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N-type voltage-dependent Ca^{2+} channel in non-excitable microglial cells in mice is involved in the pathophysiology of neuropathic pain

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ABSTRACT

Peripheral nerve injury induces neuropathic pain which is characterized by tactile allodynia and thermal hyperalgesia. N-type voltage-dependent Ca^{2+} channel (VDCC) plays pivotal roles in the development of neuropathic pain, since mice lacking $Ca_v2.2$, the pore-forming subunit of N-type VDCC, show greatly reduced symptoms of both tactile allodynia and thermal hyperalgesia. Our study on gene expression profiles of the $Ca_v2.2$ knockout (KO) spinal cord after spinal nerve ligation (SNL)-injury revealed altered expression of genes known to be expressed in microglia, raising an odd idea that N-type VDCC may function in not only excitable (neurons) but also non-excitable (microglia) cells in neuropathic pain state. In the present study, we have tested this idea by using a transgenic mouse line, in which suppression of $Ca_v2.2$ expression can be achieved specifically in microglia/macrophage by the application of tamoxifen. We found SNL-operated transgenic mice exhibited greatly reduced signs of tactile allodynia, whereas the degree of thermal hyperalgesia was almost the same as that of control. Immunohistochemical analysis of the transgenic lumbar spinal cord revealed reduced accumulation of Iba1-positive cells (microglia/macrophage) around the injured neurons, indicating microglial N-type VDCC is important for accumulation of microglia at the lesion sites. Although the mechanism of its activation is not clear at present, activation of N-type VDCC expressed in non-excitable microglial cells contributes to the pathophysiology of neuropathic pain.

1. Introduction

Voltage-dependent Ca^{2+} channels (VDCCs) are heteromultimers consisting of several subunits (α_1, α_2, β, and δ) and classified into 6 types (L, N, P, Q, R, and T types) according to physiological and pharmacological criteria [1,2]. N-type VDCC, whose core component is $Ca_v2.2$, is mainly expressed in neurons and known for its critical role in the control of neurotransmitter release [3]. We have been studying the physiological roles played by N-type VDCC by analyzing the mice deficient for $Ca_v2.2$ and have found that this $Ca_v2.2$ KO mouse exhibited greatly reduced symptoms of neuropathic pain after peripheral nerve injury [4]. To gain further insights into the signaling mechanism, we performed cDNA array analysis to identify the downstream genes responsible for the alleviated neuropathic pain symptoms in the $Ca_v2.2$ KO mice [5–9]. In this line of study, we have found that expression patterns of several genes, which are known to be expressed in microglial cells, changed in the $Ca_v2.2$ KO mice with peripheral nerve injury compared to that in control mice [6]. Expression of VDCC in non-excitable cell in normal state is generally marginal or negligible and believed to be non-functional. Microglia originated from yolk sac is essential for immune defense of central nervous system (CNS) [10]. Microglia categorized as a non-excitable cell has been shown to express several voltage-dependent channels including L-type VDCC [11]. However, evidence showing the existence of VDCC in microglia is very limited [12], suggesting VDCCs are not always present in the membrane of microglia [11]. Thus our results of array analysis may suggest that the ablation of $Ca_v2.2$ in neuron indirectly affects microglial properties. However, studies using $Ca_v2.2$ KO mice have provided evidence that suggests close relationship between microglial $Ca_v2.2$ and microglial functions. In the lumbar spinal cord from $Ca_v2.2$ KO mice with SNL-injury, reduced accumulation of microglia in the dorsal horn ipsilateral to the lesion was observed [8]. Tokuhara and colleagues reported that $Ca_v2.2$ KO mice exhibited less severe symptoms in an experimental autoimmune encephalopathy (EAE) model, possibly through reduced expression of MCP-1 in microglia, where $Ca_v2.2$ expression was confirmed [13]. Collectively, these data raise a possibility that N-type VDCC may play some physiological functions in microglia as well as in neurons. It has been shown that

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Ca^{2+} signaling properties in isolated microglia (*in vitro*) differ from those in the CNS (*in vivo*) [11,12,14] and Ca^{2+} current through VDCC in microglia is not always observed even in *in vitro* conditions [11]. So the present study aims at obtaining direct evidence for the microglial $Ca_v2.2$ functions in the pathophysiological states, by analyzing tissue-specific inducible $Ca_v2.2$ knockdown transgenic mice in a neuropathic pain model.

2. Materials and methods

2.1. Animal experiments

All the animal experiments were approved (No. 2012-054C) by the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University. Pain-related experiments were performed according to the ethical guidelines for investigations of experimental pain in conscious animals published by the International Association for the Study of Pain [15].

2.2. Generation of conditional $Ca_v2.2$ knockdown transgenic mice

A 2.0 kb *Eco*RI fragment of pCre-ERT2 (a generous gift from Dr. P. Chambon, [16]) was blunt-ended by T4 DNA polymerase and ligated to *Bam*HI-digested pB202 (kindly donated by Dr. D.G. Tenen [17]) after filling-in by T4 DNA polymerase to produce pCD11b-CreERT2. Correct ligation was confirmed by DNA sequencing at the junctions. *Apa*I – digested pBS/U6-ploxPneo (kindly provided by Dr. C.X. Deng [18]) was blunt-ended by T4 DNA polymerase and digested with *Eco*RI and then the resultant fragment was ligated with double-stranded oligonucleotide (Oligo1 and Oligo2) designed to target $Ca_v2.2$. The sequences of these oligonucleotides are as follows: Oligo1, 5′-GCCGTAATGTATCATGGGATTAAGCTTAATCCCATGATACATTACGGCCTTTTTG-3′; Oligo2, 5′-AATTCAAAAAGGCCGTAATGTATCATGGGATTAAGCTTAATCCCATGATACATTACGGC-3′. The correct clones, named pCav22sh2, were confirmed by DNA sequencing. A 5.8 kb *Kpn*I-*Not*I fragment from pCD11b-CreERT2 and a 2.3 kb *Not*I-*Kpn*I fragment from pCav22sh2 were ligated to *Kpn*I-digested pUC18 to produce pCav22sh2-CD11bCreET. To generate transgenic mice, an 8.1 kb *Kpn*I fragment from pCav22sh2-CD11bCreET was prepared and pronuclear injection was performed using C57Bl/6J zygotes. Transgenic founder mice were identified by polymerase chain reaction (PCR) and Southern blot analyses of DNA from tail biopsies. The sequences of the primers, which amplify a ~0.5 kb fragment of Cre transgene, are shown below: 5′-GGTGCAAGTTGAATAACCGG-3′ and 5′-GCTAACCAGCGTTTTCGTTC-3′. Founder mice were crossed to C57Bl/6 J mice to obtain F1 mice hemizygous for the transgene. Mouse genotyping was routinely performed using the primer pair described above with crude lysate from ear or tail tips as templates.

2.3. Southern blot analysis

Southern blot analysis was performed essentially as previously described [19]. The 2.0 kb CreERT2 cDNA fragment was labeled with digoxigenin (DIG) and used as a probe. Hybridization signals obtained with the use of CDP-Star (Tropix Inc.) as a substrate for alkaline phosphatase (AP), conjugated with anti-DIG-antibody (Roche), were detected by an image analyzer (GeneGnome HR, SYNGENE).

2.4. Tamoxifen treatment

Mice of both sexes were used at the age of 26–41 weeks at the start of tamoxifen treatment. Mice received intraperitoneal injection of tamoxifen (Sigma, 40 mg/kg) dissolved in 9:1 mixture of sunflower seed oil (Sigma) and ethanol once a day for 5 consecutive days.

2.5. Spinal nerve ligation (SNL)

SNL-operation was performed 2–4 days after the final tamoxifen injection. Mouse spinal nerves (5th lumbar level (L5) and L6) on the right side were ligated with fine silk thread according to the procedure described previously, with those on the left side kept intact for control [6,20].

2.6. Pain-related behavioral tests

From the start of tamoxifen treatment until the end of behavioral tests, mice were individually housed under temperature- and light-controlled environments (23 ± 1 °C, light and dark cycle of 12 h:12 h with the light on at 8:00 a.m.). Behavioral tests were performed 2–4 weeks after the SNL operation during the light phase in sound-proof rooms with the temperature (24–27 °C) and humidity (40–50%) controlled in essentially the same method as described previously [6]. Tactile allodynia was evaluated by determining the threshold to withdraw hindpaw from increasing mechanical stimuli. Briefly, the threshold for paw withdrawal was determined by applying mechanical stimuli (linearly increasing from 0 to 5 g over 20 s, with the cut-off force 5 g) to plantar surface of hindpaw using Dynamic Plantar Aesthesiometer (Ugo Basile, Italy). Thermal hyperalgesia was evaluated by determining the latency to withdraw a hindpaw from heat stimulus. The withdrawal latency was determined by Paw Thermal Stimulator (UCSD, San Diego), with the cut-off time 20.5 s.

2.7. Immunohistochemistry (IHC)

Mouse spinal cord was perfusion-fixed with 4% paraformaldehyde in phosphate buffered saline (PBS), post-fixed in the same fixative over night at 4 °C, washed with PBS, and finally embedded in OCT compound (Sakura Finetek Japan, Tokyo, Japan). Frozen sections through L5 spinal cord (10 μm thick) were thaw-mounted onto MAS-coated slides (Matsunami Glass Ind., Osaka, Japan).

IHC was performed in a conventional method. The antibodies used were rabbit anti-Iba1 antibody (Wako Pure Chemical Industries, Osaka, Japan, 1:500) and Cy-3 labeled anti rabbit IgG antibody (Jackson ImmunoResearch Laboratories, 1:800) as a secondary antibody. Immunofluorescence was examined with BZ-9000 microscope (Keyence, Osaka, Japan) and the number of Iba1-positive cells was counted using ImageJ software.

2.8. RNA in situ hybridization (ISH)

A ~0.7 kb cDNA fragment corresponding to the cytoplasmic loop II–III of $Ca_v2.2$ was amplified by PCR from mouse brain cDNA and cloned into pCRII vector (Invitrogen). The sequences of the primers are as follows: 5′-AGCAAGTCAAAGCCTGAAGG-3′ and 5′-TGTCAGTGTCACTGGGACAT-3′. The resultant plasmid was linearized and used as templates for synthesis of DIG-labeled riboprobes. The procedure for ISH to spinal cord frozen sections was essentially the same as that reported previously [21], except for the use of highly sensitive signal detection system. Briefly, after the final probe wash in 0.1× SSC at 50 °C, sections on slides were rinsed with PBS and then blocked using biotin-blocking system (Dako). The hybridized probe was then detected with peroxidase-labeled anti-DIG antibody (Roche), followed by biotin-TSA system (Perkin-Elmer) together with AP-labeled streptavidin (Roche), according to the manufacturer's instructions. NBT/BCIP (Roche) was used for substrates of AP for color development.

2.9. Statistical analysis

Data are presented as mean ± s.e.m. Tukey–Kramer test was used to evaluate statistical significance. *P* value less than 0.05 was considered statistically significant.

3. Results

3.1. Conditional knockdown of $Ca_v2.2$ in microglial cells

To address the issue whether $Ca_v2.2$ in microglia play some roles in pathophysiology of neuropathic pain, we generated transgenic mice, where $Ca_v2.2$ expression can be suppressed specifically in microglia/macrophage. Structure of the transgene is shown schematically in Fig. 1A. The transgene consists of two units: (1) CreERT2, a modified Cre recombinase that is activated by tamoxifen, under the control of CD11b promoter [16,17]; (2) expression unit for shRNA targeting $Ca_v2.2$ mRNA driven by the U6 promoter, the activity of which is suppressed by insertion of neomycin resistance (neoR) cassette flanked by two loxP sequences and therefore is recovered by excision of neoR cassette by Cre recombinase [22]. Thus, by treating the transgenic mice with tamoxifen, $Ca_v2.2$ expression can be suppressed specifically in microglia/macrophages. The transgene fragment excised from the vector sequence was microinjected to pronucleus of C57Bl/6 zygotes. After injection into 249 zygotes, 18 F0 mice were eventually obtained, of which 5 were found to be transgenic (Fig. 1B). These transgenic founder mice were crossed to C57Bl/6 mice but only one of them (F0#17) transmitted the transgene through the germ-line (Fig. 1C).

To confirm the suppression of $Ca_v2.2$ expression in microglial cells from tamoxifen-treated transgenic mice, we first attempted quantitative PCR analysis on isolated microglia from whole brains. However, the amounts of $Ca_v2.2$ mRNA detected in samples from wild-type and transgenic mice varied substantially, possibly due to low level expression of microglial $Ca_v2.2$. Thus it was not possible to quantitatively analyze the effect of tamoxifen on $Ca_v2.2$ expression. So we decided to examine the effect of tamoxifen on $Ca_v2.2$ expression qualitatively using the combination of ISH and IHC. We performed ISH experiments on frozen sections of the lumbar spinal cord from tamoxifen-pretreated wild-type and transgenic mice with SNL-injury. As shown in Fig. 2A, B, the major cell type that expresses $Ca_v2.2$ was neurons, consistent with previous studies [23,24], with no obvious differences in the signal intensi-

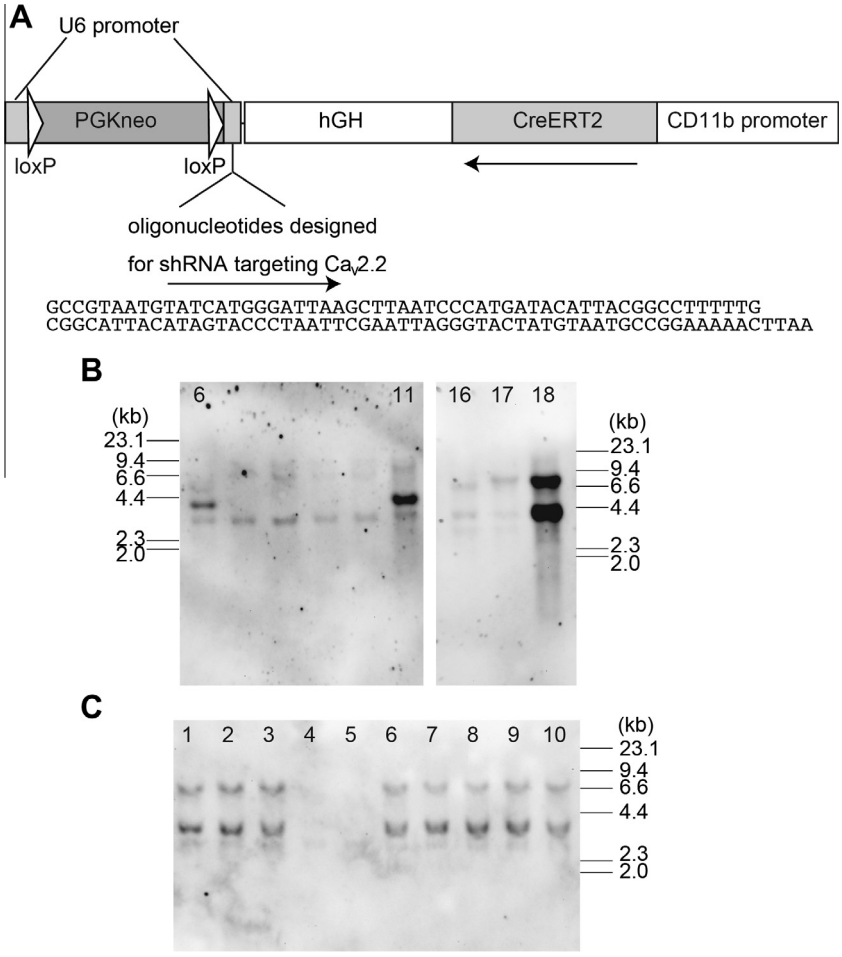

Fig. 1. Generation of conditional $Ca_v2.2$ knockdown transgenic mice. (A) Schematic presentation of the transgene. Oligonucleotides designed to knockdown $Ca_v2.2$ by RNAi were inserted downstream of the U6 promoter, whose activity was inhibited by the insertion of loxP-neoR cassette (PGKneo). Excision of loxP-PGKneo by Cre recombinase leads to expression of shRNA against $Ca_v2.2$. Tamoxifen-inducible Cre (CreERT2) is expressed in microglia/macrophages under the control of CD11b promoter. hGH, a part of human growth hormone gene. Arrows indicate the direction of transcription. Southern blot analysis of tail DNA from F0 founder mice (B) and F1 offspring obtained by crossing between a C57Bl/6 female and F0#17 founder mouse (C). *Eco*RI-digested tail DNA was resolved through 0.7% agarose gel and blotted onto a nylon membrane. The blot was hybridized with DIG-labeled CreERT2 probe. Band with the size of 4.1 kb observed is the correct size of transgene. Band with the size of ~3 kb observed are thought to be due to cross-hybridization to mouse endogenous estrogen receptor sequences. In (B), numbered lanes (6, 11, 16–18) indicate transgenic mice. In (C), lanes 1–3 and 6–9, transgenic F1 offspring; lane 10, F0#17 founder; lanes 4 and 5, wild-type F1 offspring.

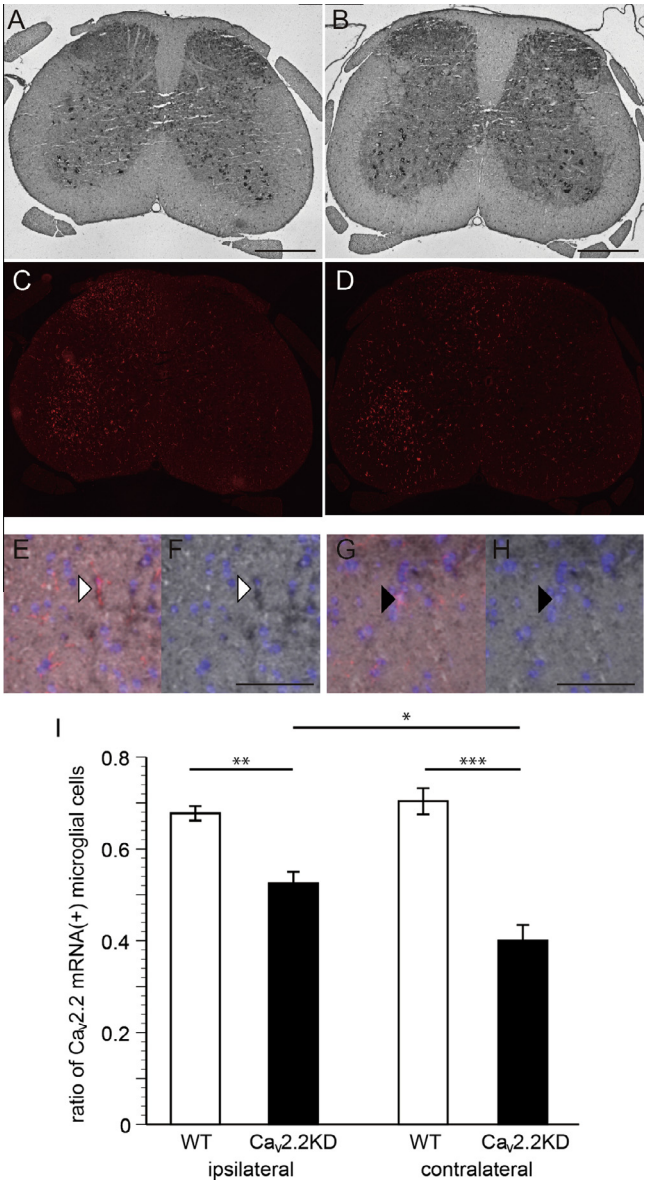

Fig. 2. ISH analysis to confirm knockdown of $Ca_v2.2$ in microglial cells. One week after SNL-operation, spinal cords of tamoxifen-pretreated mice were fixed and processed for ISH. Sections through lumbar spinal cord were *in situ* hybridized with DIG-labeled antisense probe for $Ca_v2.2$ (A and B). After signal development for ISH, the same sections were subjected to IHC with anti-Iba1 antibody to detect microglial cells (C and D). (A) and (C), wild-type spinal cord; (B) and (D), $Ca_v2.2$ knockdown transgenic spinal cord. (E) and (F), an example of microglia expressing $Ca_v2.2$ mRNA (white arrow head); (G) and (H) an example of microglia where $Ca_v2.2$ mRNA cannot be detected (black arrow head). (E) and (G) are triple-overlay of signals of ISH for $Ca_v2.2$, IHC for Iba1 and nuclear staining with Hoechst 33258, while (F) and (H) are double-overlay of signals of ISH and nuclear staining. Scale bars, 500 μm in (A) and (B); 100 μm in (F) and (H). (I) Summary of the ratio of microglia positive for the ISH signal to the total number counted on each side of the spinal cord. Open column (WT), wild-type ($n = 5$); closed column ($Ca_v2.2$KD), conditional $Ca_v2.2$ knockdown transgenic ($n = 5$). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

ties between wild-type and conditional $Ca_v2.2$ knockdown mice. However, examination of sections labeled with both ISH for $Ca_v2.2$ mRNA (Fig. 2A and B) and IHC for Iba1 (Fig. 2C and D) revealed that approximately 70% of the microglia in the spinal cord expressed $Ca_v2.2$ mRNA in the wild-type spinal cord on both sides with respect to the SNL-injury (Fig. 2E–I). On the other hand, in the conditional $Ca_v2.2$ knockdown mice, number of $Ca_v2.2$ mRNA-positive microglia on the contralateral side to the SNL-injury was reduced to 40% (Fig. 2I). The ratio of $Ca_v2.2$ mRNA-positive microglia on the ipsilateral side was 10% higher compared to the contralateral side in the knockdown mice, suggesting up-regulation of $Ca_v2.2$ expression in activated microglial cells, though the ratio of $Ca_v2.2$ mRNA-positive microglia on the ipsilateral side of knockdown mice was still 20% lower than that of wild-type mice. At present, we cannot evaluate to what degree $Ca_v2.2$ expression was decreased in transgenic microglia quantitatively but microglial $Ca_v2.2$ expression was surely suppressed in conditional $Ca_v2.2$ knockdown mice.

3.2. Tactile allodynia is greatly alleviated in conditional $Ca_v2.2$ knockdown mice

In wild-type mice, SNL-operation induced a marked decrease in the threshold for paw withdrawal from the mechanical stimulation on the ipsilateral side, reminiscent of tactile allodynia (Fig. 3A). In conditional $Ca_v2.2$ knockdown mice, however, the threshold was only slightly decreased on the ipsilateral side compared to the control, showing tactile allodynia is greatly alleviated (Fig. 3A).

As for thermal hyperalgesia, wild-type mice exhibited shortened withdrawal latency on the side ipsilateral to the lesion (Fig. 3B). In conditional knockdown mice, withdrawal latency on the ipsilateral side was shortened to almost the same level as that of wild-type (Fig. 3B). Therefore, thermal hyperalgesia occurs almost normally in the conditional knockdown mice.

3.3. Reduced accumulation of spinal microglia in conditional $Ca_v2.2$ knockdown mice

We previously reported that accumulation of microglia in the lumbar spinal cord ipsilateral to the nerve injury was attenuated in $Ca_v2.2$ KO mice [8]. To address the question whether the attenuated microglial accumulation was cell-autonomous, i.e. whether the deficit in $Ca_v2.2$ in microglia by itself causes the reduced microglial accumulation in the spinal cord, we examined the number of microglial cells. After the behavioral tests, mice were sacrificed and their spinal cords were subjected to IHC with Iba1 antibody. In wild-type mice, the number of microglia 1 month after SNL operation was still significantly larger on the ipsilateral side than on the contralateral side (Fig. 4A and C). In the conditional $Ca_v2.2$ knockdown mice, the number of microglia on the ipsilateral side was also significantly larger than the contralateral side, but was significantly smaller than that of wild-type (Fig. 4B and C). Therefore, microglial $Ca_v2.2$ seems to be directly involved in the microglial activities such as migration and proliferation.

4. Discussion

Nerve injury causes an intractable chronic pain, called neuropathic pain. Patients with neuropathic pain suffer from not only stimulus-independent spontaneous pain but also two types of stimulus-dependent pain, i.e. allodynia (in which normally innocuous stimuli can cause pain) and hyperalgesia (enhanced responses to noxious stimuli) [25]. Results of recent studies have clearly demonstrated that microglia plays crucial roles in the development of several neurological disorders including neuropathic pain [26,27]. In the present study, we have established a transgenic mouse line, where $Ca_v2.2$ expression can be suppressed by tamoxifen-inducible RNAi specifically in microglia/macrophages. ISH experiments on the spinal cord sections from conditional $Ca_v2.2$ knockdown mice showed that the number of microglial cells where $Ca_v2.2$ mRNA was detected, decreased on both contralateral and ipsilateral sides compared to the wild-type control. Especially, on the contralateral side, which represents nor-

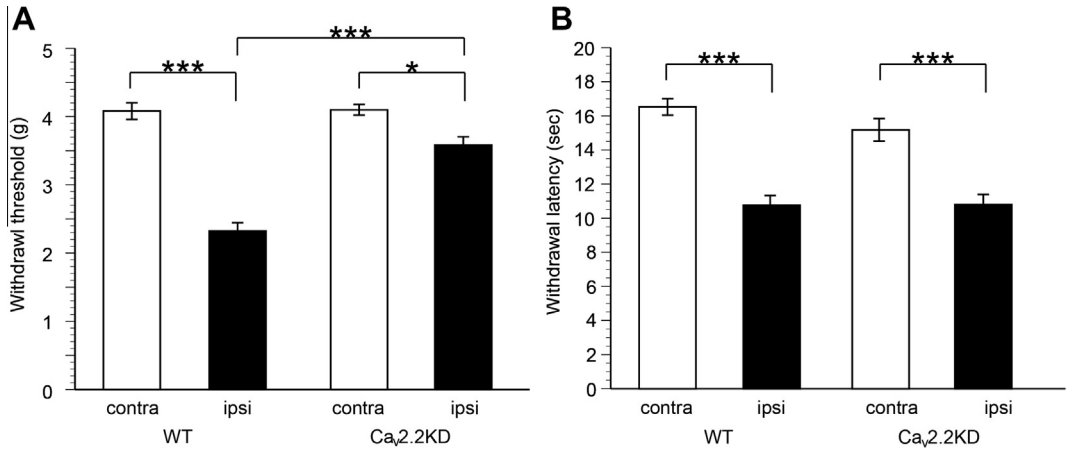

Fig. 3. Pain related behavioral tests in tamoxifen-pretreated conditional $Ca_v2.2$ knockdown mice. (A) Threshold for paw withdrawal from mechanical stimulus. (B) Latency for paw withdrawal from heat stimulus. Open column, contralateral to the SNL operation site; closed column, ipsilateral to the operation site ($n = 9$ and 8 for wild-type and conditional $Ca_v2.2$ knockdown transgenic, respectively) $^{*}p < 0.05$, $^{***}p < 0.001$.

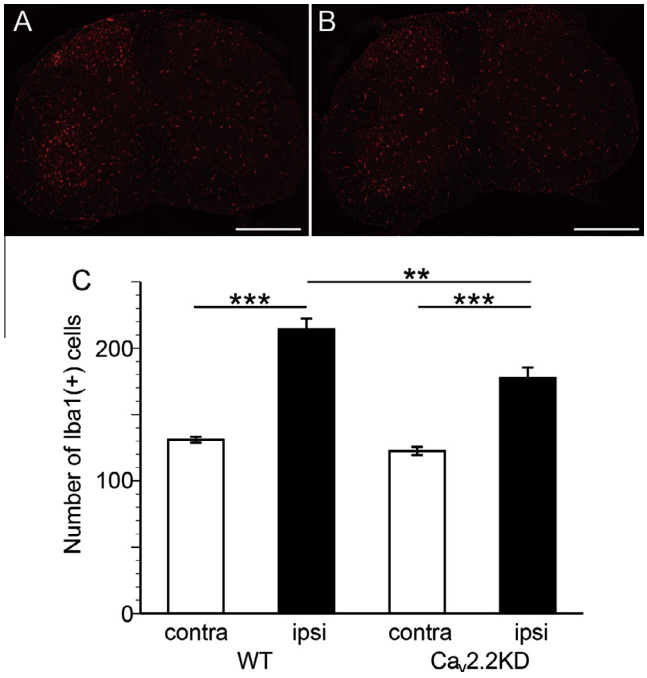

Fig. 4. Reduced accumulation of microglial cells in the spinal cord in conditional $Ca_v2.2$ knockdown mice with SNL-injury. After the pain-related behavioral tests (~1 month after SNL-operation), the spinal cord was fixed and frozen sections through lumbar spinal cord were prepared. Sections were then subjected to IHC with anti-Iba1 antibody. (A), wild-type; (B), conditional $Ca_v2.2$ knockdown transgenic. (C) Total number of Iba1-positive cells on each side with respect to the SNL-operation. Open column, contralateral to the SNL-operation; closed column, ipsilateral to the SNL-operation ($n = 7$ and 6 for wild-type and conditional $Ca_v2.2$ knockdown transgenic, respectively). Scale bars, 500 μm $^{**}p < 0.01$, $^{***}p < 0.001$.

mal state, marked decrease in the number of $Ca_v2.2$ mRNA-positive microglia was observed, suggesting that in nearly half of the microglial cells which were to express $Ca_v2.2$, $Ca_v2.2$ expression was blocked. Since the ISH method used in the present study is highly sensitive [28], it is probable that we counted the cells as $Ca_v2.2$-positive even though $Ca_v2.2$ expression was reduced, clearly leading to an underestimation of the $Ca_v2.2$ suppression. However, at present it may be safe to conclude that in nearly half of the microglial cells, $Ca_v2.2$ expression was decreased to non-detectable level.

We previously reported that $Ca_v2.2$KO mice lacking N-type VDCC in both neurons and non-neuronal cells exhibited greatly reduced signs of tactile allodynia and thermal hyperalgesia after SNL-injury [4]. In the present study, conditional $Ca_v2.2$ knockdown mice showed greatly alleviated signs of tactile allodynia but robust thermal hyperalgesia after SNL-injury. Therefore, contribution of microglial N-type VDCC to the development of tactile allodynia seems larger compared to the neuronal N-type VDCC. On the contrary, contribution of microglial N-type VDCC to the development of thermal hyperalgesia seems to be marginal, suggesting a larger contribution of neuronal N-type VDCC. Thus, pathogenic mechanisms underlying neuropathic pain seem to contain two independent signaling pathways from the perspective of N-type VDCC activation, one in neurons and the other in microglia. However, exact role played by N-type VDCC in microglia remains to be elucidated. To speculate the functions of microglial N-type VDCC, the results of analysis on the accumulated microglia in the spinal cord from the conditional $Ca_v2.2$ knockdown mice are suggestive. The reduced accumulation of microglia in the conditional $Ca_v2.2$ knockdown spinal cords is thought to reflect the deficits in microglial activities including migration and/or proliferation. Migration of microglia is thought to be controlled by several chemoattractants including cytokine MCP-1 [29]. MCP-1 is known to be critical for monocyte recruitment in some inflammatory diseases [30]. Furthermore, activated microglia from $Ca_v2.2$KO mice were found to express and release smaller amount of MCP-1 compared to wild-type control in an EAE model [13]. Thus, it is possible that microglial $Ca_v2.2$ controls production and release of MCP-1. If this is also the case with the SNL model of neuropathic pain, reduced number of microglia in the spinal cord from conditional $Ca_v2.2$ knockdown mice may be explained by the reduced recruitment of monocytes, though monocyte recruitment into the CNS remains controversial [31,32]. On the other hand, 30% of the microglial cells accumulated in the spinal cord were found to be $Ca_v2.2$-negative in wild-type mice with no change in the percentage after SNL-injury. Thus other VDCC like L-type VDCC which is known to be present in some of the microglia might also be involved in microglial migration and/or proliferation. Ca^{2+} currents through N- and L-type VDCCs have not been observed in microglia *in situ* or *in vivo* [11,12], though we have detected $Ca_v2.2$ mRNA, albeit very low level, in ~70% of the microglia. Furthermore, sensitivities to mechanical and thermal stimuli in the contralateral hindpaw of conditional $Ca_v2.2$ knockdown mice did not differ from those in wild-type mice. These results combined may suggest the following possibility: N-type

VDCC is present in microglia but may not be functional in normal condition. But the microglial N-type VDCC becomes functional through as yet unknown mechanism in pathological situations like neuropathic pain state and contributes to the seriousness of the disease *in vivo*. N-type and/or L-type VDCC blockers have been considered as candidates for treating several neurological disorders [3]. Our results indicate the importance of taking the contribution of microglial VDCCs into account in case of using these blockers for clinical purposes, because voltage dependent channels could be functional even in non-excitable cells in pathological conditions.

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