ン酸カスケードとの関連性を示すものであり，THCの退薬症候発現にアラキドン酸カスケードの不活性化が関与している可能性を示唆している．

3. 今後の展望

植物である大麻活性成分の薬理作用解明から始まったカンナビノイド研究は，脳内にカンナビノイド受容体とその内在性カンナビノイドの存在が明らかにされてから，生体におけるカンナビノイドの機能的役割を解明する研究へと方向舵を変えている．大麻乱用における精神障害の知見(38) からも，脳内の内在性カンナビノイドの病的な増加または減少は精神機能の異常をきたす可能性が推測される．まだまだ不明な点が多いが，統合失調症やアルツハイマー病の患者脳でのカンナビノイド受容体・内在性カンナビノイドの異常性はすでに散見される(39, 40)．中枢神経系における脳内カンナビノイドの機能的役割が明らかになれば，意欲減退，情動障害および精神障害との関連性も解き明かされる事が期待される．また内在性カンナビノイドと食欲や脂質代謝との関連性も，肥満を含む生活習慣病の病因解明に迫る上で新たな糸口を与えるものとして注目されている．

“快楽”と“忘却”を司る内在性カンナビノイドは，ストレスの多い現代社会に生きる脆弱な脳が己の存在を懸けて放った防人であるかもしれない．

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Direct Evidence for the Ongoing Brain Activation by Enhanced Dynorphinergic System in the Spinal Cord under Inflammatory Noxious Stimuli

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ABSTRACT

Background: Dynorphin A in the spinal cord is considered to contribute to nociceptive stimuli. However, it has not yet been determined whether activation of the spinal dynorphinergic system under nociceptive stimuli plays a role in direct acceleration of the ascending nociceptive pathway. In this study, the authors investigated the role of spinal dynorphinergic transmission in ongoing brain activation under noxious stimuli in mice.

Methods: The changes in prodynorphin messenger RNA expression and dynorphin A (1–17)-like immunoreactivity in the mouse spinal cord were determined after the intraplantar injection of complete Freund's adjuvant in mice. The signal intensity in different brain regions after the intraplantar injection of complete Freund's adjuvant or intrathecal injection of dynorphin A (1–17) was measured by a pharmacological functional magnetic resonance imaging analysis.

Results: Complete Freund's adjuvant injection produced pain-associated behaviors and induced a dramatic increase in signal intensity in the mouse cingulate cortex, somatosensory cortex, insular cortex, and thalamic nuclei. These effects were not seen in prodynorphin knockout mice. Prodynorphin messenger RNA expression and dynorphin A (1–17)-like immunoreactivity on the ipsilateral side of the spinal cord were markedly increased in complete Freund's adjuvant-injected mice. Furthermore, intrathecal injection of dynorphin A (1–17) at relatively high doses caused pain-associated behaviors and a remarkable increase in the activities of the cingulate cortex, somatosensory cortex, insular cortex, and medial and lateral thalamic nuclei in mice.

Conclusions: These findings indicate that spinally released dynorphin A (1–17) by noxious stimuli leads to the direct activation of ascending pain transmission.

What We Already Know about This Topic

- Spinal dynorphin release contributes to hypersensitivity to stimuli in rodents, but whether it activates supraspinal structures related to pain is not known

What This Article Tells Us That Is New

- Injection of complete Freund's adjuvant into the paw of mice increased dynorphin in the spinal cord and increased activity in several cortical regions associated with pain processing
- These effects were not present in genetically altered mice lacking dynorphin

ENDOGENOUS opioid peptides are efficient analgesics that bind to opioid receptors. These peptides are typically produced to counteract chronic pain. They are classified as enkephalins, endorphins, and dynorphins.[1] Dynorphins are neuropeptides that inhibit neuronal activity through κ-opioid receptors.[2] Dynorphin A (1–17) is one of the major proteolytic fragments of prodynorphin[2] and has been shown to be distributed widely throughout the central nervous system.[2–5] Many experimental models of pathologic pain, including inflammatory pain,[6–10] neuropathic pain,[10–12] bone cancer pain,[13] and abnormal pain (hyperalgesia),[14] show a

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significant induction of dynorphin A in the spinal cord. Relatively low doses of dynorphin A produce analgesia by virtue of acting as an inhibitory opioid peptide and preferentially activating κ-opioid receptors.[10,15,16] In contrast, high doses of dynorphin A elicit pronociceptive behaviors, such as biting, licking, and scratching,[17] or allodynia.[3,18,19] In such cases, the actions are not sensitive to opioid antagonist but are blocked by intrathecal pretreatment with MK-801, a noncompetitive antagonist of *N*-methyl-D-aspartic acid (NMDA) receptor channels.[19] Taken together, increased dynorphin A in the spinal cord may play an important role in the nociceptive state in terms of its paradoxical effects on neurotransmission.

Functional magnetic resonance imaging can be used to investigate spatial and temporal brain activation. Functional magnetic resonance imaging has been used to evaluate pain perception in the central nervous system in healthy humans and in those with various kinds of pain.[20,21] Noxious heat stimulation in humans or repetitive heat stimulation through peltier elements in animals activates several brain regions, so-called pain matrix.[22–25] Recent studies have demonstrated that neuroimaging in humans and animals can detect changes in regional activity initiated by the administration of drugs that induce or modulate pain, such as morphine, ketamine, formalin, and capsaicin.[26–29] Furthermore, these studies have introduced the new term pharmacological functional magnetic resonance imaging (phMRI) for this technique, which promises to become an important new tool for the researcher who is interested in mapping and understanding the pain mechanism. We previously demonstrated that neuropathic pain-like transmission evoked by the spinal activation of protein kinase C caused a significant increase in the activity of several brain regions using a mouse phMRI assay.[30]

In this study, we investigated whether a deletion of the gene that encodes the prodynorphin could affect the brain regions activated after unilateral intraplantar injection of complete Freund's adjuvant (CFA) injection using phMRI method in mice and whether direct intrathecal administration of dynorphin A (1–17) at relatively high doses could facilitate brain activity in regions that are associated with pain perception *via* an NMDA receptor-mediated pathway.

Materials and Methods

Animals

This 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. This study was approved by the Animal Research Committee of Hoshi University. The first series of experiments using 41 mice, either wild-type (C57BL/6 and 129S4/SvJ mixed genetic background, 21 males, 8–10 weeks old, 18–23 g; The Jackson Laboratory, Bar Harbor, ME) or prodynorphin gene-knockout (C57BL/6 and 129/SvJ mixed genetic backgrounds, 20 males, 8–10 weeks old, 18–23 g; The Jackson Laboratory) mice was also performed. Ninety-seven male mice (8–10 weeks old, 18–23 g) were used for another series of experiments in C57BL/6J mice (CLEA Japan, Inc., Tokyo, Japan). Animals were kept in a room with an ambient temperature of 23° ± 1° C and a 12-h light-dark cycle (lights on 8:00 AM to 8:00 PM). Food and water were available *ad libitum*. All animals were individually housed, and all behavioral studies were performed during the light period. At the end of the experiments, animals were humanely killed by a rising concentration of ethyl ether.

Intrathecal Injection

Intrathecal injection was performed as described by Hylden and Wilcox[31] using a 25-μl Hamilton syringe with a 30 ½-gauge needle. The needle was inserted into the intervertebral space between L5 and L6 level of the spinal cord. A reflexive flick of the tail was considered to be an accuracy of each injection. The injection volume was 4 μl for intrathecal injection.

Inflammatory Pain Model

A persistent inflammatory pain model was produced by unilateral intraplantar injection of CFA (mycobacterium tuberculosis; Sigma-Aldrich Co., St. Louis, MO) in a volume of 50 μl into the plantar surface of the right hind paw (ipsilateral side) of mice during anesthesia with isoflurane.[32–34] Control mice were given saline in a volume of 50 μl into the plantar surface of the right hind paw.

Pain-like Behaviors

To observe the pain-like behaviors induced by the intrathecal injection of dynorphin A (1–17) in normal mice, groups of mice were individually placed in an observation cage immediately after intrathecal injection of dynorphin A (1–17) (0.3, 3, and 30 pmol/mouse; Peptide Institute, Inc., Osaka, Japan) or saline. Intrathecal pretreatment with MK-801 (0.3 or 1 nmol/mouse) was performed 30 min before the intrathecal injection of dynorphin A (1–17) (30 pmol/mouse).

To assess the pain-like behaviors induced by inflammatory pain, groups of mice were individually placed in an observation cage immediately after the intraplantar injection of CFA or saline. Intrathecal pretreatment with antiserum against dynorphin A (1–17) (1:100; Peninsula Laboratories, Inc., San Carlos, CA) or control serum (normal rabbit serum; Vector Laboratories, Inc., Burlingame, CA) was performed 30 min before the intraplantar injection of CFA.

The number of licking or flinching behaviors was counted, and the duration of licking and flinching behaviors was measured for 20 min after treatment.

Functional Imaging

Functional imaging was performed, as previously described.[30] To investigate the effect of the intraplantar injection of CFA, prodynorphin gene-knockout mice or wild-

type mice were anesthetized using isoflurane immediately after the intraplantar injection of CFA or vehicle. To investigate the effect of a single intrathecal treatment with dynorphin A (1–17) in C57BL/6J mice, mice were anesthetized using isoflurane immediately after intrathecal injection of dynorphin A (1–17) (30 pmol/mouse). Animals were then transferred to a cradle that was designed to fit inside the probe of the magnetic resonance system and supplied with 1% isoflurane *via* a fitted mask. A continuous phMRI scanning protocol was used to study the changes in brain signal intensity using T2 star-weighted blood oxygenation level-dependent (BOLD) contrast. BOLD responses were measured hourly from 30 min to 6 h at all brain levels.

Experiments were performed with a Unity Inova spectrometer (Varian, Palo Alto, CA) that was interfaced to a 9.4-T/31-cm horizontal bore magnet equipped with actively shielded gradients capable of 300 mT/m in a rise time of 500 s (Magnex Scientific, Abingdon, United Kingdom). High-resolution anatomical scans were collected using a fast spin echo pulse sequence (repetition time = 2000 ms, echo time = 45 ms, field of view = 25.6 × 25.6 mm^2, 1. 0-mm slice thickness, 256 × 256 data matrix).

Functional images were obtained with the two-slice gradient-echo fast imaging sequence (echo time = 25 ms, repetition time = 70 ms, 30-° flip angle, 128 × 128).[35] One-millimeter-thick slices were simultaneously acquired over a field of view of 25.6 mm^2, number of average of 2, and an acquisition time of 32 s. Typically, 3–6 images were collected at baseline, followed by intrathecal injection of dynorphin A (1–17) or intraplanter injection of CFA. Intrathecal pretreatment with MK-801 (1 nmol/mouse) was performed 30 min before the intrathecal injection of dynorphin A (1–17). The regions of interest were selected, and statistical analyses were performed using the image-analysis software ImageJ (National Institutes of Health, Bethesda, MD). The regions of interest were drawn according to an atlas of the mouse brain.[36] BOLD signal intensity values in each regions of interest were extracted and normalized to the time of baseline (expressed as a percent change from baseline). Statistical analysis was performed to compare percent changes in BOLD signal intensity and activated pixels between baseline and each time point after CFA and dynorphin A (1–17) injection.

RNA Preparation and Semiquantitative Analysis by Reverse Transcription–Polymerase Chain Reaction (PCR)

Total RNA obtained from the spinal cord of mice was extracted using the SV Total RNA Isolation System (Promega Co., Madison, WI). The lumbar spinal cord was quickly removed after mice were decapitated and homogenized in ice-cold lysis buffer containing β-mercaptoethanol following the manufacturer's instructions. First-strand complementary DNA was prepared as described,[37] and the prodynorphin gene was amplified in 50 μl of a PCR solution containing $MgCl_2$, dNTP mix, and DNA polymerase with either synthesized primers (prodynorphin: 5′-GTG CAG TGA GGA TTC AGG ATG GG-3′ [sense] and 5′-GAG CTT GGC TAG TGC ACT GTA GC-3′ [antisense], glyceraldehyde-3-phosphate dehydrogenase: 5′-CCC ACG GCA AGT TCA ACG G-3′ [sense] and 5′-CTT TCC AGA GGG GCC ATC CA-3′ [antisense]). Samples were heated to 94° C for 2 min, 55° C for 2 min, and 72° C for 3 min and cycled 29 times through 94° C for 1 min, 55° C for 2 min, and 72° C for 3 min. The final incubation was at 72° C for 7 min. The mixture was subjected to 1% agarose gel for electrophoresis with the indicated markers and primers for the internal standard (glyceraldehyde-3-phosphate dehydrogenase). Each sample was applied to more than two lanes in the same gel. The agarose gel was stained with ethidium bromide and photographed with ultraviolet transillumination. The intensity of the bands was analyzed and quantified by computer-assisted densitometry using ImageJ (free download software developed by National Institutes of Health). For the control, the different intensities of each band obtained from mice treated with saline were analyzed, and the average intensity was calculated. Each control intensity was then compared again with the average intensity to calculate the standard error. Under these conditions, the intensities of bands for samples obtained from CFA-treated mice were analyzed and compared with the average intensity for mice treated with saline. Finally, the percent of control with standard error for each sample was quantified.

Quantitative Analysis by Real-time PCR

Fast SYBR Green Master Mix (2 ×; Applied Biosystems, Inc., Foster City, CA) was used as the basis for the reaction mixture in the real-time PCR assay. Each gene prepared by the above procedure was amplified in 20 μl of a PCR solution containing 10 μl of the Fast SYBR Green Master Mix (2 ×) with synthesized primers for PDYN (sense: 5′-TTT GGC AAC GGA AAA GAA TC-3′, antisense: 5′-CAT AGC GTT TGG CCT GTT TT-3′) or β-actin (sense: 5′-CAG CTT CTT TGC AGC TCC TT-3′, antisense: 5′-TCA CCC ACA TAG GAG TCC TT-3′). In addition to each sample, each test run included a no-target control that contained reaction mixture and PCR-grade water. PCR with a StepOnePlus (Applied Biosystems, Inc., Foster City, CA) was performed with the following cycling conditions: 95° C for 20 s, followed by cycled 45 cycles of 95° C for 3 s and 60° C for 30 s. Fluorescence detection was conducted after each extension step.

Spinal Cord Sample Preparation and Immunohistochemistry

Sample preparation and immunohistochemistry were performed following the methods previously described.[38] Six hours after intraplantar injection, mice were deeply anesthetized with isoflurane and intracardially perfusion fixed with freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer saline (PBS), pH 7.4. After perfusion, the lumbar spinal cord was quickly removed and postfixed in 4% paraformaldehyde for 2 h and then 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. The L5 lumbar spinal cord segments were then frozen in an embedding compound (Sakura Finetechnical, Tokyo, Japan) on isopentane using liquid nitrogen and stored at −30° C until use. Frozen spinal cord segments were cut with a freezing cryostat (Leica CM 1510; Leica, Wetzlar, Germany) at a thickness of 10 μ m and thaw mounted on poly-L-lysine-coated glass slides.

The spinal cord sections were blocked in 20% normal goat serum with 0.1% Triton X in 0.01 M PBS for 1 h at room temperature. The primary antibody [1:600 dynorphin A (1–17) (Phoenix Pharmaceuticals, Inc., Belmont, CA)] was diluted in 0.01 M PBS containing 20% normal goat serum with 0.1% Triton X and incubated for two nights at 4° C. The samples were then rinsed and incubated with an appropriate secondary antibody conjugated with Alexa 546 for 2 h at room temperature. Because the staining intensity might vary between experiments, control sections were included in each run of staining. The slides were then coverslipped with PermaFluor aqueous mounting medium (Immunon; Thermo Electron, Pittsburgh, PA). All sections were observed with a fluorescence microscope (Olympus BX-80; Olympus, Tokyo, Japan) and photographed with a digital camera (CoolSNAP HQ; Olympus).

Statistical Analysis

Dat a are expressed as the mean with SEM. One-and two-way ANOVAs with independent and repeated measures, as well as planned comparisons or Student *t* tests, were used as appropriate for the experimental design. Multiple comparisons were performed using Dunnett or Bonferroni *post hoc* test, where appropriate. All statistical analyses were performed with Prism version 5.0a (GraphPad Software, Inc., San Diego, CA).

Results

Pain-like Behaviors Induced by CFA Injection in Prodynorphin Knockout and Wild-type Mice

Genotyping of the offspring from prodynorphin knockout (−/−) mice was confirmed by PCR analysis using DNA extracted from the ear (data not shown). W e investigated whether lack of the prodynorphin gene could influence inflammatory pain using these genotype mice. In wild-type mice, CFA injection caused significant flinching or licking behaviors (fig. 1). These behaviors started just after CFA injection and lasted for more than 20 min. However, the numbers and durations of these behaviors were clearly decreased in prodynorphin knockout mice with CFA injection (fig. 1). Two-way ANOVA showed a significant interaction between genotype and treatment (flinching, $F(1,14) = 4.736$, $P = 0.0471$; licking, $F(1,14) = 6.699$, $P = 0.0215$; duration, $F(1,14) = 31.72$, $P < 0.0001$), a significant effect of treatment (flinching, $F(1,14) = 19.39$, $P = 0.0006$; licking, $F(1,14) = 17.53$, $P = 0.0009$; duration, $F(1,14) = 59.56$, $P < 0.0001$), and a significant effect of genotype (flinching, $F(1,14) = 5.291$, $P = 0.0373$; licking, $F(1,14) = 10.94$, $P = 0.0053$; duration, $F(1,14) = 43.78$, $P < 0.0001$). *Post hoc* comparison indicated a significant difference between the saline

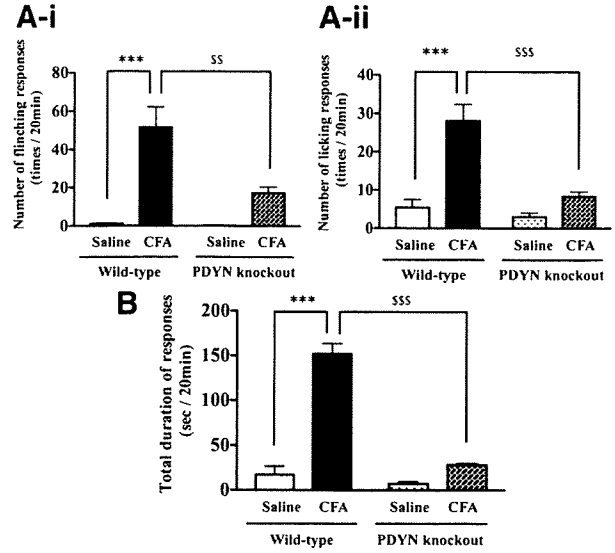

Fig. 1. Effect of an intraplantar injection of complete Freund's adjuvant (CFA) on spontaneous pain-like behaviors in wild-type (+/+) and prodynorphin (PDYN) knockout (−/−) mice. Immediately after intraplantar injection, the number of flinching (*A-i*) or licking (*A-ii*) behaviors was counted, and the total duration of flinching and licking behaviors was measured (*B*) for 20 min after the injection of CFA into the right hind paw of mice. Each point indicates the mean ± SEM of 3–5 mice. Bonferroni test: *** $P < 0.001$, wild-type mouse with intraplantar injection of saline *versus* wild-type mouse with intraplantar injection of CFA; $$ $P < 0.01$, $$$ $P < 0.001$, wild-type mouse with intraplantar injection of CFA *versus* PDYN knockout mouse with intraplantar injection of CFA.

in wild-type group and the CFA in wild-type group (flinching response: $F(3,12) = 24.87$, $P < 0.0001$; licking response: $F(3,12) = 70.84$, $P < 0.0001$; total duration of responses: $F(3,12) = 58.98$; $P < 0.0001$) and a significant difference between CFA in wild-type group and CFA in prodynorphin knockout group (flinching response: $F(3,12) = 24.87$, $P = 0.0019$; licking response: $F(3,12) = 70.84$, $P < 0.0001$; total duration of responses: $F(3,12) = 58.98$, $P < 0.0001$).

Time Course of the Effect of Intraplantar Injection of CFA on BOLD Signal Intensity in Several Brain Regions in Wild-type and Prodynorphin Knockout Mice

Next, we investigated the changes in BOLD signal intensity in two brain regions after intraplantar injection of CFA. As shown in figures 2A–C, injection of CFA caused positive signal activity in the cingulate cortex (A-i), somatosensory cortex (B-i), and insular cortex (C-i) of wild-type mice compared with prodynorphin knockout mice. In the cingulate cortex, injection of CFA produced a n increase in signal intensity (fig. 2A-ii). In the somatosensory cortex of wild-type mice, injection of CFA also produced an increase in signal intensity after injection (fig. 2B-ii). In the insular cortex of wild-type mice, similar to somatosensory area, injection of CFA produced a n increase in signal intensity after injection (fig. 2C-ii). These effects were not seen in mice that lacked the prodynorphin gene. Statistical analyses were done with two-way ANOVA followed by Bonferroni test (cingu-

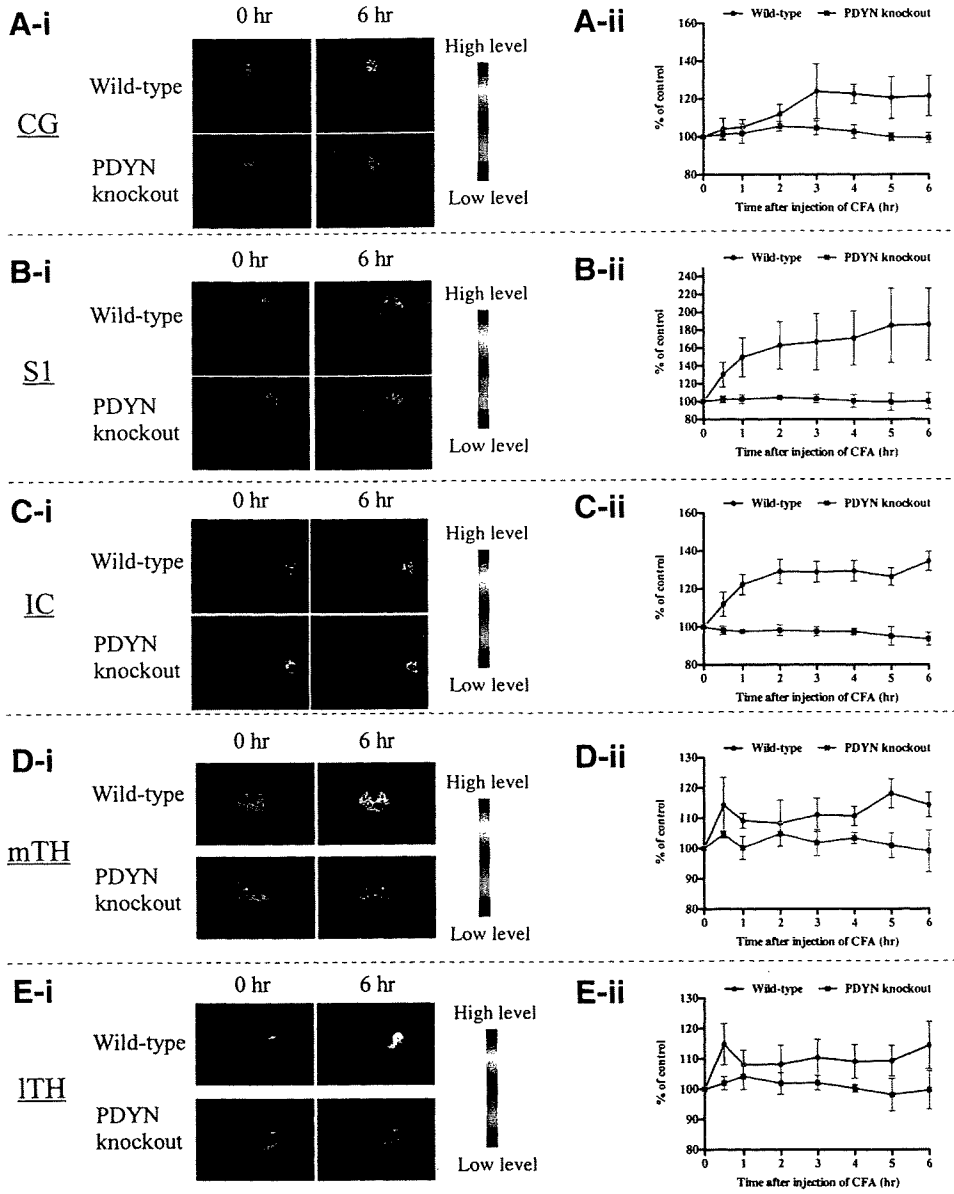

Fig. 2. Time course of the effect of intraplantar injection of complete Freund's adjuvant (CFA) on blood oxygenation level-dependent (BOLD) signal intensity in the cingulate cortex (CG), somatosensory cortex (S1), insula cortex (IC), medial halamic region (mTH), and lateral thalamic region (lTH) in wild-type (WT) and prodynorphin (PDYN) knockout (KO) mice. Representative activation maps (overlaid on anatomy) correspond to composite images at 0 or 6 h (*A-i*: CG, *B-i*: S1, *C-i*: IC, *D-i*; mTH, *E-i*; lTH) after intraplantar injection of CFA in WT and KO mice. Intraplantar injection of CFA produced a significant increase in BOLD signal in the CG (*A-ii*), S1 (*B-ii*), IC (*C-ii*), mTH (*D-ii*), and lTH (*E-ii*) of wild-type mice. Data are expressed as percentages of the corresponding baseline levels with mean ± SEM for five mice.

late cortex: interaction between genotype and time: $F(7,48) = 1.138$, $P = 0.356$; effect of genotype, $F(1,48) = 14.28$, $P = 0.0004$; effect of time, $F(7,48) = 1.348$, $P = 0.2491$; somatosensory cortex: interaction between genotype and time: $F(7,48) = 0.996$, $P = 0.446$; effect of genotype, $F(1,48) = 27.77$, $P < 0.0001$; effect of time, $F(7,48) = 0.984$, $P = 0.4543$; insular cortex: interaction between genotype and time: $F(7,48) = 4.799$, $P = 0.0004$; effect of genotype, $F(1,48) = 151.3$, $P < 0.0001$; effect of time, $F(7,48) = 2.933$, $P = 0.0122$) (figs. 2A-ii–C-ii). As shown in figures 2D and E, injection of CFA caused positive signal activity in the medial thalamic nuclei (D-i) and the lateral thalamic nuclei (E-i) of wild-type mice compared with prodynorphin knockout mice. The medial thalamic nuclei and lateral thalamic nuclei were also activated by CFA injection in wild-type mice but not in prodynorphin knockout mice. Statistical analyses were done with two-way ANOVA followed by Bonferroni test (medial thalamic nuclei: interaction between genotype and time: $F(7,48) = 0.730$, $P = 0.6475$; effect of genotype, $F(1,48) = 14.62$, $P = 0.0004$; effect of time, $F(7,48) = 0.856$, $P = 0.5476$; lateral thalamic nuclei: interaction between genotype and time: $F(7,48) = 0.501$, $P = 0.8289$; effect of genotype, $F(1,48) = 11.72$, $P = 0.0013$; effect of time, $F(7,48) = 0.563$, $P = 0.7821$) (figs. 2D-ii and E-ii). Under these conditions, control

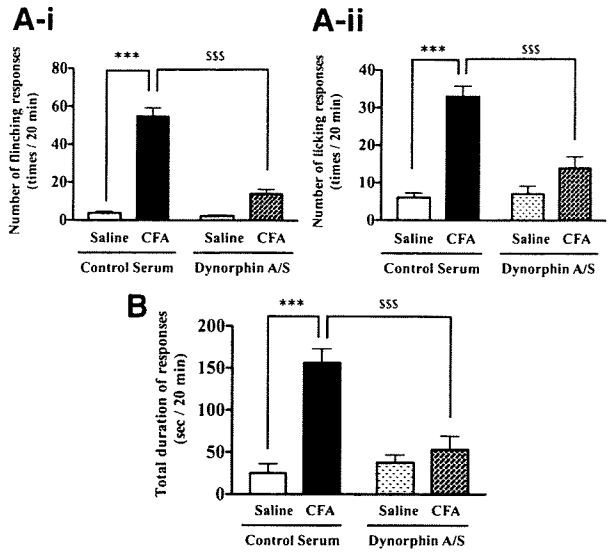

Fig. 3. Effect of intrathecal pretreatment with antiserum against dynorphin A (1–17) on pain-like behaviors evoked by intraplantar injection of complete Freund's adjuvant (CFA). Groups of mice were treated intrathecal with antiserum against dynorphin A (1–17) (Dynorphin A/S; 1:100) or control serum 30 min before the intraplantar injection of CFA. Immediately after the intraplantar injection of saline or CFA, the number of flinching (*A-i*) or licking (*A-ii*) behaviors was counted, and the duration of flinching and licking behaviors was measured (*B*) for 20 min after the injection of CFA into the right hind paw of mice. Each point indicates the mean ± SEM of 3–5 mice. Bonferroni test: *** $P < 0.001$, control serum-saline group *versus* control serum-CFA group; $$$ $P < 0.001$, control serum-CFA group *versus* Dynorphin A/S-CFA group.

mice of both genotypes that had been injected with saline failed to show activation in any brain regions (data not shown).

Effects of Intrathecal Injection of Antiserum to Dynorphin A (1–17) on the Response Induced by CFA Injection

To confirm the possible involvement of spinal dynorphin A (1–17) in the pain-related response induced by CFA injection, we performed intrathecal injection of antiserum against dynorphin A (1–17) and evaluated its effect on CFA-mediated pain-related responses. Dynorphin A (1–17) antiserum significantly decreased the number of and shortened the duration of flinching or licking responses compared with those in control serum-treated mice (fig. 3). The two-way ANOVA indicated a significant interaction between pretreatment and posttreatment (flinching, $F(1,23) = 48.89$, $P < 0.0001$; licking, $F(1,23) = 14.83$, $P = 0.0008$; duration, $F(1,23) = 14.19$, $P = 0.001$), a significant effect of pretreatment (flinching, $F(1,23) = 122.8$, $P < 0.0001$; licking, $F(1,23) = 42.02$, $P < 0.0001$; duration, $F(1,23) = 22.43$, $P < 0.0001$), and a significant effect of posttreatment (flinching, $F(1,23) = 56.6$, $P < 0.0001$; licking, $F(1,23) = 12.03$, $P = 0.0021$; duration, $F(1,23) = 8.825$, $P = 0.0068$). *Post hoc* comparison indicated a significant difference between the control serum-saline group and control serum-CFA group (flinching response: $F(3,23) = 76.95$, $P < 0.0001$; licking response: $F(3,23) = 23.04$, $P < 0.0001$; total duration of responses: $F(3,23) = 15.38$; $P < 0.0001$) and a significant difference between control serum-CFA group and dynorphin A (1–17) antiserum-CFA group (flinching response: $F(3,23) = 76.95$, $P < 0.0001$; licking response: $F(3,23) = 23.04$, $P < 0.0001$; total duration of responses: $F(3,23) = 23.04$, $P = 0.00025$).

Increase in the Expression of Spinal Prodynorphin Messenger RNA at the Early Phase of Inflammatory Pain after Intraplantar Injection of CFA in Mice

In the reverse transcription–PCR assay, the expression of prodynorphin messenger RNA (mRNA) on the ipsilateral side of the spinal cord obtained from CFA-treated mice was significantly increased at 1, 3, and 6 h after CFA injection compared with that in saline-treated mice (1 h: $P = 0.014$, 3 h: $P = 0.0002$, 6 h: $P = 0.0008$ *vs.* saline-treated group; figs. 4A and B). In the contralateral spinal cord obtained from CFA-treated mice, there were no significant differences in the expression level of prodynorphin mRNA compared with those on the contralateral side of saline-treated mice (data not shown). In agreement with the results of reverse transcription–PCR, we confirmed that the expression of PDYN mRNA was significantly increased in the spinal cord of mice with CFA treatment compared with that in saline-treated mice at 6 h after CFA injection using real-time PCR ($P = 0.042$ *vs.* saline-treated group; fig. 4C).

Changes in Dynorphin A (1–17)–like Immunoreactivity after Intraplantar Injection of CFA in the Superficial Dorsal Horn of the Mouse Spinal Cord

To investigate a possible change in the level of dynorphin A (1–17) in the superficial dorsal horn of CFA-treated mice, immunohistochemical studies were performed. Dynorphin A (1–17)-like immunoreactivity was detected on the superficial laminae of the ipsilateral side of the L5 lumbar spinal dorsal horn with saline treatment (fig. 4D-i). Six hours after CFA injection, dynorphin A (1–17)-like immunoreactivity on the superficial laminae of the ipsilateral side of the L5 lumbar spinal dorsal horn was markedly increased compared with that with saline injection (fig. 4D-ii).

Pain-like Behaviors Induced by Intrathecal Injection of Dynorphin A (1–17) in Mice

Next, we observed pain-like behaviors induced by intrathecal dynorphin A (1–17) in mice. As shown in figure 5, intrathecal injection of dynorphin A (1–17) produced a significant increase in both the number of biting or licking responses and the duration of these responses in a dose-dependent manner. Statistical analyses were done with one-way ANOVA followed by Dunnett test (biting or licking responses: $F(3,17) = 6.084$, 0.3 pmol: $P = 0.6584$, 3 pmol: $P = 0.039$, 30 pmol: $P = 0.0033$; total duration of responses: $F(3,17) = 10.94$, 0.3 pmol: $P = 0.2108$, 3 pmol: $P = 0.0018$, 30 pmol: $P = 0.0002$; *vs.* intrathecal vehicle

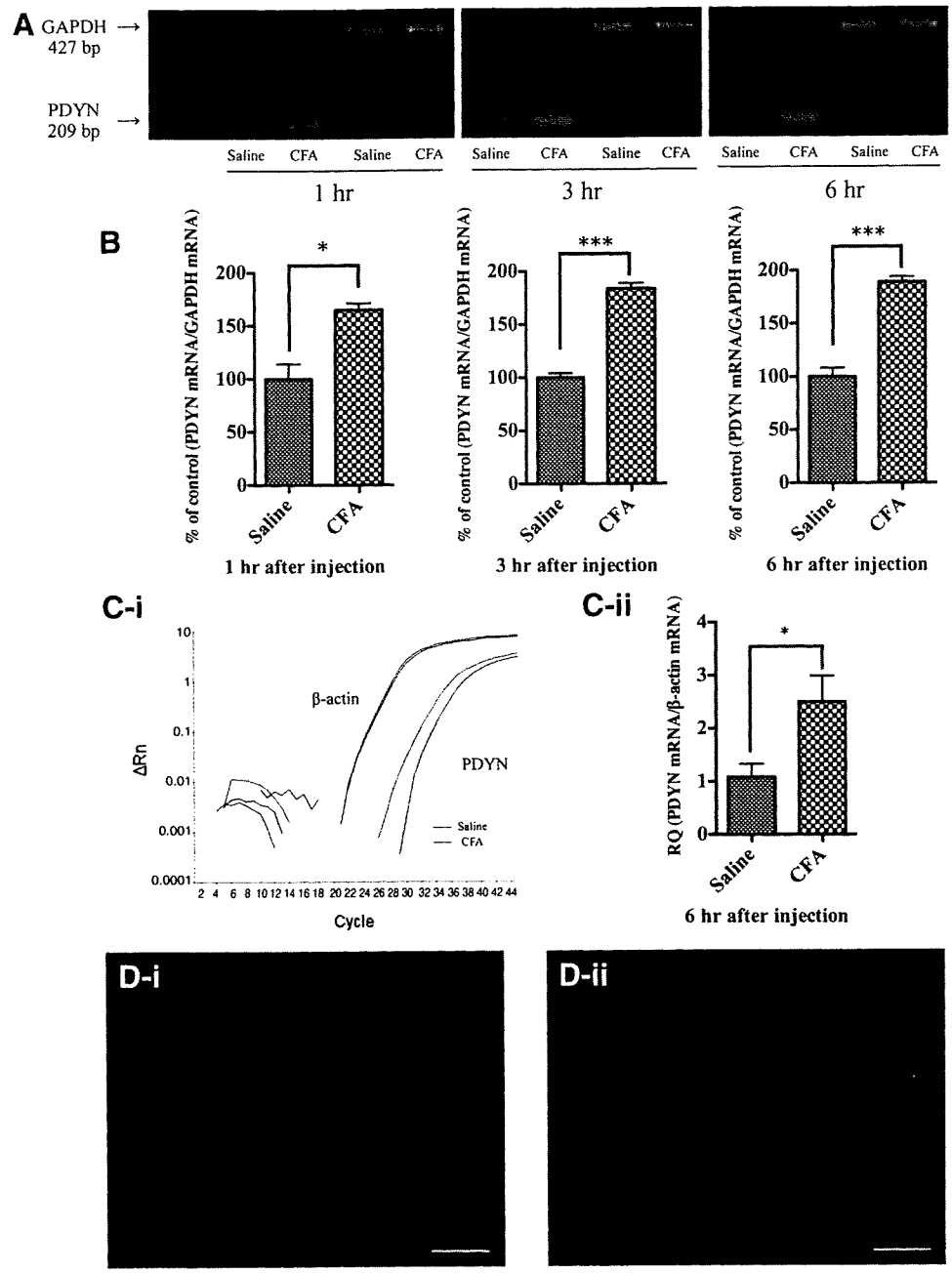

Fig. 4. (*A*) Representative reverse transcription–polymerase chain reaction for prodynorphin (PDYN) messenger RNAs on the ipsilateral side of spinal cords obtained from saline-or complete Freund's adjuvant (CFA)-injected mice. Spinal cord samples were prepared at 1, 3, and 6 h after saline or CFA injection (*B*). The intensity of the bands was determined semiquantitatively using ImageJ (National Institutes of Health). The values for PDYN messenger RNA were normalized by the value for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) messenger RNA. The value in CFA-injected mice is expressed as a percentage of the increase in saline-injected mice. Each column represents the mean ± SEM of three samples. Student *t* test: ** $P < 0.01$, *** $P < 0.001$, *versus* saline group. (*C*) Quantitative analysis of PDYN messenger RNA on the spinal cord of saline-or CFA-treated mice at 6 h after the injection. (*C-i*) Amplification plots of fluorescence intensities *versus* PCR cycle numbers in each sample are displayed. (*C-ii*) Each column represents the mean ± SEM of three samples. * $P < 0.05$ *versus* saline-treated group. (*D*) Change in dynorphin A (1–17)-like immunoreactivity on the superficial laminae of the ipsilateral dorsal horn after CFA injection. Photomicrographs show immunofluorescent staining of dynorphin A (1–17) on the superficial layers of the ipsilateral spinal dorsal horn at 6 h after saline (*D-i*) or CFA injection. Dynorphin A-like immunoreactivity observed on the superficial laminae of the ipsilateral dorsal horn after CFA injection (*D-ii*) was significantly increased compared with that after saline injection (*D-i*). Scale bars = 50 μ m.

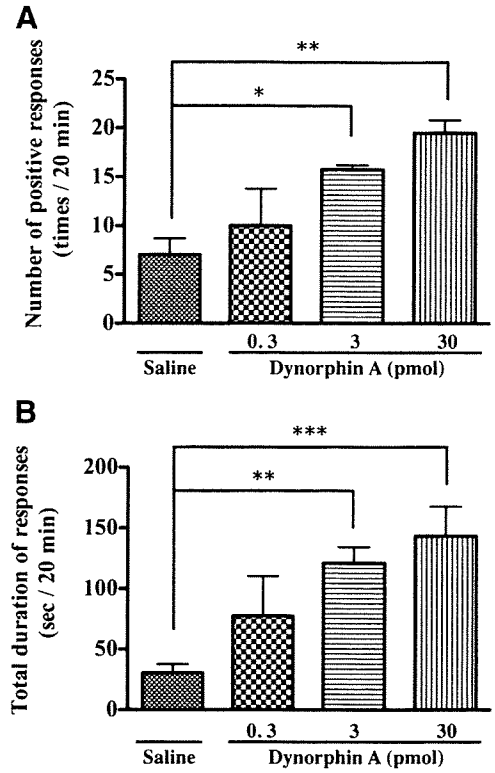

Fig. 5. Effect of a single intrathecal injection of dynorphin A (1–17) on pain-like behavior. Groups of mice were treated intrathecal injection with dynorphin A (1–17) (0.3, 3, 30 pmol) or saline. Immediately after intrathecal injection, the number of licking or biting behaviors was counted (*A*), and the duration of licking or biting behaviors was measured (*B*) for 20 min after the intrathecal injection of dynorphin A (1–17). The intrathecal injection of dynorphin A (1–17) produced a dose-dependent increase in pain-like behaviors compared with control mice injected intrathecal with vehicle. Each point indicates the mean ± SEM of four to six mice. Dunnett test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, *versus* intrathecal vehicle group.

group; figs. 5A and B). These responses started a pproximately 4 or 5 min after intrathecal injection and lasted for more than 20 min. The control group-injected intrathecal with vehicle did not exhibit these behaviors.

Time Course of the Effect of Intrathecal Injection of Dynorphin A (1–17) on BOLD Signal Intensity in Different Brain Regions in Mice

We also investigated the changes in BOLD signal intensity in different brain regions after the direct injection of dynorphin A (1–17) in the spinal cord of mice. Intrathecal injection of 30-pmol dynorphin A (1–17), which induced maxim u m pain-related responses in the behavior study, caused a significant increase in BOLD signal intensity in the cingulate cortex, somatosensory cortex, and insular cortex of mice compared with the basal activity. Although each of the signal intensities was increased compared with the basal intensity, there were slight differences in the time course of the increase between these regions. The cingulate cortex was significantly activated after intrathecal injection of dynorphin A (1–17) (fig. 6A). In the somatosensory cortex, intrathecal injection of dynorphin A (1–17) produced a significant increase in signal intensity (fig. 6B). In the insular cortex, the activation appeared after intrathecal injection of dynorphin A (1–17) (fig. 6C). In the medial and lateral thalamic nuclei, both BOLD signal intensities were significantly changed after intrathecal injection of dynorphin A (1–17) (figs. 6D and E). These activations reverted to their basal levels at 6 h after dynorphin A (1–17) injection. Control mice that had been injected intrathecal with saline did not show such activation in these regions (fig. 6). Statistical analys e s were done with two-way ANOVA followed by Bonferroni test (cingulate cortex: interaction between treatme n t and time: $F(7,64) = 0.965$, $P = 0.4642$; effect of treatment, $F(1,64) = 19.70$, $P < 0.0001$; effect of time, $F(7,64) = 0.938$, $P = 0.4837$; somatosensory cortex: interaction between treatment and time: $F(7,64) = 5.413$, $P < 0.0001$; effect of treatment, $F(1,64) = 19.85$, $P < 0.0001$; effect of time, $F(7,64) = 6.933$, $P < 0.0001$; insular cortex: interaction between treatment and time: $F(7,64) = 2.474$, $P < 0.026$; effect of treatment, $F(1,64) = 15.79$, $P = 0.0002$; effect of time, $F(7,64) = 2.977$, $P = 0.0091$; medial thalamic nuclei: interaction between treatment and time: $F(7,64) = 1.655$, $P = 0.1362$; effect of treatment, $F(1,64) = 28.97$, $P < 0.0001$; effect of time, $F(7,64) = 2.242$, $P = 0.0419$; lateral thalamic nuclei: interaction between treatment and time: $F(7,64) = 1.500$, $P = 0.1834$; effect of treatment, $F(1,64) = 12.78$, $P = 0.0007$; effect of time, $F(7,64) = 1.581$, $P = 0.1571$).

Effect of Pretreatment with MK-801 on Intrathecal Dynorphin A (1–17)–induced Pain-like Behaviors

To determine the possible involvement of spinal NMDA receptors in dynorphin A (1–17)-induced pain-like behaviors, we observed the influence of intrathecal pretreatment with the NMDA receptor antagonist, MK-801. As shown in figure 7, MK-801 pretreatment produced a dose-dependent inhibition of pain-like responses that could be caused by after intrathecal dynorphin A (1–17) injection. Statistical analys e s were done with one-way ANOVA followed by Dunnett test (biting or licking responses: $F(2,14) = 15.65$, 0.3 nmol: $P = 0.0022$, 1 nmol: $P = 0.0002$; total duration of responses: $F(2,14) = 13.28$; 0.3 nmol: $P = 0.0052$, 1 nmol: $P = 0.0003$; *vs.* vehicle–dynorphin A (1–17)-treated group). Under these conditions, control mice of both, that is, pretreatment with saline and MK-801 that had been injected with saline, failed to show these behaviors (data not shown).

Effect of Pretreatment with MK-801 on the Increase in BOLD Signal Intensity Induced by Intrathecal Injection of Dynorphin A (1–17)

Because the cingulate cortex, somatosensory cortex, insular cortex, and the medial and lateral thalamic nuclei were all activated by intrathecal injection of dynorphin A (1–17) in a time-dependent manner, we further investigated whether the activation of these brain regions could be changed by pretreatment with MK-801. After intrathecal injection of MK-801 (1 nmol) and before dynorphin A (1–

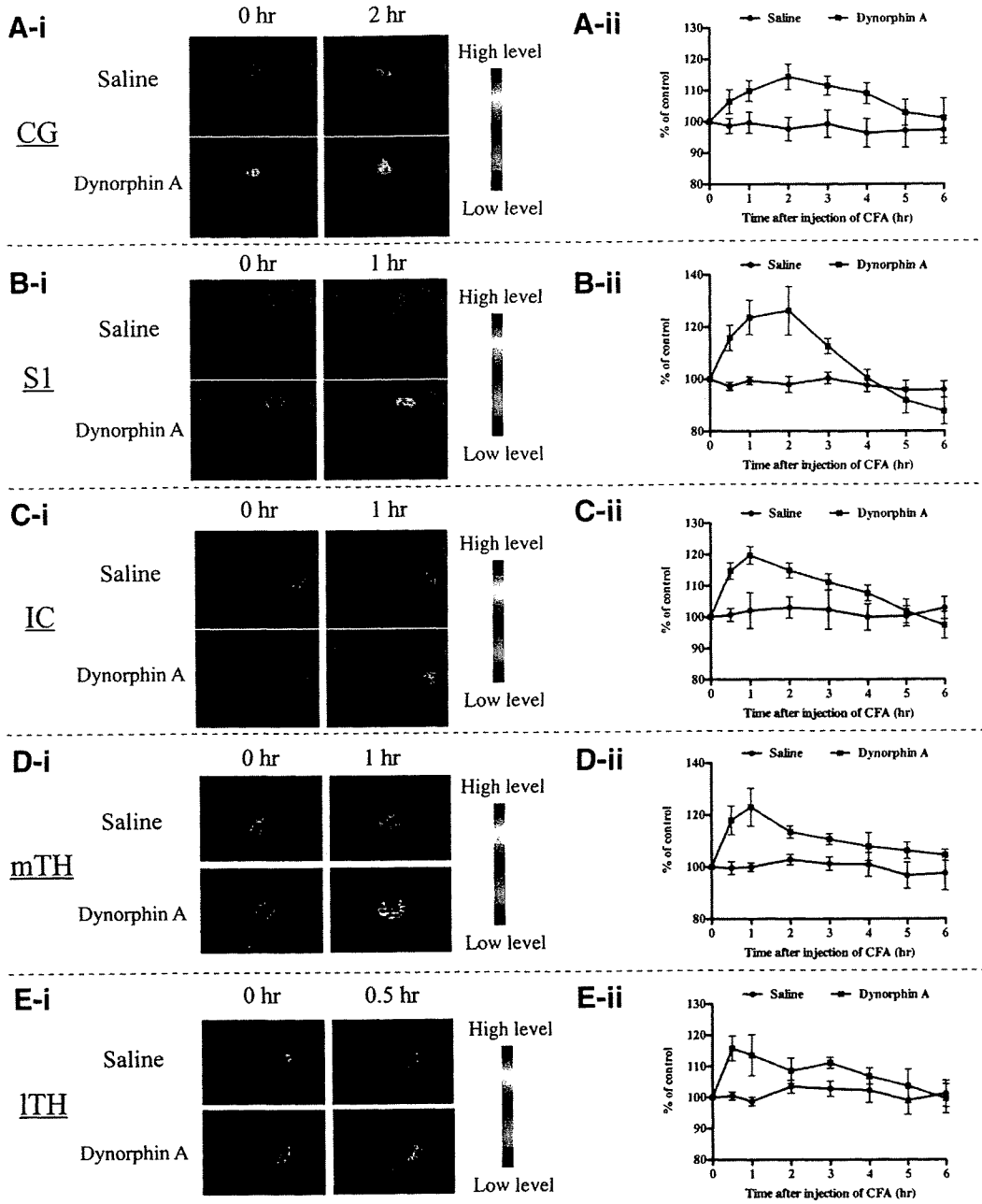

Fig. 6. Time course of the effects of intrathecal injection of dynorphin A (1–17) (30 pmol) on blood oxygenation level-dependent (BOLD) signal intensity in the cingulate cortex (CG), somatosensory cortex (S1), insula cortex (IC), medial thalamic region (mTH), and lateral thalamic region (lTH) in mice. Representative activation maps (overlaid on anatomy) correspond to composite images at 0 or 2 h (*A-i*: CG), 0 or 1 h (*B-i*: S1, *C-i*: IC, *D-i*; mTH), and 0 or 0.5 h (*E-i*: lTH) after the intrathecal injection of dynorphin A (1–17) in mice. The intrathecal injection of dynorphin A (1–17) produced a significant increase in BOLD signal compared with basal intensity (*A-ii*: CG; *B-ii*: S1; *C-ii*: IC; *D-ii* mTH; *E-ii*: lTH). Each point indicates the mean ± SEM for five mice. CFA = complete Freund's adjuvant.

17) administration, we compared the changes in the increased levels of signal intensity in brain regions after intrathecal injection of dynorphin A (1–17), when the most significant increase in BOLD signal intensity was observed. As for the cortex regions, the increased levels of BOLD intensity at 2 h (in the cingulate cortex) or 1 h (in somatosensory cortex and the insular cortex) induced by intrathecal dynorphin A (1–17) were significantly suppressed by pretreatment with MK-801 (figs. 8A–C). In the medial and lateral thalamic nuclei, increased levels of BOLD intensity induced by dynorphin A (1–17) were also blocked by pretreatment with MK-801 (figs. 8D and E). Two-way ANOVA showed a significant interaction between phase and pretreatment (cingulate cortex, $F(1,16) = 6.517$, $P = 0.0213$; somatosensory cortex, $F(1,16) = 13.06$, $P = 0.0023$; insular cortex, $F(1,16) = 26.31$, $P = 0.0001$; medial thalamic nuclei, $F(1,16) = 9.884$, $P = 0.0063$; lateral thalamic nuclei, $F(1,16) = 8.402$, $P = 0.0105$), a significant

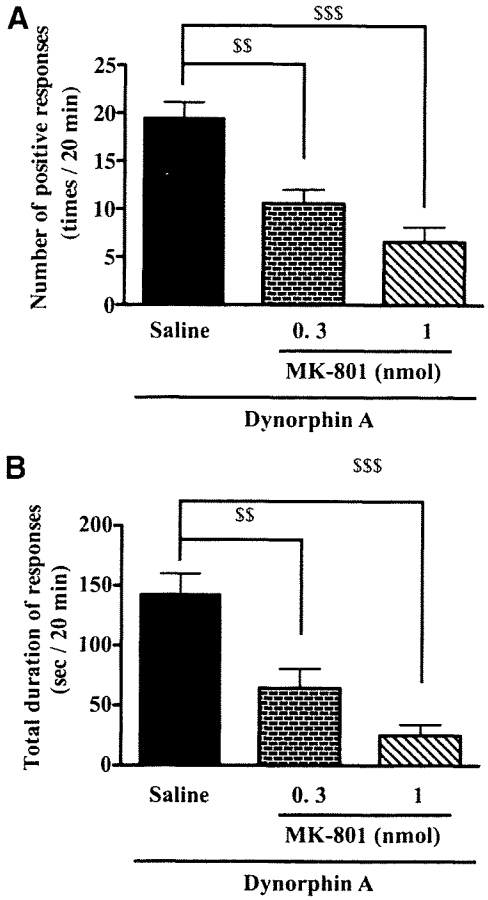

Fig. 7. Effect of intrathecal pretreatment with MK-801 on dynorphin A (1–17)-mediated pain-like response. Groups of mice were treated intrathecal with a noncompetitive *N*-methyl-D-a spartic acid receptor antagonist, MK-801 (1 nmol/mouse), or vehicle 30 min before the intrathecal injection of dynorphin A (1–17) (30 pmol). Immediately after the intrathecal injection of dynorphin A (1–17), the number of licking or biting behaviors was counted (*A*), and the duration of licking or biting behaviors was measured (*B*) for 20 min after the intrathecal injection of dynorphin A (1–17). Dunnett test: \$\$ $P < 0.01$, \$\$\$ $P < 0.001$, *versus* saline-dynorphin A (1–17) group.

effect of phase (cingulate cortex, $F(1,16) = 10.17$, $P = 0.0057$; somatosensory cortex, $F(1,16) = 29.52$, $P < 0.0001$; insular cortex, $F(1,16) = 34.81$, $P < 0.0001$; medial thalamic nuclei, $F(1,16) = 15.30$, $P = 0.0012$; lateral thalamic nuclei, $F(1,16) = 6.496$, $P = 0.0215$), and a significant effect of pretreatment (cingulate cortex, $F(1,16) = 6.427$, $P = 0.0221$; somatosensory cortex, $F(1,16) = 13.35$, $P = 0.0021$; insular cortex, $F(1,16) = 25.20$, $P = 0.0001$; medial thalamic nuclei, $F(1,16) = 11.64$, $P = 0.0036$; lateral thalamic nuclei, $F(1,16) = 9.030$, $P = 0.0084$). *Post hoc* comparison indicated a significant difference between the basal intensity of saline-pretreated groups *versus* dynorphin challenge in saline-pretreated groups (cingulate cortex, $F(3,16) = 7.703$, $P = 0.0048$; somatosensory cortex, $F(3,16) = 18.64$, $P < 0.0001$; insular cortex, $F(3,16) = 28.77$, $P < 0.0001$; medial thalamic nuclei, $F(3,16) = 12.27$, $P = 0.0008$; lateral thalamic nuclei, $F(3,16) = 7.976$, $P = 0.0085$) and a significant difference between the dynorphin challenge in saline-pretreated groups *versus* dynorphin challenge in MK-801-pretreated groups (cingulate cortex, $F(3,16) = 7.703$, $P = 0.0144$; somatosensory cortex, $F(3,16) = 18.64$, $P < 0.0001$; insular cortex, $F(3,16) = 28.77$, $P < 0.0001$; medial thalamic nuclei, $F(3,16) = 12.27$, $P = 0.00165$; lateral thalamic nuclei, $F(3,16) = 7.976$, $P = 0.0043$).

Discussion

Many studies have confirmed that endogenous opioid neuropeptides are associated with nociceptive transmission. In addition to endogenous opioid peptides, dynorphin A has been implicated in both pronociceptive and antinociceptive actions and is widely expressed throughout the central nervous system.[3] Several studies have provided evidence that the nociceptive action of spinal dynorphin plays a role in the long-term maintenance of chronic pain, such as inflammatory pain[8,21] and neuropathic pain.[39] However, little, if any, is known about the role of dynorphin A in the acute phase of inflammatory pain. Therefore, in this study, we first investigated whether dynorphin A could be involved in pain-associated behaviors and brain activity in the acute phase of the peripheral inflammatory pain-like state using prodynorphin gene knockout mice and their wild-type mice.

Role of Dynorphin A in the Acute Phase of Inflammatory Pain

Prodynorphin gene knockout mice were indistinguishable from wild-type mice in terms of general behavior and development. Intraplantar injection of CFA increased the duration and number of spontaneous pain-associated behaviors such as flinching or licking in wild-type mice but not in prodynorphin gene knockout mice. Under the present condition, phMRI showed that intraplantar injection of CFA caused robust positive signal activity in the cingulate cortex, somatosensory cortex, insular cortex, and medial and lateral thalamic nuclei of wild-type mice. In contrast, these effects were diminished in prodynorphin knockout mice. Because cortical areas are activated by the receipt of noxious information from the spinothalamic tract, many neuroimaging studies have shown activity by demonstrating brain circuitry.[40–42] These cortical representations of pain are called the pain matrix, including the somatosensory cortex, insular cortex, cingulate cortex, and prefrontal cortex.[43] Among these areas, the cingulate cortex area is an affective-motivational component of pain and mainly receives information from the medial system of the spinothalamic tract.[44,45] On the other hand, the somatosensory cortex area is a sensory-discriminative component of pain and mainly receives information from the lateral system of the spinothalamic tract. The insular cortex is basically considered to be not only in charge of the sensory-integrative component *via* the lateral system but also involved in limbic integration by virtue of the medial system.[43] The medial and lateral thalamic nuclei are also categorized as centers for pain perception, which relay sensory information to those cortical areas. Taken together, the present findings

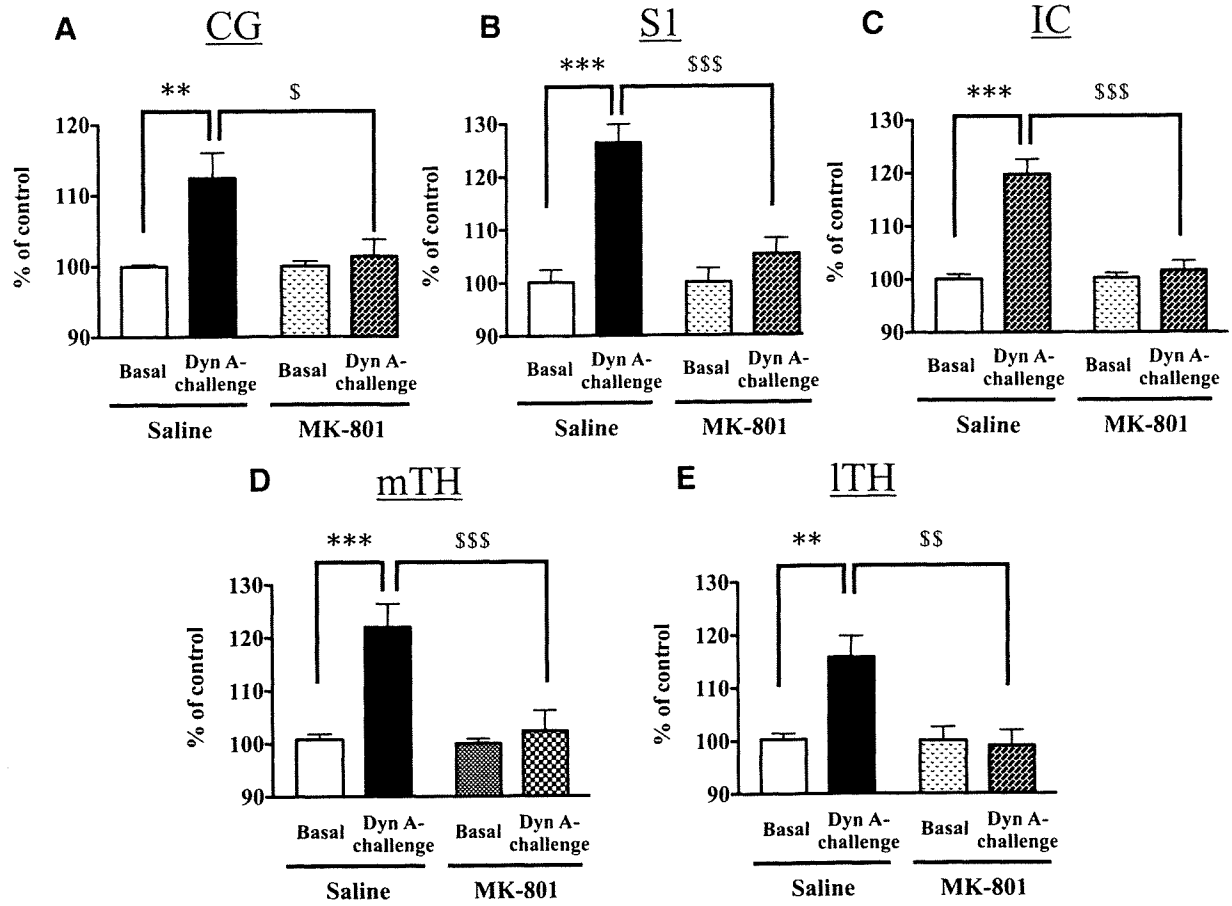

Fig. 8. The effect of the pretreatment of the MK-801 on increase in dynorphin-induced signal intensity (*A*: cingulate cortex (CG); *B*: somatosensory cortex (S1); *C*: insula cortex (IC); *D*: medial thalamic region (mTH); *E*: lateral thalamic region (lTH)). Data are expressed as percentages of the corresponding baseline levels with mean ± SEM for five mice. Bonferroni test: ** $P < 0.01$, *** $P < 0.001$, the basal intensity of saline-pretreated groups *versus* dynorphin challenge in saline-pretreated groups; \$ $P < 0.05$, \$\$ $P < 0.01$, \$\$\$ $P < 0.001$, the dynorphin challenge in saline-pretreated groups *versus* dynorphin challenge in MK-801-pretreated groups.

suggest that dynorphin A regulates spontaneous pain-associated behaviors and may play a crucial role in the activation of these pain-related brain regions in the acute phase of inflammatory pain.

Evidence for a Role of Spinal Dynorphin A

In the spinal cord, dynorphin A is principally localized in laminae 1 and 2, and in an inflammatory state it spreads to laminae 3 and 4 and deeper laminae.[20,46] Furthermore, it has been reported that spinal dynorphin A immunoreactivity is increased in CFA-injected mice, [20,46] and the upregulation of spinal preprodynorphin mRNA is induced by peripheral inflammation.[6,8,21,47,48] It was reported that in rats, incisional surgery increased the spinal cord dynorphin expression but did not drive microglial prostaglandin production or mechanical hypersensitivity.[49] Although further investigation is required, these findings suggest that the involvement of spinal dynorphin in the different types of pain might be, in some cases, the species difference. In this study, the level of prodynorphin mRNA in the lumbar spinal cord of mice was increased at 1, 3, and 6 h after CFA injection. Furthermore, 6 h after intraplantar injection, CFA produced a marked increase in dynorphin A (1–17)-like immunoreactivity on the superficial layers of the ipsilateral side of the spinal cord. In this study, we demonstrated that CFA-induced pain-associated behaviors were suppressed by intrathecal injection of a specific antiserum for dynorphin A (1–17). These findings suggest that peripheral inflammation may release dynorphin A (1–17) with an increase in the expression of prodynorphin in the spinal cord, resulting in the induction of an early phase of an inflammatory pain-like state.

Multiple Mechanism of Spinal Dynorphin A

Prodynorphin transcription is regulated by several factors in a tissue-specific manner.[50] It has been proposed that dynorphin expression is determined by a balance between gene transactivation and repression.[51] In the spinal cord, the binding of transactivators including cyclic adenosine monophosphate response element-binding protein to cyclic adenosine monophosphate response element,[52] the binding of c-*fos* with c-*jun* to an AP-1 element in the dynorphin promoter,[53] and the derepression of DREAM[54] play important roles in prodynorphin gene transcription. Several studies have reported that dynorphin A can affect several ion channels or

nociceptive receptors through nonopioidergic mechanisms.[3,15,18,19,55] In addition, those mechanisms involve the theory that dynorphin A may play a role in both antinociceptive and pronociceptive effects depending on its concentration: the former is due to endogenous κ-opioidergic action and the latter is due to glutaminergic actions through NMDA receptors.[3] At physiologically lower concentrations, dynorphin A reduces calcium influx *via* κ-opioid receptor activity, whereas at pathologically higher concentrations, dynorphin A causes increased intracellular calcium levels by activating NMDA receptors.[15] Although further experiment is required for the measurement of released dynorphin A in the present case after CFA injection, we propose here that the noxious stimuli after CFA injection into the hind paw may consistently release high concentrations of dynorphin A in the spinal cord, leading to excitatory neurotransmission.

Involvement of NMDA Receptors in Spinal Dynorphin A–dependent Ascending Pain Transmission

It is well known that NMDA receptors initiate increases in the excitability of spinal neurons and cause the central sensitization of pain. Furthermore, a previous study suggested that NMDA receptors in the spinal cord play a major role in the increased BOLD signal in the somatosensory cortex by way of the spinothalamic pathway.[56] Therefore, a subsequent study was undertaken to investigate whether NMDA receptors could be involved in spontaneous pain-associated behaviors or changes in brain activation induced by the intrathecal injection of dynorphin A. A single intrathecal injection of dynorphin A (1–17) caused an increase in pain-associated behaviors, and this effect was suppressed by pretreatment with MK-801. Under these conditions, we found here for the first time that dynorphin A produced a significant increase in signal intensity in the cingulate cortex, somatosensory cortex, insular cortex, and medial and lateral thalamic nuclei at 0.5–2 h after injection compared with the basal intensity, and this effect was abolished by pretreatment with MK-801. If we consider these findings, the present data suggest that an increase in spinal dynorphin A (1–17) may activate the transmission of ascending nociceptive information in the central nervous system *via* NMDA receptors. Although additional research is needed to understand the mechanism of the activation of dynorphin A in the early phase of inflammatory pain, it seems likely that increased spinal dynorphin A may contribute not only to signals of acute pain but also to induction of chronic pain *via* spinal NMDA receptors.

In view of the clinical application of the present information, we propose that spinal dynorphin may be an unique biomarker for pain.

Conclusion

The present data constitute novel evidence that loss of the prodynorphin gene prevents the ascending transmission of nociceptive information from the dorsal horn of the spinal cord to brain areas after the intraplantar injection of CFA. Moreover, dynorphin A within the spinal cord is directly involved in the early phase of an inflammatory pain-like state through NMDA receptors. This study is the first to clarify the ongoing brain activation in the early phase of inflammatory pain associated with an endogenous dynorphinergic pathway in an animal model of pain by means of pharmacological neuroimaging technology.

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Cell-dependent physiological synaptic action of morphine in the rat habenular nucleus: Morphine both inhibits and facilitates excitatory synaptic transmission

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ABSTRACT

Although several lines of evidence have suggested that the activity of thalamic neurons is modulated by opioids, the mechanism by which morphine in the thalamus regulates the release of excitatory neurotransmitters remains unclear. In the present study, we investigated the synaptic modulation of morphine to regulate excitatory synaptic transmission, probably glutamatergic transmission, in habenular nucleus (Hb) and centrolateral nucleus (CL) neurons in the rat thalamus. Using the whole-cell patch-clamp technique, we found dual modulation by morphine in Hb neurons: morphine caused either inhibition or facilitation of the miniature excitatory postsynaptic current (mEPSC) frequency in the Hb. In Hb neurons that showed a morphine-induced decrease in the mEPSC frequency, the mEPSC amplitude was also decreased in the presence of morphine. In contrast, the mEPSC amplitude was markedly increased in Hb neurons that showed a morphine-induced increase in the mEPSC frequency. We also observed a significant decrease in the mEPSC frequency with morphine in CL neurons without any change in the mEPSC amplitude, whereas morphine did not facilitate the mEPSC frequency in CL neurons. These results suggest that morphine may induce cell-dependent dual modulation of glutamatergic synaptic transmission in the Hb.

There is broad agreement on the general outlines of the afferent transmission pathways from primary afferent nociceptors through the spinal dorsal horn to the thalamus. Indeed, noxious stimulation activates thalamic neurons including the centrolateral nucleus (CL) in the medial thalamus and the habenular nucleus (Hb) in the epithalamus, as revealed by electrophysiological studies [1,8,26,27,31,32]. Studies on the supraspinal projection of spinothalamic pathways have suggested that the medial thalamus including the CL relays peripheral nociceptive information to the frontal part of the cortex and plays an important role in the affective and motivational aspects of pain processing [3,11,37]. In contrast, the Hb receives input from the limbic forebrain and pallidum and, in turn, projects to numerous midbrain structures [12,13]. Previous works have shown that analgesia could be achieved by electrical or chemical stimulation of the Hb [5,6,19,33,34]. These results suggest that a potential antinociceptive effect may originate via the Hb.

Autoradiographic, mRNA and immunohistochemical studies have shown the localization of μ-opioid receptors (MORs) in the thalamus, including the Hb and the CL [21–23]. Stimulation of MORs by the MOR-selective agonist D-Ala^2, N-MePhe^4, Gli-ol^5-enkephalin (DAMGO) results in an increase of an inwardly rectifying potassium conductance, which hyperpolarizes the cell and changes its firing pattern on the postsynaptic membrane in the CL [2]. Our previous study showed that stimulation of MOR by morphine in the Hb may be involved in activation of a descending antinociceptive pathway via excitatory synaptic transmission [28]. Although these results have suggested that the activity of thalamic neurons may be either positively or negatively modulated by morphine, the effect of morphine in thalamic transmission remains unclear. Therefore, the present study was undertaken to investigate the role of morphine in the presynaptic modulation of excitatory synaptic transmission in rat Hb and CL neurons. We observed the dual modulation of excitatory synaptic transmission by morphine in Hb neurons: morphine in the rat Hb caused either inhibition or facilitation of the miniature excitatory postsynaptic current (mEPSC) frequency. In contrast, morphine only produced a reduction of the mEPSC frequency in CL neurons.

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.

After young Sprague Dawley rats (10–18 days old) were decapitated, a block of tissue containing the thalamus was trimmed.

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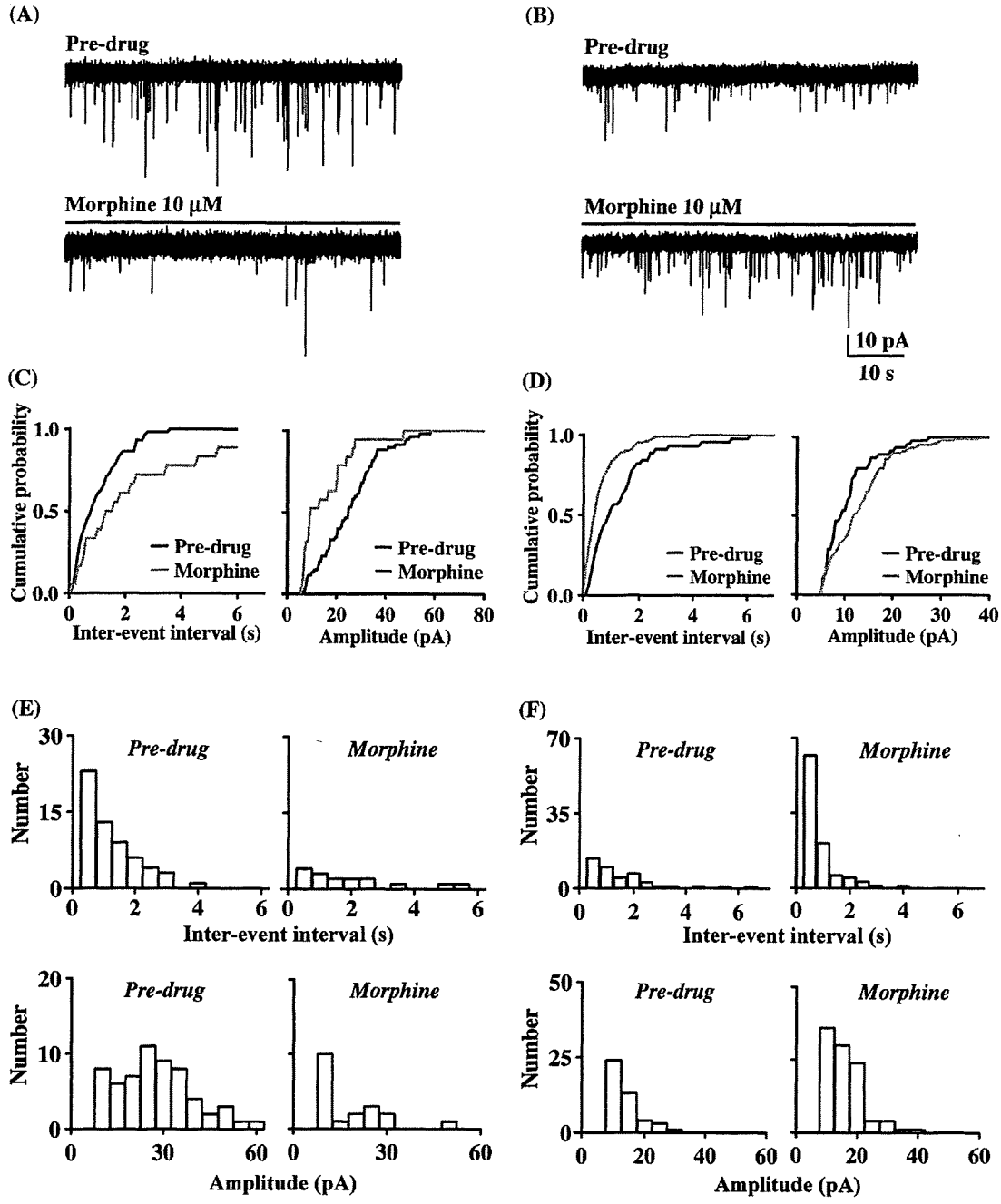

Fig. 1. Presynaptic effects of morphine in the habenular nucleus (Hb). (A and B) Representative mEPSCs recorded in the absence (pre-drug; top) or presence of 10 μM morphine (bottom) at a holding potential of −70 mV. Bath application of morphine for 3 min induced a marked decrease (A) or increase (B) in the mEPSC amplitude and frequency in Hb neurons. (C and D) Cumulative distributions of the mEPSC inter-event interval or amplitude obtained from the same neuron as shown in (A) or (B), respectively, in the absence (pre-drug) or presence of morphine. (E and F) Histograms of the number of events per minute plotted against the inter-event interval (top) or amplitude (bottom) in the absence (pre-drug; left) or presence of morphine (right). Data were obtained from the same neuron as shown in (A) or (B), respectively.

Thalamic coronal slices (150 μm thick) were cut from the block using a microslicer (DTK-1000; Dosaka, Kyoto, Japan) and perfused at a rate of 3 ml/min with artificial cerebrospinal fluid (in mM: NaCl 117, KCl 3.6, $CaCl_2$ 2.5, $MgCl_2$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 11) along with the sodium channel blocker tetrodotoxin (TTX) 0.3 μM (Wako Chemicals, Osaka, Japan), which was saturated with 5% CO_2 and 95% O_2 at room temperature. Standard whole-cell voltage-clamp recordings were made from thalamic neurons using an EPC8 amplifier (Heka, Lambrecht, Germany). Neurons were visualized under Nomarski optics with a water-immersion lens (BX-50WI; Olympus, Tokyo, Japan). The microelectrode solution had the following composition (in mM): potassium gluconate 136, KCl 5, $CaCl_2$ 0.5, $MgCl_2$ 2, EGTA 5, HEPES 5 and MgATP 5. The patch microelectrodes were made from borosilicate capillary glass and had resistances of 5–10 MΩ for the whole-cell recording. Under voltage-clamp recording conditions, the series resistance was 4–7 MΩ. The signals filtered at 3 kHz were directly digitized and stored on a personal computer. These sampled measurements were analyzed using the pCLAMP8 program (Axon Instruments). Miniature excitatory postsynaptic currents were analyzed using the MiniAnalysis Program (Synaptosoft Inc., Decatur, GA, USA).

Whole-cell patch-clamp recordings were performed in Hb neurons of P10-18 thalamic slices. In the voltage-clamp mode, mEPSCs were observed in 17 Hb neurons and 11 CL neurons at a hold-

ing potential of −70 mV. In the present study, these mEPSCs were completely blocked by 10 μM CNQX and 10 μM MK-801 (data not shown), indicating that they were mediated by glutamate receptors. We first investigated whether morphine changes the properties of mEPSCs in the Hb. Bath application of morphine induced a marked decrease ($n = 6$, Fig. 1A, C and E) or increase ($n = 5$, Fig. 1B, D and F) in the mEPSC amplitude and frequency in Hb neurons. The morphine-induced decrease or increase in mEPSC frequency was observed in approximately 35% or 30% of Hb neurons that showed mEPSCs, respectively. The average decrease or increase in the mEPSC number per minute was 58.4 ± 11.6% or 191.5 ± 48.1% of the control, respectively ($p < 0.05$, Student's t-test). In Hb neurons that showed a morphine-induced decrease in the mEPSC frequency, the mEPSC amplitude was also decreased in the presence of morphine (4 of 6 neurons) (Fig. 1C and E). The average decrease in the mEPSC amplitude per minute was 69.8 ± 6.0% of the control ($n = 4$, $p < 0.05$, Student's t-test). Interestingly, the mEPSC amplitude was markedly increased in Hb neurons that showed a morphine-induced increase in the mEPSC frequency (2 of 5 neurons, Fig. 1 D and F). In the remaining neurons tested (6 of 17 neurons), bath application of morphine failed to change the mEPSC frequency.

We next investigated whether morphine affected the properties of mEPSCs in the CL. The application of morphine reduced the mEPSC frequency in CL neurons (Fig. 2A). This morphine-induced decrease in the mEPSC frequency was observed in approximately 35% of the CL neurons examined (4 of 11 neurons). The average decrease in the mEPSC frequency was 60.2 ± 5.9% of the control ($n = 4$, $p < 0.05$, Student's t-test). Application of morphine prolonged the inter-event interval of the mEPSC frequency without affecting its amplitude in CL neurons (Fig. 2B and C). In the remaining CL neurons tested (7 of 11 neurons), the bath application of morphine did not change the mEPSC frequency. Morphine did not facilitate the mEPSC frequency in CL neurons.

It has been reported that MOR agonists presynaptically inhibit glutamatergic synaptic transmission in the spinal dorsal horn [15–17], midbrain periaqueductal gray (PAG) [36] and cultured hippocampal neurons [18]. In the present study, morphine decreased the frequency of mEPSC that was blocked by CNQX and MK-801 in both Hb and CL neurons. The frequency of mEPSCs depends on the probability of neurotransmitter release from presynaptic terminals [10]. The present data suggest that morphine may act presynaptically to inhibit glutamate release in the Hb and CL.

The CL in the medial thalamus is a primary receiving area for somatosensory input, presumably affective and motivational aspects of pain processing, and contains MORs [21,22] along with endogenous opioid peptides [9,25]. Stimulation of MORs in the CL hyperpolarized the cell and changed its firing pattern on the postsynaptic membrane [2]. Based on these findings and those in the present study, morphine may act presynaptically or postsynaptically to reduce excitatory synaptic transmission to CL neurons from the terminals of the spinothalamic tract, and could modulate nociceptive transmission projecting to the frontal cortex from the CL.

In the present study, morphine induced a marked decrease in the mEPSC frequency while decreasing the mEPSC amplitude in Hb neurons. In contrast, morphine reduced the mEPSC frequency without changing its amplitude in CL neurons. It has been considered that the mEPSC amplitude depends on several factors, including, but not limited to, the amount of transmitter released, postsynaptic sensitivity, and the driving force for the ions that mediate the synaptic current [35]. Thus, the fact that the mEPSC amplitude was decreased by morphine in the Hb but not the CL seems to result from differences in the MOR distribution: Hb neurons express MORs at high density, whereas CL neurons contain lower levels of MOR. This contention can be supported by the previous finding that morphine decreased the mEPSC amplitude in cultured rat hippocampal

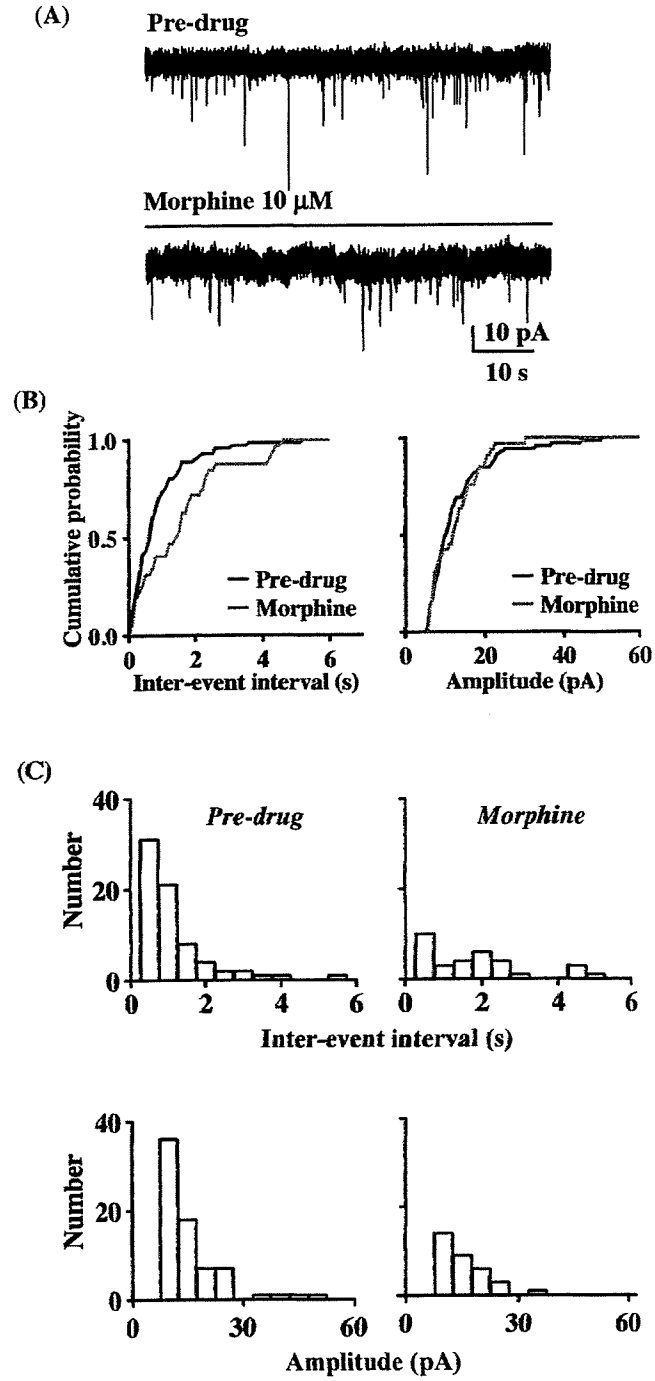

Fig. 2. Presynaptic effect of morphine in the centrolateral thalamic nucleus (CL). (A) Representative mEPSCs recorded in the absence (pre-drug; top) or presence of 10 μM morphine (bottom) at a holding potential of −70 mV. (B) Cumulative distributions of the mEPSC inter-event intervals or amplitude obtained from the same neuron as shown in (A) in the absence (pre-drug) or presence of morphine. (C) Histograms of the number of events per minute plotted against the mEPSC inter-event interval (top) or amplitude (bottom) in the absence (pre-drug; left) or presence of morphine (right). Data were obtained from the same neuron as shown in (A).

neurons overexpressing MORs, whereas it had no effect on intact neurons expressing normal levels of MORs [18].

In the present study, we found that the application of morphine caused either suppression or potentiation of the mEPSC amplitude in respective Hb neurons. These results are also consistent with a previous observation of the complex MOR modulation of glutamatergic synaptic transmission: activation of MOR is associated with either a decrease or increase in NMDA currents in individual

nucleus accumbens neurons [24]. We previously demonstrated that the application of morphine to cultured thalamic neurons evoked a potentiation of postsynaptic glutamate receptor-related currents [28]. Several physiological studies have indicated that opioid receptors can elicit excitatory, as well as inhibitory, modulation through protein kinase C (PKC)-dependent signals [7,28,29]. It has been shown that the activation of PKC enhances mEPSC frequency in the hippocampus [4,14,20,30]. Taken together, these findings suggest that the morphine-induced increase in the mEPSC frequency and amplitude in the Hb may be associated with a PKC pathway via G-protein transduction systems.

Recently, we found that the microinjection of morphine into the Hb enhanced the antinociceptive effect induced by intra-Hb injection with glutamate, which was related to the phenomenon that the application of morphine to cultured thalamic neurons evoked a potentiation of postsynaptic glutamate receptor-related currents [28]. Although the exact mechanisms that underlie this facilitation by morphine are still unclear, our present data suggest that morphine can directly modulate postsynaptic Hb neurons that receive glutamatergic input. It has been demonstrated that Hb neurons receive input from the limbic forebrain and pallidum and, in turn, project to numerous midbrain structures related to the descending pain-control pathway [12,13]. Therefore, the stimulation of excitatory synaptic transmission in the Hb by morphine may be involved in activation of the descending antinociceptive pathway.

In conclusion, we have shown that morphine in the Hb promotes both the inhibition and facilitation of excitatory synaptic transmission, whereas morphine only promotes its reduction in the CL. Our data may provide a cellular basis for the synaptic action of morphine at the thalamus level and support a physiological role for opioids as important inhibitors of sensory information processing in the thalamus. Furthermore, the present data add to the growing list of excitatory synaptic transmission that can be directly regulated by morphine.

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μ-Opioid receptor-independent fashion of the suppression of sodium currents by μ-opioid analgesics in thalamic neurons

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ABSTRACT

Most reports in the literature have shown that the effects of opioid analgesics are primarily mediated by μ-opioid receptor (MOR), whereas other potential targets of opioid analgesics have not been thoroughly characterized. In this study, we found that extracellular application of morphine, fentanyl or oxycodone, which are all considered to be MOR agonists, at relatively high concentrations, but not endogenous μ-opioid peptides, produced a concentration-dependent suppression of sodium currents in cultured thalamic neurons. These effects of opioids were not affected by either a MOR antagonist naloxone or a deletion of MOR gene. Among these opioids, fentanyl strongly suppressed sodium currents to the same degree as lidocaine, and both morphine and oxycodone slightly but significantly reduced sodium currents when they were present extracellularly. In contrast, the intracellular application of morphine, but not oxycodone, fentanyl or lidocaine, reduced sodium currents. These results suggest that morphine, fentanyl and oxycodone each produce the MOR-independent suppression of sodium currents by distinct mechanisms in thalamic neurons.

μ-Opioid receptor (MOR) is the principle physiological target for most clinically important opioid analgesics, including morphine, fentanyl and oxycodone [9,12]. It is well known that opioid receptors transduce signals through pertussis toxin-sensitive G_i/G_o proteins to inhibit adenylyl cyclase, increase membrane K^+ conductance and reduce Ca^{2+} current, which leads to cell hyperpolarization and exerts an inhibitory effect [3,4,14]. Several physiological studies have also demonstrated that opioid receptors can activate phospholipase C/protein kinase C-linked pathways [15] in a diverse range of opioid-modulated events, such as pain regulation [11,13] and the response to neuronal excitability [11]. Although there are interesting pharmacological differences in the analgesic potency and the frequency and intensity of adverse events among opioid analgesics classified as MOR agonists, the potential targets of opioid analgesics other than MOR have not been thoroughly characterized.

There is broad agreement on the general outlines of the afferent transmission pathways from primary afferent nociceptors through the dorsal horn to the thalamus. Although the thalamus constitutes the main gateway through which the cerebral cortex receives external sensory signals, there have been relatively few studies on the mechanism of the analgesic effect of opioid analgesics on the thalamus.

Voltage-gated sodium channels play an important role in excitable cells such as nerve and muscle cells. Sodium channels generate rapid and transient inward currents that permit neuronal firing and axonal conduction. Sodium channels are also the target of several classes of drugs, including anesthetics, analgesics, antiepileptics, antidepressants and antiarrhythmics. Therefore, in this study we investigated whether clinically used opioid analgesics could affect voltage-gated sodium channels in rat thalamic neurons, and examined the mechanism by which they affect this channel.

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. Thalamic neuron/glia co-cultures were grown as follows. The thalamic region was obtained from Sprague–Dawley rat (Tokyo Laboratory Animals Science, Tokyo, Japan) embryos on embryonic day 17 or $MOR^{-/-}$ mice [16] at postnatal 1 day, minced, and treated with papain (9 U/ml, Worthington Biochemical, Lakewood, NJ, USA). After being

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