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Direct evidence for the involvement of brain-derived neurotrophic factor in the development of a neuropathic pain-like state in mice

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Abstract

Thermal hyperalgesia and tactile allodynia induced by sciatic nerve ligation were completely suppressed by repeated intrathecal (i.t.) injection of a TrkB/Fc chimera protein, which sequesters endogenous brain-derived neurotrophic factor (BDNF). In addition, BDNF heterozygous (+/–) knockout mice exhibited a significant suppression of nerve ligation-induced thermal hyperalgesia and tactile allodynia compared with wild-type mice. After nerve ligation, BDNF-like immunoreactivity on the superficial laminae of the ipsilateral side of the spinal dorsal horn was clearly increased compared with that of the contralateral side. It should be noted that a single i.t. injection of BDNF produced a long-lasting thermal hyperalgesia and tactile allodynia in normal mice, and these responses were abolished by i.t. pre-treatment with either a Trk-dependent tyrosine kinase inhibitor K-252a or a selective protein kinase C (PKC) inhibitor Ro-32-0432. Supporting these findings, we demonstrated here for the first time that the increase in intracellular Ca^{2+} concentration by application of BDNF in cultured mouse spinal neurons was abolished by pre-treatment with either K-252a or Ro-32-0432. Taken together, these findings suggest that the binding of spinally released BDNF to TrkB by nerve ligation may activate PKC within the spinal cord, resulting in the development of a neuropathic pain-like state in mice.

Keywords: brain-derived neurotrophic factor, brain-derived neurotrophic factor heterozygous (+/–) knockout mouse, neuropathic pain, protein kinase C, spinal cord, TrkB.

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Neuropathic pain is well characterized by spontaneous burning pain, hyperalgesia (an exaggerated pain in response to painful stimuli) and allodynia (a pain evoked by normally innocuous stimuli). A growing body of evidence suggests that sensory nociceptive processing in the spinal dorsal horn appears to undergo significant plastic changes following peripheral nerve injury, leading to the development of neuropathic pain (Ji and Woolf 2001). In addition, it has been demonstrated that the functional changes in the ascending pain pathway from the dorsal horn to brain areas such as thalamus and cortex and the descending inhibitory pathway from the brainstem to the spinal cord may also contribute to the development of neuropathic pain (Lin 1996; Sun *et al.* 2001; Back *et al.* 2003). However, the mechanisms underlying neuropathic pain remain largely unclear.

Brain-derived neurotrophic factor (BDNF), a neurotrophin, presents in a subpopulation of small- to medium-diameter sensory dorsal root ganglia (DRG) neurons (Zhou *et al.* 1999; Obata *et al.* 2003). A synthesized BDNF in the DRGs is anterogradely transported to the primary afferent terminals in the dorsal horn where it is involved in the modulation of painful stimuli (Zhou and Rush 1996; Michael *et al.* 1997). In laminae I and II of the spinal cord, BDNF is localized in synaptic vesicles of sensory nerve terminals, but

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Abbreviations used: BSS, balanced salt saline; bp, base pairs; BDNF, brain-derived neurotrophic factor; DMSO, dimethylsulfoxide; DRG, dorsal root ganglion; GFAP, glial fiblillary acidic protein; IR, immunoreactivity; i.t., intrathecal; MAP2a/b, microtubule-associated protein 2a/b; NGS, normal goat serum; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PGK, phosphoglycerate kinase; PKC, protein kinase C; PLC, phospholipase C; Ro-32-0432, 2-{8-[(dimethylamino)methyl]-6,7,8,9-tetrahydropyrido[1,2-a]indol-3-yl}-3-(1-metyl-1H-indol-3-yl)maleimide; TrkB/Fc, recombinant human TrkB/Fc chimera protein.

not in postsynaptic neurons (Luo *et al.* 2001). There is considerable evidence demonstrating that BDNF is released from primary afferent neurons as an endogenous neurotransmitter/neuromodulator (Kaftz *et al.* 1999; Kerr *et al.* 1999; Lever *et al.* 2001; Poo 2001; Pezet *et al.* 2002), and the release of BDNF within the dorsal horn of the spinal cord is dependent on the pattern of primary afferent activity (Lever *et al.* 2001). Recently, it has been documented that the expression of BDNF is increased in the ipsilateral DRGs following nerve injury (Shen *et al.* 1999; Zhou *et al.* 1999), and the intensity of BDNF-like immunoreactive neurons is increased in the ipsilateral DRGs and the superficial dorsal horn of the spinal cord in nerve-injured animals (Zhou *et al.* 1999; Ha *et al.* 2001; Obata *et al.* 2003). Further investigation has been reported that rats exhibiting thermal hyperalgesia after loose ligation of the sciatic nerve revealed a significant increase in the concentration of BDNF in the lumbar spinal dorsal horn (Miletic and Miletic 2002). These results are consistent with our finding that repeated intrathecal (i.t.) injection of specific antibody to BDNF produced a marked suppression of thermal hyperalgesia associated with an increase in protein levels of membrane-located TrkB receptor following sciatic nerve ligation in mice (Yajima *et al.* 2002). These findings raise the fascinating possibility that the increased release of endogenous BDNF induced by nerve injury may cause a facilitation of the excitability of dorsal horn neurons through the activation of TrkB receptor, resulting in a neuropathic pain-like state.

In the present study, we investigated whether BDNF heterozygous (+/–) mutant mice could exhibit any neuropathic pain-like behaviours induced by sciatic nerve ligation. Furthermore, to clarify a substantial role of the spinal BDNF/TrkB receptor-mediated signalling pathway in the development of a neuropathic pain-like state, we demonstrated whether a sequestration of endogenous BDNF within the spinal cord by repeated i.t. treatment with TrkB receptor chimera protein could affect neuropathic pain-like behaviours in mice with nerve ligation.

Materials and methods

The present study was conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, as adopted by the Committee on Animal Research of Hoshi University, which is accredited by the Ministry of Education, Culture, Sports, Science and Technology of Japan. Every effort was made to minimize the numbers and any suffering of animals used in the following experiments. Animals were used only once in the present study. All behavioural experiments were conducted in a single-blind fashion in order to avoid the effect of subjectivity.

Animals

Breeding pairs of mice (inbred mixed BALB/c-B6-129/Sv background) heterozygous for a null mutation in the BDNF gene (STOCK $Bdnf^{tm1Jae}$) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). The animals used in the present study were litter-mate wild-type controls (+/+) and BDNF heterozygous (+/–) knockout [BDNF (+/–) knockout] mice raised within our colony. In addition, male ICR mice (Tokyo Laboratory Animals Science, Tokyo, Japan) were also used in the present study. Animals were housed in a room maintained at 22 ± 1°C with an alternating 12-h light-dark cycle. Food and water were available ad libitum.

Genotyping

The genotype of offspring from the BDNF (+/–) knockout mice was determined by polymerase chain reaction (PCR) using tail DNA obtained from pentobarbital (60 mg/kg, i.p.) anaesthetized mice.

PCR analysis was performed using BD Advantage^{TM}-GC 2 PCR kit (BD Biosciences Clontech, Palo Alto, CA, USA) according to the manufacture's manual with the extracted DNA template (10 ng/μL) and synthesized BDNF primers as described by Ward and Hagg (2000): a sense primer of BDNF (5′-ATGAAAGAAGTAAACGTCCAC-3′), an antisense primer of BDNF (5′-CCAGCAGAAAGAGTAGAGGAG-3′) and an antisense primer of phosphoglycerate kinase (PGK) (5′-GGGAACTTCCTGACTAGGGG-3′), which includes a neo expression cassette inserted into a deleted exon 5 of the BDNF gene (Ernfors *et al.* 1994).

After denaturation at 94°C for 5 min, 30 cycles of amplification were performed using the following conditions: 94°C, 1 min; 55°C, 30 s; 68°C, 1 min. The PCR products were separated by electrophoresis on 2% agarose gels and were visualized by ethidium bromide staining.

PCR products generated using the primers of antisense PGK and sense BDNF revealed a single band of 340 base pairs (bp) (targeted deficient allele), and those generated using the primers of sense BDNF and antisense BDNF revealed a single band of 277 bp (wild-type allele). Therefore, BDNF (+/–) knockout mice yielded both two amplification products (Ward and Hagg 2000).

Measurement of body temperature, basal hot-plate latency, basal tail-flick latency and rota-rod performance in BDNF (+/–) knockout mice

Body temperature in BDNF (+/–) knockout mice was evaluated by the measurement of rectal temperature using a monitoring thermometer (Thermalert TH-5, Physitemp, Clifton, NJ, USA). Basal nociceptive responses were assessed by either hot-plate (55 ± 0.5°C; Analgesia Meter Model hot-plate MK350B, Muromachi Kikai, Tokyo, Japan) or tail-flick analgesic apparatus (Analgesia Meter Model Tail-Flick MK330B, Muromachi Kikai). Either hot-plate or tail-flick latency was determined as the average of two measurements. The motor coordination was evaluated by the measurement of latency until fall-off from a rota-rod (3 cm in diameter) at 8 r.p.m. (KN-95; Natsume Seisakusyo, Tokyo, Japan). Initially, mice were trained five times to walk on the rota-rod. The time until fall-off from the rota-rod was measured at 1 h after the training.

Neuropathic pain model

The mice were anaesthetized with sodium pentobarbital (60 mg/kg, i.p.). We produced a partial sciatic nerve injury by tying a tight ligature with 7-0 or 8-0 silk suture around approximately one-third to one-half the diameter of the sciatic nerve located on the right side (ipsilateral side) under light microscope (SD30, Olympus, Tokyo,

Japan) as described previously (Malmberg and Basbaum 1998). In sham-operated mice, the nerve was exposed without ligation.

Measurement of paw withdrawal latency to a thermal stimulus

To assess the sensitivity to thermal stimulation, each of the hind paws of the mice were tested individually using a thermal stimulus apparatus (model 33 Analgesia Meter; IITC/Life Science Instruments, Woodland Hills, CA, USA). The intensity of the thermal stimulus was adjusted to achieve an average baseline paw withdrawal latency of approximately 8 to 10 s in naive mice. Only quick hind paw movements (with or without licking of hind paws) away from the stimulus were considered to be a withdrawal response. Paw movements associated with locomotion or weight shifting were not counted as a response. The paws were measured, alternating between left and right with an interval of more than 3 min between the measurements. The latency of paw withdrawal after the thermal stimulus was determined as the average of three measurements per paw. Before testing of the behavioural responses to the thermal stimulus, mice were habituated at least 1 h in an acrylic cylinder (15 cm high and 8 cm in diameter). Under these conditions, the latency of paw withdrawal in response to the thermal stimulus was tested. The measurement of latency of paw withdrawal to the thermal stimulus was performed before the surgery and 1, 3, 5 and 7 days after the surgery.

In the experiment of a single i.t. treatment with BDNF, the measurement of thermal paw withdrawal latency was performed before and after the injection until the latency returned to the baseline. The latency of paw withdrawal to the thermal stimulus was determined as the average of both paws.

Measurement of paw withdrawal response to a tactile stimulus

To quantify the sensitivity to tactile stimulus, paw withdrawal response to tactile stimulus was measured using two different bending forces (0.02 g for BDNF (+/–) knockout mice or 0.02 and 0.16 g for ICR mice) of von Frey filaments (North Coast Medical, Morgan Hill, CA, USA). Each von Frey filament was applied to the plantar surface of hind paw for 3 s and repeated three times at a minimum of 5-s intervals. Each of the hind paws of the mice were tested individually. The paw withdrawal responses to the tactile stimulus were evaluated by the scoring as follows: 0, no response; 1, a slow and/or slight response to the stimulus; 2, a quick withdrawal response away from the stimulus without flinching and licking; 3, a intense withdrawal response away from the stimulus with brisk flinching and/or licking. The paw withdrawal response to each filament was determined as the average of two scores per paw. Paw movements associated with locomotion or weight shifting were not counted as a response. The paws were measured, alternating between left and right with an interval of more than 3 min between the measurements. Before testing of the behavioural responses to the tactile stimulus, mice were habituated at least 1 h on an elevated nylon mesh floor. Under these conditions, the paw withdrawal response to the tactile stimulus was tested. The measurement of paw withdrawal threshold to the tactile stimulus was performed before the surgery and next day after the measurement of thermal threshold (day 2, 4, 6 and 8).

In the experiment of a single i.t. treatment with BDNF, the measurement of paw withdrawal responses to the tactile stimulus was performed before and after the injection until the response returned to the baseline. The paw withdrawal score to the tactile stimulus was determined as the average of both paws.

Intrathecal injection

Intrathecal injection was performed as described by Hylden and Wilcox (1980) using a 25-μL Hamilton syringe with a 30 1/2-gauge needle. The needle was inserted into the intervertebral space between the L5 and L6 level of the spinal cord. A reflexive flick of the tail was considered to be a sign of the accuracy of each injection. The injection volume was 4 μL for i.t. injection.

Groups of mice were repeatedly i.t. treated with a recombinant human TrkB/Fc chimera protein (10 ng/mouse; TrkB/Fc) 1 h before the surgery and once a day for 8 consecutive days after the surgery. A single i.t. injection of a recombinant human BDNF (BDNF; 50 ng/mouse) was performed in naive mice. Either i.t. pre-treatment with a Trk-dependent tyrosine kinase inhibitor K-252a or a selective protein kinase C (PKC) inhibitor 2-{8-[(dimethylamino)methyl]-6,7,8,9-tetrahydropyrido[1,2-a]indol-3-yl}-3-(1-metyl-1H-indol-3-yl)maleimide (Ro-32-0432) was performed 30 min before a single i.t. injection of BDNF (50 ng/mouse).

Immunohistochemistry

Sample preparation

Seven days after nerve ligation, mice were deeply anaesthetized with sodium pentobarbital (70 mg/kg, i.p.) and intracardially perfusion-fixed with freshly prepared 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.4). After perfusion, the lumbar spinal cord was quickly removed and post-fixed in 4% paraformaldehyde for 2 h, and permeated with 20% sucrose in 0.1 M PBS for 1 day and 30% sucrose in 0.1 M PBS for 2 days with agitation. Then, the L5 lumbar spinal cord segments were frozen in an embedding compound (Sakura Finetechnical, Tokyo, Japan) on isopentane using liquid nitrogen and stored at −30°C until used. Frozen spinal cord segments were cut with a freezing cryostat (Leica CM 1510, Leica Microsystems AG, Wetzlar, Germany) at a 10-μm thickness and thaw-mounted on a poly-L-lysine-coated glass slides.

The spinal cord sections were blocked in 10% normal goat serum (NGS) in 0.01 M PBS for 1 h at 23°C. The primary antibody was diluted in 0.01 M PBS containing 10% NGS 1 : 1500 chicken anti BDNF (R & D Systems, Minneapolis, MN, USA) and incubated twice overnight at 4°C. The antibody was then rinsed and incubated with secondary antibody for 2 h at room temperature. For BDNF single staining assay, Alexa 488-conjugated goat anti-chicken IgG (Molecular Probes, Eugene, OR, USA) was diluted 1 : 400 in PBS containing 10% NGS. Because staining intensity might vary between experiments, control sections were included in each run of staining.

The slides were then cover-slipped with PermaFluor Aqeous mounting medium (Immunon™; ThermoShandon, Pittsburgh, PA, USA). Fluorescence immunolabelling was detected using an U-MNIBA filter cube (Olympus) for Alexa 488. All sections were observed with the aid of light microscope (Olympus BX-80; Olympus) and photographed with a digital camera (CoolSNAP HQ; Olympus). Digitized images of superficial laminae of the spinal dorsal horn sections were captured at a resolution of 1316 × 1035 pixels by camera. The density of BDNF labelling was measured with a computer-assisted imaging analysis system (NIH Image). The

upper and lower threshold density ranges were adjusted to encompass and match the immunoreactivity (IR) to provide an image with immunoreactive material appearing in black pixels, and non-immunoreactive material as white pixels. A standardized rectangle was positioned over the superficial laminae of the contralateral dorsal horn of the spinal cord area from nerve-ligated mice. The area and density of pixels within the threshold value representing IR were calculated and the integrated density was the product of the area and density. The same box was then 'dragged' to the corresponding position on the superficial laminae of the ipsilateral dorsal horn of the spinal cord area from nerve-ligated mice and the integrated density of pixels within the same threshold was again calculated.

Confocal Ca^{2+} imaging

Tissue processing

Primary spinal cultured neurons were grown as follows: spinal cord was obtained from newborn ICR mice (Tokyo Laboratory Animals Science), minced, and treated with papain (9 U/mL, Worthington Biochemical, Lakewood, NJ, USA) dissolved in PBS solution containing 0.02% L-cysteine monohydrate (Sigma-Aldrich, St Louis, MO, USA), 0.5% glucose and 0.02% bovine serum albumin (Wako Pure Chemical, Osaka, Japan). After enzyme treatment at 37°C for 15 min, cells were seeded on poly-L-lysine (Sigma-Aldrich) with fibronectin (10 μg/mL; Sigma-Aldrich)-coated glass coverslips with a silicon rubber well (Flexiperm®, Heraeus Biotechnology, Hanau, Germany) at a density of 3×10^6 cells/cm^2. The cells were maintained for 7 days in Dulbecco's modified Eagle's medium (Invitrogen, Grand Island, NY, USA) supplemented with 10% precolostrum newborn calf serum (Invitrogen), 10 U/mL penicillin and 10 μg/mL streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

Cells were loaded with 10 μM fluo-3AM (Wako Pure Chemical) for 90 min at room temperature. After a further 20–30 min of de-esterification with the acetoxymethyl ester, the coverslips were mounted on a microscope equipped with a confocal Ca^{2+} imaging system (Radiance 2000, Bio-Rad, Richmond, CA, USA). Fluo-3AM was excited with the 488-nm line of an argon-ion laser and the emitted fluorescence was collected at wavelengths >515 nm. To compensate for the uneven distribution of fluo-3AM, self-ratios were calculated ($R_s = F/F_0$).

BDNF 1 ng/mL was perfused for 40 s at a rate of 4–5 mL/min at room temperature in cultured spinal neurons followed by superfusion of balanced salt saline (BSS, pH 7.4) containing 150 mM NaCl, 5.0 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM $MgCl_2$, 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid and 10 mM D-glucose. K-252a (15 and 150 nM) or Ro-32-0432 (1 and 10 μM) was pre-treated in cultured spinal neurons 10 min prior to BDNF application at room temperature.

Drugs

The drugs used in the present study were TrkB/Fc (R & D Systems), BDNF (R & D Systems), selective PKC inhibitor Ro-32-0432 (Calbiochem-Novabiochem) and K-252a (Calbiochem-Novabiochem). TrkB/Fc and BDNF were dissolved in sterile PBS and 0.9% sterile physiological saline, respectively. Ro-32-0432 and K-252a were dissolved in 0.9% sterile physiological saline containing 3% dimethylsulfoxide (DMSO). In confocal Ca^{2+} imaging assay, Ro-32-0432 and K-252a were dissolved in BSS containing 0.12 and 0.0018% DMSO, respectively.

Statistical analysis

All data are presented as the mean ± SEM. The statistical significance of differences between groups was assessed with repeated measures analysis of variance (ANOVA) and one-way ANOVA followed by the Bonferroni–Dunn multiple comparison test or Student's *t*-test.

Results

Effect of repeated i.t. injection of TrkB/Fc on thermal hyperalgesia and tactile allodynia induced by sciatic nerve ligation in mice

Partial ligation of the sciatic nerve caused a marked decrease in the latency of paw withdrawal after the thermal stimulus only on the ipsilateral side in nerve-ligated ICR mice (vs. PBS–sham group, $F_{1,16} = 32.384$, $p < 0.001$, Fig. 1a). In contrast, paw withdrawal latencies induced by a thermal stimulus on the contralateral side in nerve-ligated mice and on both sides in sham-operated mice were not changed (Fig. 1b). The thermal hyperalgesia observed on the ipsilateral side in nerve-ligated mice was markedly reversed by repeated i.t. injection of TrkB/Fc (10 ng/mouse) just before the ligation and once a day for 7 consecutive days after the nerve ligation (vs. PBS–ligation group, $F_{1,21} = 17.76$, $p < 0.001$, Fig. 1a).

The mice with sciatic nerve ligation also revealed a marked increase in the paw withdrawal response to the tactile stimulus only on the ipsilateral side in nerve-ligated mice (vs. PBS–sham group, $F_{1,16} = 40.082$, $p < 0.001$, Fig. 2a; vs. PBS–sham group, $F_{1,16} = 23.323$, $p < 0.001$, Fig. 2c). The contralateral side of nerve-ligated mice and both sides of sham-operated mice did not show any responses to the tactile stimulus (Figs 2b and d). Under these conditions, repeated i.t. injection of TrkB/Fc (10 ng/mouse) markedly suppressed the increase in paw withdrawal response to the innocuous tactile stimulus induced by nerve ligation in mice (vs. PBS–ligation group, $F_{1,15} = 12.261$, $p < 0.001$, Fig. 2a; vs. PBS–ligation group, $F_{1,16} = 10.241$, $p < 0.001$, Fig. 2c).

Repeated i.t. injection of TrkB/Fc at doses of which used in the present study failed to affect thermal and tactile threshold on the contralateral side in nerve-ligated mice and on both sides in sham-operated mice (Figs 1 and 2).

Genotyping and general behaviour in BDNF (+/–) knockout mice

The genotype of offspring from the BDNF (+/–) knockout mice was confirmed by PCR analyses using DNA extracted from the tail. Mice having the targeted deficient allele and the wild-type allele revealed a single band of 340 bp (lanes 2 and 4) and 275 bp (lanes 1 and 3), respectively. As shown in Fig. 3, BDNF (+/–) knockout mouse yielded both two

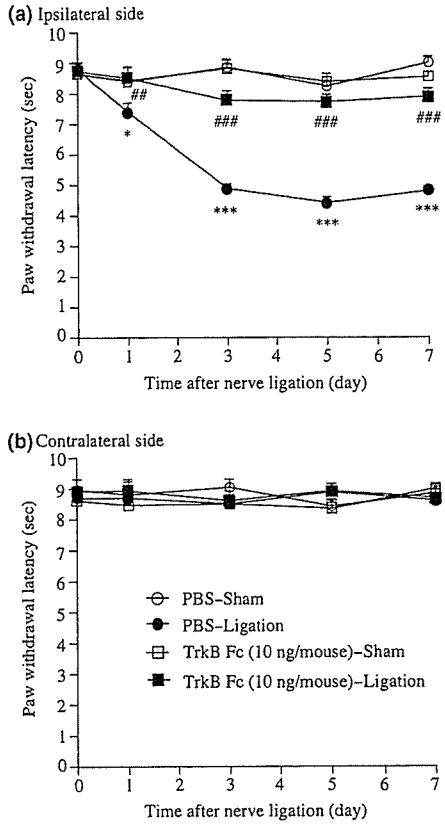

Fig. 1 Effect of repeated i.t. injection of a TrkB receptor chimera protein (TrkB/Fc) on paw withdrawal latency to the thermal stimulus in the ipsilateral (a) and contralateral (b) sides of sham-operated or nerve-ligated mice. Groups of mice were repeatedly i.t. treated with TrkB/Fc (10 ng/mouse) or PBS 1 h prior to surgery (day 0) and once a day for 7 consecutive days after surgery. Each point represents the mean ± SEM of 8–13 mice. $*p < 0.05$ and $***p < 0.001$ versus PBS–sham group, $\#\#p < 0.01$ and $\#\#\#p < 0.001$ versus PBS–ligation group.

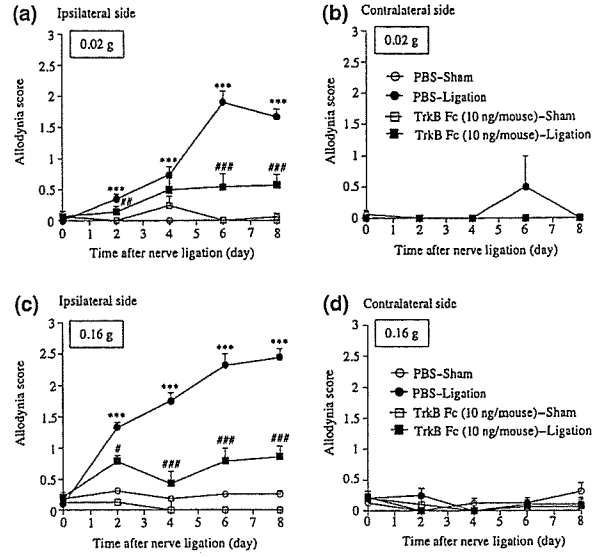

Fig. 2 Effect of repeated i.t. injection of TrkB/Fc on paw withdrawal responses to the tactile stimulus in the ipsilateral (a, c) and contralateral (b, d) sides of sham-operated or nerve-ligated mice. Groups of mice were repeatedly i.t. treated with TrkB/Fc (10 ng/mouse) or PBS 1 h prior to surgery (day 0) and once a day for 8 consecutive days after surgery. Tactile stimulus was performed by two different bending forces [0.02 g (a, b) and 0.16 g (c, d)] of filaments. Each point represents the mean ± SEM of 5–7 mice. $***p < 0.001$ versus PBS–sham group, $\#p < 0.05$, $\#\#p < 0.01$ and $\#\#\#p < 0.001$ versus PBS–ligation group.

amplification products (lanes 1 and 2). In contrast, wild-type mouse showed a single amplification product (lanes 3 and 4).

There were no significant differences between BDNF (+/–) knockout and its wild-type mice in body weight [wild-type: 29.0 ± 1.0 g, BDNF (+/–) knockout: 28.7 ± 0.5 g], body temperature [wild-type: 36.6 ± 0.2°C, BDNF (+/–) knockout: 36.7 ± 0.1°C], basal hot-plate latency [wild-type: 13.9 ± 0.6 s, BDNF (+/–) knockout: 13.0 ± 0.8 s], basal tail-flick latency [wild-type: 2.7 ± 0.1 s, BDNF (+/–) knockout: 2.8 ± 0.1 s] and the latency until fall-off from the rota-rod [wild-type: 55 ± 2.9 s, BDNF (+/–) knockout: 48 ± 9.0 s].

Changes in thermal and tactile thresholds induced by sciatic nerve ligation in BDNF (+/–) knockout mice

Baseline latency of paw withdrawal to the thermal stimulus to the plantar surface of BDNF (+/–) knockout mice did not differ from that of wild-type mice [right side, wild-type: 10.1 ± 0.4 s, BDNF (+/–) knockout: 9.3 ± 0.3 s; left side, wild-type: 9.6 ± 0.4 s, BDNF (+/–) knockout: 9.1 ± 0.3 s]. In addition, the baseline paw withdrawal response after the tactile stimulus to the plantar surface of BDNF (+/–) knockout mice did not differ from that of wild-type mice [right side, wild-type: 10.1 ± 0.4 s, BDNF (+/–) knockout: 9.3 ± 0.3 s; left side, wild-type: 9.6 ± 0.4 s, BDNF (+/–) knockout: 9.1 ± 0.3 s].

In wild-type mice, either the thermal or tactile threshold observed on the ipsilateral side was markedly decreased by nerve ligation (vs. wild-type–sham group, $F_{1,16} = 23.755$, $p < 0.001$, Fig. 4a; vs. wild-type–sham group, $F_{1,8} = 4.356$, $p < 0.01$, Fig. 5a). In contrast, either the thermal or tactile threshold on the contralateral side of nerve-ligated wild-type mice and on both sides of sham-operated wild-type mice was not changed (Figs 4b and 5b).

Under these conditions, either the decreased thermal or tactile threshold on the ipsilateral side in BDNF (+/–) knockout mice with nerve ligation was significantly suppressed compared with those observed in wild-type mice (vs. the wild-type–ligation group, $F_{1,18} = 5.781$, $p < 0.001$, Fig. 4a and Fig. 5a). In BDNF (+/–) knockout mice, the paw withdrawal response to either thermal or tactile stimulus on the contralateral side in the nerve-ligated group and on both sides in the sham-operated group was not altered (Figs 4b and 5b).

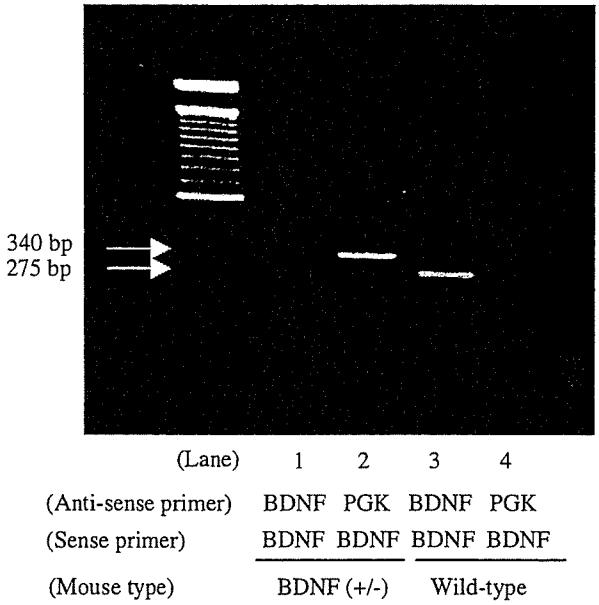

Fig. 3 Representative PCR for BDNF using DNA extracted from the tail of either wild-type or BDNF (+/–) knockout mouse. Mice having the targeted deficient allele and the wild-type allele revealed a single band of 340 bp (lanes 2 and 4) and 275 bp (lanes 1 and 3), respectively (arrows). BDNF (+/–) knockout mouse yielded both amplification products (lanes 1 and 2). In contrast, wild-type mouse showed a single amplification product (lanes 3 and 4).

Changes in BDNF-like IR by sciatic nerve ligation in the superficial dorsal horn of the mouse spinal cord

The BDNF-like IR was detected on the superficial laminae of the contralateral side of the L5 lumbar spinal dorsal horn of nerve-ligated mice (Fig. 6a). At 7 days after sciatic nerve ligation, BDNF-like IR on the superficial laminae of the ipsilateral side of the L5 lumbar spinal dorsal horn was significantly increased compared with that of the contralateral side ($p < 0.001$, Figs 6b and c).

Changes in thermal and tactile thresholds following an exogenous single i.t. treatment with BDNF in normal mice

We next investigated whether an exogenous i.t. treatment with BDNF could cause a hyperalgesic or allodynic response in normal mice. A single i.t. injection of BDNF (50 ng/mouse) produced a marked thermal hyperalgesia in normal mice after the injection, and this effect lasted for 5 days after the injection (vs. vehicle–saline group, $F_{1,13} = 10.493$, $p < 0.001$, Fig. 7a). The long-lasting thermal hyperalgesia caused by an exogenous single i.t. injection of BDNF was abolished by i.t. pre-treatment with a Trk-dependent tyrosine kinase inhibitor K-252a (1 nmol/mouse; vs. vehicle–BDNF group, $F_{1,12} = 10.178$, $p < 0.001$, Fig. 7a). In addition, the mice treated i.t. with BDNF (50 ng/mouse) revealed a marked and long-lasting tactile allodynia (vs. vehicle–saline

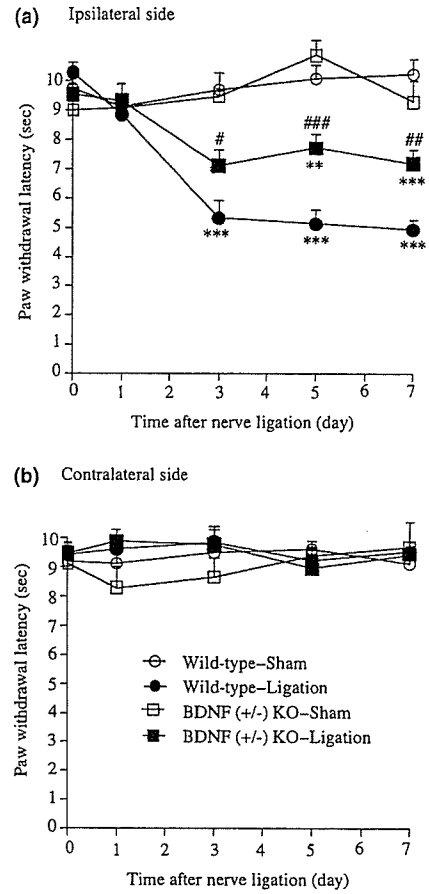

Fig. 4 Changes in paw withdrawal latencies to the thermal stimulus induced by nerve ligation on the ipsilateral (a) and contralateral (b) sides in wild-type and BDNF (+/–) knockout (KO) mice. Each point represents the mean ± SEM of 7–10 mice. $^{**}p < 0.01$ and $^{***}p < 0.001$ versus wild-type–sham group, $\#p < 0.05$, $\#\#p < 0.01$ and $\#\#\#p < 0.001$ versus wild-type–ligation group.

group, $F_{1,14} = 4.662$, $p < 0.001$, Fig. 7b). The persistent tactile allodynia caused by an exogenous single i.t. injection of BDNF was also abolished by i.t. pre-treatment with K-252a (1 nmol/mouse; vs. vehicle–BDNF group, $F_{1,14} = 3.483$, $p < 0.01$, Fig. 7b).

An i.t. pre-treatment with a selective PKC inhibitor Ro-32-0432 (1 nmol/mouse) also completely suppressed thermal hyperalgesia and tactile allodynia induced by an exogenous single i.t. injection of BDNF (50 ng/mouse; vs. vehicle–BDNF group, $F_{1,12} = 5.51$, $p < 0.001$, Fig. 7a; vs. vehicle–BDNF group, $F_{1,13} = 2.64$, $p < 0.05$, Fig. 7b).

Effect of PKC inhibitor on the BDNF-induced Ca^{2+} responses in cultured mouse spinal neurons

To clarify whether PKC is a downstream effector for a BDNF-mediated signalling event, we next investigated the effect of PKC inhibitor on the BDNF-induced Ca^{2+} responses in cultured mouse spinal neurons. Application of

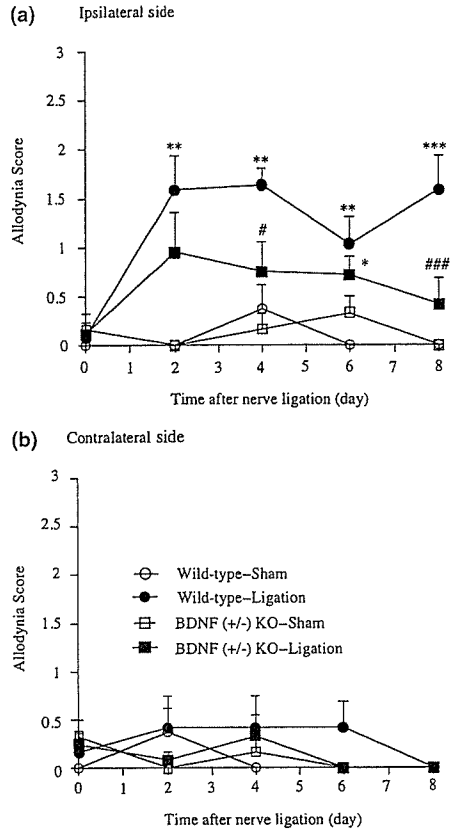

Fig. 5 Changes in paw withdrawal responses to the tactile stimulus induced by nerve ligation on the ipsilateral (a) and contralateral (b) sides in wild-type and BDNF (+/–) knockout (KO) mice. Tactile stimulus was performed by 0.02 g of von Frey filament. Each point represents the mean ± SEM of 3–6 mice. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ versus wild-type–sham group, $\#p < 0.05$ and $\#\#\#p < 0.001$ versus wild-type–ligation group.

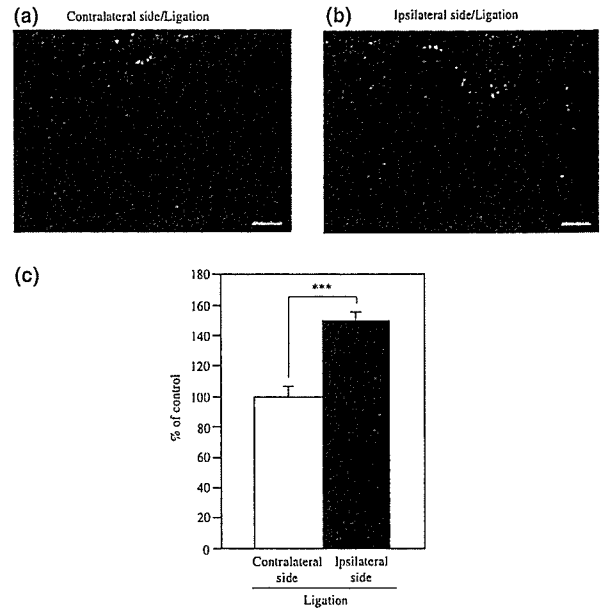

Fig. 6 Photomicrographs of immunofluorescent staining of BDNF on the superficial layers of the contralateral (a) or ipsilateral (b) spinal dorsal horn of ICR mice at 7 days after nerve ligation. (c) The density of BDNF-like IR of each side of the spinal cord slice was measured using an NIH image. The level of BDNF on the superficial dorsal horn of the ipsilateral spinal cord in nerve-ligated mice is expressed as a per cent increase (mean ± SEM) with respect to that in the contralateral side. BDNF-like IR observed on the superficial laminae of the ipsilateral dorsal horn (b) was significantly increased compared with that of the contralateral side (a). $^{***}p < 0.001$ versus contralateral side. Three independent sets of experiment were performed in this study. Scale bars = 50 μm.

BDNF (1 ng/mL) into the cultured spinal neurons produced a transient increase in the intracellular Ca^{2+} levels (Fig. 8a). The Ca^{2+} response induced by BDNF (1 ng/mL) was abolished by pre-treatment with K-252a in a concentration-dependent manner [15 and 150 nM; vs. BDNF-treated cells, $p < 0.01$ (15 nM) and $p < 0.001$ (150 nM), Fig. 8bi and c]. In addition, pre-treatment with Ro-32-0432 (1 and 10 μM) exhibited a concentration dependent suppression of the increased intracellular Ca^{2+} concentration induced by BDNF [1 ng/mL; vs. BDNF-treated cells, $p < 0.001$, Figs 8bii and c].

Discussion

We previously reported that the sciatic nerve ligation-induced thermal hyperalgesia was completely reversed by repeated i.t. treatment with specific antibodies to BDNF and the full-length TrkB receptor, and the inhibitor of Trk-dependent tyrosine kinase K-252a in mice (Yajima *et al.* 2002). In the present study, we further investigated the substantial role of the spinal BDNF/TrkB receptor pathway in the development of the neuropathic pain-like state in mice using TrkB receptor chimera protein (TrkB/Fc), which sequesters endogenous BDNF, and BDNF (+/–) mutant mice.

Thermal hyperalgesia and tactile allodynia induced by sciatic nerve ligation were markedly suppressed by repeated i.t. treatment with TrkB/Fc. The TrkB/Fc used in the present study is bivalent homodimers that contain the extracellular ligand-binding domain of a given TrkB receptor followed by the hinge and Fcγ region of human IgG1 (Binder *et al.* 1999). This compound acts as TrkB receptor body to sequester endogenous BDNF and neurotrophin-4/5 (NT-4/5), which also preferentially binds to TrkB. It has been reported that TrkB/Fc specifically abolished Trk phosphorylation induced by BDNF in primary cortical cultures (Binder *et al.* 1999), suggesting that this molecule is a highly potent and specific inhibitor for endogenous BDNF and NT-4/5. Considering these findings, the present data indicate the possibility that the release of BDNF and NT-4/5 within the spinal cord by nerve ligation may be implicated in

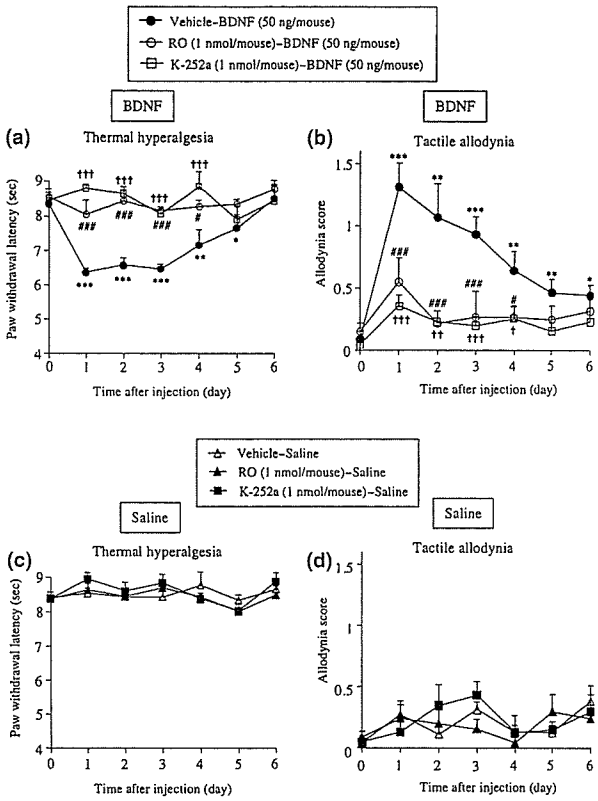

Fig. 7 Effect of a single i.t. injection of BDNF on paw withdrawal responses to the thermal (a, c) and tactile (b, d) stimulus in normal mice. Tactile stimulus was performed using 0.02 g bending force of filament. Groups of mice were pre-treated i.t. with either a Trk-dependent tyrosine kinase inhibitor K-252a (1 nmol/mouse) or a selective PKC inhibitor Ro-32-0432 (RO; 1 nmol/mouse) 30 min before a single i.t. injection of BDNF (50 ng/mouse; a, b) or saline (c, d). Each point represents the mean ± SEM of 6–9 mice. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ versus day 0 (vehicle–BDNF group), †$p < 0.05$, ††$p < 0.01$ and †††$p < 0.001$ vehicle–BDNF group versus K-252a–BDNF group, #$p < 0.05$ and ###$p < 0.001$ vehicle–BDNF group versus RO–BDNF group.

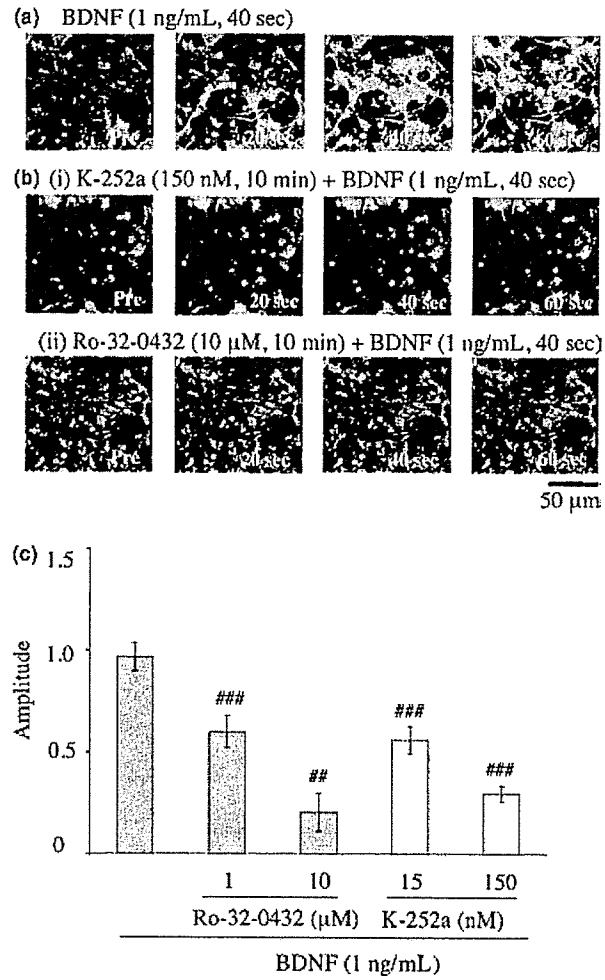

Fig. 8 Effect of K-252a or Ro-32-0432 on the BDNF-induced increase in intracellular Ca^{2+} concentration in cultured spinal neurons. (a, bi, bii) Sequential images of increase in intracellular Ca^{2+} concentration induced by BDNF (1 ng/mL) in cultured spinal neurons. BDNF at 1 ng/mL was applied by superfusion for 40 s. K-252a (15 and 150 nM) or Ro-32-0432 (1 and 10 μM) was pre-treated in cultured spinal neurons 10 min prior to BDNF (1 ng/mL) application. (c) Amplitude of the BDNF-induced increases in intracellular Ca^{2+} concentration in spinal neurons. K-252a (15 and 150 nM) or Ro-32-0432 (1 and 10 μM) was pre-treated in cultured spinal neurons 10 min prior to BDNF (1 ng/mL) application. Data represent the mean ± SEM of 54–126 cells from three separated observations. ##$p < 0.01$ and ###$p < 0.001$ versus BDNF-treated cells.

the development of a neuropathic pain-like state in mice. However, we already found that repeated i.t. injection of a specific antibody to NT-4, unlike BDNF, failed to suppress thermal hyperalgesia caused by sciatic nerve ligation in mice (Yajima *et al.* 2002). In addition, there are no differences in the spinal cord reflex properties between NT-4-deficient and its wild-type mice (Heppenstall and Lewin 2001). Taken together, these findings indicate that the BDNF released within the spinal cord by sciatic nerve injury may trigger the inducing of the development of a neuropathic pain-like state in mice.

Then, the direct approach using BDNF (+/–) knockout mice was performed to investigate whether a mutation of the BDNF gene could influence the development of a neuropathic pain-like state induced by sciatic nerve ligation in mice. As shown in Fig. 3, there were no differences in some physiological and behavioural responses, including nociceptive properties and motor coordination, between BDNF (+/–) knockout and its wild-type mice. Under these conditions, it should be noted that BDNF (+/–) knockout mice revealed a significant suppression of nerve ligation-induced thermal hyperalgesia and tactile allodynia compared with those observed in wild-type mice. Moreover, exogenous treatment with BDNF increases the excitability of

nociceptive inputs in the spinal neurons (Kerr *et al.* 1999; Thompson *et al.* 1999; Groth and Aanonsen 2002), while the spinal reflex elicited by stimulation of primary afferents is reduced in BDNF-deficient mice (Heppenstall and Lewin 2001). Consistent with these observations, we demonstrated here that an exogenous single i.t. injection of BDNF produced a long-lasting thermal hyperalgesia and tactile allodynia in normal mice. These responses were abolished by i.t. pre-treatment with a Trk-dependent tyrosine kinase inhibitor K-252a (Doherty and Walsh 1989). These findings provide further evidence for the critical role of BDNF within the spinal cord in neuropathic pain processing.

In terms of the distribution of BDNF-like IR (Ha *et al.* 2001; Groth and Aanonsen 2002), we confirmed that BDNF-like IR was detected on the superficial laminae of the dorsal horn of the spinal cord. In our preliminary study, the BDNF-like IR was clearly overlapped with a specific neuronal marker microtubule-associated protein 2a/b (MAP2a/b), but not a specific astroglial marker glial fiblillary acidic protein (GFAP; unpublished observation), indicating that BDNF within the spinal cord may be predominantly located in neurons. Under these conditions, we next investigated whether sciatic nerve ligation could affect BDNF-like IR on the superficial dorsal horn of the spinal cord. Here, we showed that sciatic nerve ligation produced a marked increase in BDNF-like IR on the superficial layers of the ipsilateral side of the spinal cord compared with that observed in the contralateral side of nerve-ligated mice. It is well documented that BDNF mRNA expression is mostly detected in small- to medium-size DRG cells, and the synthesized BDNF in the DRGs is anterogradely transported to the spinal cord through the primary afferents (Zhou *et al.* 1999; Obata *et al.* 2003). On the contrary, there is no expression of BDNF mRNA throughout the spinal cord (Conner *et al.* 1997; Heppenstall and Lewin 2001) and BDNF-like IR was not found in postsynaptic neurons (Luo *et al.* 2001), indicating that the distributed BDNF in the spinal cord may be derived from primary afferent neurons, but not from postsynaptic cells in the spinal cord. Recently, it has been shown that BDNF synthesis is increased in the ipsilateral DRGs (Shen *et al.* 1999; Zhou *et al.* 1999) and the anterogradely transported BDNF is substantially enhanced by sciatic nerve lesion (Tonra *et al.* 1998). It is noteworthy that there is a close association in the time course of the development and disappearance of behavioural signs of neuropathic pain with changes in BDNF levels in the lumbar spinal dorsal horn (Miletic and Miletic 2002). Moreover, DRG-derived neurotrophins, including BDNF, trigger allodynia after spinal nerve injury in rats (Zhou *et al.* 2000). These findings provide further explanation for the implication of spinally released BDNF in the development of a neuropathic pain-like state in mice.

Several lines of evidence have demonstrated that the activation of PKC in the spinal cord plays a critical role in the sensitization of spinal dorsal horn (Coderre 1992; Munro *et al.* 1994; Lin 1996). There is substantial evidence supporting a role for PKC expressed in dorsal horn neurons in regulating pain hypersensitivity in a number of differential neuropathic pain models (Mao *et al.* 1995; Malmberg *et al.* 1997; Hua *et al.* 1999; Ohsawa *et al.* 2000, 2001). Consistent with previous reports, we found that mice with nerve ligation showed a spinal PKC-dependent neuropathic pain-like behaviour (Ohsawa *et al.* 2000; Yajima *et al.* 2003). In addition, it is of interest to note that direct activation of spinal PKC by a single i.t. injection of the specific PKC activator phorbol 12,13-dibutyrate caused a persistent thermal hyperalgesia in normal mice (Narita *et al.* 2004; Oe *et al.* 2004). It has been well documented that autophosphorylated TrkB receptor by BDNF leads to the enhancement of phospholipase C (PLC)γ_1 activity through the src homology 2 (SH2) domain of PLCγ_1 (Rhee and Bae 1997; Sekiya *et al.* 1999; Yuen and Mobley 1999), leading to facilitating the activation of PKC cascades and an increase in the intracellular Ca^{2+} levels (Zirrgiebel *et al.* 1995). Our recent findings showed that the membrane-bound TrkB receptor was clearly increased in the ipsilateral side of spinal cord of nerve-ligated mice (Narita *et al.* 2000; Yajima *et al.* 2002). It should be mentioned that the increase in the level of membrane-located TrkB receptor on the ipsilateral spinal cord in nerve-ligated mice was paralleled with a time course of the increase in the membrane-bound PKCγ isoform on the ipsilateral spinal cord in nerve-ligated mice (Narita *et al.* 2000). Although there is substantial evidence supporting the idea of the importance of spinal PKC in the development of a neuropathic pain-like state, little is known about the direct interaction between BDNF and PKC under a chronic pain-like state. In the present study, we demonstrated here that single i.t. administration of BDNF-induced long-lasting thermal hyperalgesia and tactile allodynia were completely reversed by i.t. pre-treatment with a selective PKC inhibitor Ro-32-0432. Supporting these findings, we found here for the first time that application of BDNF in primary spinal neurons increased the intracellular Ca^{2+} concentrations, and this effect was abolished by pre-treatment with Ro-32-0432. In addition, pre-treatment with K-252a also abolished both the BDNF-induced pain-like state and Ca^{2+} responses in the cultured spinal neurons, indicating that the TrkB receptor/PKC-mediated signalling pathway within the spinal cord may be involved in this event. Collectively, these findings strongly support the idea that BDNF activates PKC through the activated TrkB receptor on the spinal dorsal horn neurons, which may in turn cause the development of a neuropathic pain-like state.

In conclusion, present data provided further evidence that the BDNF/TrkB receptor-mediated signalling pathway within the spinal cord is involved in the development of a neuropathic pain-like state induced by sciatic nerve ligation in mice. Considering these observations, a hypothesis may be

advanced that the BDNF released within the spinal cord by nerve injury can eventually activate PKC through the TrkB receptor located on the spinal dorsal horn neurons, resulting in the development of a neuropathic pain-like state.

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Chronic morphine treatment increases the expression of the neural cell adhesion molecule in the dorsal horn of the mouse spinal cord

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Abstract

It is well known that prolonged exposure to morphine results in tolerance to morphine-induced antinociception. In the present study, we found that mice that were tolerant to morphine-induced antinociception exhibited an increase in immunoreactivity for the neural cell adhesion molecule in the dorsal horn of the spinal cord, which was highly overlapped with immunoreactivity for the increased metabotropic glutamate receptor 5 (mGluR5) induced by morphine. These findings support the idea that repeated stimulation of μ-opioid receptors increases the expression of neural cell adhesion molecule and metabotropic glutamate receptor 5. This phenomenon leads to the enhanced excitatory synaptic transmission in the dorsal horn of the spinal cord, and in turn suppresses the morphine-induced antinociception.

Keywords: Morphine; Tolerance; Neural cell adhesion molecule (NCAM); Metabotropic glutamate receptor 5 (mGluR5)

In the central nervous system, neuronal transmission relies on signals transmitted through a vast array of excitatory and inhibitory neuronal synaptic connections. During the formation of synaptic connections, neurons extend their processes to specific locations where they find appropriate targets and form contacts through transcellular adhesive interactions. Contact and synapse formation are commonly thought to occur between an axonal growth cone and dendritic shaft. Initial contact establishment is followed by spatially and temporally controlled changes to form a mature synapse characterized by the specific accumulation of synaptic vesicles and active zone components within the axon, in close apposition to dendritic membrane studded with receptors [10].

The neural cell adhesion molecule (NCAM), a member of the Ig superfamily expressed on the surface of most neural cells, is involved in cell–cell interactions during brain development, synaptic plasticity, and regeneration [13]. It was further determined that NCAM expression regulates both the number of synapses and strength of excitatory synaptic connections in a glutamate receptor-dependent manner [4].

Long-term exposure to morphine results in tolerance to opioids-induced antinociception. It has been well established that glutamate receptors are critical for the development and maintenance of opioid tolerance [2,12]. Therefore, the present study was undertaken to determine the effects of repeated exposure to morphine injection on NCAM expression associated with changes in excitatory synaptic connections in the mouse spinal cord.

The present study was conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, Hoshi University, as adopted by the Committee on Animal Research of Hoshi University, which is accredited by the Ministry of Education, Culture, Sports, Science and Technology of Japan. Male ICR mice (Tokyo Laboratory Animals Science Co., Ltd., Tokyo, Japan) were used. Animals were kept in a room with an ambient temperature of 23 ± 1 °C and a 12-h light–dark cycle (lights on 08:00–20:00 h). Food and water were available ad libitum.

The drugs used were morphine hydrochloride (Sankyo Co., Tokyo, Japan).

Intrathecal (i.t.) injection was performed according to the method described by Hylden and Wilcox [5] using a 25 μL

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Hamilton syringe with a 30 1/2-gauge needle. The volume for i.t. injection was 4 μL per mouse.

To investigate the development of antinociceptive tolerance following repeated treatment with morphine, mice were repeatedly s.c. injected with morphine (10 mg/kg s.c.) or saline (10 mL/kg s.c.) once a day for 7 consecutive days. Twenty-four hours after the last repeated injection, groups of mice were challenged i.t. with morphine (0.0056–10 nmol/mouse). The antinociceptive response was evaluated by the tail-flick test 10 min after morphine injection (Muromachi Kikai Co., Tokyo, Japan). To prevent tissue damage, we established a 10 s cut-off time. Antinociception was calculated as percentage of the maximum possible effect (%MPE) according to the following formula; %MPE = (test latency − pre-drug latency)/(cut-off time − pre-drug latency) × 100. Antinociceptive response represents as the mean ± S.E.M. of percentage antinociception. The ED_{50} value was calculated by GraphPad Prism Programs version 3.0 (GraphPad Software Inc., CA, USA). Statistical analysis was performed using *F*-test.

Groups of mice were repeatedly injected with morphine (10 mg/kg s.c.) and saline (10 mL/kg s.c.) once a day for 7 consecutive days. Twenty-four hours after the last injection, mice were sacrificed by decapitation. Their whole spinal cords were removed quickly and homogenized in ice-cold buffer A containing 20 mM Tris–HCl (pH 7.5), 2 mM EDTA, 0.5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 25 μg of leupeptin per mL, 0.1 mg of aprotinin per mL and 0.32 M sucrose. The homogenate was centrifuged at 1000 × *g* for 10 min and the supernatant was ultracentrifuged at 100,000 × *g* for 30 min at 4 °C. The pellets were washed with buffer B (buffer A without sucrose) and then ultracentrifuged at 100,000 × *g* for 30 min. The final pellets were retained as the membranous fraction for Western blotting at −80 °C until the assay. Total protein concentration in each sample was determined by the Bradford assay [3]. Sample aliquots containing 10–20 μg total proteins were separated by size on 5–20% SDS–polyacrylamide gradient gel and transferred to nitrocellulose membranes. The membrane was incubated with primary antibody diluted in Tris-buffered saline (TBS) (1:1000 neural cell adhesion molecule (NCAM, Chemicon), 1:30,000 metabotropic glutamate receptor 5 (mGluR5, Upstate)) containing 5% nonfat dried milk. Immunoreactive bands were detected using SuperSignal West Dura chemiluminescent kit (Pierce, Rockford, IL, USA) and quantified using NIH Image. Statistical analyses were performed using Student's *t*-test.

Mice were repeatedly injected with morphine (10 mg/kg s.c.) and saline (10 mL/kg s.c.) once a day for 7 consecutive days. Twenty-four hours after the last injection, mice were deeply anesthetized with sodium pentobarbital (70 mg/kg, i.p.) and prepared for immunostaining as described previously [9]. The L5 lumbar spinal cord segments were cut with a freezing cryostat (Leica CM 1510) at the thickness of 10 μm and thaw-mounted on poly-L-lysine-coated glass slides. The spinal cord sections were blocked in 10% normal goat serum (NGS) in 0.01 M phosphate-buffered saline (PBS) and incubated with each primary antibodies (1:100 NCAM (Chemicon), 1:3000 mGluR5 (Upstate)) and incubated 48 h at 4 °C. The antibody was then rinsed and incubated with each secondary antibodies conjugated with Alexa 564 and Alexa 488. Fluorescence immunolabeling was observed with a light microscope (Olympus BX-80; Olympus, Tokyo, Japan) and photographed with a digital camera (CoolSNAP HQ; Olympus). Digitized images of superficial laminae of the spinal dorsal horn sections were captured at a resolution of 1316 × 1035 pixels with camera.

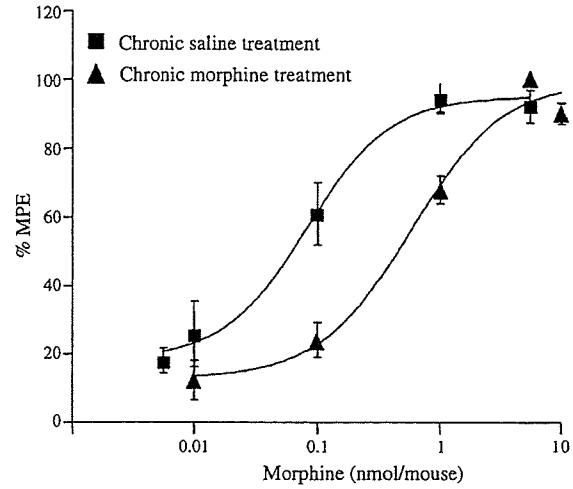

Fig. 1. Development of tolerance to morphine-induced antinociception. Mice were injected repeatedly once a day for 7 consecutive days with saline or morphine 10 mg/kg (s.c.). Twenty-four hours after the last repeated injection, groups of mice were challenged i.t. with morphine (0.0056–10.0 nmol/mouse). The dose–response curve for the antinociceptive effect of morphine in morphine-treated mice (triangle, ED_{50}: 0.36 (0.15–0.87) nmol/mouse, i.t.) was shifted to the right compared to saline-treated mice (square, ED_{50}: 0.05 (0.03–0.08) nmol/mouse, i.t.). Each point represents the mean ± S.E.M. of 6–16 mice. $p < 0.001$, $F_{1,52} = 64.3$, chronic saline treatment vs. chronic morphine treatment.

To investigate the development of antinociceptive tolerance, mice were repeatedly injected with morphine (10 mg/kg, s.c.) or saline once a day for 7 consecutive days. Repeated s.c. injection of morphine significantly suppressed the spinal antinociceptive effect induced by i.t.-administered morphine (0.0056–10.0 nmol/mouse). The dose–response curve for antinociceptive effect of morphine was clearly shifted to the right following repeated s.c. treatment with morphine, indicating the development of tolerance to morphine-induced spinal antinociception (Fig. 1: $p < 0.001$, $F_{1,52} = 64.3$).

Twenty-four hours after the last repeated injection of morphine, the IRs for mGluR5 and NCAM in the spinal cord were observed by immunohistochemical analysis. In saline-treated mice, the intense NCAM-IR was confined to laminae I–II in the dorsal horn of the spinal cord (Fig. 2A). Furthermore, the most intense mGluR5-IR appeared to be concentrated in inner part of lamina II (lamina IIi). Interestingly, repeated treatment with morphine produced the increase in both NCAM-IR and mGluR5-IR in the dorsal horn of the spinal cord (Fig. 2B). It should be mentioned that the increased mGluR5-IR by morphine was extended to the outer part of laminae II and III as well as IIi. Moreover, the red labeling for NCAM-IR and green labeling for mGluR5-IR showed apparent co-localization in the laminae II–III in the spinal cord of morphine-treated mice. In high-magnification image of the lamina IIi, the neuropil and

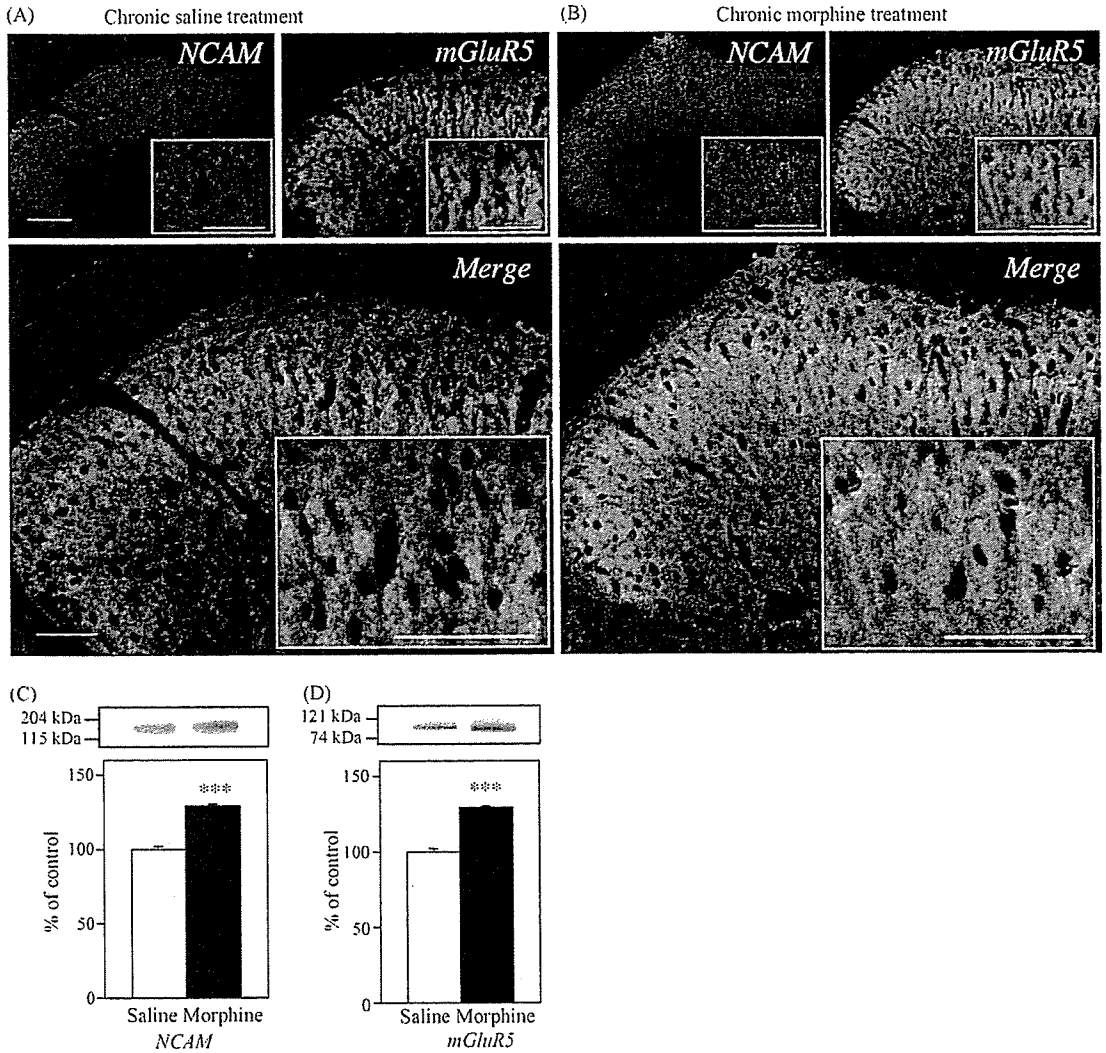

Fig. 2. (A and B) Increase in levels of NCAM-IR and mGluR5-IR in the dorsal horn of the spinal cord following repeated treatment with morphine. The increased mGluR5-IR (green) was more apparent co-localization with NCAM-IR (red) in the spinal cord of morphine-treated mice (B), as compared to saline-treated mice (A). (C and D) Quantitative analysis for levels of NCAM (C) and mGluR5 (D) in the mouse spinal cord after repeated morphine treatment. Upper: representative Western blot of NCAM and mGluR5. Lower: changes in immunoreactivities for NCAM and mGluR5 in membranes of spinal cords obtained from saline- or morphine-treated mice. Mice were repeatedly injected with saline or morphine (10 mg/kg, s.c.) once a day for 7 consecutive days. The membrane fraction was prepared at 24 h after the last injection. Each column represents the mean ± S.E.M. of three independent samples. $^{***}p < 0.001$ vs. saline-treated mice. Scale bars: 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

granular immunolabelings for the NCAM and mGluR5 were observed surrounding neuronal somata. As shown in Fig. 2C and D, Western blots showed that the protein levels of NCAM and mGluR5 in the spinal cord of morphine-treated mice were significantly increased compared to those in saline-treated mice (Fig. 2C: NCAM; 129.0 ± 1.1% of control, Fig. 2D: mGluR5; 136.9 ± 2.4% of control, $p < 0.001$).

The key finding in the present study was that repeated in vivo treatment with morphine induced a significant increase in the expression of NCAM in the mouse spinal cord, as detected by both immunohistochemical study and Western blots using the specific NCAM antibody, which recognizes at the neural cell surface of glycoproteins. NCAM is expressed by all neural cell types, subserving neuron–neuron and neuron–glia adhesion via homophilic and heterophilic interactions with other adhesion and extracellular matrix molecules. In the present study, double-immunofluorescence analysis revealed that the increased NCAM-IR located in the laminae I–II was highly co-localized with the increased mGluR5-IR in outer part of laminae II and I as well as IIi following repeated treatment with morphine. It has been reported that mGluR5-IR in the dorsal horn of the spinal cord is mostly observed in the dendritic shafts and cell body of the postsynaptic neurons, but not in astrocytes [1,8]. In addition, several lines of evidence demonstrate that the selective mGluR5 antagonist prevents the development of tolerance to morphine-induced antinociception [7,8,11]. Furthermore, it is considerable that the relative level of postsynaptic, but not presynaptic, NCAM expression controls synaptic strength in an activity-dependent manner by regulating the number of synapses [4]. A recent study has shown that repeated treatment with mor-

phine alters the expression levels of the polysialylated NCAM in the adult rat hippocampus [6]. Taken together, these findings raise the possibility that repeated treatment with morphine promotes the expression of NCAM and mGluR5 at the postsynaptic regions to increase the excitatory synaptic transmission in opposition to excessive activation of inhibitory neurons in the dorsal horn. Although additional studies are needed to further demonstrate the effect of chronic morphine treatment on direct changes in excitatory or inhibitory synaptic transmission, the present data support the idea that repeated stimulation of μ-opioid receptors may initially cause the long-lasting suppression of the release of glutamate, and in turn may increase the expression of NCAM and mGluR5 at the postsynaptic regions in order to enhance the glutamate synaptic transmission. This phenomenon may be, at least in part, responsible for the suppression of the morphine-induced antinociception, which is called tolerance to spinal antinociception induced by morphine.

In conclusion, repeated in vivo treatment with morphine induced a significant increase in the expression of NCAM associated with the up-regulation of mGluR5 in the dorsal horn of mouse spinal cord. This may be linked to the development of morphine tolerance and the induction of neuronal plasticity by chronic morphine treatment.

Acknowledgments

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Short communication

Role of gap junction in the expression of morphine-induced antinociception

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Abstract

The present study was undertaken to investigate whether gap junctional communication could be involved in morphine-induced antinociceptive response using blockers of the gap junctional channel, carbenoxolone and Gap27. Intrathecal pretreatment with either carbenoxolone or Gap27 caused a dose-dependent attenuation of morphine-induced antinociception. Furthermore, the dose–response line for morphine-induced antinociception was shifted to the right by 2.53-fold following intrathecal treatment with carbenoxolone. These findings suggest that gap-junctional-dependent communication in the mouse spinal cord may play, at least in part, a role in the expression of morphine-induced antinociception.

Keywords: Morphine; Gap junction; Antinociception

1. Introduction

A gap junction is a unique channel that directly connects cells with the interior of other cells. A gap junction enables the intercellular diffusion of molecules with a molecular mass of up to 1 kDa, including ions, cAMP, inositol (1,4,5)-triphosphate (IP_3), ATP and small peptides (Giaume and Venance, 1998; Zonta et al., 2003). A gap junction is composed of a clustered hemichannel, called a connexon, which forms aqueous conduits that link the intracellular compartments of coupled cells. Each connexon consists of a hexametric arrangement of gap junction proteins called connexin (Kamermans et al., 2001; Bennett et al., 2003). Connexin forms at least 20 gene family members that are expressed in many different cell types (Rash et al., 2001; Willecke et al., 2002). These channels can be gated in response to various stimuli, including changes in voltage and intracellular pH (Saez et al., 2003). In the central nervous system, gap-junction-mediated intercellular communication between glial cells has long been thought to contribute to tissue homeostasis in the brain. The homeostatic function includes the transport of nutrients from the bloodstream to neurons, 'spatial buffering' of K^+ released into the extracellular space during neuronal excitation and uptake and dissipation of glutamate through gap-junction channels among astrocytes (De Pina-Benabou et al., 2001).

The stimulation of μ-opioid receptor by morphine can regulate a number of signaling pathways, including inhibition of adenylate cyclase activity, activation of inwardly rectifying K^+ channels and blockade of Ca^{2+} entry through voltage-dependent Ca^{2+} channels (Childers, 1991). We previously demonstrated that the endoplasmic IP_3 receptor-mediated intracellular signaling pathway is implicated in the expression of antinociceptive effect of morphine (Aoki et al., 2003). Therefore, the aim of the present study was to investigate whether the gap junctional channel in the spinal cord could be implicated in the expression of morphine-induced antinociception.

2. Materials and methods

The present study was conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, Hoshi University, as adopted by the Committee on Animal Research of Hoshi University, which is accredited by the Ministry of Education, Culture, Sports, Science and Technology of Japan. Male ICR mice (Tokyo Laboratory Animals Science Co., Ltd.) were used in the present study.

Morphine (Sankyo) and blockers of the gap junction, 3β-hydroxy-11-oxoolean-12-en-30-oic acid 3-hemisuccinate

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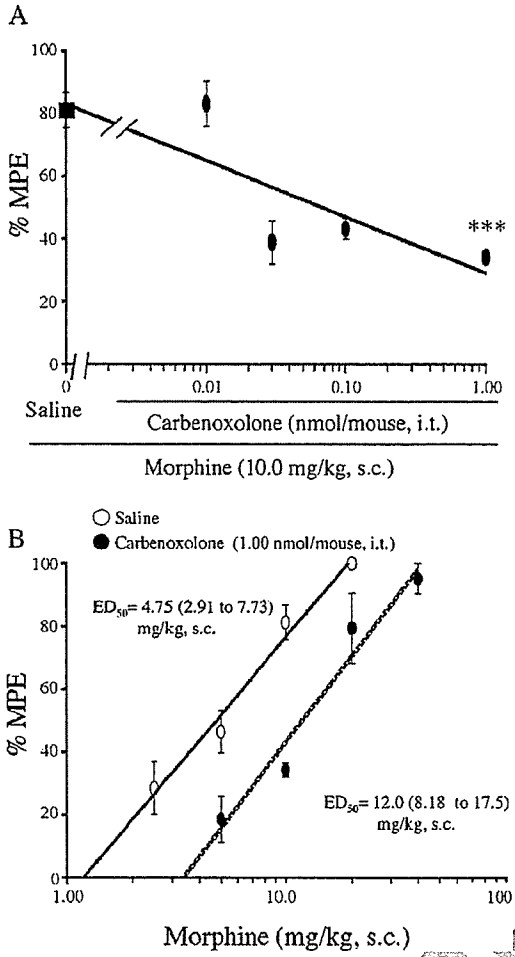

Fig. 1. Effects of carbenoxolone on the antinociception produced by subcutaneous (s.c.) injection of morphine. Intrathecal (i.t.) pretreatment with saline and carbenoxolone was performed at 30 min before morphine injection. (A) Intrathecal pretreatment with carbenoxolone (0.03–1.00 nmol/mouse) attenuated the antinociception produced by morphine (10.0 mg/kg, s.c.) in a concentration-dependent manner. ***$P<0.001$: saline vs. 1.00 nmol carbenoxolone. (B) The dose–response curve for the antinociceptive effect of morphine in carbenoxolone-pretreated mice (closed circle, carbenoxolone; 1.00 nmol/mouse, ED_{50}: 12.0 (8.18–17.5) mg/kg, s.c.) was significantly shifted to the right compared to saline-pretreated mice (open circle, ED_{50}: 4.75 (2.91–7.73) mg/kg, s.c.). Antinociception was calculated as percentage of the maximum possible effect (% MPE) according to the following formula; % MPE = (test latency − pre-drug latency)/(cut-off time − pre-drug latency) × 100. Each point represents the mean ± S.E.M. of 8–16 mice.

(carbenoxolone: Sigma-Aldrich) and Gap27 (amino-acid sequence SRPTEKTIFII, TOCRIS), were dissolved in 0.9% sterile saline.

Intrathecal (i.t.) injection was performed as described by Hylden and Wilcox (1980) using a 25 μl Hamilton syringe with a 30 gauge 1/2 in. needle. The injection volume was 4 μl for i.t. injection.

The morphine-induced antinociceptive response was evaluated by recording the latency to paw licking or tapping in the hot-plate test (55 ± 0.5 °C). To prevent tissue damage, we established a 30 s cut-off time. The test was performed 30 min after morphine treatment. Each animal served as its own control, and the latency to responses was measured both before and after drug administration. Antinociception was calculated as percentage of the maximum possible effect (% MPE) according to the following formula; % MPE = (test latency − pre-drug latency)/(cut-off time − pre-drug latency) × 100. Antinociceptive response represents as the mean ± S.E.M. of % MPE. The ED_{50} value was calculated by GraphPad Prism Programs version 3.0 (GraphPad Software Inc.). The statistical significance of differences between groups was assessed with one-way analysis of variance (ANOVA) followed by the Bonferroni/Dunn multiple comparison test.

3. Results

To investigate the role of gap junctional communication in morphine-induced antinociception, we examined the effects of blockers of the gap junctional channel, carbenoxolone and Gap27, on the μ-opioid receptor-mediated antinociceptive response using the hot-plate test. Intrathecal (i.t.) pretreatment with carbenoxolone (0.03–1.00 nmol/mouse) significantly attenuated the antinociceptive response produced by subcutaneous (s.c.) treatment with morphine (10.0 mg/kg) in a concentration-dependent manner (***$P<0.001$: saline-pretreated mice vs. 1.00 nmol carbenoxolone-pretreated mice, Fig. 1A). Furthermore, the dose–response curve for the antinociceptive effects of morphine was significantly shifted to the right by 2.53-fold following i.t. pretreatment with carbenoxolone (1.00 nmol/mouse, Fig. 1B: *** $P<0.001$). Under these conditions, carbenoxolone at the doses used in the present study had no effects on the basal hot-plate latency after s.c. treatment with saline (data not shown). Like carbenoxolone, intrathecal pretreatment with Gap27 (3.00 and 10.0 nmol/mouse)

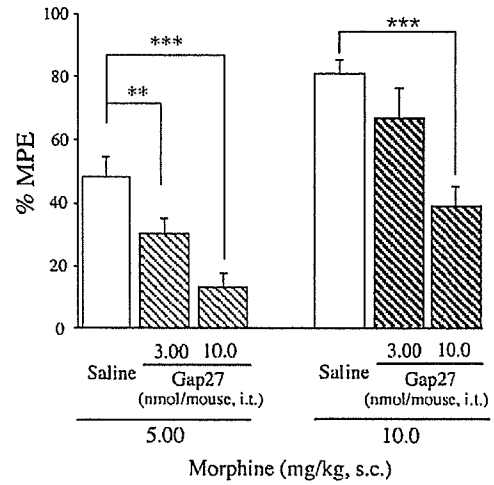

Fig. 2. Effect of Gap27 on the antinociception produced by s.c. injection of morphine. Intrathecal pretreatment with saline and Gap27 was performed at 30 min before morphine injection. Intrathecal pretreatment with Gap27 (3.00 and 10.0 nmol/mouse) attenuated the antinociceptive response produced by s.c. treatment with morphine in a concentration-dependent manner. **$P<0.01$ and ***$P<0.001$ vs. saline-pretreated 5.00 mg/kg morphine group, ***$P<0.001$ vs. saline-pretreated 10.0 mg/kg morphine group. Antinociception was calculated as percentage of the maximum possible effect (% MPE) according to the following formula; % MPE = (test latency − pre-drug latency)/(cut-off time − pre-drug latency) × 100. Each point represents the mean × S.E.M. of 8–16 mice.

significantly attenuated the antinociceptive response produced by s.c. treatment with morphine (5.00 and 10.0 mg/kg) in a concentration-dependent manner (**$P<0.01$ and ***$P<0.001$ vs. saline-pretreated 5.00 mg/kg morphine group, ***$P<0.001$ vs. saline-pretreated 10.0 mg/kg morphine group, Fig. 2).

4. Discussion

The central nervous system is composed of a closely associated network of neurons and glial cells. For a long time, astrocytes were considered to be limited to the structural, trophic and metabolic support of neurons. Recent accumulating evidence suggests that glial cells display rapid electrical responses to neuronal activity via gap junctional channels, which can trigger the propagation of Ca^{2+} waves (Alvarez-Maubecin et al., 2000; Zonta et al., 2003). Ca^{2+} waves are mediated by the diffusion of IP_3 through gap junctions between cells and are also regulated by the release of ATP though hemichannels (De Pina-Benabou et al., 2001; Galarreta and Hestrin, 2001).

Carbenoxolone is a moderately lipophilic glycyrrhetinic acid derivative that has been shown to act directly on gap junctions in brain and other tissues to reduce conductance by up to 80% (Rozental et al., 2001). Furthermore, it has been reported that Gap27 peptides target the second extracellular loop of Cx40 and Cx37/Cx43, which interrupt direct intracellular coupling in a connexin-specific fashion without disrupting the structural integrity of gap junction plaques at points of intercellular contact (Chaytor et al., 1997, 1998). In the present study, we found that i.t. pretreatment with two different gap-junction blockers carbenoxolone and Gap27, which did not have any effects on the nociceptive threshold of the hot-plate response when injected alone, caused a dose-dependent attenuation of morphine-induced antinociception. Recent in vitro study using the purified astrocytes' primary culture shows that morphine failed to affect the junctional permeability determined by dye transfer technique (Mantz et al., 1993). Consistent with these results, we previously reported that morphine had no direct effect on astrocytic activation in purified astrocytes, whereas it caused astrocytic activation in neuron/glia co-cultures (Narita et al., 2005, in press). Taken together, these findings suggest that the gap junctional channel in the spinal cord might be affected by morphine though the stimulation of μ-opioid receptor in neurons. Although gap-junction blockers do not clearly distinguish the two pathways, hemichannels and coupling between cells, these findings provide novel evidence that gap-junction channels in the spinal cord may be, at least in part, implicated in the expression of morphine-induced antinociception.

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Short communication

Chronic morphine treatment increases the expression of vesicular glutamate transporter 1 in the mouse spinal cord

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Abstract

Long-term exposure to morphine results in tolerance to morphine-induced antinociception. Here, we found that mice tolerant to morphine exhibited the significant increase in the protein levels of the vesicular glutamate transporter 1 and the synaptic vesicle-specific small G protein Rab3A, but not vesicular glutamate transporter 2 and vesicular γ-aminobutyric acid transporter. These findings suggest that repeated treatment with morphine enhances excitatory synaptic transmission in the spinal cord, and in turn suppresses the morphine-induced antinociception.

Keywords: Morphine; Tolerance; Vesicular glutamate transporter

1. Introduction

In the central nervous system, the normal function of neural networks depends on a delicate balance between excitatory and inhibitory synaptic inputs. Vesicular glutamate transporter 1 and 2 (VGluT1 and VGluT2) are known to underlie the transport of glutamate into excitatory vesicles in the glutamatergic nerve terminals, which influence the strength of excitatory transmission (Wilson et al., 2005). Vesicular γ-aminobutyric acid (GABA) transporter (VGAT) is highly concentrated in the nerve endings of GABA- and glycine-containing neurons (Chaudhry et al., 1998).

The administration of morphine directly into the spinal cord produces a powerful antinociception/analgesia, which is mediated by opioid receptors located in the marginal layer of the dorsal horn of the spinal cord. It has been proposed that morphine acts at μ-opioid receptors localized on C-fiber terminals, which inhibits neurotransmitter release by inactivating voltage-gated calcium channels (Aicher et al., 2000).

Long-term exposure to morphine results in tolerance to opioid-induced antinociception. It has been well established that glutamate receptors are critical in the development and maintenance of opioid tolerance (Trujillo and Akil, 1991; Narita et al., 2005). Here, we determined the effect of repeated treatment with morphine on the expression of VGluT1, VGluT2 and VGAT in the mouse spinal cord.

2. Materials and methods

The present study was conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, Hoshi University, as adopted by the Committee on Animal Research of Hoshi University, which is accredited by the Ministry of Education, Culture, Sports, Science and Technology of Japan. Male ICR mice (Tokyo Laboratory Animals Science Co., Ltd.) weighing 25–28 g were used in the present study. The drugs used were morphine hydrochloride (Sankyo Co., Tokyo, Japan).

Groups of mice were repeatedly injected with morphine (10 mg/kg s.c.) and saline (10 ml/kg s.c.) once a day for 7 consecutive days. Twenty-four hours after the last injection, mice were sacrificed by decapitation. Their whole spinal cords were removed quickly and homogenized in ice-cold buffer (20 mM Tris–HCl, 2 mM EDTA, 0.5 mM EGTA, pH 7.5) containing protease inhibitors cocktail. The homogenate was centrifuged at 1000×*g* for 10 min and the supernatant was

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