Research Paper

PAIN

Peripheral tetrahydrobiopterin is involved in the pathogenesis of mechanical hypersensitivity in a rodent postsurgical pain model

Hirokazu Arai[a,b,*], Rina Takahashi[a], Yoshiaki Sakamoto[a], Tatsuya Kitano[b], Okishi Mashita[c], Satoshi Hara[a], Satoru Yoshikawa[b], Koh Kawasaki[b], Hiroshi Ichinose[a]

Abstract

Because treatment for postsurgical pain (PSP) remains a major unmet medical need, the emergence of safe and innovative nonopioid drugs has been strongly coveted. Tetrahydrobiopterin (BH4) is an interesting molecule for gaining a better understanding the pathological mechanism of neuropathic pain. However, whether BH4 and its pathway are involved in the pathogenesis of PSP remains unclear. In this study, we found that early in a rat paw incision model, the gene expression of GTP cyclohydrolase 1 (GTPCH) and sepiapterin reductase (SPR), BH4-producing enzymes in the de novo pathway, were significantly increased in incised compared with naive paw skin. Although a significant increase in GTPCH protein levels was observed in incised paw skin until only 1 day after incision, a significant increase in BH4 levels was observed until 7 days after incision. In vivo, *Spr*-knockout mice showed an antinociceptive phenotype in the hind paw incision compared with the wild-type and *Spr* heterozygote groups. Furthermore, QM385, the SPR inhibitor, showed a significant dose-dependent, antinociceptive effect, which was supported by a reduction in BH4 levels in incised skin tissues, with no apparent adverse effects. Immunohistochemical analysis demonstrated that macrophages expressing GTPCH protein were increased around the injury site in the rat paw incision model. These results indicate that BH4 is involved in the pathogenesis of PSP, and that inhibition of the BH4 pathway could provide a new strategy for the treatment of acute PSP.

Keywords: Postsurgical pain, Tetrahydrobiopterin, GTP cyclohydrolase 1, SPR knockout mouse, SPR inhibitor, Nitric oxide, Macrophages

1. Introduction

Postsurgical pain (PSP) remains a significant clinical problem. Nearly 230 million operations are performed every year globally,[57] with about 313 million operations in 2012.[56] Despite progress in pain research and the development of various analgesics and clinical care, approximately 80% of surgical patients still experience PSP,[16,17] and about 25% of patients who receive analgesics experience side effects.[1] In the treatment of PSP, opioids are commonly used because they are available in a variety of formulations and highly effective for relieving PSP.[35,42] In contrast to their high analgesic efficacy, opioids have many dose-limiting side effects, such as respiratory depression, nausea, vomiting, pruritus, and bowel dysfunction.[16,59] The adverse side effects of opioids, as well as abuse and fatal overdoses due to their misuse, have become social problems throughout the world.[43,47] Recently, multimodal analgesia, which is pharmacological treatment using more than one class of nonopioid analgesics, such as nonsteroidal anti-inflammatory drugs, has been recommended for the treatment of PSP to reduce side effects or opioid use.[8,20,58] Several combination therapies have shown clinically relevant analgesic properties and succeeded in reducing the dose of opioids in the treatment of PSP.[2,12,29,38] However, concerns remain that each nonopioid analgesic also has specific dose-limiting side effects,[58] and further systematic reviews or meta-analyses are needed to clarify which combination is effective for reducing the opioid dose when treating PSP.[42] Effective pain relief for surgical patients is needed to maintain their quality of life, reduce medical costs, and prevent the development of chronic PSP.[9,16,28,45,57] Within this context, to provide new nonopioid treatments in the clinic, it is very important to clarify the mechanism of PSP.

BH4 is an essential cofactor for the biosynthesis of monoaminergic neurotransmitters such as dopamine and serotonin (5-HT), and for the production of nitric oxide (NO). Recent studies have suggested that excess BH4 production may lead to pain hypersensitivity. BH4 stimulation increases calcium influx into primary cultured neurons from dorsal root ganglia (DRG) by the production of intracellular NO, which sensitizes both transient receptor potential ankyrin 1 and transient receptor potential vanilloid 1 ion channels.[30,52,63] The increase of calcium influx

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[a] *School of Life Science and Technology, Tokyo Institute of Technology, Yokohama, Japan,* [b] *Laboratory for Pharmacology, Pharmaceuticals Research Center, Asahi Kasei Pharma Corporation, Shizuoka, Japan,* [c] *Laboratory for Safety Assessment and ADME, Pharmaceuticals Research Center, Asahi Kasei Pharma Corporation, Shizuoka, Japan*

**Corresponding author. Address: Laboratory for Pharmacology, Asahi Kasei Pharma Corporation, 632-1, Mifuku, Izunokuni 410-2321, Shizuoka, Japan. Tel.: +81-558-76-8260; fax: +81-558-76-7117. E-mail address: arai.hk@om.asahi-kasei.co.jp (H. Arai).*

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activates intracellular signaling and promotes neuronal excitability, leading to pain in vitro,[5,7] and both intraplantar and intrathecal injection of BH4 can evoke pain hypersensitivity in mice.[31,52] Conversely, inhibiting BH4-producing enzymes, GTP cyclohydrolase 1 (GTPCH, encoded by *GCH1*) or sepiapterin reductase (SPR), has been reported to attenuate both neuropathic and inflammatory pain in rodents.[4,14,23,25,32,40,52] Moreover, human genetic studies have shown that the loss of functional GCH1 haplotype is associated with reductions in several kinds of pain, including PSP.[22,24,26,27,52,62] Although the lines of evidence noted above suggest that the BH4 pathway could be a therapeutic target, the effect inhibiting BH4 production in a PSP model has not been clarified. Therefore, the elucidation of a new signaling pathway in the PSP condition is indispensable for creating new medications as alternatives to opioids in the clinic. Here, we investigated the involvement of BH4 in the pathogenesis of PSP using a rodent plantar incision pain model.

2. Methods

2.1. Animals

For the *Spr*-knockout mouse study, *Spr* heterozygous knockout mice were originally established by Lexicon Pharmaceuticals Inc. (Woodlands, TX) and back-crossed with C57BL/6J or BALB/cCr mice for more than 10 generations. Homozygous *Spr*-knockout mice were generated by crossing male heterozygous C57BL/6J mice with female heterozygous BALB/cCr mice, because the homozygous *Spr*-knockout mice on the mixed background of BALB/c-C57BL/6 survived longer than those on the pure strains.[48] All the animals used in this study, including wild-type (WT) and heterozygous mice, had the same mixed background. Although mixed-background *Spr*-knockout mice also show parkinsonian phenotypes,[48,49,60] *Spr*-knockout mice responded to the von Frey test similarly to the WT mice. Three male WT mice (average age, 27 weeks; age range, 22-37 weeks; body weight, 42.7-46.0 g), 5 male *Spr* heterozygote mice (average age, 26.4 weeks; age range, 22-33 weeks; body weight, 32.2-46.5 g), and 6 male *Spr*-knockout mice (average age, 30.7 weeks; age range, 21-37 weeks; body weight, 8.2-15.2 g) were used in this behavioral study. All mice were housed in plastic cages in groups of 2 to 3 with Paper-clean (Japan SLC Inc, Shizuoka, Japan) as bedding under a controlled environment (room temperature [RT], 22.4 ± 0.6°C; relative humidity [RH], 55% ± 6%) on a 12-hour light/dark cycle (from 08:00 to 20:00). Animals could freely access standard food and sterile mineral water. The mouse study was conducted in accordance with the general guidelines for animal experiments at the Tokyo Institute of Technology.

For the rat von Frey filament study after QM385 treatment, 50 male Sprague-Dawley (SD) rats (age, 6-7 weeks; body weight, 170-200 g) obtained from Hylasco Bio-Technology Pvt. Ltd. (Medchal Dist, India) were used. All rats in this study were housed in plastic cages in groups of 3 to 4 with soft bedding (shredded paper) under a controlled environment (RT, 22.2 ± 2°C; RH, 50% ± 10%) on a 12-h light/dark cycle (from 07:00 to 19:00). Animals could freely access standard food and sterile mineral water. Only the von Frey filament test using the rat PSP model was performed by TCG Lifesciences Private Limited (Kolkata, India) under a protocol approved by the Institutional Animal Care Committee of TCG Lifesciences Private Limited.

For rat studies other than the von Frey filament assay, a total of 129 male SD rats (age, 7 weeks; body weight, 210-260 g) obtained from Charles River Laboratory Japan Inc were used. All rats were housed in plastic cages in groups of 2 to 3 with Paper-clean (Japan SLC Inc) under controlled conditions (RT, 23 ± 3°C; RH, 40%-70%) on a 12-hour light/dark cycle (from 07:00 to 19:00). Animals could freely access standard food and sterile mineral water. All rat experiments were conducted in accordance with the general guidelines for animal experiments at Asahi-Kasei Pharma Corporation (Shizuoka, Japan).

All animals were acclimatized to the laboratory conditions for 1 week and were in a healthy condition before study initiation. In addition, the animals were not administered any drugs or used in any experiments before each study. All animals were promptly euthanized by cervical dislocation or exsanguination under 2% to 3% isoflurane anesthesia at the end of each study.

2.2. Drug administration

QM385 is an orally available, highly potent, specific SPR inhibitor. Detailed information about QM385 has been reported by Cronin et al.[11] QM385 was provided by Asahi-Kasei Pharma Corporation, and tramadol hydrochloride was purchased (42965; Merck, Kenilworth, NJ). QM385 was suspended (2 or 6 mg/mL) in 1% Tween-80 plus 0.5% hydroxypropyl methylcellulose in 50 mM carbonate buffer and administered orally once daily (5 mL/kg/day) for 3 days into PSP model rats. Tramadol hydrochloride was dissolved (10 mg/mL) in water and administered i.p. before behavioral testing (2 mL/kg). The doses of QM385 and vehicle were based on Cronin et al.[11] No adverse effects, such as abnormal behaviors (eg, changes in locomotor activities, sedative effect) or body weight loss, were observed after QM385 dosing in this study.

2.3. Measurement of QM385 concentrations in plasma and brain samples

Blood samples were collected from the caudal veins of 3 intact rats at 0.25, 0.5, 1, 2, 4, 8, and 24 hours after 3 p.o. administrations of QM385 30 mg/kg. In the study collecting brain samples after QM385 treatment, 15 rats were used; these rats were divided into 3 groups based on body weight (n = 5 each). Brain samples were collected after collecting blood from the abdominal aorta at 4 hours after 2 and 3 days of QM385 30 mg/kg p.o. administration and at 4 hours after 3 days of vehicle solution p.o. administration. Blood samples were centrifuged at 11,740*g* for 10 minutes at 4°C, and supernatant (plasma) was stored at −20°C before analysis. Brain samples were divided into right brain and left brain and stored at −20°C before analysis. Right brain samples were used for the measurement of QM385 concentrations and left brain samples for the measurement of dopamine levels. The concentration of QM385 in rat plasma was determined by liquid chromatography tandem mass spectrometry (LC-MS/MS) after protein precipitation with acetonitrile/methanol (9/1) solution.

For plasma bioanalysis, a seven-point calibration curve, ranging from 0.03 to 30 μM, was prepared. A 300 μM solution of QM385 in dimethyl sulfoxide (DMSO) was serially diluted in 100% DMSO, and then 2 μL each of standard solution were added to 18 μL of rat plasma. Proteins were precipitated from 5 μL of a plasma sample by the addition of 300 μL of acetonitrile/methanol (9/1) solution containing internal standard (5 nM buspirone, 20 nM tolbutamide, and 10 nM reserpine). Samples were mixed for 5 minutes in a shaker and spun in a centrifuge for 15 minutes at 2000*g* at 4°C. An aliquot of the supernatant was analyzed by LC-MS/MS.

For brain bioanalysis, an eight-point calibration curve ranging from 0.01 to 30 μM was prepared. A 300 μM solution of QM385 in DMSO was serially diluted in 100% DMSO, and then 2 μL of each standard solution were added to 18 μL of rat brain homogenate. Proteins were precipitated from 5 μL of a brain

homogenate sample by the addition of 300 μL of acetonitrile/methanol (9/1) solution containing internal standard. Samples were mixed in a shaker and spun in a centrifuge at 2000*g* for 15 minutes at 4°C. An aliquot of the supernatant was filtered with MultiScreen Filter Plates (0.45 μm Hydrophobic PTFE; Millipore, Burlington, MA), and then the filtered aliquots were analyzed by LC-MS/MS. Kp values were calculated from the total plasma and brain concentrations of QM385.

2.4. Plantar incision

The hind paw plantar incision for induction of PSP was performed following the protocols outlined by Cowie and Stucky[10] for mice and by Brennan et al.[3] for rats. Briefly, animals were given an intramuscular injection of penicillin (60,000 units/mouse and 30,000 units/rat) and 2% to 3% isoflurane anesthesia. For mice, a 5-mm, longitudinal incision was made 2 mm from the end of the heel using a sterile disposal scalpel (No.11; Feather Safety Razor Co, Ltd, Osaka, Japan). For rats, a 1-cm, longitudinal incision was similarly made from 0.5 cm from the end of the heel. The plantaris muscle was elevated and incised longitudinally. After hemostasis, the skin incision was sutured by 5-0 silk suture (Ethicon Inc, Cincinnati, OH), and 10% povidone-iodine solution was then applied to the wound site. After surgery, the animals were allowed to recover in their cages.

2.5. von Frey filament test

Animals were allowed to habituate in the experimental room and acclimatize into plastic chambers with a wire mesh platform for 1 hour before study initiation. Measurements of mechanical allodynia for the hind paw were obtained using the up/down methods of Dixon and Chaplan[6,13] with a series of von Frey monofilaments (0.02, 0.04, 0.07, 0.16, 0.4, 0.6, 1.0, and 1.4 g for the mouse study, and 0.4, 0.6, 1, 2, 4, 6, 8, 10, 15, and 26 g for the rat study; North Coast Medical Inc, Morgan Hill, CA). These monofilaments were applied individually from under the chambers through openings in the wire mesh to the medial–posterior plantar aspect for the mouse study and the middle of the plantar surface for the rat study and pressed until bending. Flinching, flicking, and shaking responses were defined as withdrawal responses.

For the *Spr*-knockout mouse study, mechanical allodynia was measured at presurgery and at 1, 2, 3, 4, and 7 days postsurgery. Three WT mice, 5 male *Spr* heterozygote mice, and 6 male *Spr*-knockout mice were used. The von Frey filament assay was not conducted under blinding due to the smaller body size of *Spr*-knockout mice.[60] For the QM385 administration study, mechanical allodynia was measured at the following time points: presurgery (day 0), 1, 2, and 4 hours after drug administration on day 1, and 1, 2, and 4 hours after drug administration on day 2. In this study, 50 male SD rats were used. Animals were randomly divided into one control group and 4 different treatment groups based on premeasured paw withdrawal threshold (PWT) values (n = 10). The experimenters taking the allodynia readout were completely blinded to the treatments given to the animals.

2.6. Reverse transcription quantitative polymerase chain reaction

In this study, a total of 32 rats were used. The rats were randomly divided into 8 groups based on body weight before paw incision (n = 4). Dorsal root ganglia and plantar skin including the epidermis, dermis, and hypodermis were collected from euthanized nonsurgical (normal) animals or from animals at 1, 3, and 6 hours, and 1, 2, 3, 4, and 7 days postsurgically. The plantar skin tissue surrounding the incision was excised with margins of approximately 5 mm. After excising these samples, they were immediately immersed in RNAlater Stabilization Solution (AM702; Thermo Fisher Scientific, Waltham, MA) and stored at 4°C for 24 hours. Total RNA was extracted and purified using the RNeasy Lipid Tissue Mini Kit (74804; Qiagen, Hilden, Germany) for skin and the RNeasy Mini Kit (74104; Qiagen) for DRG, following the manufacturer's instructions. Each RNA sample was reverse transcribed into cDNA using a Super Script VILO cDNA Synthesis Kit (11754-050; Thermo Fisher Scientific). Then, 100 ng of cDNA were amplified with the THUNDER BIRD Probe qPCR Mix (QPS-101; Toyobo, Osaka, Japan) and a TaqMan Gene Expression Assays custom probe (Thermo Fisher Scientific) for gene encoding in the ViiA 7 real-time PCR system (Thermo Fisher Scientific) as follows: GTP cyclohydrolase 1 (Rn00577450_m1), sepiapterin reductase (Rn01425678_g1), eukaryotic 18S rRNA (Hs99999901_s1), inducible nitric oxide synthase (Rn00561646_m1), and tryptophan hydroxylase 1 (Rn01476867_m1). The amplification reaction was run with an initial heating step at 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and at 60°C for 1 minute. All reverse transcription quantitative polymerase chain reaction (RT-qPCR) reactions were run in duplicate. The ΔΔCt method was used for data analysis, and eukaryotic 18S rRNA (Hs99999901_s1) was used as a reference gene to normalize the other target genes because alterations in the expression level of 18S rRNA were least common among several other genes used for normalization, such as beta-actin, GAPDH, and HPRT in the skin tissue after paw incision in the preliminary study (data not shown).

2.7. Western blot

In this study, a total of 16 rats were used. The rats were randomly divided into 4 groups based on body weight after paw incision (n = 4). Plantar skin including the epidermis, dermis, and hypodermis was collected from both sides of hind paws of euthanized animals at 6 hours and at 1, 2, and 3 days postsurgically. The plantar skin tissue surrounding the incision was excised in the same way as for the RT-qPCR experiment, with margins of approximately 5 mm. After excising these samples, they were immediately immersed in liquid nitrogen and stored at −80°C. These tissues were homogenized with 500 μL CelLytic MT Cell Lysis Reagent (C3228; Merck) and 5 μL protease inhibitor cocktail (25955-11; Nacalai Tesque, Kyoto, Japan) in the presence of a 5-mm zirconia bead using Micro Smash MS-100 (TOMY SEIKO Co, Ltd, Tokyo, Japan) under the following homogenizing condition: 10 cycles of 4000 r/minute for 30 seconds with intervals of 30 seconds at 4°C. Homogenized samples were centrifuged at 17,700*g* for 10 minutes at 4°C, and the amount of total protein in the supernatant was quantified using a Pierce BCA Protein Assay Kit (23227; Thermo Fisher Scientific). Then, 15 μg of total protein were loaded on a 12.5% real gel plate (MDG-236; Bio Craft, Tokyo, Japan) and transferred to nitrocellulose membranes (Amersham Protran Premium NC 0.45; GE Healthcare, Chicago, IL). The membranes were blocked with 2.5% skim milk (198-10605; Fujifilm Wako, Osaka, Japan) for 1 hour at RT and then incubated with primary antibodies against GAPDH (SC32233; mouse anti-GAPDH monoclonal; Santa Cruz Biotechnology, Santa Cruz, CA; dilution, 1:1000), GTP cyclohydrolase 1 (NBP1-84949; rabbit anti-GCH1 polyclonal; Novus Biologicals, Centennial, CO; dilution 1:100), or inducible NOS (NOS2) (ab3523; rabbit anti-iNOS polyclonal; Abcam, Cambridge, United Kingdom; dilution 1:200) overnight at 4°C. The membranes were washed and incubated with horseradish

peroxidase (HRP)-labeled sheep anti-mouse IgG secondary antibody (NA931; GE Healthcare; dilution 1:10,000 for skin samples, 1:20,000 for muscle samples) or HRP-labeled goat anti-rabbit IgG secondary antibody (ab6721; Abcam; dilution 1:1000 for GCH1, 1:10,000 for NOS2) for 1 hour at RT. The HRP signal was enhanced using Immobilon ECL Ultra Western HRP Substrate (WBUL0100; Merck) and detected using a ChemiDoc touch imaging system (Bio-Rad, Hercules, CA). Quantification of the protein contents was performed using Image J software (version 1.50i; National Institutes of Health, Bethesda, MD), and then the relative quantity of protein with respect to GAPDH was calculated.

2.8. BH4, dopamine, 5-HT, and nitrite/nitrate measurements

For the study using nontreatment paw-incision rats, 30 rats were used and randomly divided into 5 groups (n = 6) after paw incision. For the study using QM385 treatment paw-incision rats, 32 rats were used and randomly divided into 2 vehicle treatment groups and 2 QM385 treatment groups (n = 8) after paw incision. The skin tissues, including the epidermis, dermis, and hypodermis, were collected from the euthanized animals at 1, 2, 3, 4, and 7 days postsurgically or the vehicle and QM385-administered animals at 3 days postsurgically. The plantar skin tissue surrounding the incision was excised with margins of approximately 5 mm. After excising the skin samples, they were immediately immersed in liquid nitrogen and stored at −80°C until use. For measurement of dopamine levels in the brain, we used left brain samples at 4 hours after 2 and 3 days of QM385 30 mg/kg p.o. administration and at 4 hours after 3 days of vehicle solution p.o. administration (n = 5). Samples were homogenized with 600 μL of 0.2 M perchloric acid containing 5 mM ascorbic acid and 0.1 mM ethylenediaminetetraacetic acid (EDTA; for BH4, dopamine, and 5-HT) or PBS (for nitrite) in the presence of a 5-mm zirconia bead using Micro Smash MS-100R (TOMY SEIKO Co, Ltd) under the following homogenizing condition: 10 cycles of 4000 r/minute for 30 seconds with intervals of 30 seconds at 4°C. Homogenized samples were centrifuged at 17,700*g* for 10 minutes at 4°C, and supernatant was used for the analysis.

BH4, dihydrobiopterin (BH2), and biopterin levels in the supernatant were measured simultaneously and separately using a postcolumn oxidation method[50] with slight modifications. These were then separated using an Inertsil ODS-3 column (i.d. 3.0 × 250 mm; GL-Science, Tokyo, Japan) using 0.1 mol/L sodium phosphate buffer (pH 3.0) containing 5% methanol, 3 mmol/L sodium 1-octanesulfonate, 0.1 mmol/L EDTA, and 0.1 mmol/L ascorbic acid to protect BH4 oxidation during separation in the high-performance liquid chromatography (HPLC) system. BH4 and BH2 were then oxidized to biopterin with 20 mmol/L $NaNO_2$ in-line and monitored by fluorescence detection (excitation 375 nm, emission 465 nm). The peak area was compared with that of the authentic sample of biopterin. Because BH4 is an unstable substance, 5 mM ascorbic acid was added to the homogenizing solution to protect oxidation during analysis, and all samples were analyzed by HPLC within 10 hours after preparation. Dopamine and 5-HT levels in the supernatant were analyzed by HPLC with an electrochemical detector using an SC5-ODS column (EICOM, Kyoto, Japan) and a mobile phase, which contained 84 mM acetic acid-citrate (pH 3.5), 5 mg/mL EDTA, 190 mg/mL sodium 1-octanesulfonate, and 16% methanol. The nitrate/nitrite levels in the supernatant were measured using the NO_2/NO_3 Assay Kit-FX (NK05; Dojindo, Kumamoto, Japan).

2.9. Immunohistochemistry

In this study, 6 rats were used. The rats were randomly divided into 2 groups after paw incision (n = 3). Hind paws were collected from the euthanized animals at 1 and 2 days postsurgically. The samples were fixed in 4% paraformaldehyde (163-20145; Fujifilm Wako) at 4°C for 24 hours and then paraffin-embedded. The tissues were sectioned at 3 μm thickness on a cryotome. For deparaffinization and rehydration, sections were immersed in Patho Clean solution (163-20145; Fujifilm Wako), ethanol with different concentrations, and water, respectively. Antigen retrieval was performed at 105°C for 10 minutes in Target Retrieval Solution (S1699; Carpinteria, CA) using an autoclave. After 1 hour of blocking with 5% bovine serum albumin (BSA) solution (A8412-100 ML; Sigma-Aldrich, St. Louis, MO), sections were incubated with the primary antibody in 5% BSA solution at 4°C overnight. The following were used as primary antibodies: anti-GCH1 antibody (NBP1-84949; Novus Biologicals, Centennial; dilution 1:50), anti-CD11b antibody (ab8879; Abcam; dilution 1:100), anti-CD68 antibody (ab955; Abcam; dilution 1:100), and anti-mast cell tryptase antibody (ab2378; Abcam; dilution 1:500). After washing with phosphate buffered saline with Tween 20 (PBST), sections were incubated with the secondary antibody in 5% BSA solution or Rhodamine Avidin DCS (A-2012; Vector Laboratories, Burlingame, CA; dilution 1:10,000) in PBST at RT for 1 hour. The following were used as secondary antibodies: goat anti-rabbit IgG (H + L) highly cross-adsorbed secondary antibody, Alexa Fluor 488 (A-11034; Thermo Fisher Scientific; dilution 1:1000), and goat anti-mouse IgG (H + L) highly cross-adsorbed secondary antibody, Alexa Fluor 568 (A-11031; Thermo Fisher Scientific; dilution 1:1000). Slides were finally washed by PBST and mounted in Fluoromount/Plus (K048; Pleasanton, CA). Microscopy and imaging were performed using a fluorescence microscope (BZ-9000; Keyence, Osaka, Japan), and composite images were created using a BZ-II Analyzer (version 1.4.2; Keyence).

2.10. Statistical analysis

Data are presented as mean ± SD or ± standard error or the mean, as indicated. One-way analysis of variance (ANOVA) followed by Dunnett multiple-comparisons post hoc test was used for the RT-qPCR analysis. The two-tailed, unpaired Student *t*-test was used to compare a single measurement between 2 groups. In the behavioral study, the Mann–Whitney *U* test was used for comparisons between 2 groups, and the Kruskal–Wallis test with a post hoc Dunn multiple pairwise comparison test was used for comparisons between the vehicle and other treatment groups at each evaluation time point. No power analysis was performed. The numbers of animals used in the experiments were based on our experience with these behavioral tests. All animal data were used for statistical analysis without exclusion. Statistical analysis was performed using Graph-Pad Prism 7 software (San Diego, CA). *P* values <0.05 were considered significant.

3. Results

3.1. Upregulation of BH4, NO, and 5-HT-producing enzymes at the incision site after surgery

First, the expressions of *Gch1* and *Spr* in the skin around the incision site over 7 days after paw injury were examined in rats. The mRNA levels of *Gch1* and *Spr* were significantly increased over 6 hours to days compared with skin without surgery (**Figs.**

1A and B), whereas they were not changed in DRG tissue over 7 days (Supplemental Fig. 1, available at http://links.lww.com/PAIN/B65). Significant changes of GTPCH protein contents in skin tissue were confirmed only at 24 hours after incision by Western blot, coincident with increased *Gch1* gene expression (**Figs. 2A and B**). Regarding the gene expression and Western blot analyses for BH4-producing enzymes, we conducted 2 experiments independently and obtained similar results regarding the time-dependent fashion (Supplemental Figs. 2 and 3, available at http://links.lww.com/PAIN/B65).

BH4 is a cofactor of NO- and 5-HT-producing enzymes. Previous studies with GTPCH transgenic mice showed that GTPCH overexpression enhances the production of BH4 and NO, and increases the release of 5-HT from mast cells.[11,55,63] In addition, other studies have reported that both NO and 5-HT are linked to the pathogenesis of PSP.[15,18,37] Nitrite and 5-HT levels at the injury site were significantly increased after paw incision in a rodent PSP model.[18,37] These findings raised the possibility that upregulation of GTPCH levels leads to increases in NO and 5-HT levels, which are related to nociception in PSP models. To test this hypothesis, the expressions of inducible NOS (*Nos2*) and tryptophan hydrolase I (*Tph1*) in the skin were measured in rats over 7 days after paw injury. As with the *Gch1* and *Spr* genes, *Nos2* mRNA levels were also markedly increased in incised skin tissues at every time point compared with skin without surgery (**Fig. 1C**). Regarding the *Tph1* gene, a significant increase in mRNA levels was observed, but this was only a transient change at a few hours after the skin incision (**Fig. 1D**). A Western blot study was conducted to confirm whether the protein contents of NOS2 also increased in incised tissues. As with the GTPCH protein contents, the results showed that the NOS2 protein contents were significantly increased in the incised skin at only 24 hours after paw incision compared with skin without surgery (**Figs. 2A and C**). Furthermore, the nitrate/nitrite levels in the incised skin were measured at 1, 2, 3, 4, and 7 days after incision. Unexpectedly, despite elevations in the gene expression and protein contents of NOS2, the nitrate/nitrite levels in the incised skin did not change throughout all time points compared with the contralateral side (data not shown).

3.2. Increased BH4 levels in the skin after incision

A previous study showed that the GTPCH level correlated with the BH4 level.[51] Owing to this evidence, with GTPCH protein levels increasing after paw incision, one would expect that the BH4 levels in tissue would also be increased. Therefore, the changes in BH4 levels in the skin tissue measured at 1, 2, 3, 4, and 7 days after paw incision were evaluated. The BH4 levels in the skin were significantly increased in the ipsilateral compared with the contralateral site at all time points (**Fig. 3**). The BH2 levels in the incised skin tissues were also assessed at the same time points. The BH4/BH2 ratio was continuously higher in the incised than in the contralateral skin at all time points (Supplemental Fig. 4, available at http://links.lww.com/PAIN/B65).

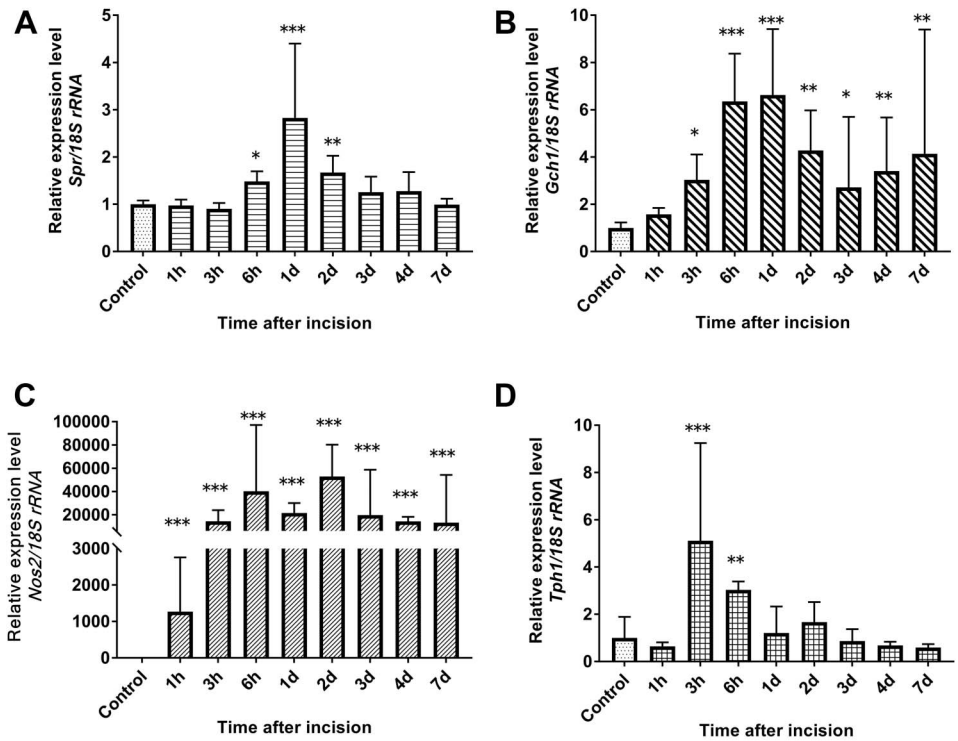


Figure 1. Increased gene expression of *Spr*, *Gch1*, *Nos2*, and *Tph1* in skin tissues after paw incision. Gene expression profiles of sepiapterin reductase (*Spr*) (A), GTP cyclohydrolase I (*Gch1*) (B), inducible nitric oxide synthase (*Nos2*) (C), and tryptophan hydrolase I (*Tph1*) (D) in skin tissues were determined by reverse transcription quantitative polymerase chain reaction (RT-qPCR). The expression of each gene was measured at baseline (control) and at 6 hours to 7 days after paw incision. The ΔΔCt method was used for data analysis to calculate relative changes in gene expression as determined by RT-qPCR. The fold-change of the gene expression level was calculated as the relative cDNA amount of a target gene in incised and nonincised skin tissues, normalized to the 18S ribosomal RNA (*18S rRNA*) reference gene. Data are expressed as mean fold-changes (incision/nonincision control) + SD. Different groups of rats were used for each time point (n = 4 per group). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs the value of control with one-way analysis of variance followed by Dunnett post hoc test.

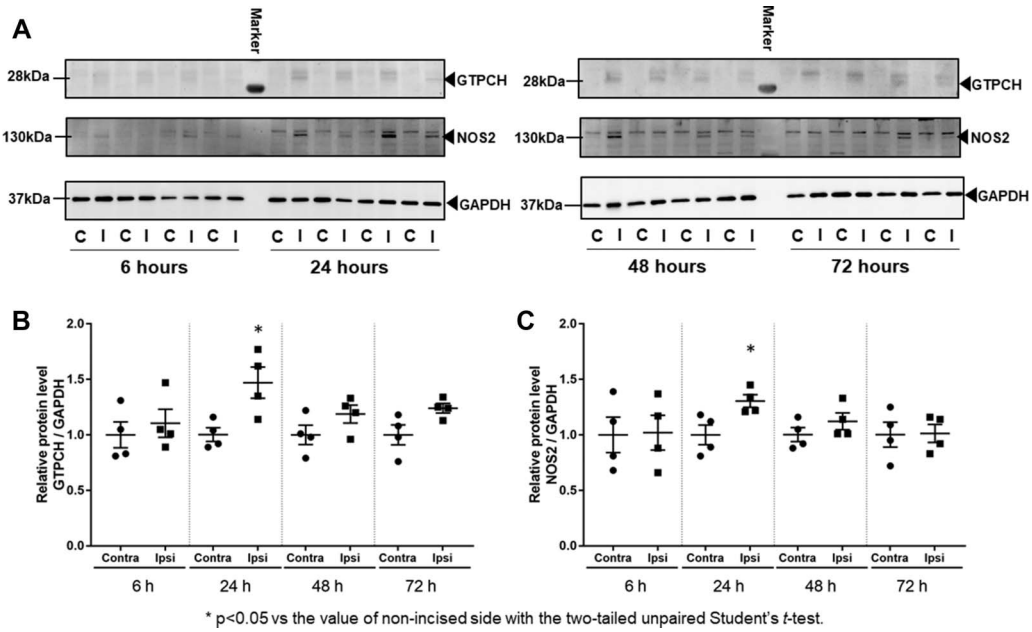


Figure 2. Increased GTPCH and NOS2 protein contents in damaged skin after paw incision. Skin tissue protein lysates were prepared and Western blot analysis was performed to detect the protein contents of GTPCH and NOS2 protein, with GAPDH serving as the loading control (A). Quantitative analysis of GTPCH and NOS2 protein contents in incised and nonincised skin tissues (B and C, respectively). The amount of each protein was quantified by correcting for the amount of GAPDH protein. Data shown are the values normalized by quantifying the average value of nonincised samples at each evaluation point. GTPCH and NOS2 protein contents in incised skin tissues were increased at 24 hours after paw incision compared with nonincised skin. Skin samples were collected at 6, 24, 48, and 72 hours after paw incision (n = 4 per each time point). The dot plots show mean fold-changes (incision/nonincision site) ± standard error. *$P < 0.05$ vs the value of the nonincised side using a two-tailed unpaired Student *t*-test. I and Ipsi: ipsilateral side (incised side), (C) and Contra: contralateral side (nonincised side). GTPCH, GTP cyclohydrolase 1.

3.3. Spr-knockout mice have an antinociceptive phenotype in the postsurgical pain model

To check that elevation of BH4 levels at the incision site was related to nociception by paw incision, behavioral testing was conducted using *Spr*-knockout mice. Because SPR is one of the BH4-producing enzymes in the de novo pathway, the BH4 levels in several tissues of *Spr*-knockout ($SPR^{-/-}$) mice were lower than those in WT ($SPR^{+/+}$) and heterozygote ($SPR^{+/-}$) mice.[48,49,60] Although it has been reported that the body size of *Spr*-knockout mice is smaller than that of WT mice, and the parkinsonian phenotype with body movement abnormalities has been observed,[49,60] the sensitivity to mechanical stimuli applied to the paw surface by von Frey filaments was found to be the same as that in WT and heterozygote mice (**Fig. 4**). Because the above findings suggested that parkinsonian motor defects were not likely to interfere with our examination to measure mechanical sensitivity, we decided to use *Spr*-knockout mice in the behavioral testing. The BH4 levels in several tissues and monoamine levels in the brain of heterozygote mice are the same as those WT mice,[49] and no studies have reported any behavioral or biochemical differences between WT and heterozygote mice; therefore, WT and heterozygote mice were included in the same group for the behavioral testing. The von Frey filament test was performed 24 hours before surgery to determine the basal PWT, and then at 6 hours and 1, 2, 3, 4, and 7 days after paw incision. No weight loss or deterioration of health condition was observed after paw incision. At every time point after paw incision, the *Spr*-knockout mouse group showed a significantly higher PWT than did the WT and heterozygote groups (**Fig. 4**). The PWT of some *Spr*-knockout mice almost reached the basal value at 4 days after paw incision. However, some potential limitations should be noted. Due to the short life-span of *Spr*-knockout mice, it was difficult to prepare a sufficient number of animals. For this reason, the number of *Spr*-knockout mice in this study was small. Furthermore, in the *Spr*-knockout mice, levels of neurotransmitters, such as dopamine, norepinephrine, and 5-HT, decreased in the brain as a result of lowering the BH4 level by *Spr* deficiency.[48,49,60] Several studies have reported that decreased 5-HT and norepinephrine levels in the brain cause depression and pain;[34] thus, it is unlikely that these decreases are related to analgesia, but these neurotransmitters are closely tied to mood and emotion.[44] Therefore, it is not possible to exclude completely the effects of decreasing monoamine levels in the brain and motor disturbance in the *Spr*-knockout mice on the behavioral testing results.

3.4. SPR inhibitor QM385 attenuates mechanical hypersensitivity in the rat postsurgical pain model

Based on the results of the *Spr*-knockout mouse study and its limitations, a drug administration study was conducted to exclude any concerns that neurotransmitters lacking in central nervous system tissues and motor disturbances affected the results of the *Spr*-knockout mice behavioral testing. The SPR inhibitor QM385 was selected for this purpose. QM385 is an orally available, highly potent, specific SPR inhibitor that decreases BH4 levels in the body in a dose-dependent manner.[11] In the pharmacokinetic study, 3 days of QM385 administration showed that free concentration in plasma was higher than the reported IC_{50} value[11]; here, we confirmed that the Kp value of QM385 relative to the brain tissue was low in rats (**Figs. 5A and B**). In the drug administration study, QM385 was administered orally for 3 days (once daily from 1 hour after operation), and von Frey filament assays were conducted after the second and third administrations; tramadol was also

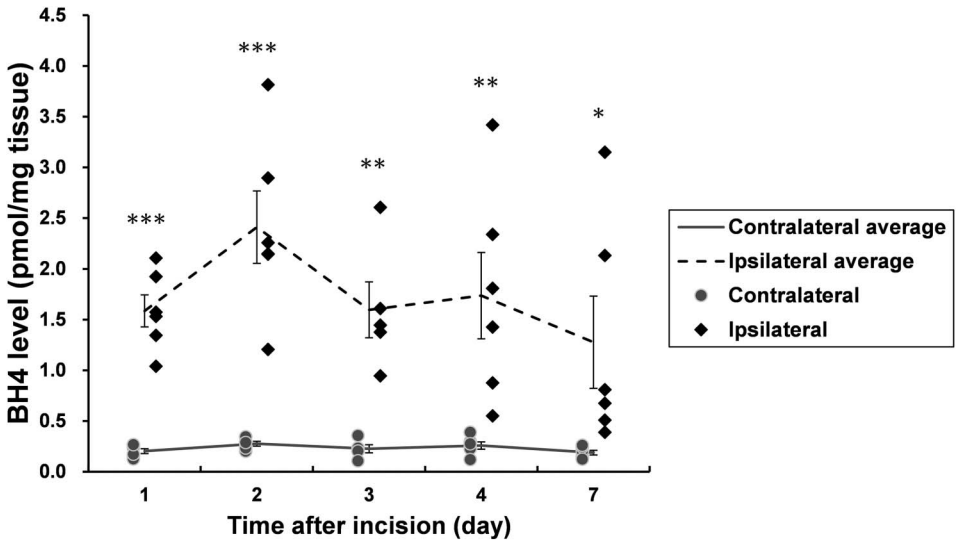
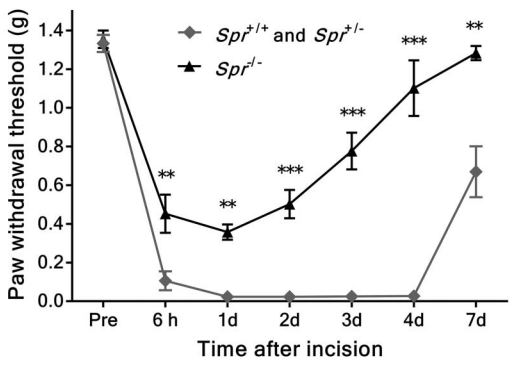


Figure 3. BH4 levels in the skin tissues were significantly increased after paw incision. BH4 levels are shown in the ipsilateral (incised) and contralateral (nonincised) skin after paw incision. BH4 levels were significantly increased in incised skin at every time point after incision (black dotted line). Both sides of skin samples were collected at 1, 2, 3, 4, and 7 days after paw incision (n = 5-6 per each time point). The dot plots show mean ± standard error of the mean. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs the value of the nonincised side using a two-tailed unpaired Student *t*-test.

administered to compare antinociceptive efficacies. QM385 significantly increased the PWT in a dose-dependent manner without any abnormal clinical signs (**Fig. 5C**). The antinociceptive efficacy of QM385 was highest at 4 hours after administration, and its effect was equivalent to that of tramadol (**Fig. 5C**). BH4 levels were measured in the injured skin tissues at 2 and 4 hours after QM385 administration to confirm the correlation between decreasing BH4 levels and antinociceptive efficacy. Significant decreases in BH4 levels were observed at both time points in the QM385 compared with the vehicle treatment group (**Fig. 5D**); average BH4 levels were reduced by 43% and 63% at 2 and 4 hours after QM385 administration, respectively. Dopamine levels in the brain tissue were not changed at 4 hours after the second and third time QM385 administrations (**Fig. 5E**).



**P<0.01, ***P<0.001 vs the value of the SPR (+/+) and SPR (+/-) group with the Mann-Whitney's U -test at each evaluating time points

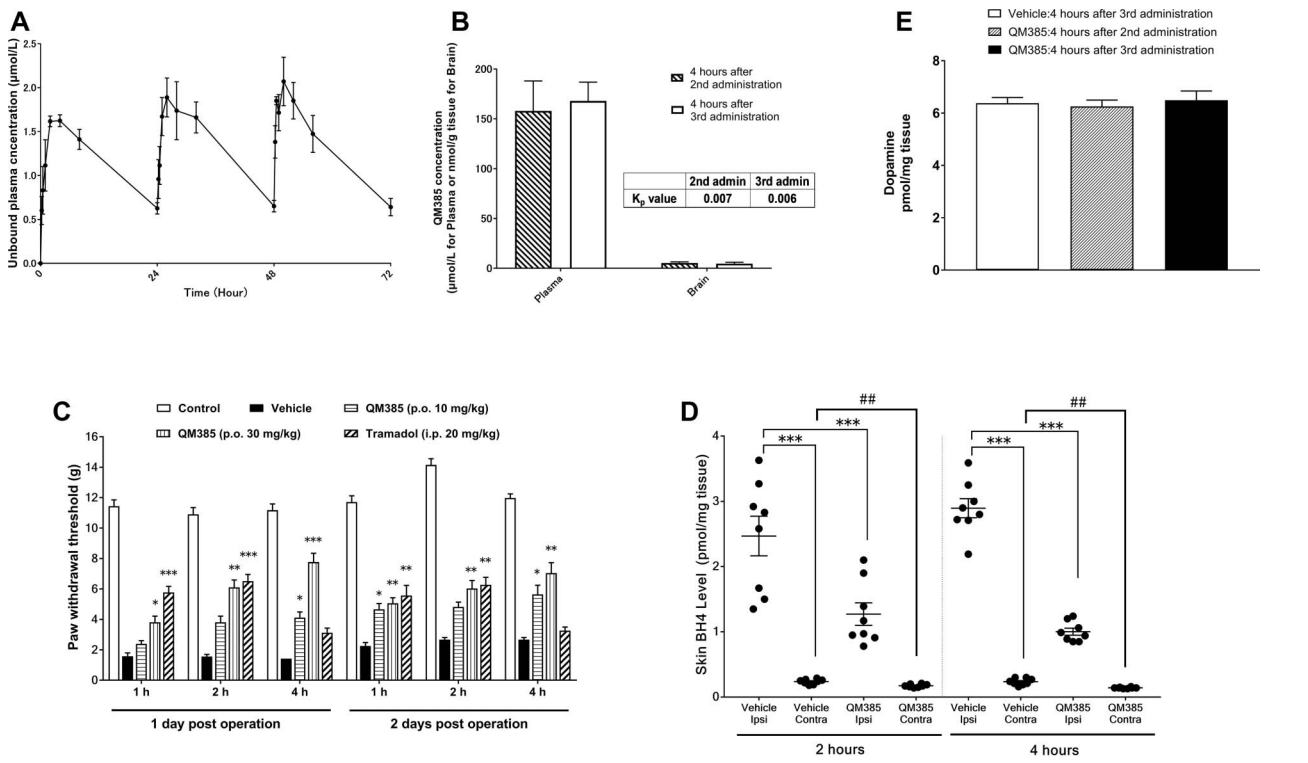
Figure 4. *Spr*-knockout mice have an antinociceptive phenotype in the postsurgical pain model. The PWT on mechanical stimulation by von Frey filaments at 24 hours before and 6 hours and 1, 2, 3, 4, and 7 days after paw incision. The basal PWT is almost the same between the *Spr*-knockout ($SPR^{-/-}$) and the WT ($SPR^{+/+}$) and *Spr*-heterozygote ($SPR^{+/-}$) mouse groups. The PWT was significantly higher in the $SPR^{-/-}$ than in the $SPR^{+/+}$ and $SPR^{+/-}$ groups after paw incision. Data are expressed as mean ± standard error of the mean (n = 8; $SPR^{+/+}$ and $SPR^{+/-}$ group, n = 6; $SPR^{-/-}$ group). $^{**}P < 0.01$ and $^{***}P < 0.001$ vs the value of the $SPR^{+/+}$ and $SPR^{+/-}$ groups using the Mann–Whitney *U* test at each evaluation time point.

3.5. GTP cyclohydrolase 1 protein was expressed in macrophages in the injured tissue after paw incision

The data presented above strongly indicated that peripheral BH4 is related to nociception in the paw incision pain model. Next, the cell types that express the BH4-producing enzyme GTPCH at the lesion site were examined to help determine how BH4 induces pain behavior after paw incision. In previous studies, *Gch1*-expressing macrophages were observed at damaged nerves in a neuropathic pain model,[25] and mast cells that secrete 5-HT produced using BH4 were involved in postoperative nociception.[37] Given these findings, this study focused on macrophages and mast cells, and an immunohistochemical analysis was performed to identify the localization of GTPCH. The immunohistochemical results showed that macrophage protein marker CD11b- and CD68-positive cells were increased in the ipsilateral side of paw tissue 1 day after incision compared with the contralateral side (**Figs. 6A and B**). Only a few CD11b- and CD68-positive cells were observed in the contralateral side. GTPCH protein was colocalized with CD11b and CD68 in the ipsilateral side of tissues at 1 and 2 days after incision (**Figs. 6C and D**, and data not shown). However, although GTPCH protein was colocalized with the mast cell protein marker mast cell tryptase (MCT), GTPCH protein-expressed cells were not clearly merged with fluorescent avidin, a protein that binds to mast cell granules (**Fig. 6E** and Supplemental Fig. 5, available at http://links.lww.com/PAIN/B65). Localization of GTPCH was observed in almost all CD11b- and CD68-expressing cells, whereas it was observed in only some MCT-expressing cells (**Figs. 6B–D**).

4. Discussion

The novel findings in this study are as follows. (1) The gene expressions of *Gch1* and *Spr*, GTPCH protein levels, and BH4 levels were significantly increased in incised tissues after paw



***p < 0.05, **P < 0.01, ***P < 0.001 vs the value of the vehicle treatment group with the Kruskal-Wallis test with post hoc Dunn Multiple pairwise comparison test at each evaluating time points.**

Figure 5. Peripherally acting SPR inhibitor QM385 attenuates mechanical hypersensitivity in a rat postsurgical pain model and decreases BH4 levels in the skin tissues. Unbound plasma concentration of QM385 after 3 administrations (A) and plasma and brain concentrations of QM385 at 4 hours after the second and third administrations (B). QM385 showed low intracerebral transferability. The Kp value, the ratio of the total concentration in brain tissue to blood, was calculated from total plasma and brain concentrations of QM385. Data are expressed as mean ± SD (n = 3 for the pharmacokinetic study and n = 5 for the brain concentration measurements). Reversal of the PWT was assessed at 2 and 4 hours postdose on days 1 and 2 after QD p.o. administration of 10 and 30 mg/kg QM385 on days 0, 1, and 2 and i.p. administration of 20 mg/kg tramadol on days 1 and 2 (C). Data are expressed as mean ± standard error of the mean (n = 10 per each group). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs the value of the vehicle treatment group using the Kruskal–Wallis test with a post hoc Dunn multiple pairwise comparison test at each evaluation time point. BH4 levels in the skin at 2 and 4 hours post QM385 dose on day 2 (D). BH4 levels were significantly decreased at both time points. Both sides of skin samples were collected from the vehicle and QM385 treatment groups at 2 and 4 hours after administration (n = 8 per each treatment group). The dot plots are shown as mean ± standard error of the mean. $^{**}P < 0.01$ and $^{***}P < 0.001$ vs the value of nonincised skin in the vehicle treatment group and $^{\#\#}P < 0.01$ vs incised skin in the vehicle treatment group using the two-tailed unpaired Student *t*-test. Ipsi: ipsilateral side (incised side), Contra: contralateral side (nonincised side). Brain dopamine levels at 4 hours after 3 days of vehicle administration and at 4 hours after 2 and 3 days of QM385 administration (E). Brain dopamine levels were not changed after QM385 administration at the time point for which the compound showed an antihypersensitivity effect. Data are expressed as mean ± SD (n = 5 for each study).

incision in rats. (2) *Spr*-knockout mice showed an antinociceptive phenotype in the paw incision model. (3) The SPR inhibitor QM385 significantly attenuated mechanical hypersensitivity in the rat paw incision model, which was accompanied by a decrease in the level of BH4 in the incised skin. Finally, (4) macrophages expressing GTPCH protein were increased at the injury site in the rat PSP model.

When evaluating gene expressions of BH4-related molecules in the skin over 7 days after paw injury of rats, significant increases of BH4-producing enzymes, especially *Gch1*, at the incised tissues were maintained for a few days (**Figs. 1A and B**). These alterations of gene expressions resulted in increased levels of GTPCH protein and BH4 (**Figs. 2A, B and 3**). These results indicate that BH4-related molecules are involved in the pathogenesis of mechanical hypersensitivity in the paw incision model. Increased BH4 levels were observed in the incised skin from 1 day after paw incision, and higher BH4 levels were maintained through 4 days after paw incision, although BH4 is an unstable substance that is easily oxidized to BH2 (**Fig. 3**).[19] Because increases in the GTPCH level were limited soon after paw incision, there may be a possible mechanism maintaining BH4 levels. BH4 levels in incised skin finally decreased 7 days after paw incision (**Fig. 3**). Because BH4 induces nociception after intraplantar injection,[31] it is believed that increased BH4 levels at the injured site contributes to evoking and maintaining mechanical hypersensitivity in the plantar incision model. In addition, linked to this idea, the rodent plantar incision model used in this study shows persistent mechanical hypersensitivity for about 7 days after incision.[3,61] The gene expressions of BH4-producing enzymes *Gch1* and *Spr* in DRG tissue were not changed for 7 days after plantar incision, whereas they were significantly increased in the incised skin (**Figs. 1A and B** and Supplemental Fig. 1, available at http://links.lww.com/PAIN/B65). Likewise, a previous gene expression study using the rat paw incision model showed that only a few genes were changed in the DRG tissues.[46] As discussed in this report, the reason the gene expressions of *Gch1* and *Spr* were not changed in DRG tissue may be because damage to sensory nerves by paw incision was small.

In the expression analysis, together with BH4-producing enzymes, the NO and 5-HT-producing enzymes, NOS2 and TPH1, which were thought to be involved in the pathogenesis of postoperative pain, were also analyzed. Our experimental results

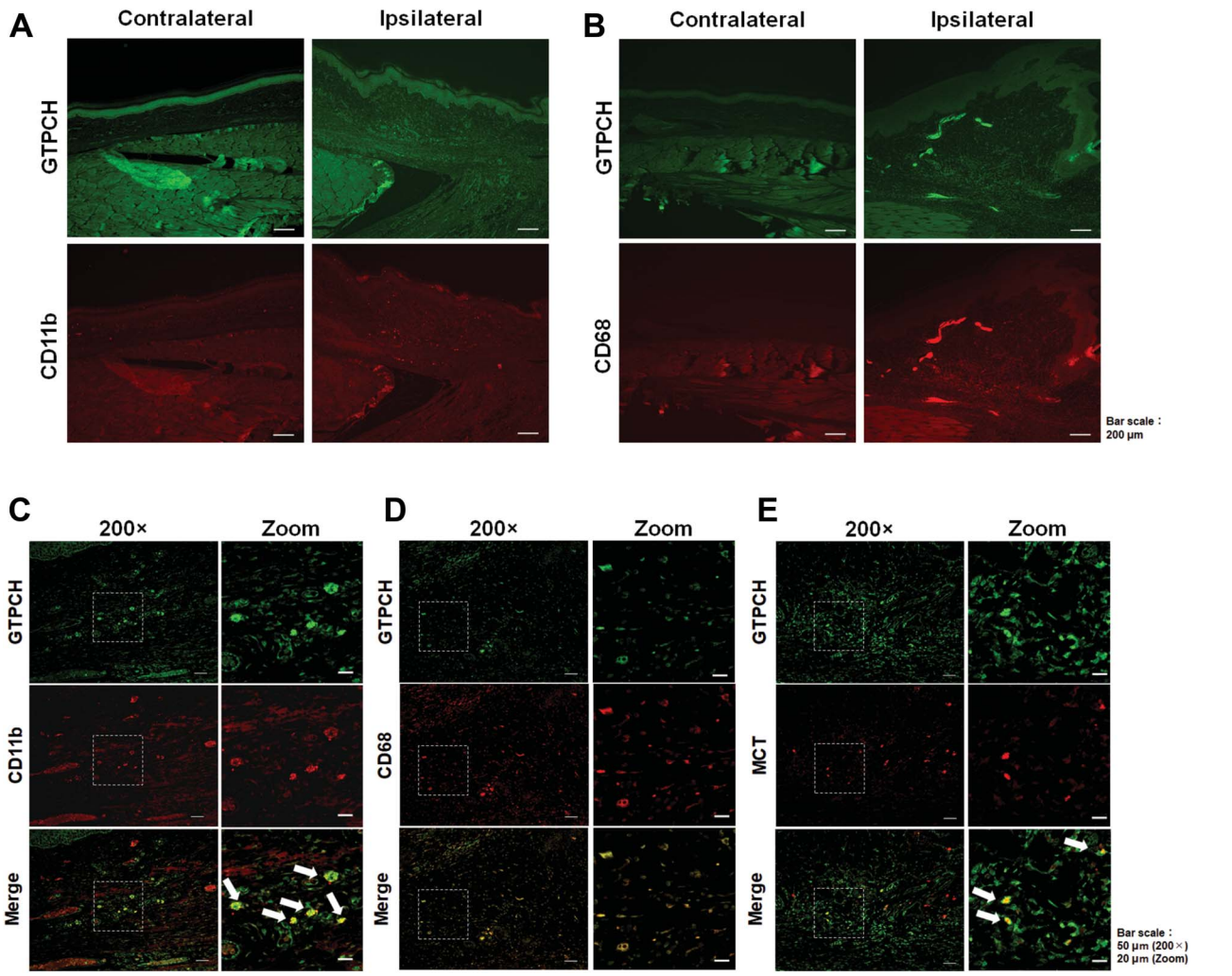


Figure 6. GTPCH is co-expressed with CD11b, CD68, and MCT in lesion tissue after paw incision. Immunofluorescent images showing GTPCH-, CD11b-, and CD68-positive cells in the ipsilateral and contralateral hind paws in a rat paw incision model (A and B). Sagittal sections of the hind paws at 1 day after paw incision were stained. Images were taken under ×40 magnification. Scale bar represents 200 μm. Coexpression of GTPCH with CD11b (C), CD68 (D), and MCT (E). Sagittal sections of the hind paws at 2 days after paw incision were stained. GTPCH (green) signals are merged with CD11b, CD68, and MCT (red) signals. The magnified views show the white dot squares in the images taken under 200× magnification. Arrows show colabeled cells. Scale bar represents 50 μm (200×) and 20 μm (zoom). GTPCH, GTP cyclohydrolase 1; MCT, mast cell tryptase.

showed increased expression of NOS2 in the incised skin (**Figs. 1C and 2A, C**). BH4 is a cofactor for all NOS isoforms and an important regulator of NOS activity because BH4 binding helps dimerization of NOS proteins.[39] Due to the function of NOS protein dimerization, overexpression of BH4 results in increased NO production.[53,55] Based on these reports, NO production seems to be induced by increased BH4 levels, and this is considered to be an important event in the pathogenesis of PSP. We conjectured that nitrate/nitrite (NO) levels would be increased in incised skin after paw incision. However, nitrate/nitrite levels were not changed for 7 days after incision in the rat incision model (data not shown); this result differs from a previous report showing that the nitrite level in the incised skin was increased in the paw incision model.[18] As a cause of the difference, NO may be continuously produced at the lesion site after incision, but it diffused rapidly and did not accumulate in skin tissues at detectable levels.[21] In good agreement with the previous report,[37] increased *Tph1* gene expression was a transient event, and was observed only at 3 and 6 hours after skin incision in this study (**Fig. 1D**). In addition, 5-HT levels in the incised skin were not increased from 1 to 7 days after paw incision (Supplemental Fig. 4, available at http://links.lww.com/PAIN/B65). We initially expected that 5-HT would contribute to mechanical hypersensitivity in the pain condition, but 5-HT may act transiently and not affect persistent mechanical hypersensitivity.

Regarding behavioral testing, *Spr*-knockout mice showed an antinociceptive phenotype after paw incision (**Fig. 4**). However, some limitations of the *Spr*-knockout mouse study should be considered, such as the lack of a sufficient number of animals due to their short life-span, and the inability to exclude the possibility that decreased central monoamines or movement disturbances affected behavior. However, in accordance with the results of the *Spr*-knockout mouse study, QM385 showed antinociceptive efficacy in the rat paw incision model, which accompanied the reduction of BH4 levels in the incised skin tissues (**Figs. 5C and D**). Because drug migration of QM385 into the brain was low, and dopamine levels in the brain were not changed (**Figs. 5B and D**), the antinociceptive effect of QM385 is thought to have occurred without any central effects. To the best of our knowledge, this is the first report that a BH4-related molecule contributes to mechanical hypersensitivity in a rodent paw incision model. The antinociceptive effect in the *Spr*-knockout group was observed

from an early time point (6 hours after paw incision), but the PWT was decreased, as in the WT and *Spr* heterozygote groups in the mouse paw incision model (**Fig. 4**). This result suggests that PSP was not completely eliminated, even in *Spr*-knockout mice. Given that BH4 levels are at a stable lower level in *Spr*-knockout mice,[48] it seems that BH4 is one of the substances that contributes to the onset and maintenance of mechanical hypersensitivity in the paw incision model. A recent investigation demonstrated that decreasing BH4 concentrations in blood were not correlated with pain sensitivity in patients with heterozygous mutations in the *GCH1* gene.[33] However, the present results regarding BH4 measurements in incised skin after QM385 treatment showed that the antinociceptive efficacy is likely correlated with the degree of the BH4 level decrease in peripheral tissues and the plasma concentration of QM385 (**Figs. 5A and D**). This result indicated that an abnormal increase in local BH4 levels in peripheral damaged tissues may cause PSP after paw incision.

In this study, we focused on only the mechanical hypersensitivity of the rat paw incision model; however, not only mechanical, but also heat hypersensitivity, is observed in a rat paw incision model.[41,61] Further studies are needed to clarify whether BH4 is also involved in the pathogenesis of heat hypersensitivity. Our QM385 administration study used tramadol, which is known as a type of opioid, and showed the potential of PSP therapeutic agents as a small molecule. The effect of the QM385 was comparable to that of tramadol at a sufficient dosage, suggesting the therapeutic potential of SPR inhibitors. We are currently planning a further comparison study against a stronger opioid, such as morphine.

In this study, we could not clarify the detailed mechanism by which BH4 evokes PSP, but the possible mechanism of BH4 evoking pain can be discussed. It has been reported that stimulation of DRG neurons by BH4 increases calcium influx, which promotes neuronal excitability leading to pain sensation.[30,52,63] This elevated calcium influx is induced by sensitized transient receptor potential ankyrin 1 and transient receptor potential vanilloid 1 channels because of NO production using BH4.[30] Although the expression of NOS1 (neuronal NOS) and NOS3 (endothelial NOS) in lesion tissues was not evaluated, the protein level of NOS2 and BH4 concentration in skin tissue were significantly increased after paw incision (**Figs. 1C, 2A, C, and 3**). NO production using BH4 possibly induces increased Ca influx in peripheral neuronal cells (nociceptors) at the lesion site, and this may lead to pain sensation in the paw incision model. In addition, because BH4 is transported into the cells using the nucleotide transporters ENT1 and ENT2,[36] there is a possibility that this may be induced by extracellular BH4 provided by surrounding cells such as macrophages because BH4 is secreted from the cell (**Figs. 6A and B**).[54] The result of our immunohistochemical study suggested that BH4 was probably produced by GTPCH expressed in macrophages that had infiltrated after paw incision, rather than the resident cells (**Figs. 6A and B**). However, more recently, in a *Gch1*-deletion study, it was reported that BH4 plays an important role in T-cell proliferation.[11] Clarifying the participation of cells, including macrophages, T cells, mast cells, and neuronal cells, at lesion sites in the PSP model, and their relationships with BH4 and pain sensations using BH4-modulating compounds and/or genetically modified mice, remains an issue for future studies.

5. Conclusion

The results of this study clearly indicated that BH4 is an important substance involved in the onset of mechanical hypersensitivity in a rodent PSP model. Accordingly, a drug capable of reducing BH4 levels peripherally, such as an SPR inhibitor, could be a new candidate for acute PSP, because such a drug may demonstrate an antinociceptive effect through a new mechanism of action.

Conflict of interest statement

The authors have no conflicts of interest to declare.

Appendix A. Supplemental digital content

Supplemental digital content associated with this article can be found online at http://links.lww.com/PAIN/B65.

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