Upregulation of casein kinase 1ε in dorsal root ganglia and spinal cord after mouse spinal nerve injury contributes to neuropathic pain

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Abstract

Background: Neuropathic pain is a complex chronic pain generated by damage to, or pathological changes in the somatosensory nervous system. Characteristic features of neuropathic pain are allodynia, hyperalgesia and spontaneous pain. Such abnormalities associated with neuropathic pain state remain to be a significant clinical problem. However, the neuronal mechanisms underlying the pathogenesis of neuropathic pain are complex and still poorly understood. Casein kinase 1 is a serine/threonine protein kinase and has been implicated in a wide range of signaling activities such as cell differentiation, proliferation, apoptosis, circadian rhythms and membrane transport. In mammals, the CK1 family consists of seven members (α, β, γ1, γ2, γ3, δ, and ε) with a highly conserved kinase domain and divergent amino- and carboxy-termini.

Results: Preliminary cDNA microarray analysis revealed that the expression of the casein kinase 1 epsilon (CK1ε) mRNA in the spinal cord of the neuropathic pain-resistant N-type Ca2+ channel deficient (Cav2.2-/-) mice was decreased by the spinal nerve injury. The same injury exerted no effects on the expression of CK1ε mRNA in the wild-type mice. Western blot analysis of the spinal cord identified the downregulation of CK1ε protein in the injured Cav2.2-/- mice, which is consistent with the data of microarray analysis. However, the expression of CK1ε protein was found to be up-regulated in the spinal cord of injured wild-type mice. Immunocytochemical analysis revealed that the spinal nerve injury changed the expression profiles of CK1ε protein in the dorsal root ganglion (DRG) and the spinal cord neurons. Both the percentage of CK1ε-positive neurons and the expression level of CK1ε protein were increased in DRG and the spinal cord of the neuropathic mice. These changes were reversed in the spinal cord of the injured Cav2.2-/- mice. Furthermore, intrathecal administration of a CK1 inhibitor IC261 produced marked anti-allodynic and anti-hyperalgesic effects on the neuropathic mice. In addition, primary afferent fiber-evoked spinal excitatory responses in the neuropathic mice were reduced by IC261.

Conclusions: These results suggest that CK1ε plays important physiological roles in neuropathic pain signaling. Therefore CK1ε is a useful target for analgesic drug development.
Background
Neuropathic pain is a complex chronic pain generated by damage to, or pathological changes in the somatosensory nervous system. Neuropathic pain is characterized by the appearance of allodynia (pain perceived in response to normally innocuous stimuli), hyperalgesia (increased responsiveness to painful stimuli) and spontaneous pain [1]. Such abnormalities associated with neuropathic pain state remain to be a significant clinical problem. However, the neuronal mechanisms underlying the pathogenesis of neuropathic pain are complex and still poorly understood [2]. Partly for this reason, attempts to develop new therapeutic agents confront difficulties and the efficacies of currently available drugs for neuropathic pain are reported to be marginal and/or variable for each patient. Thus, development of new strategies leading to pharmacological treatment of neuropathic pain is eagerly awaited. For this purpose, it would be essential to understand the molecular mechanism of the induction and maintenance of neuropathic pain.

In the present study, we have utilized mice lacking N-type voltage-dependent Ca\(^{2+}\) channels (VDCCs) and searched for new neuropathic pain-related molecules. These mice exhibit markedly reduced symptoms of neuropathic pain after spinal nerve injury [3], suggesting a critical role of N-type VDCCs (Ca\(_{\alpha2.2}\)) in the development of neuropathic pain. It is generally believed that changes of gene expression induced by nerve injury contribute substantially to the initiation and maintenance of long lasting neuropathic pain state [4]. Therefore, we have searched for the genes whose expression was altered by spinal nerve injury in the wild-type (Ca\(_{\alpha2.2}\)^+/+) and N-type VDCC-deficient (Ca\(_{\alpha2.2}\)^−/−) spinal cord using microarray techniques and compared these gene expression profiles. From this preliminary comparative cDNA microarray analysis, we found that the spinal nerve injury down-regulated the expression of \textit{casein kinase 1 epsilon} (CK1\(_{\epsilon}\)) mRNA in the spinal cord of Ca\(_{\alpha2.2}\)^−/− mice but not of the Ca\(_{\alpha2.2}\)^+/+ mice. CK1\(_{\epsilon}\) is a serine/threonine protein kinase and has been implicated in a wide range of signaling activities such as cell differentiation, proliferation, apoptosis, circadian rhythms and membrane transport [5-7]. In mammals, the CK1\(_{\epsilon}\) family consists of seven members (α, β, γ1, γ2, γ3, δ, and ε) with a highly conserved kinase domain and divergent amino- and carboxy-termini. CK1\(_{\epsilon}\) isoforms were shown to be associated with cytosolic vesicles including small synaptic vesicles and phosphorylated several small synaptic vesicle-associated proteins in neuronal cells [6,8,9]. In the present study, we have tested a possibility that CK1\(_{\epsilon}\) plays a role in the maintenance of neuropathic pain state. We first quantified the expression of CK1\(_{\epsilon}\) protein and then examined the distribution of this protein in dorsal root ganglia and the spinal cords. Next, we have tested the effects of a CK1 inhibitor on neuropathic pain behaviors. We have also analyzed the effects of the CK1 inhibitor on the excitatory responses in the spinal dorsal horn elicited either by direct activation of postsynaptic glutamate receptors or by presynaptic primary afferent fiber stimulation.

Results
Expression of CK1 in the spinal cord
Using cDNA microarray technique, we previously reported that the expression of ~900 genes out of ~10,000 genes in the Ca\(_{\alpha2.2}\)^+/+ spinal cord was increased more than 1.20-fold by the lumbar L5 and L6 spinal nerve ligation (L5/L6 SNL) injury as compared with sham-operated Ca\(_{\alpha2.2}\)^+/+ mice. Among these genes, we recently suggested that glucocorticoid receptor [10] and peripheral-type benzodiazepine receptor [11] are useful targets in the management of neuropathic pain. On the other hand, we also observed that the expression of ~1,300 genes out of ~10,000 genes was reduced more than 1.20-fold by L5/L6 SNL injury in the Ca\(_{\alpha2.2}\)^−/− spinal cord when compared with sham-operated Ca\(_{\alpha2.2}\)^+/+ mice. Because Ca\(_{\alpha2.2}\)^−/− mice showed markedly reduced symptoms of the SNL-induced neuropathic pain [3], we speculated that these down-regulated genes have some contribution to the reduced neuropathic pain symptoms. Among these down-regulated genes, we focused on CK1\(_{\epsilon}\) in this study, because there has been no information about the expression and function of this molecule in sensory pathway up to this moment, in spite of the fact that CK1 is one of the first serine/threonine protein kinases that were isolated and characterized [5]. The expression of CK1\(_{\epsilon}\) mRNA was not changed in Ca\(_{\alpha2.2}\)^+/+ mice (1.01-fold) but decreased by 2.81-fold in Ca\(_{\alpha2.2}\)^−/− mice 2 weeks after SNL injury. Quantitative real-time PCR analysis of C57BL/6j mice 2-3 weeks after sham or SNL operation also showed no difference of the CK1\(_{\epsilon}\) mRNA expression in the spinal cord (n = 4 for sham and SNL, data not shown).

We next examined the expression of CK1\(_{\epsilon}\) protein in the spinal cord by immunoblot analyses (Figure 1A and 1B). The CK1\(_{\epsilon}\) expression was significantly enhanced in the Ca\(_{\alpha2.2}\)^+/+ spinal cord 2 weeks after SNL injury. In contrast, CK1\(_{\epsilon}\) expression in the SNL-operated Ca\(_{\alpha2.2}\)^−/− spinal cord was significantly reduced, which is consistent with the cDNA microarray results.

Immunohistochemical analysis of CK1\(_{\epsilon}\) expression in the spinal cord after spinal nerve injury
We further characterized the distribution of CK1\(_{\epsilon}\)-positive cells in the spinal cord of sham and neuropathic mice by immunofluorescence techniques. As shown in Figure 1C-E, the CK1\(_{\epsilon}\) protein was found to be broadly expressed in the spinal cord.

Consistent with the immunoblot data, the intensity of CK1\(_{\epsilon}\)-immunoreactivity (CK1\(_{\epsilon}\)-IR) in the dorsal horn of
the spinal cord was increased on the ipsilateral side to the nerve injury.

To further characterize the localization profile of the CK1ε, we performed double staining of CK1ε with cell-type specific markers. CK1ε-IR was colocalized with a neuronal marker, neuronal specific nuclear protein (NeuN) (Figure 2Ai-Ci), but not with an astrocytic marker, glial fibrillary acid protein (GFAP) (Figure 2Aii-Cii) or a microglial marker, ionized calcium binding adaptor molecule 1 (Iba 1) (Figure 2Aiii-Ciii). Both GFAP and Iba 1 immunostainings were significantly enhanced on the side ipsilateral to SNL injury (Figure 2Aii-Cii). Confocal microscopic analyses indicated that the expression of CK1ε protein was indeed up-regulated in the ipsilateral superficial dorsal horn neurons of C57BL/6 j mice 2 weeks after SNL injury, whereas the same injury slightly reduced the CK1ε expression in C57BL/6 j mice (Figure 3C-F). CK1ε-IR was detected mostly in the cytoplasm, with only faint staining in the nucleus.

**Immunohistochemical analysis of CK1ε expression in the dorsal root ganglia after spinal nerve injury**

To further address the feature of CK1ε expression at neuropathic pain state, we have investigated whether CK1ε is expressed in primary sensory neurons and whether the expression pattern of CK1ε is altered by the SNL injury by immunofluorescence analyses. In agreement with the previous reports [14-16], we observed marked loss (50%) of ipsilateral L5 DRG neurons 2 weeks after the nerve injury when compared with the ipsilateral side of sham operated mice (Table 1). Most dramatic decrease (87%) was observed in large-sized group, and 53% and 38% losses were observed in small- and medium-sized groups, respectively (Table 1). Similar level of cell loss was also observed in SNL-operated Cav2.2+/− mice (Table 1).

In L5 DRG, the CK1ε-IR was mainly observed in small- and medium-sized neurons with IR appearing largely in cytoplasm with weak staining in the nucleus (Figure 4). The percentages of CK1ε-positive neurons ipsilateral to sham operation were 68.4%, 29.1% and 15.1% in small-, medium- and large-sized neurons, respectively (Figure 4D). The percentages of CK1ε-positive neurons in the DRG ipsilateral to sham operation were similar to those in contralateral to sham operation and SNL injured, and in naive DRG (data not shown). On the other hand, significant increases in the percentages of CK1ε-positive neurons in the ipsilateral L5 DRG compared with sham group were observed in all size groups 2 weeks after SNL injury (Figure 4B and 4D). Similar changes of CK1ε-positive cell population were also observed in SNL-operated Cav2.2+/− mice (Figure 4C and 4D). Expression level of CK1ε protein was also analyzed by a computerized image analysis out significant changes in the contralateral side (data not shown). In Cav2.2+/− spinal cord, SNL injury, in contrast, significantly reduced the percentage of the CK1ε-positive neurons in both ipsilateral superficial and middle layers (Figure 3A and 3B). There were no overt differences in the numbers between right and left sides in naive control and between ipsilateral and contralateral sides in sham-operated animals (data not shown).

Although the increase of CK1ε-positive neurons after SNL injury would explain the upregulation of CK1ε protein revealed by the immunoblot experiments, we also tested another possibility that SNL injury increased the expression level of CK1ε protein in the dorsal horn neurons. Conclusive microscopic analyses indicated that the expression of CK1ε protein was indeed up-regulated in the ipsilateral superficial dorsal horn neurons of C57BL/6 j mice 2 weeks after SNL injury, whereas the same injury slightly reduced the CK1ε expression in C57BL/6 j mice (Figure 3C-F). CK1ε-IR was detected mostly in the cytoplasm, with only faint staining in the nucleus.

**Figure 1**

SNL upregulates CK1ε expression in the spinal cord.

(A) Immunoblot performed with a rabbit polyclonal antibody to CK1ε in the Ca2.2+/+ and Ca2.2−/− spinal cord. (B) Expression of CK1ε protein, which is normalized by GAPDH loading control. **p < 0.05, SNL Ca2.2+/+ compared with sham Ca2.2+/+** (Student’s t test), **# p < 0.05, SNL Ca2.2−/− compared with sham Ca2.2−/−** (Student’s t test); n = 7 in each group. (C-E) Immunofluorescent micrographs of CK1ε-IR in L5 spinal cord from sham- and SNL-operated (D) C57BL/6 j mice, and SNL-operated Ca2.2−/− mice (E). Scale bar, 100 μm.

![Image](image-url)
When compared with sham group, significant increases in the intensity of CK1ε-IR were observed in the small- and medium-sized neurons of ipsilateral L5 DRG 2 weeks after SNL injury.

Similar extent of increase was also observed in SNL-operated Cav2.2-/- mice (Figure 4E). In contrast, the intensity of CK1ε-IR in large-sized neurons was not significantly changed following SNL injury. To clarify the expression profiles of CK1ε protein in sham and neuropathic mice L5 DRGs, we carried out the double immunofluorescence analysis using CK1ε antibodies together with antibodies against two DRG neuron markers. Small and medium-sized DRG neurons were generally classified into two subgroups, which have been designated as peptidergic and non-peptidergic [17]. The former expresses two major...
peptidergic neuromodulators, substance P and calcitonin gene-related peptide (CGRP), and the latter expresses isolectin B4 (IB4)-binding glycoprotein. CK1ε protein was found in both CGRP- and IB4-positive populations (Figure 5). The CK1ε expression was detected in 66.4% of CGRP-positive neurons and 44.9% of CK1ε-positive neurons were CGRP-positive in sham operated DRG neurons (Figure 5). Similarly, the CK1ε expression was detected in 82.3% of IB4-positive neurons and 38.3% of CK1ε-positive neurons were IB4-positive. Similar results were also obtained from naive DRG neurons (data not shown). Two weeks after SNL, a marked decrease of IB4 binding (both the cell number and the intensity) was observed in ipsilateral L5 DRGs (Figure 5D), which is similar to that shown by a previous study [16]. Relatively lesser extent of decrease occurred in the number and the intensity of CGRP-positive small- and medium sized neurons (Figure 5B). In injured DRG neurons, CK1ε-IR was detected in 94% and 91% of CGRP- and IB4-positive neurons, respectively. CGRP-IR was detected in 65% of CK1ε-positive neurons, however, IB4 binding was observed in only 3% of CK1ε-positive neurons. The proportions of CK1ε-expressing cells within CGRP- and IB4-positive neurons in the contralateral DRGs were not different from those of naive and sham-operated DRGs (data not shown). The colocalization of CK1ε with CGRP observed in the superficial dorsal horn area of spinal cord slice preparations (Figure 5E and 5F) suggest that part of the CK1ε protein detected by immunoblot analyses (Figure 1A and 1B) and CK1ε-IR in the spinal cord (Figure 1D) originated from the CK1ε present at the primary afferent fibers and terminals, because CGRP is generally accepted as a pure primary afferent marker [18]. It seems clear that the intensity of CK1ε protein in the primary afferent fibers and terminals is enhanced in CGRP-positive DRG neurons after SNL injury (Figure 5F). Because enhanced protein expression of CK1ε was observed in DRG neurons, we have analyzed mRNA level by quantitative real-time PCR method and found that the 2.03 fold increase of CK1ε mRNA was observed 2 weeks after SNL operation (n = 4 for sham and SNL, p < 0.001, data not shown).

### Table 1: Number of L5 ipsilateral DRG neurons 2 weeks after L5 and L6 spinal nerve injury.

|            | Small-sized | Medium-sized | Large-sized | Total    |
|------------|-------------|--------------|-------------|----------|
| Sham C57BL/6j | 371.6 ± 31.2  | 116.2 ± 10.7  | 18.6 ± 2.90 | 506.3 ± 42.3 |
| SNL C57BL/6j  | 176.2 ± 18.4*** | 72.3 ± 6.64*** | 2.33 ± 0.59*** | 250.8 ± 21.9*** |
| SNL Ca2.2−/− mutant | 225.3 ± 25.2*** | 67.7 ± 8.62*** | 3.17 ± 0.72*** | 296.1 ± 30.2*** |

Each value represents the mean ± SEM (n = 12 in each group). ***p < 0.001, compared with Sham C57BL/6j (one-way ANOVA followed by Tukey’s post hoc test). Numbers were obtained using conventional profile counting methods.
Intrathecal injection of saline or dimethyl sulfoxide (DMSO; 3% in saline) used as a solvent for the drugs did not show any effects on mechanical allodynia and thermal hyperalgesia (Figure 6C and 6D).

**CK1 inhibitor reduced presynaptic primary afferent fiber-evoked spinal excitatory responses**

To explore the mechanism of CK1 inhibitor-induced analgesia at the spinal level, we made L5 dorsal root attached-spinal cord slice preparation from adult mice (9-12 weeks old) and carried out spatio-temporal analyses of the primary afferent fiber-evoked excitatory responses in the dorsal horn by means of imaging techniques using a voltage-sensitive dye 4-(2-(6-(dibutylamino)-2-naphthalenyl)ethyl(enyl)-1-(3-sulfoethyl)-5-(4-ANPEPPS). Repetitive stimulation (10 pulses at 20 Hz) of L5 dorsal root produced prolonged (lasting for 3-4 s) and widely propagating excitatory responses extending from superficial layer to deeper laminae within the ipsilateral L5 spinal dorsal horn (Figure 7). In sham and SNL animals, the magnitude of integrated area of the optical response recorded in the superficial layer of each animal group were significantly larger than those recorded in the corresponding middle layer (Figure 7). Remarkably, the optical responses were not reduced by SNL injury (Figure 7C and 7D), in spite of the fact that 50% of the neurons were lost in DRG. Excitatory synaptic transmission evoked by primary afferents is known to be mainly mediated by glutamate [18,21], and glutamate receptors, especially NMDA receptor, are considered to play important roles in development and maintenance of neuropathic pain [22,23]. Application of an NMDA-receptor antagonist, D-APV (50 μM), suppressed the optical responses in the superficial and middle layers elicited by the nerve stimulation in sham and SNL animals. A perfusion of a solution containing both D-APV (50 μM) and a non-NMDA-receptor antagonist, CNQX (20 μM), further reduced the optical responses in both layers in sham and SNL animals (Figure 7). These results suggest that the activation of glutamate receptors is largely responsible for the induction of the excitatory responses evoked by the repetitive electrical stimulation of the primary afferents. To identify a role of CK1ε in neuropathic pain-related spinal nociceptive transmission, we investigated the effects of the CK1 inhibitor on the dorsal root-evoked optical responses (Figure 8). IC261 (1 and 2 μM) showed significant inhibitory effects on the optical responses in the SNL dorsal horn (Figure 8B and 8D). Similar results were obtained when using another CK1 inhibitor CKI-7 (data not shown).

Interestingly, IC261 did not reduce the dorsal root-evoked optical responses elicited in the dorsal horn of sham mice (Figure 8A and 8C). Vehicle control (0.01 and 0.03%
DMSO in ACSF) did not change the optical responses evoked in both superficial and middle layers of naïve dorsal horn (data not shown). Thus CK1 inhibitor was found to be effective in reducing spinal excitatory response elicited by the presynaptic electrical stimulation only in neuropathic mice.

**CK1 inhibitor reduced direct NMDA-evoked excitatory responses**

To test whether the observed effects of CK1 inhibitor originated from the direct effects on the postsynaptic glutamate receptors, we further examined the effect of IC261 on the optical responses induced by NMDA and glutamate in the SNL spinal cord in the presence of tetrodotoxin (TTX; 0.3 μM).

In both superficial and middle layer of the SNL spinal cord, excitatory optical responses evoked by the bath-application of NMDA (300 μM) for 30 s was not inhibited by 1 μM of IC261 but significantly inhibited by 3 μM of IC261 (Figure 9A). These results suggest that blockade of primary afferent fiber-evoked excitatory responses in the dorsal horn by IC261 (Figure 8B and 8D) was partly caused by the blockade of NMDA evoked responses, because only higher concentration of IC261 was effective. On the other hand, 3 μM of IC261 did not show any effect on the excitatory optical responses evoked by the bath-application of glutamate (3 mM) for 1 min (Figure 9B).

**Discussion**

CK1 family constitutes one of the eight major groups of protein kinases in the human and mouse genome [24,25]. However, few physiological roles have been described for CK1 in synaptic transmission. It has been recently reported that metabotropic glutamate receptors down-regulated NMDA receptor-mediated synaptic currents through CK1 dependent activation of protein phosphatases in the striatum [26]. In the present study, we have shown several lines of evidence that CK1ε plays a key role in the maintenance of neuropathic pain induced by spinal nerve injury. Thus, CK1 isoforms expressed in central and peripheral nervous system might display region- and individual cell-specific regulation of synaptic transmission in normal and pathological states.

To our knowledge, this is the first report demonstrating the alteration of the expression pattern of CK1ε in the spinal cord and DRG following spinal nerve injury. After spinal nerve injury, number of CK1ε-positive neurons and the expression level of CK1ε protein were both increased in the superficial and middle layers of ipsilateral L5 dorsal horn. These SNL-induced changes observed in wild-type mice were completely reversed in Cav2.2-/- mice. Furthermore NMDA-evoked excitatory responses and neuropathic pain behaviors were inhibited by IC261. These findings may point the importance of CK1ε-positive neurons within the spinal dorsal horn in neuropathic pain state.

We also found that enhanced GFAP expression possibly reflecting astroglial activation and enhanced Iba1 expression possibly reflecting microglial migration identified in L5 spinal dorsal horn from neuropathic mice were strongly suppressed in SNL-operated Cav2.2-/- mice. The reason why the glial activation accompanying spinal injury is suppressed in Cas2.2-/- mice is not known at this moment, but this seems to be a reason why Cav2.2-/- mice did not show neuropathic pain symptoms after SNL, because these glial activations have been shown to induce neuropathic pain [12,13]. It would be interesting to explore the mechanism by which N-type Ca2+ channel activation induced by the SNL injury leads to enhanced CK1ε expression in the spinal neuron and glial activation at the spinal dorsal horn in the future study. One plausible mechanism for the enhanced CK1ε expression would be that CK1ε expression may be regulated directly or indirectly by the calcium entry through N-type Ca2+ channel both at the transcriptional and at the translational levels. The calcium dependent transcriptional regulation may include up-regulation of CK1ε mRNA and some miRNAs regulating the translation of CK1ε. If the SNL injury down regulates some of the gene expression including CK1ε besides the activation of N-type Ca2+ channel, mRNA level may not be changed in Cav2.2-/-/+ mice but will be reduced in Cav2.2-/-/- mice. Furthermore, if the activation of Cav2.2 channel induces translation of CK1ε by the up-regulated regulatory miRNAs or other unknown mechanism, protein level will be increased in Cav2.2-/-/+ mice but will be
decreased in Cav2.2−/− mice. Above presumptive results are exactly what we observed in the actual experiments. However these results are based on the several assumptions that have to be proven experimentally. Furthermore, expression levels of mRNA and protein in DRGs were found to be proportional. Thus further rigorous study would be necessary to clarify the role of N-type Ca2+ channel on the expression of CK1ε.

The effects of nerve injury on the number of DRG neurons have been examined in many different injury models. Although the degree varies in each model presumably due to the differences of the experimental manipulations and the counting methodologies, previous studies in rat [14-16,27] and mouse [28] indicate a loss of neurons in L5 DRG after spinal nerve injury. Our results were found to be consistent with these previous reports. More importantly, we found that both the percentage of CK1ε-positive neurons and the expression level of CK1ε protein were significantly increased in ipsilateral small- and medium-sized L5 DRG neurons after SNL. Furthermore intense expression of CK1ε was found in the primary afferent fibers possibly including presynaptic boutons after SNL injury. These enhanced expression and following activation of CK1ε may be responsible for the apparently normal level of spinal excitatory responses in SNL mice in spite of the fact that more than 50% of the DRG neurons were lost after SNL injury.

Small- and medium-sized DRG neurons are generally considered to correspond to C- and Aδ-fiber neurons, whose axons terminate in the superficial layer of the dorsal horn. On the other hand, large-sized DRG neurons are generally considered to correspond to Aβ-fiber neurons, whose
axons terminate in the middle layer of the dorsal horn. Compensation of excitatory responses in the superficial layer of SNL-dorsal horn may be caused by the facilitation of nociceptive transmitter release from the CK1ε-positive C- and Aδ-fiber terminals. In contrast, since the cell loss of large-sized DRG neurons are prominent, the compensation of excitatory responses observed at the middle layer of SNL-dorsal horn may be caused by the indirect effect from the enhanced excitation of superficial layer by the increased input through interneurons linking laminae I-II and laminae III-IV neurons. However, further rigorous study is necessary to verify this hypothesis.

On the other hand, the nature of apparently recovered spinal excitatory responses seems to be very different from those found in normal animals. Firstly, CK1 inhibitor had no effect on the excitatory responses in sham-operated mice but SNL injury turned CK1 inhibitor effective in blocking excitatory responses. Secondly, CK1 inhibitor is effective in blocking neuropathic pain in injured hindpaw.
without showing any appreciable effect on uninjured hindpaw. Naturally, it is important to identify the target proteins of CK1ε that would induce these changes. Our preliminary experiments trying to identify CK1ε targets resulted in many candidate proteins (data not shown). Further rigorous study is necessary to narrow down and identify the causative proteins.

Conclusions
The present study demonstrated that SNL injury enhanced the expression of CK1ε protein in spinal dorsal horn and DRG neurons. Furthermore CK1 inhibitor was found to be effective in blocking excitatory synaptic responses in the spinal dorsal horn and neuropathic pain symptoms. These results suggest that CK1ε plays important physiological roles in neuropathic pain signaling, which makes it a useful target for analgesic drug development.

Methods
Animals
Male C57BL/6J mice (7-8 weeks old at the time of operation) were purchased from Clea Japan, Inc. (Tokyo, Japan) and housed under controlled temperature (24 ± 1°C) and humidity (55 ± 10%) with a 12-h light-dark cycle with food and water freely available. Experiments were conducted with the approval of the Animal Care Committee of Tokyo Medical and Dental University (approval No. 0909173), and according to the ethical guidelines for the study of experimental pain in conscious animals published by the International Association of the Study of Pain [29].

Animal model of neuropathic pain
L5/L6 SNL was carried out as described previously [3,10].

Intrathecal injection
I.t. injection was given in a volume of 5 μl by percutaneous puncture through an intervertebral space at the level of the 5th or 6th lumbar vertebra, according to a previously reported procedure [30,31].

Behavioral studies
Behavioral studies were conducted in a sound proof room during the light cycle (8:00 a.m.-8:00 p.m.) 2-4 weeks after spinal nerve ligation as described [32]. An investigator, who was unaware of the drug treatment, performed all of the behavioral experiments.

cDNA microarray analysis
Ca_{2.2^{-/-}} mice were generated and housed as previously reported [3]. Seven Ca_{2.2^{+/+}} and Ca_{2.2^{-/-}} mice were used for L5/L6 SNL surgery and five Ca_{2.2^{+/+}} and Ca_{2.2^{-/-}} mice
were used for sham surgery. cDNA microarray analysis was performed using the CodeLink™ UniSet Mouse 10K I (GE Healthcare, Piscataway, NJ) following the protocol provided by the manufacturer. Four data sets (Cav2.2+/+ sham, Cav2.2+/+ SNL, Cav2.2−/− sham, Cav2.2−/− SNL) were compared using the CodeLink™ System Software.

**Immunoblot analysis**

Proteins were separated by SDS-PAGE (7.5% gel) and then transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA). Anti-CK1ε antibody was used (rabbit; 1: 1000; Santa Cruz Biotechnology, Santa Cruz, CA). We have also tested other anti-CK1ε antibody (mouse; 1: 500; BD Transduction Laboratories, Franklin Lakes, NJ) and found that they showed similar results (single band with same size in immunoblot analysis, data not shown). Immunoreactivity was detected by using the ECL system (GE Healthcare, Buckinghamshire, UK). An anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (mouse, 1:20,000; Chemicon, Temecula, CA) was used to normalize protein loading. Relative intensities of the bands were quantified by using an image analysis system with Image J software, version 1.40 g (National Institutes of Health, Bethesda, MD).

**Immunohistochemistry**

Transverse spinal and DRG sections (10 μm) were used. The antibodies used are as follows: CK1ε (rabbit, 1:50; Santa Cruz Biotechnology; mouse, 1:50; BD Transduction Laboratories), CGRP (rabbit, 1:1000; Sigma), Neun (mouse, 1:100; Chemicon), GFAP (mouse, 1:400; Chemicon) and Iba 1 (rabbit, 1:500, Wako Pure Chemical Industries, Ltd., Osaka, Japan). The sections were then incubated for 2 h at room temperature with Alexa Fluor 488-labeled donkey anti-rabbit IgG (1:1000; Invitrogen, Paisley, UK) or Cy3-conjugated donkey anti-mouse IgG (1:1000; Jackson ImmunoResearch, West Grove, PA). For the detection of IB4 binding, biotinylated IB4 (1:400; Vecto Laboratories, Burlingame, CA) and FITC-conjugated extravidin (1:500; Sigma) were used. Sections of a set of control and experimental tissues were concurrently immunostained and images were captured under the same conditions. Control tissue sections, in which the primary antibody was omitted, showed no specific staining. The experiments were carried out at least three times.

Immunofluorescent preparations were examined with a fluorescence microscope (BIOREVO BZ-9000; Keyence Corp, Osaka, Japan).
The numbers of CK1ε-positive neurons were counted in L5 DRGs. On average, five to seven non-adjacent sections of each DRG, where the CK1ε-positive neurons with visible nuclei were equally distributed throughout the rostrocaudal length of the DRG, were randomly selected from 3 to 4 animals in naive control, sham- and SNL-operated groups and numbers of neurons that showed distinctive CK1ε-labeling compared with background labeling were counted as CK1ε-positive by two investigators blinded to the surgical treatment and averaged. The fluorescence intensity was quantified using a 255-level gray scale [33]. To determine the percentage of immunoreactive neurons in each DRG, a threshold of average fluorescence intensity level (for example, 30 in 255-level gray scale for CK1ε) was set by observing several images of normal DRGs. The fluorescence intensity threshold was then applied to all other sections of ipsilateral and contralateral DRGs. The fluorescence intensity and cross-sectional area of CK1ε-positive neurons were quantified using BZ-II analyzer (BZ-H1C software; Keyence Corp, Osaka, Japan). To distinguish cell size-specific changes, we divided the DRG into small-sized (< 600 μm²) medium-sized (600-1200 μm²), and large-sized (> 1200 μm²) groups based on their cross-sectional areas [34,35]. For the co-localization analyses, double stained DRG sections were similarly selected from 3-4 mice, and CK1ε, CGRP- and Iba1-positive cells were counted.

For counting the dorsal horn cells, five to seven sections from the L5 spinal cord segment were randomly selected from each mouse and numbers of distinctive CK1ε-, NeuN-, GFAP- and Iba1-positive cells in the superficial and middle layers of the dorsal horn were counted by two investigators blinded to the surgical treatment and averaged. The border between superficial and middle layers was delineated according to a representative immunostaining images of protein kinase C-type γ (PKCγ) prepared in our laboratory. PKCγ is present in a subpopulation of neurons in the inner part of lamina II and allows a good localization of the border between laminae II and III. The middle layer was defined as dorsal half of deep dorsal horn (laminae III-VI). The number of cells per one section was averaged in each naive control, sham- and SNL-operated animal and the overall means were calculated.

Proportions of CK1ε-positive cells and intensities of CK1ε-IR measured in single- and double-staining were not significantly different in any of the experimental groups. Therefore, data from both single- and double-labeled cells were combined.

**Confocal laser-scanning microscopy**

Images of spinal cords stained with the rabbit anti-CK1ε antibody or double labeled with CK1ε (mouse) and CGRP (rabbit) antibodies were collected using a Zeiss LSM 5 Pascal confocal microscope with argon and helium neon lasers (Carl Zeiss Microscopy, Jena, Germany). A × 63, 1.2 NA water-immersion C-achromatic objective and 2 × zoom value were used for high magnification. The digital images of 20 consecutive z-scan sections (step size approximately 0.5 μm) were analyzed on a computer equipped with an image analysis system (Image J version 1.40 g). CK1ε-IR was quantified using a 255-level gray scale [33]. To quantify CK1ε-IR of the superficial dorsal horn neurons, the immunofluorescence intensities of five randomly selected neurons were quantified using a 255-level gray scale. For each spinal cord section, the ratio of the immunofluorescence intensities of the ipsilateral to the contralateral side was calculated. The ratios for 3-4 non-adjacent sections were averaged in sham- and SNL-operated groups.

**Preparation of spinal cord slices**

Spinal cord slices were prepared according to the method described previously [21,36]. Transverse slices (thickness, 600-750 μm) of the L5 spinal segments with the L5 dorsal root attached were prepared and stained with a fluorescent voltage-sensitive dye di-4-ANEPPS (Invitrogen).

**Optical recording**

Changes in voltage-sensitive dye fluorescence in the spinal cord were detected using an optical recording system MiCAM02 (Brainvision Inc., Tsukuba, Japan) [37,38]. Repetitive electrical stimulation (current: 1.0 mA, duration: 1 ms) comprised of 10 pulses at 20 Hz were applied through a suction electrode attached to the dorsal root, as described previously [39,40] to induce a long-lasting excitatory component including NMDA receptor transmission. Both A- and C-fibers were thought to be activated by the stimulation mode [41]. For the recording of glutamate receptor agonist-evoked responses in the presence of TTX (0.3 μM), short exposure mode of MiCAM02 recording and analyzing software (BV analyzer; Brainvision Inc., Tsukuba, Japan) was used to minimize dye bleaching according to the manufacturer's guide. The effect of a pharmacological agent on the nerve- or glutamate receptor agonist-induced responses was evaluated by comparing the averaged magnitude of two or three control responses with the magnitude of response after 25-30 min equilibration for each drug. The concentrations of glutamate receptor antagonists and TTX used were determined according to the previous studies [40,42,43] and our preliminary study.

**Drugs**

D-APV, CNQX and IC261 were purchased from Tocris Bioscience, Bristol, UK. Glutamate and NMDA were from Sigma, St. Louis, MO, USA. TTX was from Sankyo Co., Ltd., Tokyo, Japan.

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Statistical analysis
Experimental data are expressed as mean ± SEM. Single comparisons were made using Student’s two-tailed paired or unpaired t-test. One-way ANOVA followed by the Dunnett’s or Tukey’s test was used for multiple comparisons. p < 0.05 was considered statistically significant.

Competing interests
Tokyo Medical and Dental University and Japan Science and Technology Agency (JST) hold a shared patent (Japan Patent No. 4227121) based on the results related to but not presented in the paper.

Authors’ contributions
ES carried out all experiments, performed statistical analysis and wrote the manuscript. TK participated in the design of the study, performed optical recording study and wrote the manuscript. KK performed behavioral and immunohistochemical analysis. HS and SZ performed molecular biological study of Ca2+2 mutant mice. TT participated in the design of the study, supervised the experiments and wrote the manuscript. All authors read and approved the final manuscript.

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References
1. Woelf CJ, Mannion RJ: Neuropathic pain: aetiology, symptoms, mechanisms, and management. Lancet 1999, 353:1959-1964.
2. Ossipov MH, Lai J, Porreca F: Mechanisms of experimental neuropathic pain: integration from animal models. In Textbook of Pain 5th edition. Edited by: McMahon SB, Koltzenburg M. Philadelphia: Elsevier; 2006:929-946.
3. Saegusa H, Kurihara T, Zong S, Kazuno A, Matsuda Y, Nonaka T, Han W, Toriyama H, Tanabe T: Suppression of inflammatory and neuropathic pain symptoms in mice lacking the N-type Ca2+ channel. EMBO J 2001, 20:2349-2356.
4. Zhang X, Xiao HS: Gene array analysis to determine the components of neuropathic pain signaling. Curr Opin Mol Ther 2005, 7:332-337.
5. Gross SD, Anderson RA: Casein kinase I: spatial organization and positioning of a multifunctional protein kinase family. Cell Signal 1998, 10:699-711.
6. Knippschild U, Gocht A, Wolff S, Huber N, Löhler J, Stoter M: The casein kinase I family: participation in multiple cellular processes in eukaryotes. Cell Signal 2005, 17:675-689.
7. Price MA: CK1, there’s more than one: casein kinase I family members in Wnt and Hedgehog signaling. Genes Dev 2006, 20:399-410.
8. Wolff S, Stoter M, Giamas G, Piesche M, Henne-Bruls D, Banting G, Knippschild U: Casein kinase 1 delta (CK1δ) interacts with the SNARE associated protein snapin. FEBS Lett 2006, 580:6477-6484.
9. Takamori S, Holt M, Stenius K, Lemke EA, Grenborg M, Riedel D, Urlaub H, Schnell S, Brügger B, Ringler P, Müller SA, Rammner B, Gräter F, Hub JS, De Groot BL, Mieskes G, Moriyama Y, Klingauf J, Grubmüller H, Heuser J, Wieland F, Jahn R: Molecular anatomy of a trafficking organelle. Cell 2006, 125:831-846.
10. Takasaki I, Kurihara T, Saegusa H, Zong S, Tanabe T: Effects of glucocorticoid receptor antagonists on allodynia and hyperalgesia in mouse model of neuropathic pain. Eur J Pharmacol 2005, 524:80-83.
11. Kochendo D, Saegusa H, Yabe R, Takasaki I, Kurihara T, Zong S, Tanabe T: Peripheral-type benzodiazepine receptor antagonist is effective in relieving neuropathic pain in mice. J Pharmacol Sci 2009, 110:55-63.
12. Ji RR, Kawasaki Y, Zhuang ZY, Wen YR, Decosterd I: Possible role of animal astrocytes in maintaining chronic pain sensitization: review of current evidence with focus on bFGF/JNK pathway. Neuroglia Bio 2006, 2:259-269.
13. Inoue K, Tsuda M: Microglia and neuropathic pain. Glia 2009, 57:1469-1479.
14. Willis JR, Coggeshall RE: Loss of dorsal root ganglion cells concomitant with dorsal root axon sprouting following segmental nerve lesions. Neuroscience 1997, 81:527-534.
15. Vestergaard S, Tandrup T, Jakobsen J: Effect of permanent axotomy on number and volume of dorsal root ganglion cell bodies. J Comp Neurol 1997, 388:307-312.
16. Hammond DL, Ackerman L, Holdsworth R, Elzey B: Effects of spinal nerve ligation on immunohistochemically identified neurons in the L4 and L5 dorsal root ganglia of the rat. J Comp Neurol 2004, 475:575-584.
17. Snider WD, McMahon SB: Tackling pain at the source: new ideas about nociceptors. Neuron 1998, 20:629-632.
18. Willis JR, Coggeshall RE: Sensory mechanisms of the spinal cord Vol. I. 3rd edition. New York: Kluwer Academic/Plenum Publishers; 2004.
19. Behrend L, Milne DM, Stoter M, Deppert W, Campbell LE, Meek DW, Knippschild U: IC261, a specific inhibitor of the protein kinases casein kinase 1-delta and -epsilon, triggers the mitotic checkpoint and induces p53-dependent postmitotic effects. Oncogene 2000, 19:5303-5313.
20. Chijiwa T, Higawara M, Hidaka H: A new synthesized selective casein kinase inhibitor, N-(2-aminoethyl)-5-chloroisouquinolo-

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(page number not for citation purposes)
32. Kondo D, Yabe R, Kurihara T, Saegusa H, Zong S, Tanabe T: Progesterone receptor antagonist is effective in relieving neuropathic pain. Eur J Pharmacol 2006, 541:44-48.

33. Ma W, Zhang Y, Bantel C, Eisenach J: Medium and large injured dorsal root ganglion cells increase TRPV-1, accompanied by increased α2C-adrenoceptor co-expression and functional inhibition by clonidine. Pain 2005, 113:386-394.

34. Fukuoka T, Tokunaga A, Tachibana T, Dai Y, Yamanaka H, Noguchi K: VR1, but not P2X3, increases in the spared L4 DRG in rats with LS spinal nerve ligation. Pain 2002, 99:111-120.

35. Takagi K, Okuda-Ashitaka E, Mabuchi T, Katano T, Ohnishi T, Matsunura S, Ohsaka M, Kaneko S, Abe T, Hirata T, Fujisawa S, Minami T, Ito S: Involvement of stem cell factor and its receptor tyrosine kinase c-kit in pain regulation. Neuroscience 2008, 153:1278-1288.

36. Yoshimura M, Jessell TM: Primary afferent-evoked synaptic responses and slow potential generation in rat substantia gelatinosa neurons in vitro. J Neurophysiol 1989, 62:96-108.

37. Tominaga T, Tominaga Y, Yamada H, Matsumoto G, Ichikawa M: Quantification of optical signals with electrophysiological signals in neural activities of Di-4-ANEPPS stained rat hippocampal slices. J Neurosci Methods 2000, 102:1-11.

38. Fuji R, Ichikawa M, Ozaki M: Imaging of molecular dynamics regulated by activities in neural circuits and synapses. Neurosignals 2008, 16:260-277.

39. Kurihara T, Suzuki H, Yanagisawa M, Yoshioka K: Muscarinic excitatory and inhibitory mechanisms involved in afferent fibre-evoked depolarization of motoneurons in the neonatal rat spinal cord. Br J Pharmacol 1993, 110:61-70.

40. Kurihara T, Yoshioka K: The excitatory and inhibitory modulation of primary afferent fibre-evoked responses of ventral roots in the neonatal rat spinal cord exerted by nitric oxide. Br J Pharmacol 1996, 118:1743-1753.

41. Park JS, Nakatsu T, Nagata K, Higashi H, Yoshimura M: Reorganization of the primary afferent termination in the rat dorsal horn during post-natal development. Dev Brain Res 1999, 113:29-36.

42. Kurihara T, Yoshioka K, Otsuka M: Tachykinergic slow depolarization of motoneurons evoked by descending fibres in the neonatal rat spinal cord. J Physiol (Lond) 1995, 485:787-796.

43. Ikeda H, Ryu PD, Park JB, Tanifuji M, Asai T, Murase K: Optical responses evoked by single-pulse stimulation to the dorsal root in the rat dorsal horn in slice. Brain Res 1998, 812:81-90.