PLA_2 to determine the subtype would be the next important issue.

On the other hand, it currently remains unknown which cell types are involved in nerve injury-induced LPA production, although there are some reports that LPA can be synthesized and secreted by primary neurons and Schwann cells in vitro (Fukushima et al., 2000; Weiner et al., 2001). It is particularly difficult to clarify whether the de novo LPA synthesis occurs in specific neurons or highly differentiated cell types. Alternatively, LPA synthesis may occur through neuron–glia interactions or in an autocrine manner. In future research, an important aim will be the evaluation of LPA production in individual or cocultured specific cell types. Another issue to be investigated in the future is the clarification of which species of LPA molecules are involved in the injury-induced de novo synthesis, because there are several subspecies of LPA (Aoki, 2004; Aoki et al., 2008). The development of an advanced method using mass spectrometry with highly efficient purification and condensation would be required for such studies.

The present study also provides information regarding the mechanisms underlying nerve injury-induced neuropathic pain. In a series of previous studies, we have demonstrated that LPA_1 receptor signaling initiates nerve injury-induced neuropathic pain and its underlying mechanisms (Inoue et al., 2004; Ueda, 2006, 2008). Recently, a pharmacological study demonstrated that LPA_1 signaling can initiate neuropathic pain within a timeframe of 2 to 4 h (Ma et al., 2009a). Therefore, our present study provides the first demonstration that the de novo biosynthesis of LPA at 2 to 3 h after nerve injury is essential for the development of neuropathic pain. Targeted inhibition of PLA_2- and ATX-mediated LPA synthesis may be a potential strategy for the prevention of nerve injury-induced neuropathic pain.

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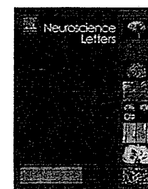

Absence of morphine analgesia and its underlying descending serotonergic activation in an experimental mouse model of fibromyalgia

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ABSTRACT

Mice exposed to intermittent cold stress (ICS), but not constant cold stress (CCS) showed sustained thermal hyperalgesia for up to 12 days. Systemic or intracerebroventricular (i.c.v.) injection of morphine caused no significant analgesia in ICS mice, but induced dose-dependent analgesia in control mice. However, significant analgesic effects were achieved by intrathecal or intraplantar injection of morphine. The i.c.v. injection of morphine significantly increased the turnover ratio (5-HIAA/5-HT) in the dorsal half of the spinal cord of control mice, but not in ICS mice. Collectively, these results indicate that the loss of descending serotonergic activation seems to be a key mechanism underlying the absence of morphine-induced analgesia.

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Fibromyalgia (FM) is a common condition with generalized or widespread allodynia that affects at least 2% of the US, European and Japanese populations. It is most common in women, but also prevalent in men and children [5,10,35,37]. Although the etiology of this disease remains to be fully understood, it has been reported that genetic and environmental factors are likely to be involved in the development of FM [3,4,33,36,38]. Regarding analgesic medications for FM, opioids and NSAIDs are not effective [5,20,28,34], but only a few existing drugs are permitted for the treatment of FM in some countries [1,13,21]. To develop new treatments for FM, experimental animal models for FM are needed. So far, several FM-like experimental animal models have been proposed and involve injecting acidic saline into the gastrocnemius muscle [32], vagotomy [18] and sound stress [17], which are believed to mimic the peripheral mechanisms underlying FM-like pain. More recently, we have reported another mouse model using intermittent cold stress (ICS model), which show a generalized, female-predominant, long-lasting pain, as often observed in FM patients [25]. The present study demonstrates that mice exposed to ICS show an absence of morphine analgesia, a common feature in FM patients, and this is accompanied by an absence of morphine-induced activation of the descending serotonergic system.

Male C57BL/6J mice weighing 18–22 g were used (total number = 249). They were kept in a room with a temperature of 22 ± 2 °C with free access to standard laboratory diet and tap water. All procedures were approved by the Nagasaki University Animal Care Committee and complied with the recommendations of the International Association for the Study of Pain [44]. ICS and CCS (constant cold stress) were induced as previously reported [25]. Briefly, for the ICS model, mice were placed in a cold room at 4 °C overnight (from 4:30 pm to 10:00 am), followed by ICS with alternating environmental temperatures between 24 and 4 °C every 30 min from 10:00 am to 4:30 pm. These procedures were repeated twice. On the day 3, the mice were returned to and adapted to the room at 24 °C for 1 h before the behavior tests. For the CCS model, mice were placed in the cold room from 4:30 pm on the day 1 to 10:00 am on day 3, followed by adaptation at 24 °C for 1 h. Mice in the control group were kept at 24 °C for all 3 days (from 4:30 pm on day 1 to 10:00 am on day 3). During the stress period, two mice were kept in each cage (120 × 150 × 105 cm), with free access to food and agar in place of fluid. There was no significant change in the amounts of food or water/agar intake during the stress period or body weight at P5 (post-stress day 5) among the three groups.

For the thermal paw withdrawal test, the nociception threshold was determined as the latency to paw withdrawal upon a thermal stimulus [11,16]. Unanesthetized animals were placed in plexiglass cages on top of a glass sheet, and allowed to adapt for 1 h. The thermal stimulator (IITC Inc., Woodland Hills, CA, USA) was positioned under the glass sheet and the focus of the projection bulb was aimed exactly at the middle of the plantar surface of the paw. A mirror attached to the stimulator permitted visualization of the plantar surface. A cut-off time of 20 s was used to prevent tissue damage. The intrathecal injection was performed between the spinal L5 and L6 segments according to the method of

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Hylden and Wilcox [15]. Morphine hydrochloride (Takeda Chemical Industries, Osaka, Japan) was dissolved in physiological saline or artificial cerebrospinal fluid (aCSF; 125 mM NaCl, 3.8 mM KCl, 2.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 1.2 mM KH_2PO_4, 26 mM $NaHCO_3$, 10 mM glucose, pH 7.4); these vehicles were injected in the control group. The doses of drug were chosen according to results of previous studies [30] and our pilot studies; the dose that produced the maximum analgesic effect without causing toxicity or motor impairment was used. For the time-course experiment, we measured the paw withdrawal latencies at every 10 min interval until 60 min after morphine injection. In the AUC (area under the curve) score for morphine analgesia was calculated by deducting the basal AUC score (Time 0 threshold × 50 min) from the score with morphine from Time 10 to 60 min.

For the determination of serotonergic turnover ratio, the spinal cord (4 mm, L4–L6 region) was isolated from each mouse at 20 min after the i.c.v. morphine (or vehicle) treatment at noon on P1 (post-stress day 1) or P5. The dorsal half of the spinal cord was then placed in 500 μL of isoproterenol and stored at −80 °C before use. The quantification of 5-HT and 5-HIAA was performed use HPLC with electrochemical detection, as previously reported [24].

All results are expressed as means ± S.E. Differences between multiple groups were analyzed by one-way ANOVA with Scheffe's *F* multiple comparison post hoc analysis. Changes in the thresholds were analyzed using unpaired Student's *t*-test. The criterion of significance was set at $p < 0.05$.

Mice exposed to ICS showed hyperalgesia that lasted for up to 12 days for the thermal paw withdrawal test, which is consistent with our recent results with the mechanical nociception test [25]. The level of hyperalgesia was comparable with that of the neuropathic pain model using partial sciatic nerve injury [16,31]. This sustained hyperalgesia is specific to intermittent cold exposure because hyperalgesia in mice exposed to constant cold stress was transient, lasting for only 5 days after the stress (Fig. 1B). In the present study, to assess the pharmacological effect of morphine in the ICS model, we administered morphine (3 mg/kg, s.c.) to mice with or without ICS. Morphine treatment quickly and significantly increased the threshold of control mice, with a peak at 20 min post-treatment (Fig. 1C). However, there was no change in the threshold for the 60 min period in mice at day 5 (P5) after ICS (Fig. 1D). The absence of analgesia at doses of morphine ranging from 0.3 to 3 mg/kg (s.c.) was also observed based on AUC scores for analgesia at P1 or P5 after ICS exposure (Fig. 1E).

To clarify the mechanisms underlying the absence of morphine analgesia, we treated mice with morphine via three different routes, intracerebroventricular (i.c.v.), intrathecal (i.t.) and intraplantar (i.pl.). As shown in Fig. 2A, i.c.v. injection of morphine between 0.03 and 0.3 nmol dose-dependently increased the analgesic AUC score in control mice, but not in ICS mice. Of note, 0.3 nmol morphine via i.c.v. showed equipotent analgesia in CCS mice to that in control mice. When 1 nmol morphine was administered via the i.t. route, the analgesia was comparable to that of 0.3 nmol morphine via i.c.v. in control and ICS mice at P5 (Fig. 2B). The i.pl. injection of 30 nmol morphine showed equivalent analgesia in control mice, but only 50% of the maximal analgesia in ICS mice (Fig. 2C). Collectively, these results suggest that supraspinal analgesia is the dominant form of analgesia after systemic administration of morphine, and the activation of the cerebrospinal descending pain-inhibitory pathway is lost, thus underlying the morphine analgesia in ICS mice.

Central serotonergic neurons are an important origin of the descending modulatory systems, which inhibit or facilitate pain at the level of the spinal cord [7]. Morphine activates the serotonergic neurons to accelerate 5-hydroxytryptamine (5-HT) turnover ratio in the spinal cord [8,23,39,40,43]. To determine whether the serotonergic neurons are activated, the levels of 5-HT and its major

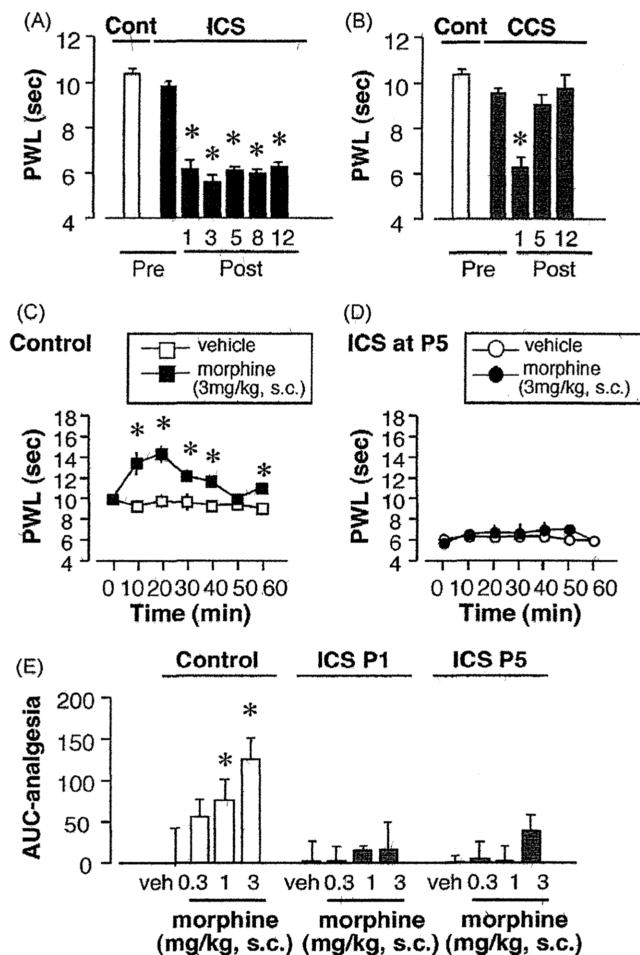

Fig. 1. Absence of morphine analgesia in a murine ICS model of fibromyalgia. (A) ICS-induced thermal hyperalgesia before stress (day 0) and at P1, 3, 5, 8 and 12 (days 3, 5, 7, 10 and 14) after stress. (B) CCS-induced thermal hyperalgesia before stress (day 0) and at P1, 5 and 12 (days 3, 7 and 14) after stress. Results are presented as paw withdrawal latency (PWL) in seconds. $^*p < 0.05$ versus the control group. Results are means ± S.E. for 4 mice. (C, D) Effects of systemic morphine analgesia on ICS-induced hyperalgesia at P5. Time-course of morphine (3 mg/kg, s.c.) analgesia in the ICS model (D). (E) Dose–response for morphine analgesia for 60 min after injection in the control and ICS model. The data are presented as AUC analgesia. $^*p < 0.05$ versus the vehicle group. Results are means ± S.E. for 6 individual mice per group.

metabolite 5-hydroxyindolacetic acid (5-HIAA) were measured at P1 and P5 after ICS exposure. For this experiment, the dorsal horn of the spinal cord was isolated 20 min after the administration of morphine (30 nmol, i.c.v.). There was no significant difference in the 5-HT level (ng/mg tissue) between control, P1 and P5 after ICS with or without morphine (Fig. 3A), while the 5-HIAA level increased slightly, although not significantly, after morphine treatment in control mice (Fig. 3B). However, morphine treatment significantly increased the turnover ratio, defined as 5-HIAA/5-HT, in control mice, but not in ICS mice at P1 or P5 (Fig. 3C).

We demonstrated that there is substantially no analgesia by morphine in a dose up to 3 mg/kg s.c. in the ICS, an FM-like animal model. In the present study we adopted the thermal paw withdrawal test, as a less stressful nociception test without requirement of animal holding in order to avoid the influence of handling stress. As the hyperlocomotion of mice given with 10 mg/kg (s.c.) of morphine makes it difficult to measure the reliable nociceptive threshold, we gave up using such higher doses. Therefore we were not able to conclude that morphine analgesia is completely absent

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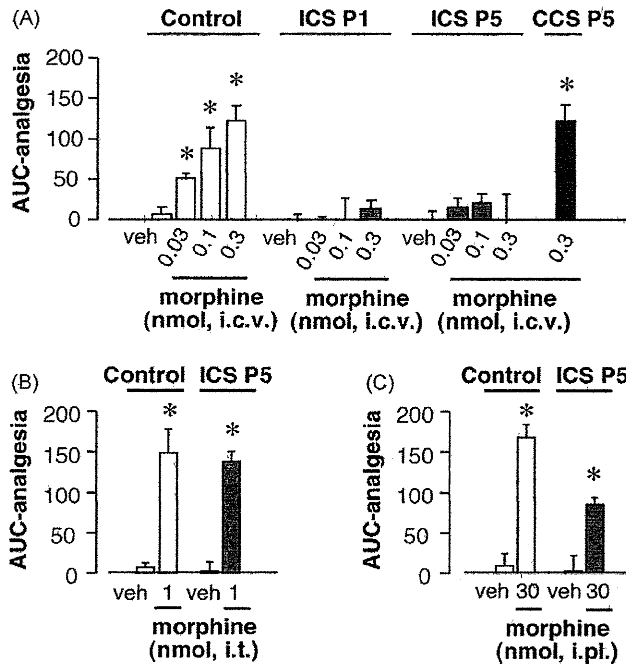

Fig. 2. Selective absence of analgesia after supra-spinal administration of morphine. (A) Analgesia induced by i.c.v. administration of different doses of morphine. Experiments were performed in control, ICS (P1 and P5) or CCS model mice. (B, C) Analgesia induced by i.t. (B) or i.pl. (C) administration of morphine. Experiments were performed in control and ICS (P5) mice. All results are represented as the AUC score. Results are means ± S.E. for 4 mice with vehicle in control group and for 9 individual mice with other treatments. $^{*}p < 0.05$ versus the vehicle group.

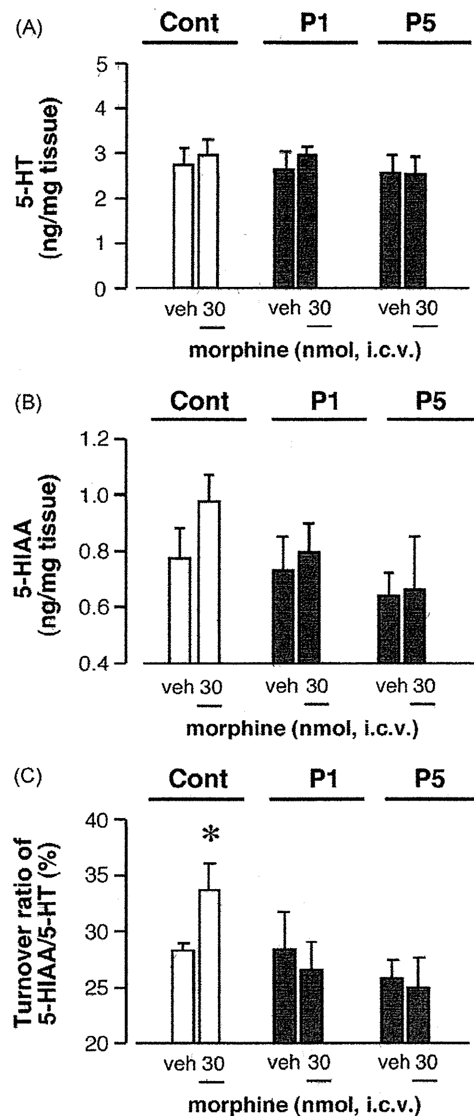

Fig. 3. Loss of morphine-stimulated serotonin turnover ratio in ICS mice. Levels (ng/mg wet weight of tissue) of 5-HT (A) and 5-HIAA (B), and turnover ratio of 5-HIAA/5-HT (C) in the dorsal half of the spinal cord from control and ICS (P1 and P5) mice treated with or without morphine (30 nmol, i.c.v.). Results are means ± S.E. for 6–10 individual mice per group. $^{*}p < 0.05$ versus the vehicle group.

in the ICS model. This absence of morphine analgesia occurs as early as P1 and lasts at least for 5 days after the stress. These findings suggest that the analgesic absence is more like related to the ICS-type stress, but not to the secondary stress caused by chronic pain.

Similar absence of morphine analgesia was also observed when it was given supra-spinally (i.c.v.), but not spinally (i.t.). According to a number of studies [9,14,26,27], major sites of actions for morphine analgesia are located at the lower brain stem, such as periaqueductal grey matter (PAG) and rostral ventromedial medulla (RVM). Specifically, descending serotonergic and noradrenergic systems from lower brain stem to spinal cord are representative pathways [9,22,23,39]. Thus, it is speculated that the failure of activation of descending pain-inhibitory system may underlie the absence of morphine analgesia. This speculation was supported by the present study, in which morphine-induced increase in serotonergic turnover ratio, a key indicator for the neuronal activity, was also absent at least for 5 days after the ICS-type stress.

It is also an interesting question whether the alteration of descending serotonergic activity is related to the cause of chronic pain, since serotonergic or noradrenergic reuptake inhibitors are widely used for FM in clinic [1,6,28,37]. However, we failed to detect any change in the serotonergic turnover ratio in ICS model mice in the absence of exogenous pain stimuli, as seen in Fig. 3C. Further studies using exogenous pain stimuli would be the next subject.

Although the mechanisms underlying absence of morphine analgesia in the ICS model remains to be determined, several investigators have attempted to explain the absence of morphine analgesia in FM patients based on the following findings: (1) enkephalin levels in the cerebrospinal fluid (CSF) are elevated, and (2) mu opioid receptor (MOP) binding in neuroimaging study is decreased in the nucleus accumbens, amygdala and dorsal anterior cingulated [6,12,29,34]. These studies suggest that high levels of endogenous opioids may down-regulate MOP, which may lower the potency of morphine. However, these studies did not describe the significant changes in midbrain periaqueductal grey or rostral ventromedial medulla, representative loci for morphine analgesia [9]. More detailed analyses might be necessary in terms of the absence of morphine analgesia in FM patients. Alternative mechanisms might be explained by the anti-opioid hypothesis, in which the analgesia is reduced by counter-adaptation through enhanced activations of glutamate-NMDA receptor system or neuropeptide systems following chronic morphine treatments [42]. Although closely related clinical evidence is not available, there are reports that pain in FM patients is alleviated by ketamine, possessing NMDA receptor antagonist activity [34].

On the other hand, in the mouse model with partial sciatic nerve injury, morphine analgesia was 10-fold less potent [30], as is often seen in patients with neuropathic pain [2,19]. In that peripheral neuropathic pain model, there was no analgesia by peripheral (i.pl.) morphine, while i.t. morphine was 10-fold less potent. These

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decreased potencies may be attributed to a decrease in MOP expression in the DRG [30,41]. However, i.c.v. morphine showed no significant change in the potency of morphine analgesia after nerve injury. These results demonstrate that the mechanisms underlying chronic pain by ICS are quite different from those underlying peripheral neuropathic pain, in terms of supra-spinal morphine analgesia.

Lastly, we have reported that there was a female predominance of chronic pain in the ICS model mice, when they had been gonadectomized [25]. However, no significant change in the pain threshold was observed between male and female mice without gonadectomy. As female mice have a short estrus cycle in 4–6 days, we decided to use male mice in the present study. Studies using gonadectomized female mice would be also needed for the further discussion of the lack of morphine analgesia.

Taking into consideration the previous findings that the chronic pain by ICS is long-lasting, generalized and female-predominant, the present study suggest that the ICS model showing absence of morphine analgesia seems to mimic a variety of clinical evidence with FM in patients [6,12,29,34]. Furthermore, the loss of descending serotonergic activation seems to be a key mechanism underlying the absence of morphine analgesia.

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NEURON-RESTRICTIVE SILENCER FACTOR CAUSES EPIGENETIC SILENCING OF K_V4.3 GENE AFTER PERIPHERAL NERVE INJURY

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Abstract—Peripheral nerve injury causes a variety of alterations in pain-related gene expression in primary afferent, which underlie the neuronal plasticity in neuropathic pain. One of the characteristic alterations is a long-lasting down-regulation of voltage-gated potassium (K_v) channel, including K_v4.3, in the dorsal root ganglion (DRG). The present study showed that nerve injury reduces the messenger RNA (mRNA) expression level of K_v4.3 gene, which contains a conserved neuron-restrictive silencer element (NRSE), a binding site for neuron-restrictive silencer factor (NRSF). Moreover, we found that injury causes an increase in direct NRSF binding to K_v4.3-NRSE in the DRG, using chromatin immunoprecipitation (ChIP) assay. ChIP assay further revealed that acetylation of histone H4, but not H3, at K_v4.3-NRSE is markedly reduced at day 7 post-injury. Finally, the injury-induced K_v4.3 downregulation was significantly blocked by antisense-knockdown of NRSF. Taken together, these data suggest that nerve injury causes an epigenetic silencing of K_v4.3 gene mediated through transcriptional suppressor NRSF in the DRG. **© 2010 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: neuropathic pain, dorsal root ganglion, transcription, epigenetic, voltage-gated potassium channel, neuron-restrictive silencer factor.

In the primary afferent, transient outward potassium current (I_A) is crucial in controlling electrical excitability (Vydyanathan et al., 2005). Voltage-gated potassium (K_v) channel K_v4 subunits (K_v4.1, K_v4.2, and K_v4.3) and K_v1.4 mediate the I_A (Vydyanathan et al., 2005; Phuket and Covarrubias, 2009). On the other hand, peripheral nerve injury is known to reduce the I_A (Everill and Kocsis, 1999) and the expression of dominant isoform K_v4.3 located at nonpeptidergic C-fibers over a long period, thereby causing neuronal hyperexcitability underlying neuropathic pain (Kim et al., 2002; Chien et al., 2007). However, the underlying transcriptional mechanisms remain unknown.

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Abbreviations: aCSF, artificial cerebrospinal fluid; AS-ODN, antisense oligodeoxynucleotide; ChIP, chromatin immunoprecipitation; DRG, dorsal root ganglion; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HDAC, histone deacetylase; I_A, transient outward potassium current; K_v, voltage-gated potassium; mRNA, messenger RNA; MS-ODN, mismatch scrambled oligodeoxynucleotide; NRSE, neuron-restrictive silencer element; NRSF, neuron-restrictive silencer factor; PCR, polymerase chain reaction.

Transcription factor-mediated epigenetic mechanisms, such as DNA methylation and histone modifications, regulate the long-lasting transcription, thereby contributing to neuronal functions (Borrelli et al., 2008). Neuron-restrictive silencer factor (NRSF, also known as REST) functions as a repressor of genes, which contain neuron-restrictive silencer element (NRSE, also called RE1). Upon binding to NRSE, NRSF recruits histone deacetylase (HDAC) for generating a repressive chromatin environment (Ballas and Mandel, 2005). Since the presence of NRSE in K_v4.3 gene is reported (Otto et al., 2007), we hypothesized that injury causes NRSF-directed epigenetic silencing of K_v4.3 gene in the dorsal root ganglion (DRG).

EXPERIMENTAL PROCEDURES

Animals and surgery

Male C57BL/6J mice weighing 20–25 g were used. They were kept in a room with a temperature of 21±2 °C with free access to standard laboratory diet and tap water. The experiments were designed to minimize the number of animals used and their suffering. All procedures were approved by the Nagasaki University Animal Care Committee and were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. Partial sciatic nerve ligation was performed as described previously (Inoue et al., 2004).

Oligonucleotide treatments

The antisense oligodeoxynucleotide (AS-ODN) against NRSF (5′-CGGAAGGGCTTGGCC-3′) and its mismatch scrambled oligodeoxynucleotide (MS-ODN; 5′-GTCGTCGGCGGAGCA-3′) were synthesized. AS-ODN and MS-ODN were freshly dissolved in artificial cerebrospinal fluid (aCSF; 125 mM NaCl, 3.8 mM KCl, 2.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 1.2 mM KH_2PO_4, 26 mM $NaHCO_3$, 10 mM glucose, pH 7.4). AS-ODN or MS-ODN was intrathecally injected in a dose of 10 μg/5 μl of aCSF on the first, third, and fifth days, then injury was done with subsequent injections of AS-ODN on days 1, 3, 5, and 6 after injury. The intrathecal injection was administered into the space between the spinal L5 and L6 segments according to the method of Hylden and Wilcox (Hylden and Wilcox, 1980). The messenger RNA (mRNA) expression was assessed at day 7 post-injury.

Quantitative real-time polymerase chain reaction (PCR)

The extraction of total RNA from the ipsilateral L4-6 DRGs and quantitative real-time PCR were performed as described previously (Uchida et al., 2009). The PCR primers used were as follows: for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-TATGACTCCACTCACGGCAAAT-3′ (forward) and 5′-GGGTCTCGCTCCTGGAAGAT-3′ (reverse); for K_v4.3, 5′-TGCATCTTTCTGGTACACCATAGT-3′ (forward) and 5′-GCTAAAGTTGGAGACTATCACAGG-3′ (reverse). GAPDH was used as an internal control for

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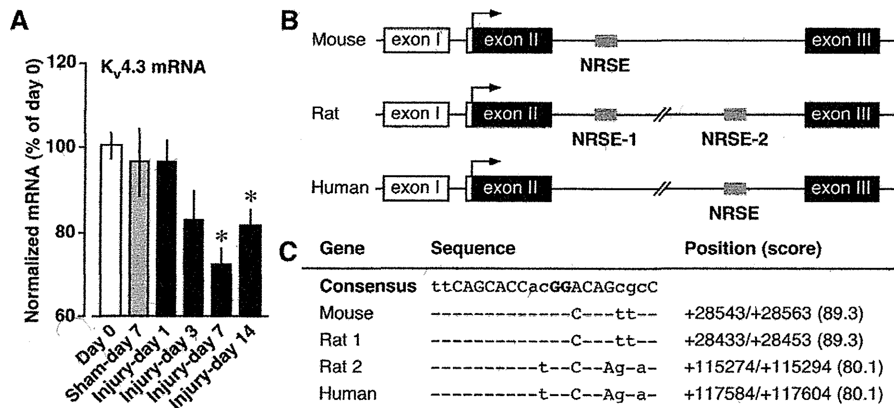

Gene	Sequence	Position (score)
Consensus	ttCAGCACCac**GG**ACAGcgcC	
Mouse	-------------C---tt--	+28543/+28563 (89.3)
Rat 1	-------------C---tt--	+28433/+28453 (89.3)
Rat 2	----------t--C--Ag-a-	+115274/+115294 (80.1)
Human	----------t--C--Ag-a-	+117584/+117604 (80.1)

Fig. 1. Downregulation of neuron-restrictive silencer element (NRSE)-containing $K_v4.3$ gene. (A) Time course of $K_v4.3$ messenger RNA (mRNA) expressions after injury. Data are calculated as percentages of day 0, and expressed as the means±SEM from at least four mice. * $P<0.05$, vs. day 0. (B) Schematic diagram of locations of $K_v4.3$-NRSE. The black arrow indicates translation initiation site. The coding exon is shown as black box, the non-coding exon as open box and NRSE sequence as gray box. (C) Deviations of $K_v4.3$-NRSEs from the consensus NRSE. The capital letters are conserved among functional NRSE sequences, and the bold capital letters are important for neuron-restrictive silencer factor (NRSF) binding. The scores are the threshold scores from the TFSEARCH program.

normalization. In all cases, the validity of amplification was confirmed by the presence of a single peak in the melting temperature analysis and linear amplification with increasing number of PCR cycles. Detailed information of PCR primers was shown in Supplemental Table S1.

Western blot

The ipsilateral L4-6 DRGs were homogenized in ice cold cell-lysis buffer (10 mM Tris–HCl, pH 8.0, 10 mM NaCl, 0.2% NP-40, 1 μM p-APMSF) twice, and then the homogenates were centrifuged to remove contaminating cytosol. Crude nuclear fraction (30 μg) was separated by on 7.5% (NRSF) or 15% (histone H3) SDS-polyacrylamide gels. The primary antibodies were used in the following dilutions: NRSF (1:500; Upstate, CA, USA) and histone H3 (1:500; Upstate, NY, USA). Immunoreactive bands were detected using enhanced chemiluminescent substrate (SuperSignal West Pico chemiluminescent Substrate; Pierce Chemical, Rockford, IL, USA).

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed by using protocols from Upstate Biotechnology and a previous report (Kubat et al., 2004) with some modifications. Briefly, chromatin extracted from L4-6 DRGs were cross-linked by 1% formaldehyde, and then sheared by sonication to 200–500 bp fragments. Ten percent of each lysate was used as the input control for normalization. Chromatin was immunoprecipitated with anti-NRSF (5 μg), anti-acetyl-H3 (5 μg; Upstate, Lake Placid, NY, USA) or anti-acetyl-H4 (5 μl antiserum; Upstate, Lake Placid, NY, USA) antibodies, or normal rabbit IgG (5 μg; Santa Cruz, CA, USA). Purified DNA was used for PCR analysis with primers for $K_v4.3$-NRSE (forward, 5′-ACAACTTAGTTTTGCGCACCAT-3′; reverse, 5′-GCGTGGAACACCTCAAATGT-3′) to amplify the region (35002–35145) of $K_v4.3$ gene (NC_000069.5). After 38 cycles of amplification (94 °C for 15 s, 59 °C for 30 s, 72 °C for 30 s), 144 bp of PCR products were analyzed on a 2% agarose gel. Quantitative real-time PCR was performed as described above.

Statistical analysis

In Fig. 3, the differences between multiple groups were analyzed using a one-way ANOVA with Tukey–Kramer multiple comparison post-hoc analysis. In Figs. 1A and 2D, mRNA and ChIP data were analyzed using the Student's *t*-test. The criterion of significance was set at $P<0.05$. All results are expressed as means±SEM.

RESULTS

To examine whether $K_v4.3$ is downregulated at the transcriptional level after injury, we quantified its mRNA expressions in the DRG by real-time PCR. There was a significant reduction in $K_v4.3$ expression starting from day 7 post-injury, which persisted at least 14 days (Fig. 1A). Using TFSEARCH program (version 1.3, available at: http://www.cbrc.jp/research/db/TFSEARCHJ.html), we found that mouse $K_v4.3$ gene contains a putative NRSE sequence within intron 2, which is completely conserved in rat (Fig. 1B, C). In addition, another putative conserved NRSE was found in intron 2 of rat and human $K_v4.3$ genes (Fig. 1B, C). Within all NRSE sequences of these genes, the GG nucleotides known to be important for NRSF binding (Mori et al., 1992), were completely conserved (Fig. 1C).

Using ChIP analysis (Fig. 2A), we found that injury causes a drastic increase in NRSF binding to $K_v4.3$-NRSE at day 7 post-injury (Fig. 2B), suggesting that this putative NRSE is capable of serving as NRSF-binding site. In the quantitative real-time PCR analysis, the fold-change was not calculated in the NRSF binding to $K_v4.3$-NRSE, since no significant signal was detected in sham-operated preparations (Fig. 2C). In contrast, negligible binding was observed when precipitated by normal IgG, confirming the specificity of the immunoprecipitation (Fig. 2B, C). Next, we assessed the acetylation of histone H3 and H4, which is correlated with transcriptional activation. Injury caused significant reduction in acetylation of histone H4, but not H3, at $K_v4.3$-NRSE at day 7 post-injury (Fig. 2D). These data suggest that injury induces repressive chromatin states around $K_v4.3$-NRSE possibly through NRSF-HDAC-mediated mechanisms.

For selective knockdown of NRSF expression, AS-ODN designed to target the mouse NRSF sequence,

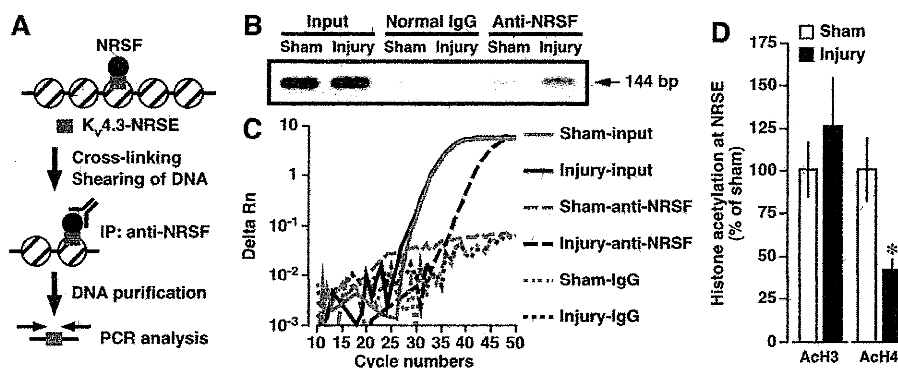

Fig. 2. Histone hypoacetylation at K_V4.3-NRSE with an increase in NRSF binding. Chromatin immunoprecipitation (ChIP) assay was performed at day 7 post-injury. (A) Schematic protocol for ChIP analysis with anti-NRSF antibody. (B) Gel image showing the increase in NRSF binding to K_V4.3-NRSE. (C) Representative amplification plots for quantitative analysis of NRSF binding to K_V4.3-NRSE. The graph shows the change of normalized reporter signal (delta Rn) plotted versus cycle numbers. (D) Quantification of acetylation of histone H3 (AcH3) and H4 (AcH4) at K_V4.3-NRSE. Data are calculated as percentages of sham-operated mice, and expressed as the means±SEM from at least five mice. * $P<0.05$, vs. sham-operated mice.

which corresponds to rat sequence previously targeted for antisense-knockdown (Calderone et al., 2003), was used. When mice were intrathecally pretreated with AS-ODN or MS-ODN, NRSF protein expression in the DRG was reduced by AS-ODN, but not by MS-ODN (Fig. 3). Moreover, the AS-ODN treatments completely blocked injury-induced K_V4.3 downregulation (Fig. 3). In contrast, AS-ODN had no effects on the basal expression level of K_V4.3 in the sham-operated mice (Fig. 3). These findings strongly suggest that NRSF-mediated mechanisms are responsible for the transcriptional suppression of K_V4.3 gene after injury.

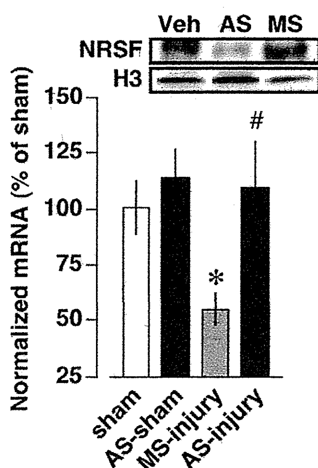

Fig. 3. Blockade of injury-induced K_V4.3 downregulation by NRSF-knockdown. Antisense oligodeoxynucleotide (AS-ODN) (AS)-induced inhibition of K_V4.3 downregulation after injury. Data are calculated as percentages of sham-operated mice, and expressed as the means±SEM from at least five mice. * $P<0.05$, vs. sham-operated mice and # $P<0.05$, vs. mismatch scrambled oligodeoxynucleotide (MS-ODN) (MS)-treated and nerve-injured mice. Inset shows the reduction of NRSF protein expression by AS-ODN, but not by MS-ODN in the dorsal root ganglion, using western blot analysis. NRSF, 200 kDa; histone H3 (H3), 17 kDa.

DISCUSSION

Given that the long-term changes in pain-related gene expression underlie the most important mechanisms responsible for injury-induced neuropathic pain (Hökfelt et al., 2006; Ueda, 2006), we focused on the critical contribution of epigenetic mechanisms (Borrelli et al., 2008). Here, we demonstrated that injury-induced K_V4.3 downregulation, which is implicated in neuronal hyperexcitability underlying neuropathic pain (Kim et al., 2002; Chien et al., 2007), is closely related to the hypoacetylation of histone H4 at K_V4.3-NRSE. As histone acetylation is known to affect other epigenetic modifications including DNA methylation, which in turn establish stable gene regulation (Abel and Zukin, 2008), such epigenetic mechanisms may underlie chronic neuropathic pain.

Upon binding to NRSE, NRSF acts as transcriptional repressor for numerous genes encoding essential for neuronal functions, such as ion channels, neurotransmitter receptors, and synaptic vesicle proteins (Bruce et al., 2004). The present study provided the evidence that injury promotes both NRSF binding and histone hypoacetylation at K_V4.3-NRSE, and that NRSF-knockdown blocks injury-induced K_V4.3 downregulation. These results strongly suggest that this site mediates the long-term silencing of K_V4.3 gene expression after injury.

The fact that antisense-knockdown of K_V4.3 located at nonpeptidergic C-fibers causes mechanical hypersensitization, implies the critical role of injury-induced K_V4.3 downregulation in neuropathic pain (Chien et al., 2007). However, we failed to detect any change in the injury-induced thermal hyperalgesia and mechanical allodynia by NRSF AS-ODN treatments (unpublished data), being consistent to the report that the ablation of the majority of C-fibers has no effects on the manifestation of neuropathic pain (Abrahamsen et al., 2008). As C-fibers could be divided at least into peptidergic and nonpeptidergic and the threshold through each fiber is differentially affected by nerve injury (Ueda, 2006), the further detailed pain study is

required to address the etiological role of $K_v4.3$ in neuropathic pain.

CONCLUSION

In conclusion, the present study demonstrated that NRSF plays a key role in injury-induced $K_v4.3$ downregulation in the DRG through epigenetic mechanisms. A study concerning the regulatory mechanisms for NRSE-NRSF system after injury is the next subject for research.

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APPENDIX

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neuroscience.2009.12.021.

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Calpain-mediated down-regulation of myelin-associated glycoprotein in lysophosphatidic acid-induced neuropathic pain

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Abstract

Lysophosphatidic acid receptor (LPA_1) signaling initiates neuropathic pain through demyelination of the dorsal root (DR). Although LPA is found to cause down-regulation of myelin proteins underlying demyelination, the detailed mechanism remains to be determined. In the present study, we found that a single intrathecal injection of LPA evoked a dose- and time-dependent down-regulation of myelin-associated glycoprotein (MAG) in the DR through LPA_1 receptor. A similar event was also observed in *ex vivo* DR cultures. Interestingly, LPA-induced down-regulation of MAG was significantly inhibited by calpain inhibitors (calpain inhibitor X, E-64 and E-64d) and LPA markedly induced calpain activation in the DR. The pre-treatment with calpain inhibitors attenuated LPA-induced neuropathic pain behaviors such as hyperalgesia and allodynia. Moreover, we found that sciatic nerve injury activates calpain activity in the DR in a LPA_1 receptor-dependent manner. The E-64d treatments significantly blocked nerve injury-induced MAG down-regulation and neuropathic pain. However, there was no significant calpain activation in the DR by complete Freund's adjuvant treatment, and E-64d failed to show anti-hyperalgesic effects in this inflammation model. The present study provides strong evidence that LPA-induced calpain activation plays a crucial role in the manifestation of neuropathic pain through MAG down-regulation in the DR.

Keywords: calpain, demyelination, dorsal root, lysophosphatidic acid, myelin-associated glycoprotein, neuropathic pain.

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Lysophosphatidic acid (LPA) is a bioactive lipid mediator that exerts diverse physiological and pathophysiological functions through its cognate LPA receptors ($LPA_{1\text{-}5}$ and P2Y5) (Noguchi *et al.* 2009). Recently, we demonstrated that LPA_1 receptor signaling initiates neuropathic pain following peripheral nerve injury, using mice lacking the *lpa_1* gene (*$Lpar1^{-/-}$*) (Inoue *et al.* 2004; Ueda 2008). Regarding the molecular bases, LPA up-regulates pain-related gene expression, such as Ca^{2+} channel α2δ-1 subunit and ephrinB1 in the dorsal root ganglion (DRG) and protein kinase C γ-isoform in the spinal cord (Inoue *et al.* 2004; Uchida *et al.* 2009). Moreover, LPA causes demyelination of the dorsal root (DR) through down-regulation of myelin-related proteins, such as myelin basic protein (MBP), peripheral myelin protein 22 (PMP22) and myelin protein zero (MPZ) in *in vivo* injury models and *ex vivo* culture models (Inoue *et al.* 2004; Fujita *et al.* 2007). As the temporal profile of down-regulation of myelin protein levels is similar to the gene expression levels (Inoue *et al.* 2004; Fujita *et al.* 2007), we hypothesized that protein-degradation and transcriptional suppression might be involved in LPA-induced demyelination. However, the details remain unclear.

Myelin-associated glycoprotein (MAG), a minor component of myelin, is predominantly located in the periaxonal membranes of Schwann cells, where it mediates glia-axon interactions (Quarles 2007). As MAG expression starts during the early stages of myelination, it has been postulated that

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Abbreviations used: CalX, calpain inhibitor X; CFA, complete Freund's adjuvant; DR, dorsal root; DRG, dorsal root ganglion; LPA, lysophosphatidic acid; *$Lpar1^{-/-}$*, mice lacking *lpa_1* gene; MAG, myelin-associated glycoprotein; MBP, myelin basic proteins; MMP, matrix metalloproteinase; MPZ, myelin protein zero; PBS, phosphate buffered saline; PMP22, peripheral myelin protein 22.

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MAG is crucial for initiation of the myelination process (Owens and Bunge 1989; Paivalainen and Heape 2007; Quarles 2009). Moreover, the sustained expression of MAG, at relatively high levels, in adulthood is also assumed to play a key role in the maintenance of myelin integrity (Garbay *et al.* 2000; Schachner and Bartsch 2000; Quarles 2009). In addition, MAG-mediated signaling from glia to axons is known to maintain the structural integrity of myelinated axons by modulating the axonal cytoskeleton and inhibiting the outgrowth of neuronal processes (sprouting) through interactions with Nogo receptors, gangliosides (such as GD1a and GT1b) and paired immunoglobulin-like receptor B (Atwal *et al.* 2008; Filbin 2008; Quarles 2009; Schnaar and Lopez 2009). Given the involvement of MAG in myelination and sprouting, we hypothesize that MAG down-regulation may play a key role in LPA-induced neuropathic pain conditions. Therefore, we attempted to examine whether LPA affects MAG expression levels in the DR. Here, we report that LPA activates calpain to down-regulate MAG expression through the LPA_1 receptor in the DR, thereby causing neuropathic pain.

Materials and methods

Animals and surgery

Male mice lacking the *lpa_1* gene (*$Lpar1^{-/-}$*) (Contos *et al.* 2000) and wild type C57BL/6J mice weighing 20–24 g were used. They were kept in a room with a temperature of 21 ± 2°C with free access to standard laboratory diet and tap water. All procedures were approved by the Nagasaki University Animal Care Committee and complied with the recommendations of the International Association for the Study of Pain (Zimmermann 1983). Partial ligation of the sciatic nerve was performed under pentobarbital (50 mg/kg) anesthesia, following the methods of Malmberg and Basbaum (Malmberg and Basbaum 1998).

Drugs

Lysophosphatidic acid (1-oleoyl-2-hydroxy-sn-3-glycerol-3-phosphate) and 1,10-phenanthroline were purchased from Sigma-Aldrich (St. Louis, MO, USA). Calpain inhibitor X (CalX; Z-Leu-Abu-CONH-ethyl) and epoxomicin were from Calbiochem (San Diego, CA, USA). z-valine-alanine-aspartate (zVAD)-fmk (carbobenzoxy-L-valyl-L-alanyl-β-methyl-L-aspart-1-yl-fluoromethane) was from Peptide Institute, Inc. (Osaka, Japan). *p*-APMSF [(*p*-amidinophenyl) methanesulfonyl fluoride hydrochloride] and complete Freund's adjuvant (CFA) were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). E-64 ([(2*S*,3*S*)-3-Carboxyoxirane-2-carbonyl]-L-leucine (4-guanidinobutyl) amide hemihydrate) and E-64d [(2S,3S)-*trans*-Epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester] were kindly provided by the Taisho Pharmaceutical Co., Ltd. (Saitama, Japan).

Drug injection

Lysophosphatidic acid and CalX were dissolved in artificial CSF (aCSF; 125 mM NaCl, 3.8 mM KCl, 1.2 mM KH_2PO_4, 26 mM $NaHCO_3$, 10 mM glucose, pH 7.4). E-64d was dissolved in 100% dimethylsulfoxide as a stock solution, and diluted in saline prior to administration. The intrathecal (i.t.) injection was administered into the space between the spinal L5 and L6 segments according to the method of Hylden and Wilcox (Hylden and Wilcox 1980). The intrathecal injection of CalX or intravenous (i.v.) injection of E-64d into the tail vein was performed 30 min prior to LPA treatment. The intraplantar (i.pl.) injections were given using a Hamilton microsyringe connected to polyethylene tubing with a 30-gauge hypodermic needle. Peripheral inflammation was induced by CFA (20 μL i.pl.) injection. In nerve injury and peripheral inflammation models, E-64d (i.v.) was injected twice a day (12-h interval) for 3 days, starting from 30 min prior to nerve injury and CFA injection.

Nociception test

In the thermal paw withdrawal test, the nociception threshold was evaluated as the latency to withdraw a paw upon a thermal stimulus (Hargreaves *et al.* 1988; Ma *et al.* 2009). Unanesthetized animals were placed in Plexiglas cages on top of a glass sheet and an adaptation period of 1 h was allowed. A thermal stimulator (IITC Inc., Woodland Hills, CA, USA) was positioned under the glass sheet and the focus of the projection bulb was aimed exactly at the middle of the plantar surface of the animal. A mirror attached to the stimulator permitted visualization of the plantar surface. A cut-off time of 20 s was set to prevent tissue damage. The mechanical paw pressure test was performed as described previously (Inoue *et al.* 2004; Matsumoto *et al.* 2006). Briefly, mice were placed in a plexiglas chamber on a 6 × 6 mm wire mesh grid floor and allowed to acclimatize for a period of 1 h. A mechanical stimulus was then delivered to the middle of the plantar surface of the right-hind paw using a Transducer Indicator (Model 1601; IITC Inc.). The amount of pressure that induced a flexor response was defined as the pain threshold. A cut-off pressure of 20 g was set to avoid tissue damage. In these experiments using mechanical and thermal tests, the thresholds were determined from three repeated challenges at 10-min intervals, and the averages of responses were evaluated.

Western blot

According to manufacturer's instructions, non-reduced protein was used for the detection of MAG, and reduced protein was used for β-tubulin. Total protein (20 μg) extracted from L4–5 DRs was separated on SDS-polyacrylamide gels (12%). Primary antibodies were used at the following dilutions: mouse anti-MAG antibody (1 : 5000; Chemicon, Temecula, CA, USA) and rabbit anti-β-tubulin polyclonal antibody (1 : 500; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Horseradish peroxidase-labeled anti-mouse IgG and horseradish peroxidase-labeled anti-rabbit IgG were used as secondary antibodies at dilutions of 1 : 1000. Immunoreactive bands were detected using an enhanced chemiluminescent substrate (SuperSignal West Pico Chemiluminescent Substrate; Pierce Chemical, Rockford, IL, USA).

Ex vivo DR cultures

Ex vivo DR cultures were performed as described previously (Fujita *et al.* 2007). Briefly, the L4–5 DRs with DRG were isolated by carefully removing the spinal vertebra without tearing. The tissue was washed with ice-cold phosphate buffered saline (pH 7.4, PBS) containing penicillin and streptomycin, and placed in Dulbecco's modified Eagle's medium without serum. The culture was carried

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out at 37°C in the presence of 5% CO_2. Protease inhibitors were added 30 min prior to LPA treatment (1 μM).

Determination of calpain activity

Calpain activities in protein lysates from L4–5 DR, DRG and spinal dorsal horn were analyzed using a commercially available calpain activity assay kit (BioVision, Mountain View, CA, USA) according to the manufacturer's instructions. This method is based on the detection of the fluorometric cleavage of the calpain substrate Ac-leucine-leucine-tyrosine-7-amino-trifluoromethyl coumarin, which emits blue light (400 nm). Upon cleavage of the substrate by calpain, free 7-amino-trifluoromethyl coumarin (AFC), which emits yellow-green fluorescence (505 nm), was quantified using a 1420 ARVOMX/Light fluorescent plate reader (PerkinElmer Japan Co., Ltd, Yokohama, Japan).

Immunostaining for MAG

Mice were deeply anesthetized with sodium pentobarbital (50 mg/kg i.p.), and perfused with potassium-free PBS (K^+-free PBS, pH 7.4), followed by 4% paraformaldehyde solution. The L4–5 DRs were isolated, post-fixed for 3 h, and cryoprotected overnight in 25% sucrose solution. The DRs were fast-frozen in cryo-embedding compound on a mixture of ethanol and dry ice and stored at −80°C until ready for use. The DRs were sectioned at a thickness of 10 μm, thaw-mounted on silane-coated glass slides, and air-dried overnight at 25°C. The DR sections were incubated with blocking buffer containing anti-mouse IgG (1 : 50; Zymed, South San Francisco, CA, USA) and 4% bovine serum albumin in 0.1% Triton X-100 in K^+-free PBS, and then incubated with anti-MAG antibody (1 : 300) overnight at 4°C. After washing, sections were incubated with Alexa Fluor 488-conjugated anti-mouse IgG (1 : 1000; Molecular Probes, Carlsbad, CA, USA) secondary antibody for 2 h at 25°C. The MAG-immunoreactivity was detected by automatic fluorescence microscopy using BZ Image Measurement software (Bio-Zero, Keyence, Tokyo, Japan).

Statistical analysis

In Figs 3(b), 4(a), 5, 6, 7 and 8(b), the differences between multiple groups were analyzed by one-way ANOVA with Tukey-Kramer multiple comparison *post-hoc* analysis. In Fig. 1, 3(a) and 8(a), data were analyzed using the Student's *t*-test. The criterion of significance was set at $p < 0.05$. Results are expressed as the mean ± SEM.

Results

Down-regulation of MAG protein expression by LPA

Previously, we have reported that LPA (1 nmol i.t.) induces demyelination of the DR 24 h post-injection (Inoue *et al.* 2004). Using western blot analysis, we found that intrathecal injection of LPA dose-dependently down-regulates MAG protein expression in the DR from 0.1 to 10 nmol at 24 h post-injection (Fig. 1a). The most prominent reduction was observed at a dose of 10 nmol (Fig. 1a), which exerts no abnormal behavior. The MAG expression levels were slightly but not significantly reduced at 3 h after LPA injection (10 nmol i.t.), however, a significant decrease in MAG levels occurred at 12 h post-injection (Fig. 1b). Maximal reduction was observed at 24 h post-injection (Fig. 1b). While the significant reduction was still observed at day 3 post-injection, it was moderate but not significant at day 7 post-injection (Fig. 1b). These results suggest that LPA induces rapid down-regulation of MAG protein expression in the DR.

Lack of MAG down-regulation in *Lpar1*$^{-/-}$ mice

In the peripheral nervous system, MAG expression has been found abundantly in paranodal regions and to a lesser extent in periaxonal Schwann cell membranes (Georgiou *et al.* 2004). Consistent with this previous study, immunohistochemical analysis revealed that strong MAG signals are located in paranodal regions and weaker ones along the axons in the DR of vehicle-treated wild type mice (Fig. 2a). LPA (10 nmol i.t.) caused a marked reduction of MAG signals at 24 h post-injection (Fig. 2b). On the other hand, the MAG signals seen in vehicle-treated *Lpar1*$^{-/-}$ mice were comparable to that of vehicle-treated wild type mice (Fig. 2c). In contrast, the LPA-induced down-regulation of

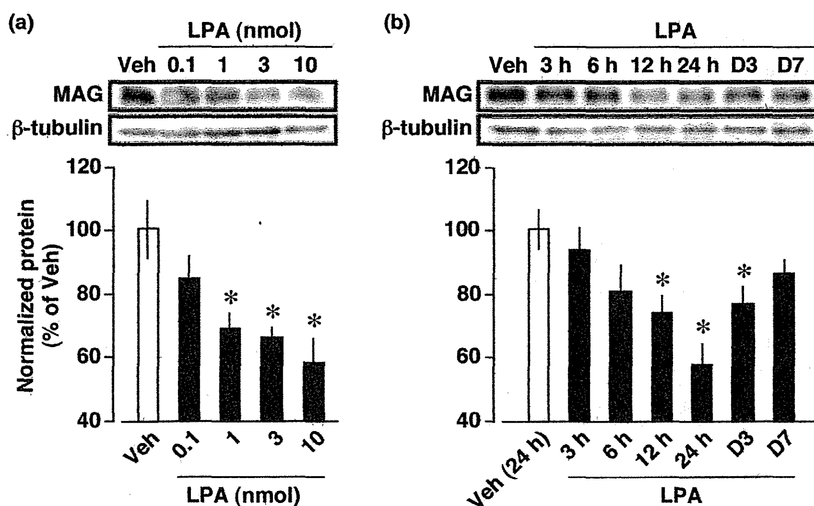

Fig. 1 LPA-induced down-regulation of MAG protein expression in the DR. The expression levels of MAG protein in the DR were assessed by western blot analysis. (a) Dose-dependent down-regulation of MAG by intrathecal injection of LPA at 24 h post-injection. (b) Time-course of LPA-induced (10 nmol i.t.) down-regulation of MAG. Photograph shows representative data. Results are normalized to β-tubulin protein expression levels, and expressed as percentage of the levels in the vehicle (Veh)-treated group. *$p < 0.05$, vs. Veh-treated group. Data represent the means ± SEM from at least three mice.

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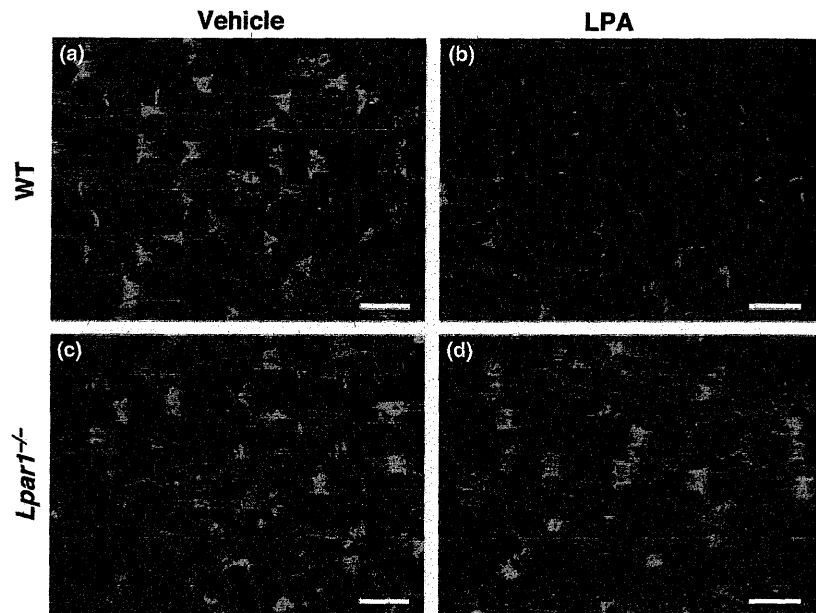

Fig. 2 LPA_1 receptor-dependent down-regulation of MAG in the DR. MAG protein expression at 24 h after injection of Veh (a and c) or LPA (10 nmol i.t.) (b and d) was assessed by immunohistochemical analysis. Wild type (WT; a and b) and *Lpar1*$^{-/-}$ (c and d) mice were used. Photographs show representative data. Scale bar = 20 μm.

MAG was absent in *Lpar1*$^{-/-}$ mice (Fig. 2d), suggesting that the LPA_1 receptor is crucial for MAG down-regulation in the DR by LPA.

Protease-mediated MAG down-regulation

Using *ex vivo* cultures of DR, we have demonstrated that LPA causes demyelination of DR via the down-regulation of MBP and MPZ at 24 h post-treatment (Fujita *et al.* 2007). Thus, we assessed whether LPA down-regulates MAG expression in cultures of DR. Western blot analysis revealed that treatment with LPA (1 μM) for 24 h causes a significant reduction of MAG protein expression (Fig. 3a). As z-valine-alanine-aspartate (zVAD)-fmk (1 μM), a broad spectrum caspase inhibitor, had no effect on LPA-induced down-regulation of MAG (Fig. 3b), it is unlikely that LPA causes apoptotic cell death of Schwann cells in the DR. To test the involvement of protease-mediated protein degradation in LPA-induced down-regulation of MAG, we used various protease inhibitors such as E-64 (100 μM) for calpains and other cysteine proteases (Ray *et al.* 2003), CalX (10 μM) for calpains (James *et al.* 1998), epoxomicin (5 μM) for proteasomes, (*p*-amidinophenyl) methanesulfonyl fluoride hydrochloride (1 μM) for serine proteases and 1,10-phenanthroline (100 μM) for matrix metalloproteinases (MMP). For all inhibition studies, cultures were treated with inhibitors 30 min prior to LPA exposure. The inhibitors alone had no effect on the MAG expression levels in the vehicle-treated DR cultures (data not shown). Among these inhibitors, only the calpain inhibitors (CalX and E-64) significantly blocked LPA-induced down-regulation of MAG (Fig. 3b), suggesting that calpain is a plausible mediator of MAG degradation by LPA in the DR.

Blockade of LPA-induced down-regulation of MAG by calpain inhibitors

We examined whether calpain is involved in LPA-induced down-regulation of MAG in the DR *in vivo*. For *in vivo* studies, we used E-64d, an esterified and more cell-permeable analog of E-64 (Ray *et al.* 2003). Western blot analysis revealed that pre-treatment with CalX (10 nmol i.t.) or E-64d (1.5 mg/kg i.v.) 30 min prior to LPA injection (10 nmol i.t.) completely blocked LPA-induced down-regulation of MAG (Fig. 4a). Similar results were obtained with immunohistochemical analysis (Fig. 4b–e). These results suggest that calpain plays a key role in LPA-induced down-regulation of MAG.

LPA-induced calpain activation

To obtain direct evidence for the activation of calpain by LPA, we assessed calpain activity in the DR *in vivo*. We isolated L4–5 DRs 24 h after LPA injection (10 nmol i.t.), and measured calpain activity in extracted protein from DRs using a fluorometric assay. We found that LPA significantly up-regulated calpain activity, and that pre-treatment with CalX (10 nmol i.t.) or E-64d (1.5 mg/kg i.v.) completely blocked the activation (Fig. 5). In contrast, CalX or E-64d alone had no effect on calpain activity in the vehicle-treated DRs (data not shown).

Attenuation of LPA-induced neuropathic pain behaviors by calpain inhibitors

Next, we assessed whether calpain inhibitors could block LPA-induced neuropathic pain-like behaviors, such as thermal hyperalgesia and mechanical allodynia. Consistent with previous data (Inoue *et al.* 2004), LPA (10 nmol i.t.)

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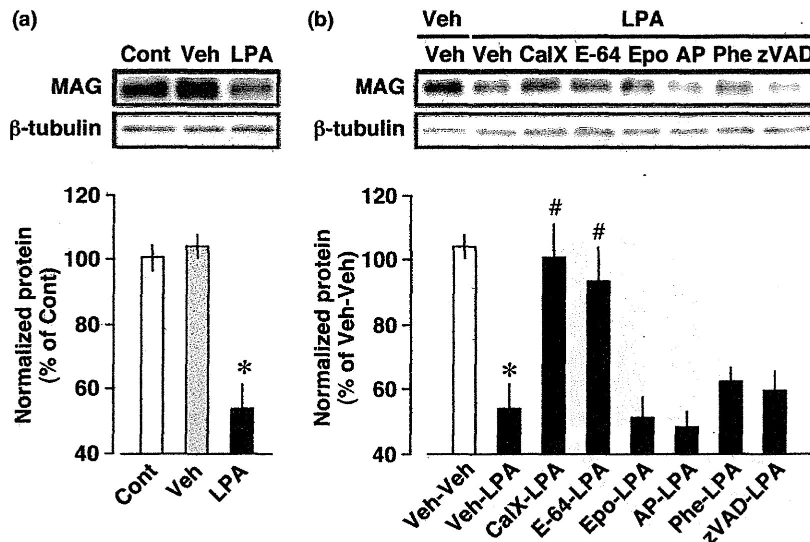

Fig. 3 LPA-induced down-regulation of MAG in *ex vivo* DR cultures. MAG expression in *ex vivo* cultures of DR at 24 h after LPA treatment (1 μM) was assessed by western blot analysis. (a) LPA-induced down-regulation of MAG. (b) Effects of various protease inhibitors on LPA-induced down-regulation of MAG. The concentration of each inhibitor was as follows: CalX (10 μM), E-64 (100 μM), epoxomicin (Epo, 5 μM), *p*-APMSF (AP, 1 μM), 1,10-phenanthroline (Phe, 100 μM), zVAD-fmk (zVAD, 1 μM). Photograph shows representative data. Results are normalized to β-tubulin protein expression levels, and expressed as a percentage of the levels in non-treated control groups (Cont) (a) or Veh-Veh-treated group (b). $^*p < 0.05$ vs. Veh-treated group (a) or Veh-Veh-treated group (b) and $\#p < 0.05$ vs. Veh-LPA-treated group. Data represent the means ± SEM from at least five mice.

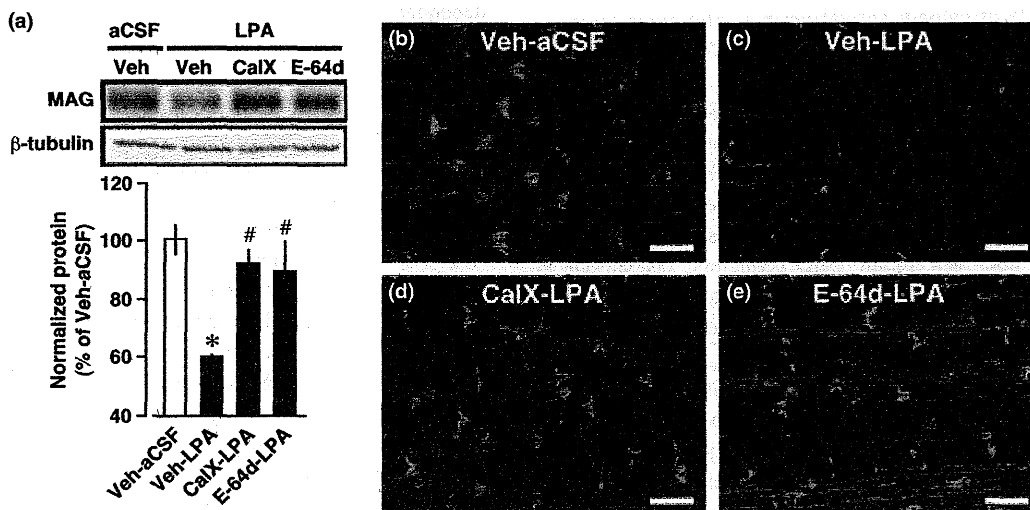

Fig. 4 Inhibition of LPA-induced down-regulation of MAG by calpain inhibitors. Blockade of LPA-induced (10 nmol i.t.) down-regulation of MAG by CalX (10 nmol i.t.) or E-64d (1.5 mg/kg i.v.) at 24 h post-injection *in vivo*, assessed by western blot (a) and immunohistochemical analysis (b–e). (a) Results are normalized to β-tubulin protein expression levels, and expressed as a percentage of the levels in the Veh-aCSF-treated group. $^*p < 0.05$ vs. Veh-aCSF-treated group and $\#p < 0.05$ vs. Veh-LPA-treated group. Data represent the means ± SEM from at least four mice. Photograph shows representative data. Scale bar = 20 μm.

significantly reduced the thermal and mechanical pain thresholds during day 1–7 post-injection (Fig. 6a–d). Pretreatment with CalX (10 nmol i.t.) showed a partial but significant inhibition of LPA-induced thermal hyperalgesia and mechanical allodynia (Fig. 6a and b). In contrast, CalX alone had no effects on pain thresholds in vehicle-treated mice (Fig. 6a and b). Similar results were obtained with E-64d (Fig. 6c and d). These results suggest that the calpain-mediated protein degradation system plays a key role in LPA-induced neuropathic pain behaviors.

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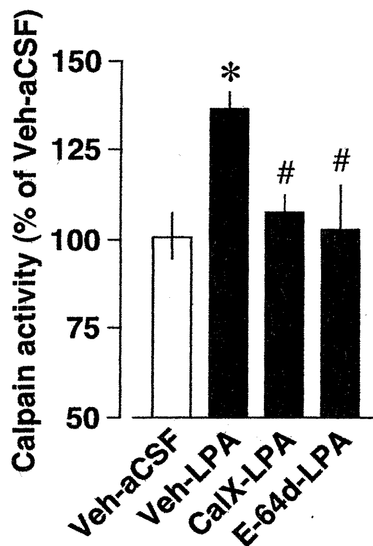

Fig. 5 Increase in calpain activity by LPA in the DR. Calpain activity in the DR was assessed at 24 h after LPA injection (10 nmol i.t.). Veh, CalX (10 nmol i.t.) or E-64d (1.5 mg/kg i.v.) were injected 30 min prior to aCSF or LPA. Results are expressed as a percentage of the Veh-aCSF-treated group. $^{*}p < 0.05$ vs. Veh-aCSF-treated group and $\#p < 0.05$ vs. Veh-LPA-treated group. Data represent the means ± SEM from at least three mice.

Critical role of calpain activation in sciatic nerve injury-induced neuropathic pain, but not in CFA-induced inflammatory pain

The intrathecal injection of LPA has been found to mimic the peripheral nerve injury-induced neuropathic pain and its underlying mechanisms (Inoue *et al.* 2004; Ueda 2006, 2008). Therefore, we next examined whether nerve injury activates calpain in the DR, thereby causing MAG down-regulation and neuropathic pain behaviors. As shown in Fig. 7(a), nerve injury markedly up-regulated calpain activity in the DR during 1–3 days post-injury. We found that nerve injury-induced calpain activation is absent in LPA_1-deficient mice (Fig. 7b), suggesting the critical contribution of LPA_1 receptor signaling. Moreover, when mice were treated E-64d for 3 days starting from 30 min prior to injury, nerve injury-induced calpain activation at day 1 post-injury (Fig. 7a) and MAG-down-regulation at day 7 post-injury were significantly blocked (Fig. 7c and d). In addition, these treatments also partially, but significantly, prevented the development of thermal hyperalgesia after nerve injury (Fig. 7e).

On the other hand, peripheral inflammation is reported to cause calpain activation in the DRG and spinal cord (Pareek *et al.* 2006). When CFA was given intraplantarly, the calpain activity was significantly increased in the spinal dorsal horn, partially increased in the DRG, but not in the DR (Fig. 8a). We found that the treatments with E-64d have no effect on the manifestation of CFA-induced thermal hyperalgesia (Fig. 8b).

Discussion

We have reported that LPA_1 signaling is crucial for the initiation of nerve injury-induced neuropathic pain, which is in part mediated through demyelination of the DR (Inoue *et al.* 2004; Fujita *et al.* 2007). Transcriptional suppression of myelin-related genes, such as MBP and PMP22, has been assumed to contribute to LPA-induced demyelination (Ueda 2006, 2008). In the present study, we provide the first evidence that LPA down-regulates MAG expression in the DR through the calpain-mediated protein degradation system.

In addition to MBP, PMP22 and MPZ (Inoue *et al.* 2004; Fujita *et al.* 2007), the present study clarified that MAG is a novel target for LPA_1 receptor signaling. The dose-range required for LPA-induced down-regulation of MAG in both *in vivo* and *ex vivo* experiments was comparable to that for the down-regulation of other myelin proteins (Inoue *et al.* 2004; Fujita *et al.* 2007). Moreover, LPA-induced down-regulation of MAG was found to commence at 3 h post-injection, and reached a maximal level at 24 h post-injection. This time-course was comparatively similar to LPA-induced down-regulation of MBP in *ex vivo* experiments (Fujita *et al.* 2007). We have reported that LPA mimics nerve-injury-induced neuropathic pain, and that nerve injury-induced down-regulation of MBP and PMP22 are LPA_1 receptor-dependent (Inoue *et al.* 2004; Ueda 2008). Consistent with these findings, the present study showed that nerve injury activates calpain in the DR through LPA_1 receptor, thereby causing MAG down-regulation.

Calpain is a cytoplasmic cysteine protease that plays a key role in physiological and pathophysiological conditions in the nervous system (Camins *et al.* 2006; Vosler *et al.* 2008). A large number of proteins are known as calpain substrates, including myelin proteins such as MAG and MBP (Vosler *et al.* 2008). Calpain-mediated down-regulation of MAG and MBP has been found in demyelinating diseases such as multiple sclerosis (Inuzuka *et al.* 1987; Shields and Banik 1999; Vosler *et al.* 2008). The present study provides the first evidence that LPA down-regulates MAG protein expression in the DR in a calpain-dependent manner, using *in vivo* and *ex vivo* experiments. While MMP is reported to degrade MAG as well as MBP (Gijbels *et al.* 1993; Proost *et al.* 1993; D'Souza and Moscarello 2006; Milward *et al.* 2008), LPA-induced down-regulation of MAG was insensitive to the MMP inhibitor. Moreover, we found that LPA markedly induces calpain activity in the DR, using a fluorometric assay. It is well known that calpain is activated by calcium (Goll *et al.* 2003; Vosler *et al.* 2008), however, further studies are required for the elucidation of the mechanisms underlying LPA-induced calpain activation in the DR.

Using behavioral studies, we found that calpain-mediated protein degradation is involved in LPA- and nerve injury-induced neuropathic pain behaviors. The fact that LPA- and

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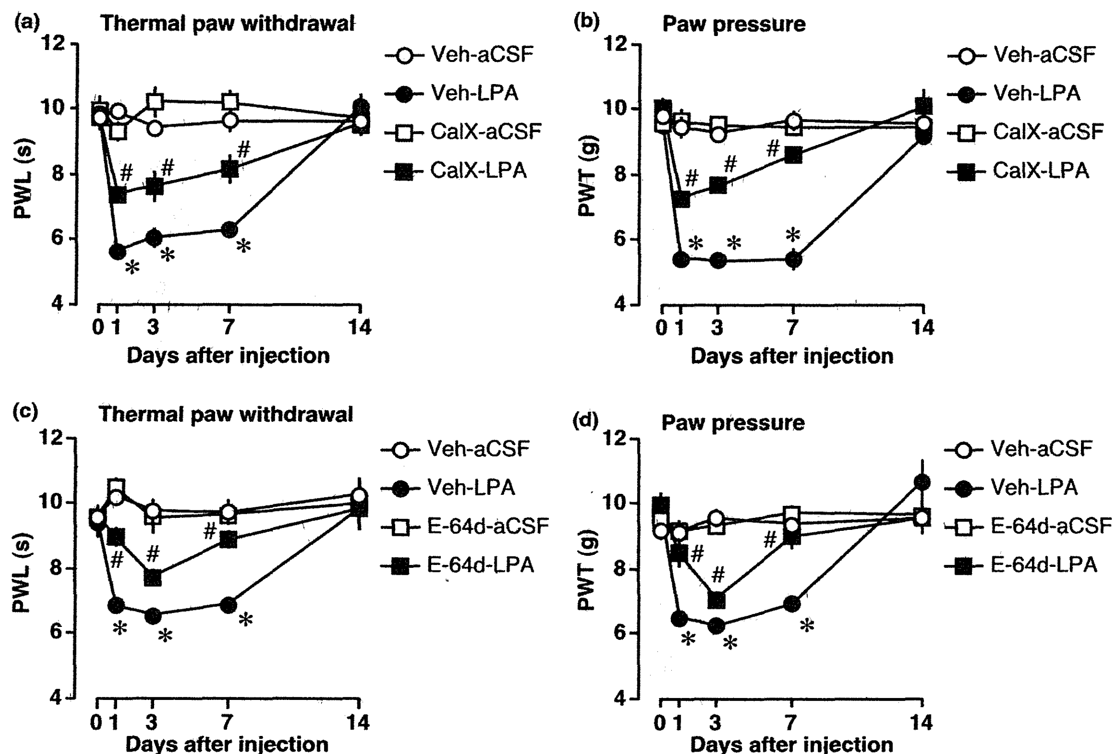

Fig. 6 Partial blockade of LPA-induced neuropathic pain by calpain inhibitors. Blockade of LPA-induced (10 nmol i.t.) thermal hyperalgesia and allodynia by CalX (a and b) and E-64d (c and d). After LPA injection, (a and c) thermal paw withdrawal latencies (PWL) in seconds were measured using the thermal paw withdrawal test, and (b and d) mechanical paw withdrawal thresholds (PWT) in grams were measured using the paw pressure test. $*p < 0.05$ vs. Veh-aCSF-treated group and $\#p < 0.05$ vs. Veh-LPA-treated group. Data represent the means ± SEM from five mice.

nerve injury-induced abnormal pain were partially blocked by calpain inhibitors indicates the presence of other mechanisms induced by LPA. In this context, we have recently identified genes, that are immediately induced by LPA_1 receptor signaling in the DRG (Uchida *et al.* 2009). One of these, ephrinB1, has been found to play a crucial role in LPA-induced neuropathic pain through activation of the *N*-methyl-D-aspartate receptor in the spinal cord (Uchida *et al.* 2009). In addition, calpain is reported to mediate early cytokine expression in the DRG, including tumor necrosis factor-α and interleukin-1β after nerve injury (Uceyler *et al.* 2007). Therefore, it is possible that LPA might induce calpain activation in the DRG, thereby causing neuropathic pain. However, the details remain to be determined.

On the other hand, calpain is found to be activated in the DRG and spinal cord after peripheral inflammation, and implicated in the sensitization of nociceptive neurons through the cleavage of neurofilament light chain and p35, an activator of cyclin-dependent kinase 5 (Kunz *et al.* 2004; Pareek *et al.* 2006). The present study showed that CFA induces calpain activation in the spinal dorsal horn and partially in the DRG. In contrast, CFA-induced calpain activation was absent in the DR, indicating the clear difference in the underlying mechanisms between neuropathic pain and inflammatory pain. Similarly, E-64d, a calpain inhibitor, markedly attenuated the neuropathic pain, but not CFA-induced chronic inflammatory pain, though there is a report that different type of calpain inhibitor shows a weak attenuation of acute inflammatory pain induced by zymosan (Kunz *et al.* 2004). Further study to examine the role of calpain activation in the manifestation of acute and chronic inflammatory pain would be the next subject.

The pathological consequences of LPA-induced down-regulation of MAG in the DR remain elusive. In addition to MBP, PMP22 and MPZ, MAG is crucial for the maintenance of myelination (Garbay *et al.* 2000). While MBP, PMP22 and MPZ are localized in compact myelin, MAG is localized in the non-compacted myelin (such as paranodal regions) and periaxonal Schwann cell membranes, where it regulates glia-axon interactions (Quarles 2007). Thus, it is likely that LPA reduces the structural and functional integrity of myelin through down-regulation of myelin proteins, thereby causing long-lasting demyelination in the DR. On the other hand, MAG is known to act as an inhibitor of neurite outgrowth

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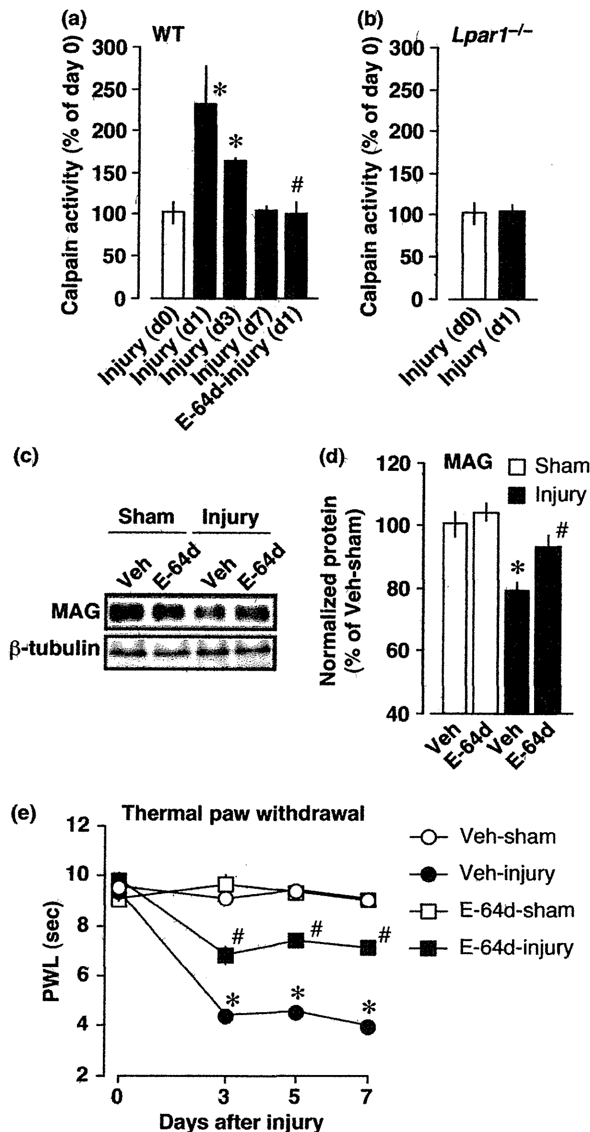

Fig. 7 Nerve injury-induced MAG down-regulation in the DR and neuropathic pain through LPA_1 receptor-dependent calpain activation in the DR. Neuropathic pain was induced by partial ligation of the sciatic nerve. (a) Calpain activation in the DR after nerve injury and its blockade by E-64d in wild type (WT) mice. Veh or E-64d (1.5 mg/kg i.v.) was injected twice a day for 3 days, starting from 30 min prior to injury. (b) Lack of nerve injury-induced calpain activation in the DR at day 1 post-injury in $Lpar1^{-/-}$ mice. (c and d) Blockade of nerve injury-induced MAG down-regulation in the DR by E-64d, assessed by western blot analysis. Photograph shows representative data (c). Results are normalized to β-tubulin protein expression levels, and expressed as a percentage of the levels in Veh-treated and sham-operated group. (e) Partial blockade of nerve injury-induced thermal hyperalgesia by E-64d. Thermal paw withdrawal latencies (PWL) in seconds were measured using the thermal paw withdrawal test. $*p < 0.05$ vs. Veh-treated and sham-operated group and $\#p < 0.05$ vs. Veh-treated and nerve-injured group. Data represent the means ± SEM from at least six mice.

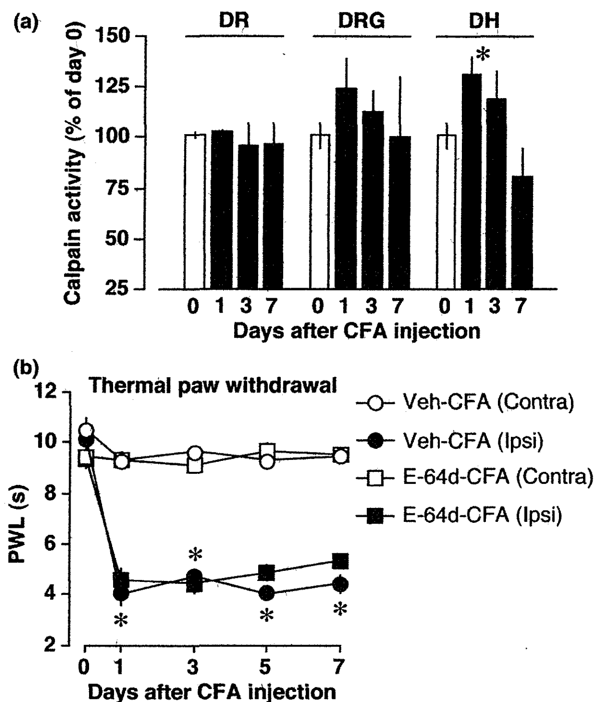

Fig. 8 Absence of calpain activation in the DR and E-64d-induced anti-hyperalgesic effects in peripheral inflammation model. Peripheral inflammation was induced by CFA (20 μL i.pl.) injection. (a) Time-course of calpain activity in the DR, DRG and spinal dorsal horn (DH) after CFA treatment. $*p < 0.05$ vs. day 0 group. (b) Normal manifestation of CFA-induced thermal hyperalgesia in the E-64d-treated mice. Veh or E-64d (1.5 mg/kg i.v.) was injected twice a day for 3 days, starting from 30 min prior to CFA injection. Thermal paw withdrawal latencies (PWL) in seconds were measured in the ipsilateral (Ipsi) and contralateral (Contra) paw of CFA-treated mice, using the thermal paw withdrawal test. $*p < 0.05$ vs. Contra paw of Veh-treated and CFA-treated group. Data represent the means ± SEM from at least three mice.

through interactions with Nogo receptors, gangliosides and paired immunoglobulin-like receptor B expressed in axons (Atwal *et al.* 2008; Filbin 2008; Quarles 2009; Schnaar and Lopez 2009). Therefore, LPA-induced down-regulation of MAG may cause sprouting of axons, which leads to physical cross-talk underlying neuropathic pain, as suggested previously (Ueda 2008). While these peripheral mechanisms are involved in the development of neuropathic pain, it is well established that central mechanisms, including cortical plasticity and reorganization, play a key role in the maintenance of neuropathic pain (Zhuo 2008; Descalzi *et al.* 2009). Considering that the feed-forward amplification within the pain pathways underlies the mechanisms for neuropathic pain (Zhuo 2007; Costigan *et al.* 2009), it would be an interesting subject to examine whether LPA-induced plastic changes between primary afferents and spinal neurons promote the cortical plasticity underlying neuropathic pain.

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In conclusion, the present study clarifies that LPA down-regulates MAG protein expression in the DR through calpain activation, thereby causing neuropathic pain. Thus, calpain inhibitors may be a novel type of analgesic drug useful for the prevention of progressive neuropathic pain.

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Development/Plasticity/Repair

Epigenetic Gene Silencing Underlies C-Fiber Dysfunctions in Neuropathic Pain

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Peripheral nerve injury causes neuropathic pain, which is characterized by the paradoxical sensations of positive and negative symptoms. Clinically, negative signs are frequently observed; however, their underlying molecular mechanisms are largely unknown. Dysfunction of C-fibers is assumed to underlie negative symptoms and is accompanied by long-lasting downregulation of $Na_v1.8$ sodium channel and μ-opioid receptor (MOP) in the dorsal root ganglion (DRG). In the present study, we found that nerve injury upregulates neuron-restrictive silencer factor (NRSF) expression in the DRG neurons mediated through epigenetic mechanisms. In addition, chromatin immunoprecipitation analysis revealed that nerve injury promotes NRSF binding to the neuron-restrictive silencer element within MOP and $Na_v1.8$ genes, thereby causing epigenetic silencing. Furthermore, NRSF knockdown significantly blocked nerve injury-induced downregulations of MOP and $Na_v1.8$ gene expressions, C-fiber hypoesthesia, and the losses of peripheral morphine analgesia and $Na_v1.8$-selective blocker-induced hypoesthesia. Together, these data suggest that NRSF causes pathological and pharmacological dysfunction of C-fibers, which underlies the negative symptoms in neuropathic pain.

Introduction

Neuropathic pain is characterized by the paradoxical sensations of positive (hyperalgesia, allodynia, and paresthesia) and negative (hypoesthesia, hypoalgesia) symptoms (Baron, 2006), and negative signs are frequently observed during clinical sensory examinations (Devigili et al., 2008; Leffler and Hansson, 2008). The molecular mechanisms underlying positive symptoms have been extensively investigated (Devor, 2006; Costigan et al., 2009); however, those underlying negative symptoms are much less well understood. A possible mechanism for negative symptoms is a dysfunction of small-diameter (C)-fibers (Taylor, 2001; Devigili et al., 2008; Costigan et al., 2009), such as a loss of C-fiber terminals, an impairment of C-fiber-mediated axon-reflex flare responses, or an increase in the threshold against C-fiber-specific stimuli (Fields et al., 1998; Ueda, 2008). Such C-fiber dysfunctions have been implicated in the manifestation of positive symptoms as well as of negative ones, possibly through a synaptic reorganization in the spinal dorsal horn (Taylor, 2001; Ueda, 2008; Costigan et al., 2009). Representative examples for negative symptoms were observed with long-lasting downregulations of $Na_v1.8$ sodium channel and μ-opioid receptor (MOP) in C-fibers (Waxman et al., 1999; Rashid et al., 2004; Kohno et al., 2005), which are essential for C-fiber functions in terms of determining pain thresholds (Akopian et al., 1999) and for the pharmacological actions of μ-opioids (Dickenson and Kieffer, 2006), respectively.

In terms of long-lasting transcriptional regulation, the transcription factor-mediated epigenetic mechanisms have been demonstrated to play a key role (Borrelli et al., 2008). Neuron-restrictive silencer factor (NRSF, also known as REST) functions as a transcriptional repressor of genes that contain neuron-restrictive silencer element (NRSE, also called RE1) (Chong et al., 1995; Schoenherr and Anderson, 1995). NRSF, when it binds to NRSE, recruits histone deacetylase (HDAC) through its corepressors, mSin3 and CoREST, for generating a repressive chromatin environment (Ballas and Mandel, 2005). It has been reported that NRSF represses transcription of MOP gene through HDAC-mediated mechanisms (Kim et al., 2004). Here, we show that nerve injury induces a long-lasting NRSF expression in the dorsal root ganglion (DRG), thereby causing epigenetic silencing of MOP gene and loss of pharmacological target for peripheral morphine analgesia. Furthermore, we also investigated the possible epigenetic silencing of $Na_v1.8$ gene, which has unique forward and reverse NRSE sequences.

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Materials and Methods

Animals and surgery. Male C57BL/6J mice weighing 20–25 g were used. They were kept in a room with a temperature of 21 ± 2°C with *ad libitum* access to a standard laboratory diet and tap water. All procedures were approved by the Nagasaki University Animal Care Committee (Nagasaki, Japan) and complied with the recommendations of the International Association for the Study of Pain (Zimmermann, 1983). Partial ligation of the sciatic nerve was performed under pentobarbital (50 mg/kg) anesthesia, following the methods of Malmberg and Basbaum (1998).

Oligonucleotide treatments. The antisense oligodeoxynucleotide (AS-ODN) was designed to target the mouse NRSF sequence and corresponds to the rat sequence targeted for antisense knockdown previously (Calderone et al., 2003). AS-ODN (5′-CGGAAGGGCTT-