Up-regulation of brain-derived neurotrophic factor in the dorsal root ganglion of the

rat bone cancer pain model

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Abstract

Metastatic bone cancer causes severe pain, but current treatments often provide insufficient pain relief. One of the reasons of this issue is because mechanisms underlying bone cancer pain are not solved completely. Our previous studies have shown that Brain-derived neurotrophic factor (BDNF), known as a member of the neurotrophic family, is an important molecule in the pathological pain state in some pain models. We hypothesized that expression changes of BDNF may be one of factors related to bone cancer pain, then in this study, we investigated changes of BDNF expression in dorsal root ganglia (DRG) in rat bone cancer pain model. As we expected, BDNF mRNA and protein were significantly increased in L3 DRG after intra-tibial inoculation of MRMT-1 rat breast cancer cells. Among the eleven splice-variants of BDNF mRNA, exon 1-9 variant increased predominantly. Interestingly, the up-regulation of BDNF is localized in small-neurons (mostly nociceptive neurons) but neither in medium- nor large-neurons (non-nociceptive neurons). Further, expression of nerve-growth factor (NGF), which is known as a specific promoter of BDNF exon 1-9 variant, was significantly increased in tibial bone marrow. Our findings suggest that BDNF is one of a key molecule in bone cancer pain and NGF-BDNF cascade possibly develops bone cancer pain.

Abbreviations:

BDNF; Brain-derived neurotrophic factor

NGF; Nerve-growth factor

TrkA; Tropomyosin receptor kinase A

RPL27; 60S ribosomal protein L27

DRG; Dorsal root ganglion

PWT; Paw withdrawal threshold

IR; immunoreactive

Introduction

Pain is one of the most feared and burdensome symptoms experienced by cancer patients.

In a recent systematic review, 64% of advanced cancer patients were suffering from severe

cancer-related pain.[1] In particular, cancer patients who develop bone metastasis experience

significant pain, and drugs such as opioids and non-steroidal anti-inflammatory drugs often

provide an insufficient analgesic effect for such severe pain.

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophic family and plays

important roles in survival, differentiation, and synaptic plasticity of neurons.[2] BDNF is also

implicated in the development of pathological pain. In the spinal dorsal horn, BDNF

modulates pain transduction by several mechanisms, such as down-regulation of potassium

chloride co-transporter (KCC2) in a partial nerve ligation model[3] and activation of

N-methyl-D-aspartate receptor in an L5 spinal nerve ligation model.[4] Between DRG neurons,

BDNF acts as autocrine and/or paracrine signal in inflammatory pain model and enhances

release of neurotransmitter such as calcitonin-gene related protein and substance P.[5] BDNF

expression is induced by nerve-growth factor (NGF) in DRG neurons[6] and by

adenosine-5'-triphosphate in spinal microglia.[3] While the mechanism of BDNF induction is

not fully understood in the bone cancer pain model, a recent study demonstrated that BDNF

mRNA expression increased in DRG, and siRNA-mediated BDNF knockdown reduced the

behavioral hypersensitivity in the rat bone cancer pain model induced by prostate cancer cell inoculation.[7]

The rat BDNF gene contains 8 non-coding exons (exons 1 through 8) and one coding exon (exon 9).[8] These non-coding exons can be spliced to the coding exon to form the following splice-variants: exons 1-9, 2a-9, 2b-9, 2c-9, 3-9, 4-9, 5-9, 6-9, 7-9, 8-9, and 9a-9. Expressions of these splice-variants are regulated in an age- and organ-dependent manner.[8] This finding would indicate that multiple promoters are regulating transcription of the splice-variants in response to diverse environmental conditions. Our previous studies have clearly demonstrated that BDNF mRNA expression in DRG was up-regulated in inflammatory and neuropathic pain models, and the exon 1-9 variant showed the greatest responses in both models.[9] We also reported that BDNF exon 1-9 was induced by NGF stimulation in cultured DRG neurons.[10] These observations indicated that a specific variant of BDNF mRNA, exon 1-9, can be a strong indicator of various pain. We hypothesized that alternative expression of BDNF and NGF-BDNF cascade is also related to bone cancer pain. In this study, to address this issue, we investigated that the expression profile of BDNF splice-variants in DRGs in rat bone cancer pain model and the relation between NGF and BDNF expression.

Materials and Methods

Cell culture

MRMT-1 rat breast cancer cells were provided by the Cell Resource Center for Biomedical

Research, Tohoku University (Miyagi, Japan). Cells were cultured in RPMI 1640

supplemented with 300 mg/mL L-glutamine, 10% fetal bovine serum, 200 U/mL penicillin,

and 200 µg/mL streptomycin, and incubated at 37°C in a humidified atmosphere of 5% CO_2.

Animals

All animal procedures were carried out in accordance with the Ethical Guidelines for the

Investigation of Experimental Pain in Conscious Animals issued by the International

Association for the Study of Pain.[11] The Board of Animal Care and Use Committee of

Okayama University Medical School approved this study on 31 March 2010 (OKU-2010103,

Chairman Prof M. Nishibori).

Male Wistar rats (CLEA Japan, Tokyo, Japan: 170-190 g at surgery, total 54 rats) were

housed under controlled conditions (12 h alternating light-dark cycle, food and water ad

libitum).

Surgical procedure

Injection of MRMT-1 cells was performed as described previously.[12] Briefly, all surgical

procedures were performed under 1-2% isoflurane inhalation anesthesia. After the induction

of anesthesia, the left leg was shaved, and the skin was disinfected with 70% v/v ethanol. A 1-cm rostro-caudal incision was made in the skin over the top half of the surface of the proximal end of the tibia. A 24-gauge needle was inserted 5 mm below the knee joint into the intramedullary cavity of tibia. A cell suspension containing 3×10^3 MRMT-1 cells in 10 µL of Hank's buffered sterile saline (HBSS) buffer (Sigma-Aldrich, St. Louis, MO, USA) was injected into the medullary space of the left tibia with a 10-µL Hamilton syringe (MRMT-1 group). Control animals were injected the same volume of HBSS buffer only (Sham group). The injection site was sealed with bone wax, and then the wound was closed and gentamicin sulfate ointment was applied. After the surgery, the rats were placed in a thermo-regulated recovery box until they had regained consciousness, and they were then returned to the home cage.

Radiological analysis

Roentgenography of the ipsilateral tibia was performed preoperatively and on postoperative days 7 and 14. Radiographs were taken by a Latheta LCT-200 X-ray imaging system (Hitachi Aloka Medical, Mitaka, Tokyo, Japan) under sodium pentobarbital anesthesia (50 mg/kg, i.p). After the examination, the rats were placed in a thermo-regulated recovery box until they had regained consciousness, and they were then returned to the home cage.

Behavioral assessment

Pain behavior was assessed by the von Frey test and measurement of hind limb weight-bearing before and 1-14 days after the surgery.

Mechanical allodynia was measured as the hind paw withdrawal threshold (PWT) with von Frey filaments (Touch-Test® Sensory Evaluator, North Coast Medical, Morgan Hill, CA, USA). The rats were placed individually in a plastic cage (13 × 10 × 15 cm³) with an elevated wire mesh bottom, allowing full access to the plantar surfaces of both hind paws. The mechanical stimuli were applied to the medial plantar aspect of each hind paw with one of a series of nine von Frey filaments (0.4, 0.6, 1.0, 1.4, 2.0, 4.0, 6.0, 8.0, and 15.0 g). Each trial was started with a von Frey force of 2 g for 1-2 s. Stimuli were presented at intervals of at least 10 s, allowing for apparent resolution of any behavioral responses to previous stimuli. On the basis of the response pattern and the force of the final filament, the 50% PWT was determined by the up-down method of Dixon[13] and calculated using the formula described by Chaplan et al.[14] If the strongest filament did not elicit a response, the PWT was recorded as 15.0 g.

Hind limb weight-bearing was measured by an Incapacitance Tester (Linton Instrumentation, Norfolk, UK) as described by Fernihough et al.[15] The rat was placed in a chamber so that each hind paw was resting on a separate force transducer pad. Each transducer pad recorded a body weight on each paw 4 times in 1 s, and the testing duration was set to 3 s.

Five readings were averaged for each hind paw, and the results were presented as the weight-bearing ratio (ipsilateral/contralateral).

Quantitative real-time Reverse Transcription-PCR (RT-PCR)

After carrying out behavioral assessment, rats were sacrificed by decapitation under deep ether anesthesia 14 days after the surgery. Ipsilateral L3, L4, and L5 DRG were dissected rapidly and dipped immediately in RNAlater (Qiagen Inc. Valencia, CA, USA). Tibial bone marrow was collected by flushing 100 µL of RNAlater in the tibial cavity. Total RNA was isolated and purified from individual DRG and the bone marrow with RNeasy Lipid Tissue Mini Kit (Qiagen Inc. Valencia, CA, USA). In this process, DNAse I was used for removal of genomic DNA according to manufacture's instruction. Removal of genomic DNA was confirmed by PCR of total RNA with BDNF exon 9 primers (no RT control). Then, 1 µg of total RNA was reverse-transcribed with the Ready-To-Go T-primed First-Strand Kit (GE Healthcare Life Sciences, Buckinghamshire, UK). cDNA solutions were diluted 10 fold with DNase-free water. cDNA was amplified in a 20 µL real-time PCR reaction mixture containing 10 µL SYBR Premix Ex Taq (Takara-Bio, Otsu, Japan), 4.2 µL DNase-free water, 0.2 µM each of forward and reverse primers, and 5 µL diluted cDNA solution. The forward primer for each BDNF splice variant was designed from each non-coding exon, and the reverse primer was designed from the coding region in exon 9 (Fig. 1). To quantify expression of total BDNF,

forward and reverse primers were both designed from exon 9. The sequences of primer sets

are shown in Table 1. Quantitiative real-time RT-PCR was performed with a LightCycler®

(Roche Diagnostics Corporation, Indianapolis, IN, USA) with the following amplification

conditions: 95°C for 10 s followed by 45 cycles of 5 s at 95°C and 20 s at 60°C. To quantify

absolute cDNA copy numbers, standard curves were created by the following method. Each

PCR product with the quantitative primers was cloned into pCRII-TOPO (Life Technologies,

Carlsbad, CA, USA), and the insert was amplified with M13 forward and reverse primers.

The amplified inserts were isolated by agarose gel electrophoresis, extracted from the gel.

The concentrations of the oligonucleotides in the gel extracts were measured by

spectrophotometry. Absolute copy numbers of the oligonucleotides were calculated from the

concentrations and diluted serially to generate standard curves. Expression of each mRNA

was normalized to expression of 60S ribosomal protein L27 (RPL27)[16]. The data were

shown as percentage to mean values of the sham group. PCR specificity was confirmed by

sequencing, agarose gel electrophoresis, and melting curve analysis.

Immunohistochemistry

Rats were anesthetized deeply with ether and perfused transcardially with 50 mL of saline,

followed by 500 mL of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The L3, L4,

and L5 DRG were dissected and postfixed in the same fixative for 30 minutes, incubated in

phosphate-buffered 20% sucrose solution overnight. These tissues were embedded in OCT

compound and 8-μm-thick frozen sections were cut. For visualization of BDNF in the DRG,

the avidin-biotin-horseradish peroxidase complex method was used. These sections were

incubated overnight with rabbit anti-BDNF antibody (1: 4000, #sc-546, Santa Cruz

Biotechnology, Santa Cruz, CA, USA), followed by incubation with biotinylated goat

anti-rabbit IgG (1: 200) and avidin-biotin-horseradish peroxidase complex (Vector

Laboratories, Burlingame, CA, USA). Immunoreaction products were visualized with

diaminobenzidine and nickel ammonium sulfate. DRG neurons were categorized as small (<

20 μm diameter), medium (20-40 μm diameter), and large (> 40 μm diameter), and the

proportion of BDNF-immunoreactive (IR) neurons was analyzed in each group. The cell

counting was performed by a blinded observer. Three representative sections that contained

over 70 neurons, total at least 210 each neurons, with distinct nuclei were randomly

selected in each of the DRG.

Statistical analysis

Data are presented as means ± standard deviation (SD). The data of hind limb

weight-bearing and tactile withdrawal thresholds with von Frey filaments were analyzed by

two-way analysis of variance (ANOVA) followed by Scheffe's F test. Data of real-time

RT-PCR analysis and the proportion of BDNF-IR neurons were analyzed by Student's t-test.

p < 0.05 was considered significant.

Results

Osteopenia and pain-related behavior after cancer cell inoculation

Osteopenia caused by growth of inoculated cells was observed in the MRMT-1 group

postoperatively (Fig. 2A). While osteopenia was gradually increased through day 7 to 14,

cortical integrity was maintained at day 14. This phenomenon represents to clinical

observation that bone metastatic cancer cells grow in tibia of a breast cancer patient. It

sometimes causes osteolysis. No radiological changes were found in the sham group.

During the observation period, rats with MRMT-1 walked with mild claudication. This

observation showed that cancer inoculation did not influence on motility nor cause bone

fractures at least in observation period as radiographs also showed. Pain related behavior

was assessed by the von Frey tests (Fig. 2B, 2C) and the hind limb weight-bearing tests (Fig.

2D). Prior to intramedullary injection of MRMT-1 or vehicle, there was no difference in

ipsilateral 50% PWT between the MRMT-1 group and the sham group. After the injection of

MRMT-1, the ipsilateral 50% PWT was decreased significantly on day 9 (MRMT-1 group 6.2

± 2.0 g vs. sham group 14.8 ± 0.4 g, $p < 0.01$) (Fig. 2B). The decreased 50% PWT was

continuously observed until day 14 at the end of the observation period. The contralateral

hind limb showed no pain-related behavior in both MRMT-1 group and sham group during

the observation period (Fig. 2C). Rats injected with MRMT-1 cells showed significant

reduction in weight-bearing on the left hind limb beginning on day 12 (MRMT-1 group 0.69 ±

0.19 vs. sham group 1.01 ± 0.03, $p < 0.05$, Fig. 2D). These pain-related behaviors

progressed along with the growth of the bone cancer seen in the radiographs (Fig. 2A).

BDNF mRNA and protein was increased in L3 DRG after cancer cell inoculation

Increased BDNF levels in DRG were reported in some pain models and were thought to be

involved in pain development. We investigated BDNF expression and localization in DRG

neurons. Total BDNF mRNA expression in L3, L4, and L5 DRG are shown in Fig. 3A. In the

MRMT-1 group, the expression of total BDNF mRNA in L3 DRG increased significantly to

119% compared to the sham group ($p < 0.05$). However, no significant differences in the

expressions of total BDNF mRNA in the L4 and L5 DRG were seen between the two groups.

To further investigate the expression profiles of BDNF mRNA, expressions of BDNF splice

variants were studied. Among all splice variants, exon 1-9 showed the greatest increase, to

207% compared to the sham group ($p < 0.01$). Expressions of exons 2a-9, 2b-9, 2c-9, 4-9,

and 6-9 also increased significantly to 161%, 159%, 133%, 142% and 137% compared to

the sham group, respectively (exon 2c-9 : $p < 0.05$, other exons : $p < 0.01$) (Fig. 3B).

Expression of exon 3-9 and 9a-9 did not change. Since the absolute copy numbers of exon

5-9 and 8-9 were each below 10 copies/μL, which was under the lower limit for quantification,

data for these exons were excluded from analysis. We couldn't amplify exon 7-9 in rat

neuronal cDNA despite our best efforts. No significant differences in the expressions of

BDNF mRNA splice variants in the L4 and L5 DRG were found between the MRMT-1 group

and the sham group, as well as in total BDNF mRNA (data not shown).

Figure 3C shows representative photomicrographs of the L3 DRG on day 14. The

percentages of BDNF-positive neurons of the left L3, L4, and L5 DRG are shown in table 2.

While the percentage of BDNF-positive neurons in the sham group was similar to a previous

report,[17] our new finding was that BDNF in L3 DRG was significantly increased by MRMT-1

inoculation in only small-sized neurons (136% vs sham group, $p < 0.01$) but not in medium-

or large-sized neurons (Table 2).

NGF is one of the key regulators of BDNF in bone cancer pain model

Since BDNF expression in DRG is reported to be induced by systemic administration of

NGF,[6] and further BDNF exon 1-9 variant contributes a considerable part of the

NGF-induced expression of BDNF mRNA,[10] we investigated NGF expression in tibial cavity.

Quantitative real-time RT-PCR revealed a robust increase of NGF mRNA in the MRMT-1

group (3.8-fold vs sham group, $p < 0.01$) (Fig. 4).

Since NGF mRNA was increased in the tibial cavity after MRMT-1 innoculation, we

investigated expression of NGF receptor, tropomyosin receptor kinase A (TrkA) mRNA in L3

DRG. In the MRMT-1 group, expression of TrkA mRNA was increased significantly to 193%

compared to the sham group ($p < 0.01$, Fig. 5).

Discussion

In this study, we found that inoculation of MRMT-1 cancer cells induced pain-related behavior, and the proportion of BDNF-IR-positive neurons were increased significantly in small neurons in the L3 DRG. Up-regulation of BDNF mRNA in the L3 DRG after MRMT-1 inoculation was observed. Further for detail, among the splice-variants, the exon 1-9 variant showed the greatest increase in this model. This report functions as the first report showing the profile of BDNF splice-variants in rat bone cancer pain. Expression of NGF, which is known to induce BDNF in DRG neurons, was increased in the intratibial cavity with MRMT-1 cells, and its high affinity receptor TrkA was increased in L3 DRG. Our results implicate that NGF up-regulation in the tibia play a role in BDNF induction.

The role of BDNF in the development of pathological pain has been well described. BDNF in the spinal dorsal horn modulates synaptic transmission by several mechanisms,[18] including down-regulation of KCC2 transporter and conversion of inhibitory GABAergic neurons to excitatory.[3] Although Coull et al. regarded spinal microglia as a source of BDNF,[3] anterograde transport of BDNF from DRG sensory neurons contributes to the increase of BDNF in the spinal dorsal horn.[19] BDNF also acts as autocrine and/or paracrine signal between DRG neurons. Up-regulation of BDNF and TrkB receptor were reported in inflammatory pain model and BDNF stimulation increased release of neurotransmitter such

as calcitonin-gene related protein and substance P in cultured DRG neurons.[5] Extensive

evidences indicate BDNF roles in development of pathological pain in inflammatory[20] and

neuropathic[21] pain models. It has also been reported that BDNF mRNA expression was

increased in L4-L6 DRG in a rat bone cancer pain model with Walker 256 mammary grand

carcinoma cell inoculation.[7] In the present study, we confirmed that BDNF-positive neurons

were increased in L3 DRG after MRMT-1 inoculation. Consistently, the report studied by

intratibial injection of retrograde tracer suggested that tibial bone marrow was innervated

mainly by L3 DRG neuron.[22] Taken together with the present study, our novel finding that

BDNF-positive neurons were increased in L3 DRG after MRMT-1 inoculation is convincing.

Blockade of BDNF reduced the pain behavior in bone cancer pain,[7] neuropathic pain,[23] and

inflammatory pain[24] models. However, suppression of BDNF was documented to result in

serious adverse effects, such as early postnatal death in homozygous knockout mouse,[25]

decrease or loss of the sensory nervous system in heterozygous knockout mouse,[26] and

learning disturbance and memory disorder after the injection of anti-BDNF antibody into the

cerebral ventricle.[27] To avoid these effects resulted from systemic blockade of BDNF, local

BDNF suppression (e.g. intrathecal injection of siRNA,[28] DRG specific vector transfer

mediated by intrathecal injection of AAV8[29]) can be a possible therapeutic tool for pain

conditions. We previously reported predominant up-regulation of BDNF exon 1-9 mRNA

among the splice-variants in L4 and L5 DRG in inflammatory and neuropathic pain models.[9]

We also reported that DNA decoy targeting BDNF exon 1 promoter activity showed the

anti-nociceptive effect in a rat neuropathic pain model.[30] Since the exon 1-9 variant showed

the highest increase in the present study, as well as in other pain models, suppression of

BDNF exon 1 transcription might be a therapeutic target for bone cancer pain.

We observed up-regulation of BDNF protein in small neurons in L3 DRG. Majority of small

neurons express TrkA, high affinity NGF receptor, and almost all TrkA positive cells express

calcitonin gene-related peptide immunoreactivity.[31] These facts indicate majority of small

neurons are nociceptive and responsive to NGF. On the other hand, most of large neurons,

which convey proprioception via A-beta fiber, do not express TrkA.[31] Our

immunohistochemical results suggest the role of BDNF in cancer induced bone pain and

possible involvement of NGF-TrkA signaling in BDNF up-regulation. Medium to large DRG

neurons are reported to change their phenotype and turn to produce BDNF in pain models

accompanying axotomy.[32] We did not observe increase of BDNF in medium and large

neurons in this model.

In the present study, NGF mRNA expression was increased in the intratibial cavity in

MRMT-1 inoculated rats. In the intratibial cavity, MRMT-1 cells are thought to produce NGF.

Several lines of evidence on NGF induction of BDNF have been reported. Cultured DRG

neurons up-regulated BDNF mRNA in response to NGF stimulation in a dose-dependent manner.[10] We also observed TrkA mRNA expression was increased in the L3 DRG after intratibial inoculation of MRMT-1. NGF-TrkA complex is internalized and transported from peripheral terminals to sensory cell bodies in the DRG[33] and activates transcription factors that control downstream gene expression.[33] Systemic administration of NGF to rats induced BDNF mRNA in DRG neurons expressing TrkA receptors.[6] Long term NGF stimulation induced TrkA mRNA and enhanced intracellular signaling in PC12 cells.[34] Sensory nerve fibers sprouting to bone cancer[35] express TrkA receptor. These findings support the involvement of the NGF-TrkA-BDNF cascade in the present study. Importantly, NGF expression in breast cancer tissue is not limited to experimental conditions. It has been reported that biopsies of human breast cancer showed strong NGF immunoreactivity in most specimens.[36,37]

The limitation of our study is that we do not directly prove involvement of cancer-derived NGF in BDNF induction and pain-related behavior. To clarify the involvement of NGF and BDNF in bone cancer pain, further study is required.

Disclosure

All authors declare that there is no conflict of interests regarding the publication of this

paper.

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Figure legends

Fig.1. BDNF gene structure and primer set.

Fig. 2. (A) Radiographs of left tibiae inoculated with MRMT-1 breast cancer cells or vehicle. The white arrow indicates the MRMT-1 injection site.

(B) Time course of the ipsilateral paw withdrawal threshold (PWT) to mechanical stimuli after intratibial inoculations of Hank's buffered sterile saline (white box, n = 6) and MRMT-1 cells (black box, n = 6). These data are reported as means ± SD. ** $p < 0.01$ vs. sham group.

(C) Time course of the contralateral paw withdrawal threshold (PWT) to mechanical stimuli after intratibial inoculations of Hank's buffered sterile saline (white box, n = 6) and MRMT-1 cells (black box, n = 6). These data are reported as means ± SD. ** $p < 0.01$ vs. sham group.

(D) Time course of the hind limb weight-bearing ratio (ipsilateral/contralateral) in rats that received intratibial inoculations of Hank's buffered sterile saline (white box) (n=6) and MRMT-1 cells (black box) (N=6). These data are reported as means ± SD. ** $p < 0.01$ vs. sham group.

Fig. 3. (A) Relative expression of BDNF mRNA in the ipsilateral L3, L4, and L5 DRG 14 days after MRMT-1 inoculation. These data are shown as percentage to mean values of the sham group. Black bars and 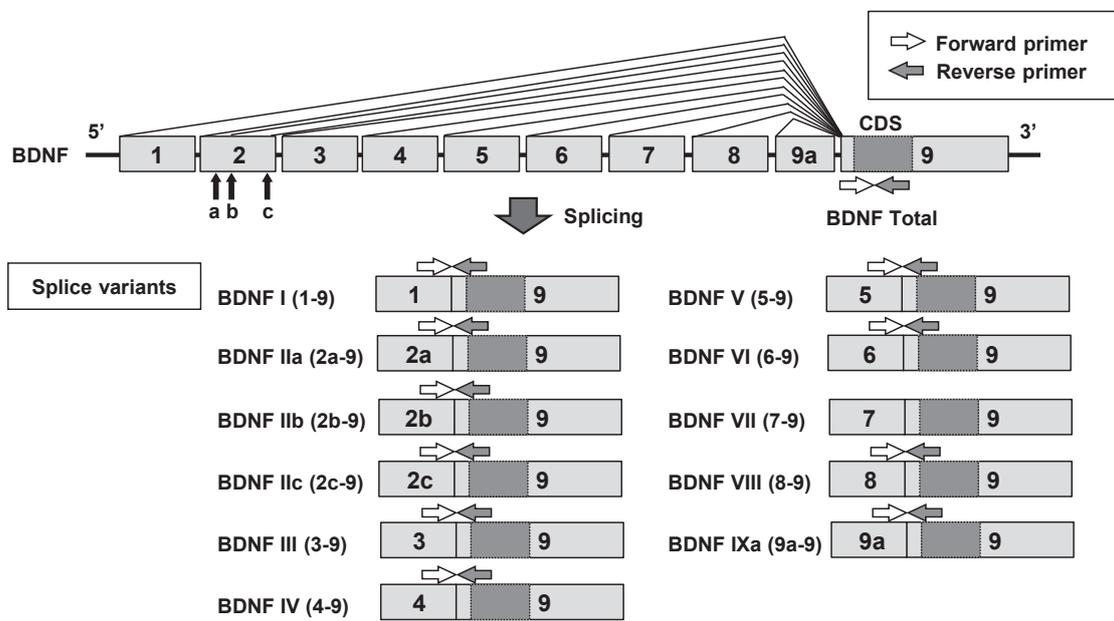gray bars indicate the MRMT-1 groups (n = 6) and sham groups (n = 6), respectively. These data are reported as means ± SD. * $p < 0.05$, vs. sham group.

(B) Relative expression BDNF splice variants in the ipsilateral L3 DRG 14 days after

MRMT-1 inoculation. These data are shown as percentage to mean values of the sham

group. Black bars and gray bars indicate MRMT-1 groups (n = 6) and sham groups (n = 6),

respectively. These data are reported as means ± SD. * $p < 0.05$, **$p < 0.01$ vs. sham group.

(C) Photomicrographs showing the ipsilateral L3 DRG in the MRMT-1 group and sham

group 14 days after surgery. Arrows indicate BDNF-positive small sized neurons (< 20 μm),

and arrowheads indicate BDNF-positive medium sized neurons (20–40 μm).

Fig. 4. Relative expression of NGF mRNA in the intratibial bone marrow 14 days after

MRMT-1 inoculation. These data are shown as percentage to mean values of the sham

group. Black bar and gray bar indicate MRMT-1 groups (n = 4) and sham groups (n = 4),

respectively. Expression of each mRNA was normalized to expression of RPL27. These

data are reported as means ± SD. **$p < 0.01$ vs. sham group.

Fig. 5. Relative expression of TrkA mRNA in the ipsilateral L3 DRG 14 days after MRMT-1

inoculation. These data are shown as percentage to mean values of the sham group. Black

bars and gray bars indicate the MRMT-1 groups (n = 6) and sham groups (n = 6),

respectively. These data are reported as means ± SD. ** $p < 0.01$, vs. sham group.

Fig. 1, Tomotsuka et al.

(A)

MRMT-1

DAY0   DAY7   DAY14

Vehicle
DAY14

5 mm

(B) ipsilateral

50% PWT (g)

15

10

5

0

0   2   4   6   8   10   12   14
(days)

■ MRMT-1 group
□ Sham group

**   **   **

(C) contralateral

50% PWT (g)

15

10

5

0

0   2   4   6   8   10   12   14
(days)

■ MRMT-1 group
□ Sham group

(D)

Weight bearing ratio
(ipsi./contra.)

1.2

1.0

0.8

0.6

0.4

0.2

0

0   2   4   6   8   10   12   14
(days)

■ MRMT-1 group
□ Sham group

**   **

Fig. 2, Tomotsuka et al.

(A)

BDNF mRNA expression (% of sham group)

- MRMT-1 group
- Sham group

*

L3DRG L4DRG L5DRG

(C)

MRMT-1 group   Sham group

50 μm   50 μm

(B)

BDNF mRNA expression (% of sham group)

- MRMT-1 group
- Sham group

** ** ** * ** **

1-9 2a-9 2b-9 2c-9 3-9 4-9 6-9 9a-9

BDNF variant
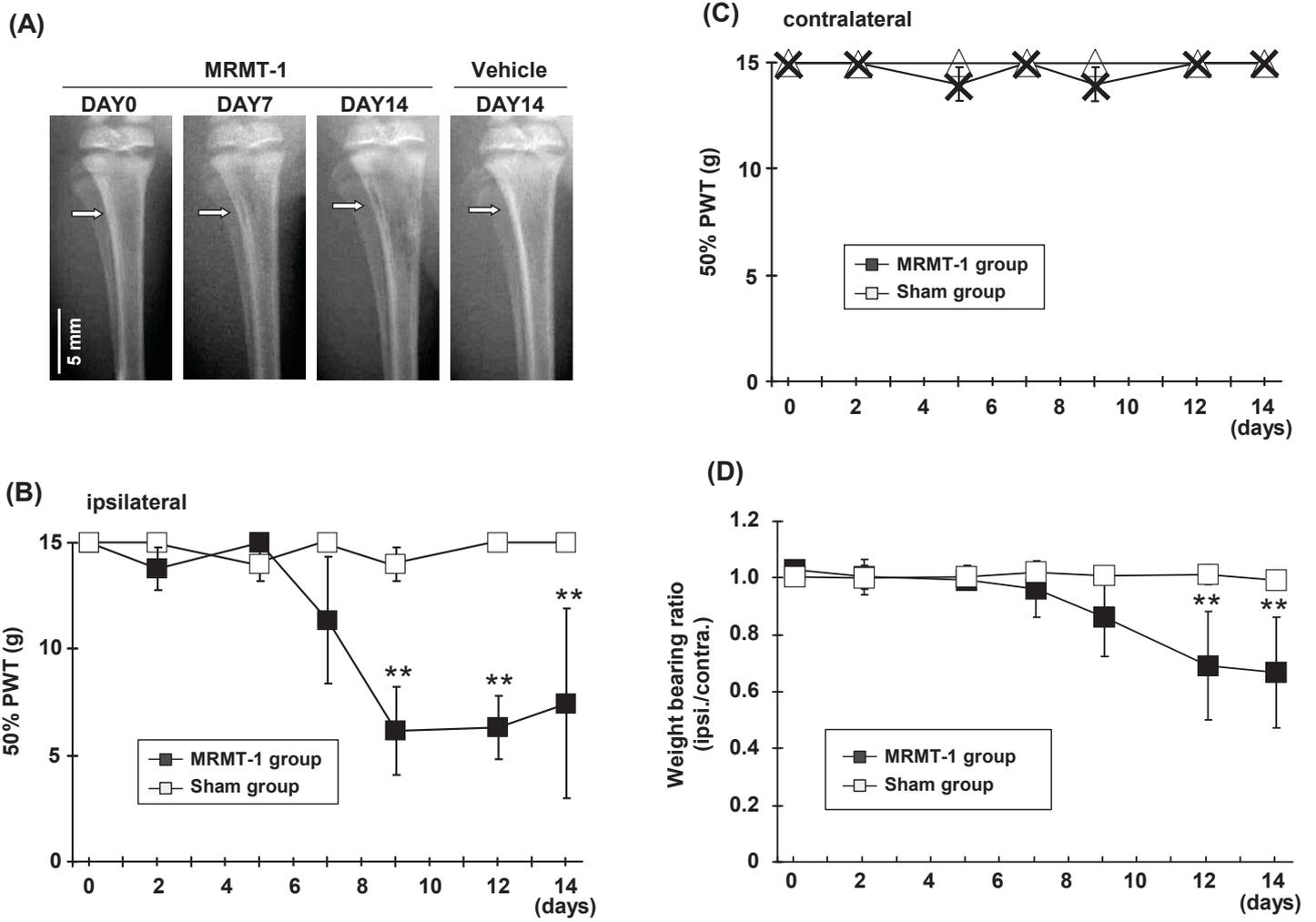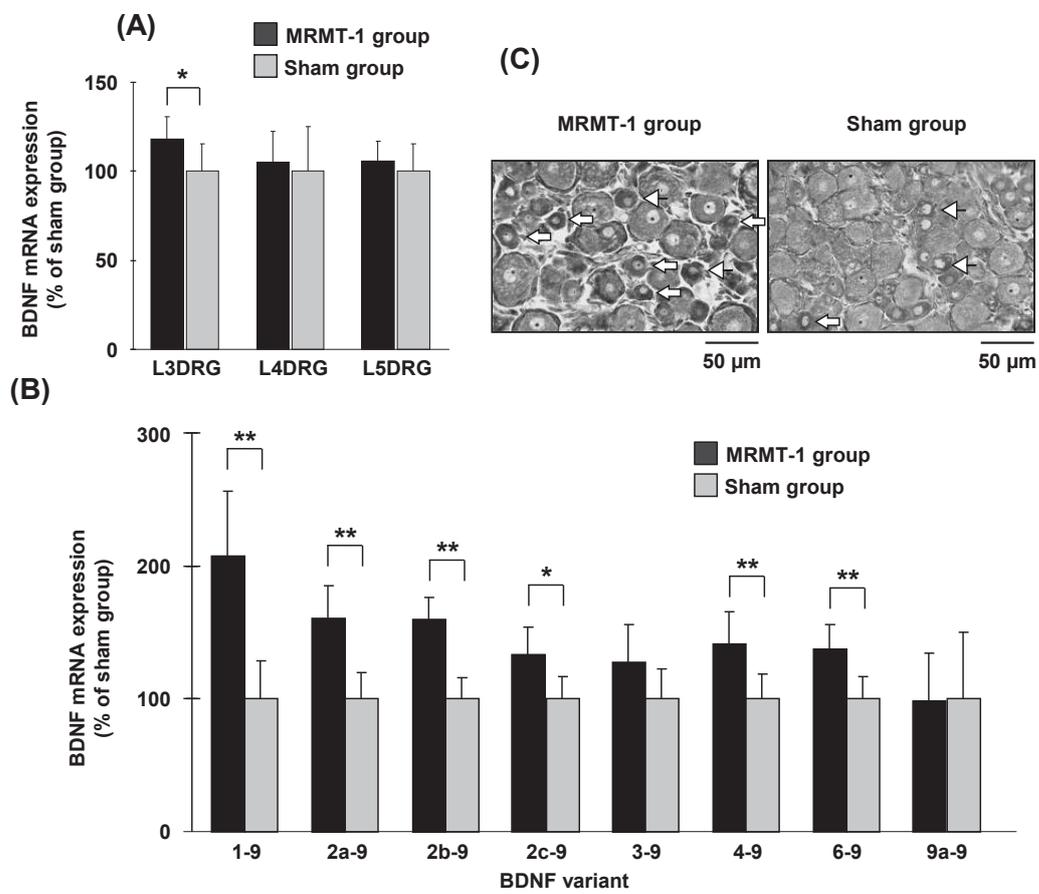
BDNF; Brain-derived neurotrophic factor

Fig. 3, Tomotsuka et al.

NGF; Nerve-growth factor

Fig. 4, Tomotsuka et al.

250

200

150

100

50

0

TrkA mRNA expression
(% of sham group)

**

■ MRMT-1 group
□ Sham group

L3 DRG
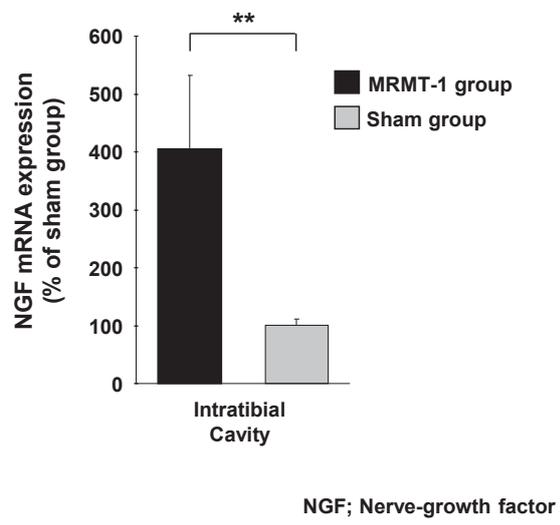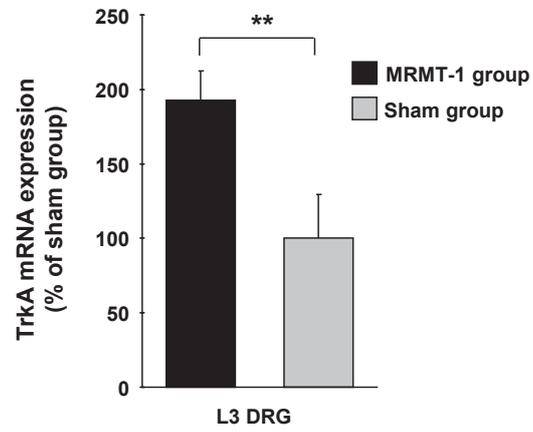
TrkA; tropomyosin receptor kinase A

Fig. 5, Tomotsuka et al.

Table 1. Oligonucleotide sequences of primers for real-time RT-PCR.

Target cDNA	GenBank Acc. No.	Forward primers (5' to 3')	Reverse primers (5' to 3')	Amplicon size (bp)
BDNF 1-9	**EF125675**	tgttggggagacgagatttt	cgtggacgtttgcttctttc	159
BDNF 2a-9	**EF125676**	tacttcatccagttccaccag	caagttgccttgtccgt	129
BDNF 2b-9	**EF125677**	aagctccggttccaccag	tgcttctttcatgggcg	102
BDNF 2c-9	**EF125678**	gtggtgtaagccgcaaaga	cgtggacgtttgcttctttc	124
BDNF 3-9	**EF125686**	ctgagactgcgctccactc	gtggacgtttgcttctttca	152
BDNF 4-9	**EF125679**	gagcagctgccttgatgttt	gtggacgtttgcttctttca	148
BDNF 5-9	**EF125687**	accccgcacactctgtgta	acagctgggtaggccaagtt	204
BDNF 6-9	**EF125680**	gatccgagagctttgtgtgg	gtggacgtttgcttctttca	130
BDNF 8-9	**EF125689**	cagtggagctgaacaaacga	gccttcatgcaaccgaagta	117
BDNF 9a-9	**EF125690**	gtctctgcttccttcccaca	cgtggacgtttgcttctttc	124
Total BDNF	**NM_012513**	gcggcagataaaaagactgc	gcagccttccttcgtgtaac	141
TrkA	**NM_021589**	ttctcaagtgggagctaggg	ctctgcctcacgatggaagt	155
RPL27	**NM_022514**	gaattgaccgctatcccaga	tcgctcctcaaacttgacct	200

The forward primer for each BDNF splice variant is designed from each non-coding

exon. The forward primer for total BDNF is designed from exon 9. All reverse primers

except TrkA and RPL27 are designed from exon 9.

BDNF; Brain-derived neurotrophic factor

TrkA; Tropomyosin receptor kinase A

RPL27; 60S ribosomal protein L27

Table 2. Expression and localization of BDNF in L3, L4, and L5 DRG

DRG Neuron[a]		MRMT-1 group (%)[b]	Sham group (%)[b]	MRMT-1/Sham (fold)	p-value[c]
L3	Total	15.59 ± 1.42	11.62 ± 0.61	1.34	0.0022 **
	Small	35.89 ± 2.11	26.32 ± 1.66	1.36	0.00038 **
	Medium	13.18 ± 2.45	11.26 ± 1.51	1.17	0.23
	Large	3.11 ± 0.99	1.55 ± 1.24	2.00	0.23
L4	Total	13.73 ± 3.79	11.80 ± 2.19	1.16	0.41
	Small	30.01 ± 4.77	26.85 ± 1.77	1.11	0.26
	Medium	13.52 ± 3.72	11.51 ± 3.46	1.17	0.45
	Large	3.56 ± 2.73	3.33 ± 1.75	1.06	0.89
L5	Total	12.73 ± 3.01	9.53 ± 1.26	1.34	0.098
	Small	26.65 ± 2.14	25.29 ± 2.95	1.05	0.48
	Medium	11.59 ± 3.97	8.69 ± 1.27	1.33	0.21
	Large	4.11 ± 2.65	2.00 ± 0.84a	2.05	0.18

[a]DRG neurons were categorized as small (< 20 µm), medium (20–40 µm), and large

(> 40 µm). [b]The data are described as a percentage of BDNF-IR neurons of each

group (N=4). The data are reported as means ± SD. ^{c}MRMT-1 vs. sham group.

**$p<0.01$ vs. sham group.

BDNF; Brain-derived neurotrophic factor

DRG; Dorsal root ganglion