereas it increased gradually in the sural zone. Moreover, the density was higher in the tibial zone than that in the sural zone before day 14. These results suggested that factors mediating proliferation and activation of microglia may be released in the tibial zone, whereby they gradually diffuse into the sural zone. Present histological examinations revealed that tibial nerve axons commonly degenerated at day 7 in both models, but degeneration in the severance model proceeded faster and finished earlier compared with the ligation model. Two events, (1) minimum withdrawal threshold and KCC2 localization, and (2) peak density of microglia, occurred earlier in the severance model compared with the ligation model. Previous studies demonstrated that central axon terminals of injured nerves release various cytokines, such as colony stimulating factor-1 (CSF1) (Guan et al., 2016; Okubo et al., 2016), chemokine ligand (CCL) 21 (Biber et al., 2008; Biber et al., 2011; de Jong et al., 2005), and CCL2 (Toyomitsu et al., 2012), which influence the activation and proliferation of microglia within the dorsal horn. As such, these factors may be released from the central terminals of degenerating axons, whereby they mediate the development of allodynia during the first post-surgical month.

Most of degeneration had finished by day 56 in both models. At day 90, nerve fibers in both models were occupied by myelinated and unmyelinated fibers similar to before surgery (data not shown), and did not contain neuroma, which was detected at the proximal position of the injured nerve in the spared nerve injury models (Chim et al., 2013; Toia et al., 2015; Valverde Guevara et al., 2014; Yarar et al., 2015). This result suggested that pathological abnormalities such as nerve degeneration, neuroma, and chronic inflammation, may be independent from persistent allodynia in the presently used ligation model. However, ligation model mice continued to suffer from allodynia for 90 days, and the density of microglia was higher and ratio of KCC2-positive area was lower in the sural zone compared with the tibial zone after day 28. This result indicated that different factors from the sural zone, rather than the tibial zone, may play key roles in activation and proliferation of microglia, which mediate persistent allodynia. Previous studies demonstrated that after injury, intact axons synthesize and release BDNF (Fukuoka et al., 2001; Sikandar et al., 2018) and vanilloid receptor (VR1) (Fukuoka et al., 2002; Hudson et al., 2001), which mediate changes in GABAergic action and activation of microglia. Collectively, these results suggested that different factors, including BDNF and VR1, might be released from intact axon terminals, whereby they may play dominant roles in proliferation of microglia after day 56, and could be involved in persistence of neuropathic pain in the sural nerve area.

Previous studies demonstrated that pathway between various cytokines, released from injured nerves, and P2X4R positive microglia may be suppressed by the female hormones, including

progesterone and estrogens, and adaptive immune system may be activated in the female mice (Rosen et al., 2017; Sorge and Totsch, 2017). Therefore, the spatial and temporal relationship between tibial nerve injury and microglial activation in the tibial and sural zones may not be detected in the female mice.

3.4 Possible mechanisms underlying the development and persistence of mechanical allodynia

The results of the present study suggested two distinct mechanisms underlying the development and persistence of mechanical allodynia, as summarized in Figure 11. Before operation, GABA negatively regulates sensory signals in the dorsal horn, as KCC2 is abundantly expressed and $[Cl^-]_i$ is sufficiently low in secondary sensory neurons (Fig. 11A). After tibial nerve injury, such as ligation and severance, the distal part of sensory axons degenerated (Fig. 11B). Factors, released from the central terminals of injured neurons whose peripheral part was degenerating, may diffuse into the sural zone in the dorsal horn and activate microglia (Biber et al., 2008; Inoue and Tsuda, 2018). Activated microglia may release BDNF, which can suppress KCC2 expression in secondary sensory neurons (Coull et al., 2005; Trang et al., 2011; Vanelderen et al., 2010) in the sural zone. Reduced KCC2 expression may elevate $[Cl^-]_i$, thus reducing GABAergic synaptic inhibition (Kaila et al., 2014; Price et al., 2006; Schulte et al., 2018). Finally, mechanical allodynia may develop in the sural nerve area (lateral part of the hind sole). During the chronic stage (Fig. 11C), nerve degeneration may be repaired, but other factors released from intact nerve terminals may continue to

activate microglia and drive the microglia-BDNF-KCC2-GABA pathway (Beggs and Salter, 2013; Ferrini and De Koninck, 2013; Inoue and Tsuda, 2018; Taves et al., 2013).

We additionally examined the changes in localization of KCC2 and Iba1 in the peroneal zone, lateral part of the dorsal horn (Lee et al., 2009), even though the withdrawal threshold could not be evaluated by von Frey filaments in the peroneal nerve area, dorsum of the foot (Bajrovic and Sketelj, 1998; Decosterd and Woolf, 2000; Swett and Woolf, 1985). We found that in the peroneal zone of the ligated side, the KCC2 localization was slightly decreased compared to the intact side, and Iba1 positive cells were slightly increased compared to that before operation at day 14 through 90 (Data not shown), although these changes were not statistically significant. Average density of the Iba1-positive cells was lower than those in the sural zone, and the percentage of KCC2-positive area was higher. These results support the above mechanisms that factors from injured nerve terminals or intact terminals may diffuse from tibial zone or sural zone into peroneal zone, respectively. During diffusion, their concentration may be reduced. As previously indicated (Mapplebeck et al., 2016; Mapplebeck et al., 2017; Rosen et al., 2017; Sorge et al., 2015; Sorge and Totsch, 2017), there is a sex difference in the mechanisms underlying neuropathic pain. Therefore, these microglia-KCC2-GABA-allodynia pathway after nerve injury could be demonstrated, because we examined the male mice in this study. In the female spinal cord, spatial and temporal relationship amongst activation of microglia, reduction of KCC2, and allodynia may not be detected, and current investigations should be examined in the female mice.

4 Experimental procedures

4.1 Animals

Male C57BL/6J mice aged 10-12 weeks (SLC, Shizuoka, Japan) were maintained at a controlled temperature and humidity under a 12/12-h light/dark cycle, and fed a standard diet. The procedures in this study were approved by the Animal Care and Use Committee of University of the Ryukyus (Permission No.; A2016054 and A2016055), and all animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals of University of the Ryukyus. Protocols for the care and handling of animals confirmed to current international laws and policies (NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85-23, 1985, revised 1996). Every effort was made to minimize the number of animals used and their suffering. Number of the mice for each experiment was summarized in the Table1.

4.2 Anesthesia

For surgical operation and transcardial fixation, mice were deeply anesthetized by an intraperitoneal injection of a mixed solution (10 μL/g body weight) containing 8% (v/v) Somnopentyl® (pentobarbital sodium, 5 mg/mL) and 20% (v/v) ethanol in saline.

4.3 Operation for nerve injury models

Under deep anesthesia, the right sciatic nerve and its branches (tibial, medial sural cutaneous, and common peroneal nerves) were exposed. The tibial nerve was ligated by 8-0 nylon thread (Polypropylene, Ethicon US, Cincinnati, OH) until its diameter was reduced to half (tibial

nerve ligation model, Fig. 1A) or it was completely severed (tibial nerve severance model, Fig. 6A).

4.4 Evaluation of mechanical withdrawal threshold by von Frey filament test

Mice were acclimatized in a transparent cup on a mesh metal floor for more than 15 min before testing. To measure the mechanical withdrawal threshold at the lateral part of hind soles, we employed von Frey hair filaments (0.04, 0.07, 0.16, 0.4, 0.6, 1, 1.4, and 2 g; 20PC AESTHE, MUROMACHI KIKAI, Tokyo, Japan) as previously described (Kami et al., 2016; Koga et al., 2004). Fifty-percent g threshold was calculated by the up-and-down method (Chaplan et al., 1994; Dixon, 1980) using the formula below.

$$50\%\ \text{g threshold} = (10^{\lceil xf+k0.224 \rceil})/10000$$

xf = value (in log units) of the final von Frey filament used

k = tabular value for the pattern of positive/negative responses

4.5 Antibody characterization

Antibodies used in this study are listed in Table 1. The specificity of antibodies against GAD (Kobayashi et al., 2018), KCC2 (Takayama and Inoue, 2006), and VGAT (Takayama and Inoue, 2004) had already reported in previous studies. Furthermore, we showed the adult mouse spinal cord sections staining with and without primary antibodies (Sup. Fig. 1). Although gray matter was densely stained by adding the primary antibodies against KCC2 (Sup. Fig. 1A) and VGAT (Sup. Fig. 1C), no significant immunolabeling was detected in the sections stained without primary antibody (Sup. Fig. 1B, D)).

4.6 Tissue preparation and immunohistochemistry

Under deep anesthesia, mice were fixed by transcardial perfusion with fixative, containing 4% paraformaldehyde in (v/v) phosphate buffer (PB, 0.1 M, pH 7.4) before and 3, 7, 14, 21, 28, 56, and 90 days after surgery. The spinal cord was rapidly removed, immersed in the same fixative overnight, and cryoprotected with 30% (w/v) sucrose in PB for 48 h at 4°C. Transverse sections of lumbar spinal cord with a 20 μm thickness were prepared using a cryostat and then mounted on gelatin-coated glass slides. Sections on glass slides were treated with 100% (v/v) methanol containing 0.3% (v/v) H_2O_2 for 30 min, followed by PB for 10 min, 3% (v/v) normal goat serum in PB for 1 h, and then primary antibodies against, GAD, VGAT, KCC2, or Iba1 overnight at room temperature. After rinsing three times with PB for 15 min, sections were visualized using the avidin-biotin-peroxidase complex (ABC) method with a Histofine kit (Nichirei, Tokyo, Japan).

4.7 Determination of tibial and sural zones in the spinal cord

To determine tibial and sural zones in the dorsal horn, central (Fig. 2A) or lateral (Fig. 2B) parts of the right hind soles of normal control mice were cauterized by laser mess under deep anesthesia. Mice were fixed three hours after cauterization, and sections were prepared as described above. Sections on gelatin-coated glass slides were reacted with c-fos antibody and visualized by the ABC method (Hunt et al., 1987; Molander et al., 1992).

4.8 Electron microscopic observation of tibial nerves

Tibial nerves around ligation or severance sites were removed from mice after fixation with 4% (w/v) paraformaldehyde in PB, then post-fixed with mixed solution, containing 4% paraformaldehyde and 0.5% glutaraldehyde in PB for 20 min. After exposure to 1% (w/v) OsO_4 in PB for 2 h at 4°C, specimens were stained with 2% (w/v) uranyl acetate aqueous solution overnight, and embedded in epoxy resin in the usual manner. Semi-thin transverse sections with a 250 nm thickness were stained with lead citrate solution (Reynolds, 1963) and observed under an TM3030 scanning electron microscope using backscattered electron mode (Hitachi, Japan). Image data shown were processed by reversing black/white.

4.9 Semi-quantitative analysis of immunohistochemistry

Percentage of immune-positive areas for GAD, VGAT, and KCC2 in tibial and sural zones of the dorsal horn were semi-quantified as follows. For image analysis, we used at least three different stained sections per mouse spinal cord, and at least three mice per group. The dorsal horn containing both sides of tibial and sural zones was imaged under light microscopy (Olympus AX-80 with 4×objective lens), and images were imported into Image J (NIH, Bethesda, MD). Each image was adjusted for color threshold to binary. Five quadrangular areas per stained section were randomly selected in the lamina I and II of each tibial and sural zone for each operated and intact sides. The lamina I was identified to be the marginal layer consisting of large elongated and smaller triangular, multipolar and fusiform neurons using the adjacent sections stained by toluidine blue. The lamina II was identified to be the layer consisting of small, rounded or slightly elongated neurons beneath the

lamina I (Sengul and Watson, 2012). We measured percentages of stained region in each quadrangular area, and calculated ratios between operated and intact sides (percentage of operated side / percentage of intact side) in each tibial and sural zone. The results are reported as mean ± standard error of mean (SEM) and were analyzed using Bonferroni test for multiple comparisons. In all cases, $p < 0.05$ was considered to be statistically significant.

Densities of Iba1-positive cells, indicating microglia, in tibial and sural zones of the dorsal horn were semi-quantified as follows, using at least three different stained sections per mouse spinal cord, and at least three mice per group. Images were also adjusted for color threshold to binary. Next, to determine the range, we chose both small and big cells, which could be activated microglia with multiple processes (Hoogland et al., 2015; Ji et al., 2013), and measured their particle pixel size. Next, numbers of stained cells were counted in each tibial and sural zone by setting the range of particle size from 10000 to 500000 pixels. Density of stained cells from day 3 to day 90 was compared with that in normal mice without surgical operation in both tibial and sural zones. The results are reported as mean ± SEM and were analyzed using Bonferroni test for multiple comparisons. In all cases, $p < 0.05$ was considered to be statistically significant.

Figure legends

Figure 1 Tibial nerve ligation and mechanical allodynia test using von Frey filaments

A) An image showing tibial nerve ligation by 8-0 nylon thread.

B) Changes in withdrawal threshold after tibial nerve ligation. Withdrawal threshold at the intact side (triangles) remained between 1.5 g and 2 g, whereas that of the ligated side (circles) markedly decreased until day 21 and did not significantly increase (ns) between day 21 and day 90. Error bars indicate SEM. *: $p < 0.05$ vs before surgery, **: $p < 0.01$ vs before surgery.

Figure 2 Identification of tibial and sural zones in the spinal cord dorsal horn

A and B) Schematic illustrations showing the cauterized place in the hind sole. Two dashed lines in A and single dashed line in B indicate the cauterized place for detection of tibial and sural zones in the dorsal horn, respectively.

C–F) Low (C, E) and high (D, F) magnification images of c-fos immunohistochemistry after cauterization of tibial (C, D) and sural nerve areas (E, F) in the hind soles. The tibial zone was medial part (C, E) and the sural zone was the central part (D, F) of the dorsal horn. Squares in C and E indicate regions shown in D and F, respectively. Scale bar = 100 μm.

Figure 3 Immunohistochemistry for GAD and VGAT after tibial nerve ligation

A–F) Low-magnification images of immunohistochemistry for GAD (A–C) and VGAT (D–F) in the dorsal horn at day 7 (A, D), day 21 (B, E), and day 90 (C, F).

G–J) Changes in ratios of GAD- (G, H) and VGAT-positive areas between ligated and intact sides in tibial (G, I) and sural (H, J) zones.

Obvious differences were not detected between intact (left) and ligated (right) sides (A–F), and these results were confirmed by objective analysis (G–J). Error bar indicates SEM. Scale bar = 100 μm.

Figure 4 Immunohistochemistry for KCC2 after tibial nerve ligation

A–I) Low (A–C) and high (D–I) magnification images of KCC2 immunohistochemistry in the dorsal horn at day 7 (A, D, E), day 21 (B, F, G), and day 90 (C, H, I). Density of KCC2 immunolabeling of the ligated side (right in B and C, G, I) was slightly lower than observed for the intact side (left in B and C, F, H) at day 21 (B, F, G) and day 90 (C, H, I). Squares in A, B, and C indicate regions shown in D–I. I and II indicate the laminae I and II, respectively. Dashed lines indicate the border between tibial, sural and peroneal zones.

J and K) Changes in ratios of KCC2-positive areas between ligated and intact sides (J, K). Objective analysis showed that the ratio significantly decreased at day 14 in both tibial (J) and sural (K) zones, but was not significantly changed (ns) between day 21 and day 90 in either zone. Error bar indicates SEM. *: $p < 0.05$ vs before surgery, **: $p < 0.01$ vs before surgery. Scale bar = 100 μm.

Figure 5 Immunohistochemistry for Iba1 after tibial nerve ligation

A–I) Lower (A–C) and higher (D–I) magnification images of Iba1 immunohistochemistry at day 7 (A, D, E), day 21 (B, F, G), and day 90 (C, H, I). Iba1-positive cells with multiple processes were more abundantly detected in laminae I (I) and II (II) on the ligated side at days 7 through 90 (right in

A–C, E, G, I) compared with the intact side (left in A–C, D, F, H). Squares in A, B, and C indicate regions shown in D–I. Dashed lines indicate the border between tibial, sural and peroneal zones.

J and K) Changes in densities of Iba1-positive cells in tibial (J) and sural (K) zones on ligated sides. Objective analysis showed that the density rapidly increased in both zones until day 7, and decreased in the tibial zone after day 14 (J). In contrast, it was sustained after day 14 in the sural zone, and was significantly higher at day 90 compared with before ligation (K). Note that the density in the tibial zone was higher than in the sural zone until day 14, but was lower after day 21. Error bar indicates SEM. *: $p < 0.05$ vs before surgery, **: $p < 0.01$ vs before surgery. Scale bar = 100 μm.

Figure 6 Electron microscopic changes of the tibial nerve before (A) and after ligation (B–F)

A–C) Before ligation, numerous myelinated and unmyelinated axons occupied nerve bundles (A). At day 7, degenerating axons (d) and macrophages (m) were often detected (B, C) in the marginal region (B) and central region (C). Scale bar = 10 μm.

D) At day 28, no pathological changes, such as neuroma, were detected in the proximal part of the nerve, although some blood vessels were expanded. Scale bar = 100 μm.

E and F) In the distal part of the nerve, myelinated fibers were abundant in the marginal region (E). In contrast, myelinated axons (arrows) were sparse and degenerating axons (d) were still detected in the central part (F). Scale bar = 10 μm.

G) At day 56, pathological changes, such as neuroma, were not detected in the proximal part of the nerve, although some blood vessels were expanded. Scale bar = 100 μm.

H and I) In the distal part, average axon diameter was higher in the marginal region (H) than in the central region (I). Note that degeneration and regeneration proceeded faster in the marginal region (B, E, H) than in the central region (C, F, I). Scale bar = 10 μm.

Figure 7 Tibial nerve severance and mechanical allodynia tests after operation

A) An image showing severance of the tibial nerve.

B) Changes in withdrawal threshold after tibial nerve severance. Withdrawal threshold on the intact side (triangles) remained between 1.5 g and 2 g, whereas that of the severed side (circles) markedly decreased until day 14, before gradually and significantly increasing until day 90. Note that the day of lowest expression was earlier than in the ligation model (Fig. 1B). Error bar indicates SEM. *: $p <$ 0.05 vs before surgery, **: $p < 0.01$ vs before surgery, §§: $p < 0.01$ between D14 and D90.

Figure 8 Immunohistochemistry for KCC2 after tibial nerve severance

A–I) Low (A–C) and high (D–I) magnification images of KCC2 immunohistochemistry in the dorsal horn at day 3 (A, D, E), day 14 (B, F, G), and day 90 (C, H, I). Density of KCC2 immunolabeling on the severed side (right in A and B, E, G) was slightly lower than observed on the intact side (left in A and B, D, F) in both tibial and sural zones at day 3 (A, D, E) and day 14 (B, F, G). Squares in A, B, and C indicate regions shown in D–I, respectively. I and II indicate the laminae I and II, respectively. Dashed lines indicate the border between tibial, sural and peroneal zones.

J and K) Changes in ratios of KCC2-positive areas between severed and intact sides. Objective analysis showed that the ratio significantly decreased until day 14 in both tibial (J) and sural (K)

zones, but gradually and significantly increased thereafter until day 90. Note that the day of lowest expression occurred earlier than in the ligation model (Fig. 4J, K). Error bar indicates SEM. *: $p <$ 0.05 vs before surgery, **: $p < 0.01$ vs before surgery, §: $p < 0.05$ between D14 and D90 in the sural zone**,** §§: $p < 0.01$ between D14 and D90 in the tibial zone. Scale bar = 100 μm.

Figure 9 Immunohistochemistry for Iba1 after tibial nerve severance

A–I) Low (A–C) and high (D–I) magnification images of Iba1 immunohistochemistry at day 3 (A, D, E), day 14 (B, F, G), and day 90 (C, H, I). Iba1-positive cells with multiple processes were more abundantly detected in laminae I and II on the severed side (right in A-C, E, G, I) at days 3 through 90 than on the intact side (left in A–C, D, F, H). Squares in A, B, and C indicate regions shown in D–I. I and II indicate the laminae I and II, respectively. Dashed lines indicate the border between tibial, sural and peroneal zones.

J and K) Changes in density of Iba1-positive cells in tibial (J) and sural (K) zones on the severed sides. Objective analysis showed that the density rapidly increased in both zones until day 14, but then gradually decreased in both zones. Statistical significance was not detected at day 90 compared with before severance. Note that density in the tibial zone was higher than in the sural zone until day 14. Error bar indicates SEM. *: $p < 0.05$ vs before surgery, **: $p < 0.01$ vs before surgery. Scale bar = 100 μm.

Figure 10 Electron microscopic changes of the tibial nerve after severance

A and B) At day 7, degenerating axons (d) and macrophages, containing fragments of neurons and myelin (m), were often detected (A). At day 28, degenerating axons were absent, and many myelinated and unmyelinated fibers were observed throughout the distal part of the nerve bundle (B). Scale bar = 10 μm.

C) No pathological changes were detected in the proximal part near the severance site (C). Scale bar = 100 μm.

Figure 11 Schematic illustration of the possible mechanism underlying the development (B) and persistence (C) of mechanical allodynia

A) Before surgery, GABA negatively regulates sensory signals from tibial (Tibial) and sural (Sural) nerves in laminae I and II of the dorsal horn (DH), as KCC2 is abundantly expressed and $[Cl^-]_i$ is sufficiently low in secondary sensory neurons.

B) After ligation of the tibial nerve, its peripheral part degenerated and some factors (triangles) may have been released from the central terminals. These factors may have diffused from the tibial zone (dense brown) into the sural zone (pale brown), whereby they induced proliferation and activation of microglia in both zones. KCC2 localization may be reduced by BDNF, released from activated microglia. Subsequently, $[Cl^-]_i$ may be increased, GABAergic synaptic inhibition may be reduced, and allodynia can develop in the sural nerve area of the hind sole.

C) Three months later, degeneration of the tibial nerve was repaired, and regeneration was proceeding. Different factors (squares) that continued to activate microglia might be released

from central terminals of the intact sural nerve. Activated microglia continued to reduce KCC2-localization by mediating BDNF release and sustaining a high level of $[Cl^-]_i$, thus permitting allodynia to continue in the sural nerve area.

Table1 Number of the mice for each experiment

Days after operation		before	D3	D7	D14	D21	D28	D56	D90
von Frey test									
Ligation model		101	34	30	23	16	12	8	8
Severance model			29	32	26	19	12	10	10
Immunohistochemistry									
Ligation model									
GAD	Tibial zone	4	3	3	3	3	3		3
	Sural zone	3	3	3	3	3	3		3
VGAT	Tibial zone	4	3	3	3	3	3		3
	Sural zone	4	3	3	3	3	3		3
KCC2	Tibial zone	6	3	3	6	5	3		7
	Sural zone	5	3	3	4	5	3		5
Iba1	Tibial zone	4	3	3	3	5	5		7
	Sural zone	3	3	3	3	5	5		5
Severance model									
KCC2	Tibial zone	6	6	6	6	6	6		5
	Sural zone	6	5	5	4	5	5		5
Iba1	Tibial zone	4	3	3	3	3	3		3
	Sural zone	4	3	3	3	3	3		3

Table2 Characterization of antibodies used in this study.

Antigen	Immunogen	Manufacturer, species, antibody type	Dilution
c-fos	Recombinant full length protein of human c-fos 1-380	Abcam, No.190289, Lot.GR2777449-1, mouse, monoclonal	1:4,000
GAD	Synthetic peptide of the C terminal of mouse GAD with the amino acid sequence [C]DFLIEEIERLGQDL	Original antibody, rabbit, polyclonal (Kobayashi et al., 2018)	1 μg/mL
Iba1	Synthetic peptide of the C terminal of Iba1	WAKO, No.019-19741, Lot.LKL0566 rabbit, polyclonal	1 μg/mL
KCC2	Synthetic peptide, aa 44-64 from N-terminals of mouse	Original antibody, rabbit polyclonal (Takayama and Inoue, 2006)	1 μg/mL
VGAT	Recombinant protein, aa 1022–1042 of the mouse VGAT	Original antibody, Guinea pig, polyclonal (Takayama and Inoue, 2004)	1 μg/mL

Supplemental Figure 1 Immunohistochemical staining with and without primary antibodies against KCC2 (A, B) and VGAT (C, D) antibodies in the adult spinal cord.

After treating as mentioned in **4.6**, normal adult spinal cord transverse sections were reacted with rabbit anti-KCC2 antibody, guinea pig anti-VGAT antibody, or PB (without primary antibody) overnight at room temperature. They were visualized by ABC method (using biotinylated anti-rabbit or anti-guinea pig secondary antibody) under the same condition.

Although dense immunolabeling was detected within the gray matter after the reaction with KCC antibody (A), no significant signal was detected in the sections stained without primary antibody (B). VGAT immunolabeling was abundantly detected within the gray matter after reaction with VGAT antibody (C), no significant immunolabeling was detected in the section without primary antibody (D).

Further, we counted the density of the immunolabeling in the dorsal horn (DH) of each section. When the primary antibody was reacted, the average percentage of KCC2 and VGAT immunolabeling was 31.4% and 51.2%, respectively. But the percentage of the immunolabeling was less than 0.1% in all sections stained without primary antibodies.

Abbreviations; Ab; antibody, cc central canal, DH: dorsal horn, PF; posterior funiculus, Scale bar=100μm

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