Involvement of Voltage-Gated Sodium Channel Na\textsubscript{v}1.8 in the Regulation of the Release and Synthesis of Substance P in Adult Mouse Dorsal Root Ganglion Neurons

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Received June 24, 2008; Accepted August 15, 2008

Abstract. This study was conducted to determine whether Na\textsubscript{v}1.8 contributes to the release and/or synthesis of substance P (SP) in adult mice dorsal root ganglion (DRG) neurons. The SP released from cultured DRG neurons of Na\textsubscript{v}1.8 knock-out mice exposed to either capsaicin or KCl was significantly lower than that from wild-type (C57BL/6) mice based on a radioimmunoassay. The SP level of L6 DRG in Na\textsubscript{v}1.8 knock-out mice was also lower than that in wild-type mice. After chronic constriction injury (CCI) of the sciatic nerve, the level of SP decreased in the L6 ipsilateral DRG of wild-type but not Na\textsubscript{v}1.8 knock-out mice. The preprotachykinin-A (PPT-A) mRNAs in L4-6 DRGs of Na\textsubscript{v}1.8 knock-out mice also fell to half their normally abundant levels of expression. There were significant increases in Na\textsubscript{v}1.8 expression of the L6 contralateral DRG from wild-type mice and in the percentage of neurons expressing neurokinin-1 receptor in the cytosol of L6 DRGs from wild-type or Na\textsubscript{v}1.8 knock-out mice. These findings suggest that Na\textsubscript{v}1.8 is involved in the regulation of the release and synthesis of SP in the DRG neurons of wild-type mice.

Keywords: dorsal root ganglion (DRG), chronic constriction injury (CCI), Na\textsubscript{v}1.8 knock-out mice, preprotachykinin-A (PPT-A) mRNA, substance P (SP)

Introduction

Na\textsubscript{1.8}, a tetrodotoxin-resistant voltage-gated sodium (Na\textsubscript{s}) channel, is an integral membrane protein that conducts sodium ions through the plasma membrane to produce the rising phase of an action potential (1–3). The primary afferent nerves expressing Na\textsubscript{1.8} contribute to the abnormal conduction of sensory information following neuropathy, facilitating repetitive firing in the dorsal root ganglion (DRG) upon sensory stimulation (4–7). Based on the distribution and pathophysiology of Na\textsubscript{1.8}, several studies have demonstrated the involvement of Na\textsubscript{1.8} in the transmission of nociceptive and neuropathic pain messages. For example, treatment with a specific antisense oligodeoxynucleotide blocking this channel reduces the neuropathic pain caused by spinal nerve ligation in rats (8). In addition, a potent and selective Na\textsubscript{1.8} sodium channel blocker (A-803467) has been shown to attenuate both neuropathic and inflammatory pain in rats (9, 10).

Substance P (SP) is a tachykinin neuropeptide well-known for its function as an important neurotransmitter and/or primary afferent modulator to relay the pain message to nerves leading to the spinal cord and brain. In particular, it plays a critical role in communicating chronic pain information from the periphery to the central nervous system (11). SP, encoded by the preprotachykinin-A (PPT-A) gene, is synthesized in the DRG and released from primary afferent neurons to convey information about various noxious stimuli...
through a complex process (12 - 16). However, it is not known whether the complex process of SP release and/or the SP synthesis requires the regulation of Na1.8 in DRG neurons. It is therefore of interest to investigate the possible relationship between Na1.8 and the release and synthesis of SP in DRG neurons.

The present study used a highly sensitive radioimmunoassay, immunofluorescence staining, and real-time PCR techniques to examine whether Na1.8 is involved in the regulation of capsaicin- and KCl-evoked SP release from cultured mice DRG neurons and to identify the potential relationship between Na1.8 and the SP synthesis in DRG neurons of mice with or without a chronic constriction injury (CCI) of the sciatic nerve.

Materials and Methods

Isolation of DRGs and culture of DRG neurons

Either adult male C57BL/6 wild-type or Na1.8 knock-out mice (kindly provided by Dr. Nobukuni Ogata, ref. 17) were divided into two groups, respectively. After the two groups were decapitated, the spinal cord sections were separated from the bodies, and DRGs attached to the spinal cords were aseptically picked up and harvested. The isolated L4 - 6 DRGs from group I were directly used to examine the SP level and the PPT-A mRNA expression. According to a previously described method (12), the isolated DRGs (four regions: cervical, thoracic, lumbar, and sacral DRGs) of wild-type and Na1.8 knock-out mice (6 - 9 weeks of age) from group II were dissociated into single isolated neurons and non-neuronal cells. The cultures were maintained at 37°C in a water-saturated atmosphere with 5% CO2 for 5 days before the initiation of the experiments. All animal procedures were performed in accordance with the Guide for Animal Experimentation, Hiroshima University and the Committee of Research Facilities for Laboratory Animal Sciences, Graduate School of Biomedical Sciences, Hiroshima University. The deletion of Na1.8 in the Na1.8 knock-out mice was confirmed by a previously described patch-clamp technique (17).

Measurement of the SP content

Except for some cultured cells treated with peptidase inhibitors alone (as a control), other cultured cells were exposed to capsaicin (10 to 1,000 nM) or KCl (30 and 50 mM) in 1 ml Krebs-HEPES buffer (110 mM NaCl, 4.5 mM KCl, 2 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4, 25 mM NaHCO3, 11.7 mM D-glucose, 5 mM HEPES) containing peptidase inhibitors (1 μM phosphoramidon, 4 μg/ml bacitracin and 1 μM captopril; Sigma Chemical Co., St. Louis, MO, USA) for 10 min at 37°C. Thereafter, the SP content collected from the Krebs-HEPES buffer was measured using a highly sensitive radioimmunoassay with a polyclonal antibody specific to the C-terminus of SP (12). Alternatively, either the total SP content collected from untreated cultured DRG neurons of wild-type or Na1.8 knock-out mice and their respective culture medium [horse serum-containing Dulbecco’s modified Eagle’s medium (DMEM; Nissui Pharmaceutical, Tokyo)] or the SP content collected from lumbar DRGs of wild-type and Na1.8 knock-out mice was lyophilized and then assayed by a radioimmunoassay.

Real-time PCR for determining PPT-A mRNA expression

Total RNA harvested from the L4 – 6 DRGs of mice by the acid guanidinium thiocyanate–phenol–chloroform extraction method was separately subjected to reverse transcription into cDNA using a Superscript kit (Life Technologies, Gaithersburg, MD, USA) according to the manufacturer’s protocol. Thereafter, 2 μg of cDNA sample was used immediately in a real-time PCR assessment of PPT-A mRNA levels with iQ SYBR Green Supermix (Bio-Rad) and forward primer (5’-GGTGCCAACGATGATCT-3’) and reverse primer (5’-GCATCCCCGTGTGCCCCATT-3’) on a DNA engine Opticon 2 real-time PCR detection system (Bio-Rad). The thermal cycler parameters were as follows: 1 cycle of 3 min at 95°C, followed by 40 cycles of 15 s at 95°C, 30 s at 60°C, and 30 s at 72°C. A glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control was run simultaneously with the same reaction recipe listed in the instruction manual for the iQ SYBR Green Supermix (Bio-Rad). All data were normalized to the GAPDH mRNA levels to account for any variation in RNA concentrations between the samples obtained from 4 separate experiments.

Preparation of mouse sciatic nerve CCI model

After wild-type and Na1.8 knock-out mice were anaesthetized with trichloroacetaldehyde monohydrate (450 mg/kg i.p.; Wako, Osaka), the left or right sciatic nerves were exposed at the mid-thigh portion, two ligatures of 4 – 0 chronic gut (Ethicon, Brussels, Belgium) were tied loosely around these nerves (18); the contralateral sciatic nerves were exposed but not manipulated. For sham-operated control mice, the sciatic nerves were exposed similarly but not manipulated. At three days after surgery, the plantar surfaces of the hind paws were probed by a series of 6 von Frey filaments (ranging from 0.16 to 2 g force). The CCI surgery produced a decrease in the mechanical pain threshold
one week after loose ligation of the sciatic nerve and this phenomenon continued for approximately one month (data not shown). To isolate the lumbar DRGs, all mice were killed two weeks after the surgeries.

**Immunofluorescence staining**

The isolated lumbar DRGs from these sham-operated and CCI model mice were embedded in paraffin blocks. Next, 4-μm-thick sections were cut and mounted on glass microslides (Matsunami, Osaka). The paraffin-embedded sections were deparaffinized in xylene and then rehydrated through a graded ethanol series to water. The sections were then washed twice in 10 mM phosphate-buffered saline (PBS) containing 0.1% Triton X-100 (Sigma) and incubated for 60 min at room temperature with rabbit anti-SP receptor (1:2,000 dilution; Sigma) alone or rabbit anti-Na,1.8 antibody (1:500 dilution; Alomone Labs, Jerusalem, Israel) plus mouse anti-MAP-2 primary antibody (1:200 dilution; Chemicon, Temecula, CA, USA) in PBS containing 1% BSA and 0.1% Triton X-100 (14), followed by washing twice with PBS and incubation for 60 min at room temperature with Alexa Fluor 546 goat anti-rabbit IgG (1:1,000 dilution; Molecular Probes, Eugene, OR, USA) alone or Alexa Fluor 488 goat anti-rabbit IgG (1:1,000 dilution, Molecular Probes) plus Alexa Fluor 546 goat anti-mouse IgG (1:1,000 dilution, Molecular Probes). Finally, the sections were washed three times in PBS and visualized by a fluorescent microscope (Biozero BZ-8000; Keyence, Osaka). Quantification of Na,1.8 immunofluorescence was performed with the use of image analysis software (VH Analyzer, Keyence) from 3 separate experiments. For the quantification in each experiment, two equal-sized fields of each photograph per group were randomly chosen.

**Statistics**

The data are each presented as the mean ± S.E.M. Statistical analyses were performed by the multiple t-test with the Bonferroni correction following ANOVA. Significance was set at a value of \( P<0.05 \) (two-tailed).

**Results**

The release and total content of SP from cultured DRG neurons

Figure 1, a and b, show a dose-dependence of SP release induced by either capsaicin (10 to 1,000 nM) or KCl (30 and 50 mM) from cultured DRG neurons. A gradual increase in either the capsaicin (10 to 100 nM)- or KCl (30 and 50 mM)-evoked SP release from cultured DRG neurons of either wild-type or Na,1.8 knock-out mice was observed, whereas either the capsaicin- or KCl-evoked response of SP release from the cultured DRG neurons of Na,1.8 knock-out mice was weaker than that from wild-type mice. A high concentration of

![Graphs showing release and content of SP from cultured DRG neurons of either wild-type or Na,1.8 knock-out mice.](image-url)
capsaicin (1 μM) induced a lower level of SP release from cultured DRG neurons of either wild-type or Na,1.8 knock-out mice, which seems to be a consequence of TRPV1 (transient receptor potential vanilloid receptor subtype 1) desensitization (16). Next, it was observed that there was no difference in terms of total SP content between the two kinds of mice (Fig. 1c).

**SP content in the isolated lumbar DRGs of wild-type and Na,1.8 knock-out mice with or without CCI**

To examine the SP level which reflects the release and synthesis of SP in the L4 – 6 DRG in both wild-type and Na,1.8 knock-out mice, the CCI model mice were prepared to analyze the SP content by using a polyclonal antibody specific to C-terminus of SP. The SP content of L4 – 6 in wild-type mice (naive) was first compared to those of sham-operated wild-type. There was no difference in the SP level of L4 – 6 (data not shown), therefore the data obtained from sham-operated wild-type mice was regarded as those of wild-type mice (naive). As shown in Table 1, the SP level in L6 DRG (233 ± 5 pg/DRG) of sham-operated wild-type mice was approximately 2.5-fold greater in comparison to the L4 (83 ± 7 pg/DRG) and L5 (93 ± 6 pg/DRG) DRGs, which was the most abundant among the L4 – 6 DRGs. In Na,1.8 knock-out mice, a similar result was observed and the SP level in L6 DRG (151 ± 15 pg/DRG) of sham-operated Na,1.8 knock-out mice was approximately 1.8-fold greater in comparison to the L4 (78 ± 6 pg/DRG) and L5 (83 ± 13 pg/DRG) DRGs.

Interestingly, the CCI of the sciatic nerve with wild-type mice produced a significant decrease of the SP level in ipsilateral L6 DRG (179 ± 14 pg/DRG) and induced a significant increase of the SP level in the contralateral L6 DRG (273 ± 8 pg/DRG) in comparison to L6 DRG (233 ± 5 pg/DRG) of sham-operated wild-type mice. The CCI of the sciatic nerve also led to a relative decrease in the SP level of either ipsilateral (50 ± 4 pg/L4 DRG, 68 ± 16 pg/L5 DRG) or contralateral (56 ± 11 pg/L4 DRG, 69 ± 2 pg/L5 DRG) L4–5 DRGs of wild-type mice in comparison to the corresponding lumbar DRGs (83 ± 7 pg/L4 DRG, 93 ± 6 pg/L5 DRG) of sham-operated wild-type mice (see Table 1).

Similar alterations in the SP levels of either ipsilateral (76 ± 10 pg/L4 DRG, 65 ± 4 pg/L5 DRG) or contralateral (64 ± 5 pg/L4 DRG, 61 ± 4 pg/L5 DRG) L4 – 5 DRGs of Na,1.8 knock-out mice were observed in comparison to the corresponding lumbar DRGs (78 ± 6 pg/L4 DRG, 83 ± 13 pg/L5 DRG) of sham-operated Na,1.8 knock-out mice (see Table 1). However, there was no significant alteration in the SP levels between ipsilateral and contralateral L6 DRGs of Na,1.8 knock-out mice with CCI, in comparison to the sham-operated Na,1.8 knock-out mice.

**PPT-A mRNA expression in the lumbar DRGs of wild-type and Na,1.8 knock-out mice with or without CCI**

Unlike the distribution pattern of the SP levels in those lumbar DRGs, the PPT-A mRNA expressions in the L4 – 6 DRGs of Na,1.8 knock-out mice were approximately 1.5-, 3-, and 2-fold higher than those in the corresponding lumbar DRG of wild-type mice, respectively (Fig. 2a). After a CCI of the sciatic nerve of Na,1.8 knock-out mice, lower levels of PPT-A mRNA expression were observed in the L4 – 6 DRGs in comparison to the corresponding lumbar DRGs of sham-operated mice (Fig. 2b). Furthermore, the levels of PPT-A mRNA expression in the ipsilateral (46%, 38%, and 47% for L4, L5, and L6 in comparison to L4 of sham-operated mice, respectively) lumbar DRGs of Na,1.8 knock-out mice were more lower than in the corresponding contralateral (62%, 52%, and 88% for L4, L5 and L6 compared to L4 of sham-operated mice, respectively) lumbar DRGs (Fig. 2b). These data suggest the CCI of the sciatic nerve induced the DRG to enhance the intra-dorsal root ganglionic SP release in lumbar DRGs.

### Table 1. The levels of SP in the L4 – 6 DRGs from control and CCI operated mice

<table>
<thead>
<tr>
<th>DRG</th>
<th>Wild-type mice</th>
<th>Na,1.8 knock-out mice</th>
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<tr>
<td></td>
<td>sham</td>
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<tr>
<td>L4</td>
<td>83 ± 7</td>
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<td>93 ± 6</td>
<td>68 ± 16</td>
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<td>233 ± 5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>179 ± 14&lt;sup&gt;d&lt;/sup&gt;</td>
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The data are each expressed as the mean ± S.E.M. (pg/DRG) of 3 – 6 mice; *P<0.01, compared to the corresponding L4 – 5 DRGs of mice; <sup>b</sup>P<0.05 and <sup>c</sup>P<0.01, compared to the contralateral L6 DRG of wild-type mice; <sup>d</sup>P<0.05, compared to the ipsilateral L6 DRG of wild-type mice; <sup>e</sup>P<0.01, compared to the corresponding lumbar DRG of wild-type mice.
Expression of Na\textsubscript{v}1.8 and NK-1R in the L6 DRGs by immunohistochemistry

To estimate the intra-dorsal root ganglionic release of SP, the levels of NK-1R in the L6 DRG of wild-type mice with or without CCI of the sciatic nerve were quantified. Since activation of the NK-1R by SP triggers SP release in cultured adult rat DRG neurons with NK-1R internalization (14), the numbers of DRG neurons expressing NK-1R protein in their cytosols were quantified by immunohistochemistry. As shown in Fig. 3d, after the CCI of the sciatic nerve, an increase was observed in the percentage of neurons expressing NK-1R in the cytosol of these neurons of either the ipsilateral (79 ± 13% for wild-type mice or 72 ± 13% for Na\textsubscript{v}1.8 knock-out mice) or contralateral L6 DRGs (78 ± 2% for wild-type mice or 73 ± 4% for Na\textsubscript{v}1.8 knock-out mice) from wild-type or Na\textsubscript{v}1.8 knock-out mice with CCI (Fig. 3d) in comparison to their respective shams (23 ± 2% for wild-type mice or 30 ± 4% for Na\textsubscript{v}1.8 knock-out mice), thus suggesting an augmentation of intra-dorsal root ganglionic SP release in both L6 in response to the sciatic nerve ligation. In the present study, the ratio of the number of neurons expressing the NK-1R in the cytosol to the total number of NK-1R-positive neurons in a randomly selected field in each image from three separate experiments was simultaneously calculated from digitally merged photomicrographs of bright-field and immunofluorescent images of NK-1R expressions. The immunofluorescent photomicrographs (Fig. 3b) of the sham indicate the DRG neurons where the NK-1Rs are mainly expressed on their membranes, but there is very little or no NK-1R expression in the cytosol of these neurons. Other photomicrographs (red) of CCI indicate the DRG neurons where the NK-1Rs are expressed on their membranes and in the cytosol.

The expression of Na\textsubscript{v}1.8 protein (Fig. 3c) tended to increase in the ipsilateral L6 DRGs (123 ± 13%) after the CCI of the sciatic nerve of wild-type mice, whereas it significantly increased in the contralateral L6 DRGs (212 ± 12%), in comparison to the L6 DRGs (100 ± 13%) of sham. These data suggest that Na\textsubscript{v}1.8 protein could be necessary to regulate SP release in L6 DRG.

Discussion

The current approach was intended to determine a possible correlation between Na\textsubscript{v}1.8 and the release and/or synthesis of SP in mouse DRG neurons. For the first time, L6 DRG was demonstrated to have the most abundant amount of SP among the lumber DRGs. This was also the first study to show that Na\textsubscript{v}1.8 plays important roles in contributing to the regulation of SP release from DRG neurons and the synthesis of SP in the L6 DRG of mice.

In the absence of a well-established blocker of Na\textsubscript{v}1.8, DRG neurons from Na\textsubscript{v}1.8 knock-out mice were used to examine changes in the levels of SP release and its total content in vitro and in vivo. During a 5-day period of primary culture, there was no difference in the morphological changes and the number of DRG neurons from either wild-type or Na\textsubscript{v}1.8 knock-out mice (data not shown). Although, there was no difference in the total amount of SP in cultured DRG neurons from wild-type or Na\textsubscript{v}1.8 knock-out mice (Fig. 1c), the cultured DRG neurons from wild-type mice exhibited a higher SP release response to two potent stimulators (KCl and capsaicin) than those from Na\textsubscript{v}1.8 knock-out mice (Fig. 1: a and b). These data suggest that Na\textsubscript{v}1.8 could be involved in the release of SP from DRG neurons when stimulated by capsaicin or KCl. Furthermore, Na\textsubscript{v}1.8 might not be involved in the storage of SP in DRG.
neurons. However, the possibility of the loss of the Na\textsubscript{1.8} activity during the 5-day period of primary culture could not be ruled out.

Alternatively, the present study indicated that the level of SP in the L6 DRGs was the most abundant in the corresponding L4 – 6 DRGs of wild-type or Na\textsubscript{1.8} knock-out mice. The phenomenon of the abundant SP in the L6 DRG may be interpreted to reflect the type of innervations in the organs because the SP-immunoreactive neurons in L6 DRG of rats may be involved in the transmission of nociception in rat penile frenulum (19). Interestingly, the SP level of L6 DRG in the sham-operated wild-type mice was much greater in comparison to that in the sham-operated Na\textsubscript{1.8} knock-out mice (Table 1). This suggests that Na\textsubscript{1.8} might be involved in the upregulation of localized biosynthesis of SP in L6 DRG of mice. To test this hypothesis, a CCI of the sciatic nerve was applied to two kinds of mice to examine the intra-dorsal root ganglionic SP release. There is extensive data on the expression of PPT-A mRNA (20) or Na\textsubscript{1.8} function (21) in DRG neurons after peripheral nerve injury. In the present study, the PPT-A mRNAs in the lumbar DRGs of the Na\textsubscript{1.8} knock-out mice with CCI also fell to half their normally

![Fig. 3. Expression of Na\textsubscript{1.8} protein and localization of NK-1R. a: Representative photomicrographs of Na\textsubscript{1.8} (green, upper panels) and MAP-2 (red, lower panels) immunofluorescence in L6 DRGs of wild-type mice with or without CCI. b: NK-1R expression in L6 DRGs. BF: bright-field photomicrograph. Scale bars: 30 µm. c: Quantification of Na\textsubscript{1.8} expression from panel a. d: Counting of neurons expressing NK-1R in the cytosol from panel b. *P<0.05, **P<0.01, and ***P<0.001 versus the corresponding sham from either wild-type or Na\textsubscript{1.8} knock-out mice.](image)
abundant levels of expression, which is similar to an observation of Nahin et al. (20). SP is derived directly from the transcripts of nuclear PPT-A genes and it is synthesized on ribosomes. Thereafter, it is stored in so-called large dense core vesicles (LDCVs) in DRG neurons to be released in response to various noxious stimuli for conveying nociceptive information. In addition, the CCI of the sciatic nerve induced the NK-1R internalization in small diameter DRG neurons of both wild-type and Na\textsubscript{v}1.8 knock-out mice, and thus significantly increased the expression level of Na\textsubscript{v}1.8 in the contralateral L6 DRGs of wild-type mice. The internalization of NK-1R is now considered to be an important index of endogenous SP release (14, 22–26). During the internalization of NK-1R, the SP/NK1-R complex dissociates in acidified endosomes. SP is degraded, whereas the NK1-R recycles to the cell surface (27). Based on our results and the observations described above, we herein propose in Fig. 4 the possible molecular mechanisms underlying the relationships among the SP content amount and its mRNA level and Na\textsubscript{v}1.8 expression in the L6 DRG during the sciatic nerve injury. These indicate the hyper-SP release from these lumbar DRGs during the sciatic nerve injury. Therefore the reduction of the SP level in the ipsilateral L6 DRGs from wild-type mice with CCI should also be partially considered to be a consequence of hyper-SP release. The increase of the SP level in the contralateral L6 DRGs of wild-type mice with CCI seems to be associated with the significant increase of the Na\textsubscript{v}1.8 expression in the contralateral L6 DRGs. However, further studies will be necessary to elucidate the exact mechanism by which DRG neurons of Na\textsubscript{v}1.8 knock-out mice exert their distinct characteristics in the inhibition of release and synthesis of SP, for example, to investigate effects of the potent and selective Na\textsubscript{v}1.8 sodium channel blocker A-803467 (9) on the release and localized synthesis of SP from the DRG neurons of wild-type mice.

In summary, the deletion of Na\textsubscript{v}1.8 attenuates the noxious stimuli-induced response of SP release from DRG neurons and also contributes to a decrease in the localized synthesis of SP in the L6 DRG of mice. These observations provide more evidence to understand the relationship between Na\textsubscript{v}1.8 sodium channels and SP in primary afferent neurons.
References