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Intracerebroventricular Morphine Produces Antinociception by Evoking γ-Aminobutyric Acid Release through Activation of 5-Hydroxytryptamine 3 Receptors in the Spinal Cord

Tomoyuki Kawamata, M.D., Keiichi Omote, M.D.,† Masaki Toriyabe, M.D.,‡ Mikito Kawamata, M.D.,§ Akiyoshi Namiki, M.D.‖*

Background: **It has been generally considered that supraspinal morphine activates the serotonergic descending inhibitory system and releases serotonin (5-hydroxytryptamine [5-HT]) in the spinal cord, producing antinociception through activation of 5-HT receptors. The involvement of a spinal γ-aminobutyric acid–mediated (GABAergic) system is also suggested in supraspinal morphine antinociception. It has been reported that spinal GABAergic system contributes to 5-HT_3 receptor-mediated antinociception. In this study, the authors investigated the contribution of spinal 5-HT_3 receptor and the GABAergic system in the intracerebroventricular morphine–induced antinociception.**

Methods: **Male Sprague-Dawley rats were used. Using the spinal microdialysis method, concentrations of 5-HT and GABA were measured after intracerebroventricular morphine administration. The effect of intracerebroventricular naloxone or spinal perfusion of a selective 5-HT_3 receptor antagonist 3-tropanyl-indole-3-carboxylate methiodide on the spinal release of GABA after intracerebroventricular morphine administration was also examined. In the behavioral study, involvement of 5-HT_3 receptors or $GABA_A$ receptors in the intracerebroventricular morphine–induced antinociceptive effect was investigated using the tail-flick test.**

Results: **Intracerebroventricular morphine (40 nmol) significantly increased spinal GABA and 5-HT release. Evoked spinal GABA release was reversed by intracerebroventricular naloxone (40 nmol) or spinal perfusion of 3-tropanyl-indole-3-carboxylate methiodide (1 mM). In the behavioral study, intracerebroventricular morphine produced significant antinociception. Intrathecal administration of either $GABA_A$ receptor antagonist bicuculine or 3-tropanyl-indole-3-carboxylate methiodide but not vehicle reversed the morphine-induced antinociceptive effect.**

Conclusion: **Intracerebroventricular morphine evokes spinal GABA release *via* the activation of 5-HT_3 receptors in the spinal cord, resulting in antinociceptive effect.**

IT is generally considered that supraspinal morphine produces its antinociceptive effect through the descending serotonergic and noradrenergic neurons projecting to spinal cord. Several spinal mechanisms of supraspinal morphine–induced antinociception have been proposed. In addition to direct inhibition of spinal neurons or primary afferent nerve terminals by noradrenaline and serotonin (5-hydroxytryptamine [5-HT]), indirect mechanisms *via* spinal interneurons have been suggested.[1,2] It has been reported that a spinal γ-aminobutyric acid type A ($GABA_A$) receptor antagonist inhibits the supraspinal morphine–induced antinociception.[1] Furthermore, a spinal GABA-mediated (GABAergic) system contributes to spinally administered 5-HT_3 receptor agonist–mediated antinociception.[3] These observations suggest a possible interaction between the serotonergic descending system and a spinal GABA system in supraspinal morphine–induced antinociception.

Among 5-HT receptor subtypes, only the 5-HT_3 receptor is a ligand-gated cation channel.[4] Electrical stimulation in the periaqueductal gray (PAG) produces inhibition of dorsal horn neuronal responses, and this PAG-induced inhibition is blocked by spinally administered 5-HT_3 receptor antagonists.[5] Behavioral studies indicated that intrathecal 5-HT–induced antinociception was mediated by 5-HT_3 receptors.[6] However, it is unlikely that the activation of 5-HT_3 receptors directly inhibits neuronal responses because the activation of 5-HT_3 receptors causes a depolarization by means of increased membrane permeability to monovalent cation.[4] Supraspinal morphine could evoke GABA release from interneurons through the activation of 5-HT_3 receptors in the spinal cord, resulting in antinociception. However, there has been no direct evidence of supraspinal morphine–induced 5-HT_3 receptor activation and GABA release in the spinal cord.

In the current study, we examined whether supraspinally administered morphine would evoke GABA release through the activation of 5-HT_3 receptors in the spinal cord. We also examined the functional roles of these neurotransmitters in supraspinal morphine–induced antinociception.

Materials and Methods

The protocol for this study was approved by Sapporo Medical University Animal Care and Use Committee (Sapporo, Japan). Experiments were conducted on male Sprague-Dawley rats (weight, 250–350 g; Japan SLC, Hamamatsu, Japan) housed individually in a temperature-controlled (21 ± 1°C) room with a 12-h light–dark cycle and given free access to food and water. Each animal was used in only one experiment.

* Instructor, † Associate Professor, ‡ Postgraduate Student, § Assistant Professor, ‖ Professor and Chairman.

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Address reprint requests to Dr. Kawamata: Department of Anesthesiology, Sapporo Medical University School of Medicine, South 1, West 16, Chuo-ku, Sapporo, Hokkaido 060-8543, Japan. Address electronic mail to: kawamata@sapmed.ac.jp.

Intracerebroventricular Cannula and Lumbar Intrathecal Catheter

Rats were anesthetized with pentobarbital (50 mg/kg administered intraperitoneally) and were placed in a stereotaxic head holder to implant an intracerebroventricular guide cannula. The skull was exposed, and a guide cannula equipped with a 32-guage stylet was directed into the left lateral ventricle according to Paxinos and Watson[7] (anterior-posterior +0.8 mm, midline +1.4 mm, dorsal-ventral 3.8 mm with respect to bregma) and was attached to the skull with three anchoring screws and cemented in place. For intracerebroventricular injection, the stylet was removed and the injection cannula was inserted.

For the behavioral study, a lumbar intrathecal catheter was implanted in the rats with an intracerebroventricular guide cannula. A polyethylene intrathecal catheter (PE-10; Becton Dickenson, Franklin Lakes, NJ) was inserted 15 mm cephalad into the lumbar subarachnoid space at the L4–L5 intervertebral level, with the tip of the catheter located near the lumbar enlargement of the spinal cord to administer the drugs intrathecally. The catheter was tunneled subcutaneously and externalized through the skin in the neck region. At least 6 days of postsurgical recovery were allowed before animals were used in the behavioral study.

Microdialysis Catheter Implantation

The spinal cord dialysis probe was prepared according to our modification of the method described by Skilling *et al.*[8] The dialysis probe was constructed from a 1-cm length of dialysis fiber (an ID of 200 μm, an OD of 220 μm, and 50-kd molecular weight cutoff; DM-22, Eicom Co., Kyoto, Japan) which was coated with a thin layer of epoxy glue (Devcon Co., Danvers, MA) along the whole length, except for a 2-mm region in the middle. To make the fiber firm enough for implantation, a Nichrome-Formvar wire with a 78-μm ID (A-M Systems, Inc., Everret, WA) was passed through the fiber. Each end of the fiber was attached to 2-cm polyethylene catheters (PE-10; Becton Dickinson, Franklin Lakes, NJ), and each end of the polyethylene catheters was then attached to an 8-cm teflon tube with an ID of 100 μm and an OD of 400 μm (JT-10; Eicom). Seven days after an intracerebroventricular guide cannula implantation, rats were anesthetized with pentobarbital (50 mg/kg administered intraperitoneally) and incised along the dorsal midline from T2 to L2. The lateral surfaces of vertebra L1 were exposed, and bilateral holes were carefully made through the bone, exposing the spinal cord laterally at the level of the dorsal horn. A dialysis tube was placed through the holes by hand, passing transversely through the dorsal spinal cord. The two distal ends of the probe were tunneled subcutaneously and externalized through the skin in the neck region. The experiments were performed 18–24 h after the implantation of the dialysis probe. After a recovery period, the animals showing any signs of limb paralysis or impaired movement were excluded in this study. After each experiment, we perfused methylene blue dye through the dialysis probe to verify the position of the dialysis fiber, and then rats were killed with an overdose of pentobarbital. The data to be reported are from rats in which methylene blue dye remained in the area of the dorsal half of the dorsal horn.

Microdialysis Study

The animals freely moved in a plastic cage with dimensions of 30 × 30 × 35 cm during the dialysis experiments. A liquid switch (SI-50; Eicom) was placed between the syringe pump and the dialysis probe to enable a different drug to be administered locally *via* the probe. The dialysis probe was perfused with artificial cerebrospinal fluid (ACSF; 140 mM NaCl, 4.0 mM KCl, 1.26 mM $CaCl_2$, 1.15 mM $MgCl_2$, 2.0 mM Na_2HPO_4, 0.5 mM NaH_2PO_4, pH 7.4) at a constant flow rate of 3 μl/min. The samples were collected as 20-min fractions and divided into two samples: 20 μl dialysate collected for analysis of GABA and 20 μl for 5-HT. The samples were frozen at −80°C until analysis. Three consecutive samples were collected for determination of basal concentrations 180 min after starting perfusion of ACSF. After obtaining three consecutive samples, 40 nmol/3 μl of intracerebroventricular morphine (Sankyo Co., Tokyo, Japan) was administered over a 120-s period. The samples were collected up to 360 min after morphine administration. To examine the naloxone sensitivity and the involvement of spinal 5-HT_3 receptor activation in morphine-induced spinal GABA release, either intracerebroventricular naloxone (Sigma, St.Louis, MO) or the selective 5-HT_3 receptor antagonist, 3-tropanyl-indole-3-carboxylate methiodide (TICM; Research Biochemical International, Natick, MA) was administered 180 min after morphine administration. Naloxone 40 nmol was injected in a volume of 3 μl over a 120-s period. TICM (1 mM) was perfused through the dialysis catheter for 60 min. All drugs used in microdialysis study were prepared in ACSF.

Analysis of Serotonin

Serotonin in the dialysate was analyzed using high-performance liquid chromatography with electrochemical detection (Degasser, DG-100; liquid chromatograph, EP-100; electrochemical detector, ECD-100; Eicom). The chromatographic conditions were as follows: column, Eicompak (CA-5ODS 2.1 × 150 mm; Eicom); mobile phase, 0.1 M phosphate buffer (pH 6.0) containing 20.0% methanol, 0.02 mM EDTA, and 0.72 mM sodium 1-octanesulfonate for the analysis of 5-HT; working electrode, glassy carbon (WE-3G, Eicom); flow rate, 0.23 ml/min. Detector voltage and detector temperature were set at 0.45 V and at 25.0°C, respectively. Retention time for

5-HT was 14.85 min. The detection limit for 5-HT analysis is 0.05 fg/20 μl.

Analysis of γ-Aminobutyric Acid

γ-Aminobutyric acid in the dialysate was analyzed using high-performance liquid chromatography with fluorescence detection (Degasser, DG-100; liquid chromatograph, EP-100; fluorescence detector, FLD-370; Eicom) after derivatization with o-phthaldialdehyde (Sigma). The o-phthaldialdehyde derivatizing reagent was prepared by dissolving o-phthaldialdehyde (54 mg) in absolute methanol (1 ml) and adding 2-mercaptoethanol (40 μl) and sodium carbonate (0.1 M, pH 9.5, 99 ml). Ten microliters of this derivatizing reagent was mixed with 20 μl of the dialysate and allowed to react for 2.5 min. Twenty microliters of this solution was analyzed. The chromatographic conditions were as follows: column, Eicompak (SC-5ODS 2.1 × 150 mm; Eicom); mobile phase, 0.1 M sodium dihydrogenphosphate and 0.1 M disodium hydrogenphosphate (pH 3.5) containing 50.0% methanol and 0.1 mM disodium EDTA; flow rate, 0.23 ml/min. Detector temperature was set at 30°C. Retention time for GABA was 14.83 min. The detection limit for GABA analysis is 0.2 pg/20 μl.

Behavioral Study

A behavioral study was performed separately from the microdialysis study. The tail-flick test was used to assess thermal nociceptive threshold. Tail-flick testing was performed by monitoring latency to withdrawal from the heat source (a 50-W projection lamp bulb) focused on a distal segment of the tail, using a thermal analgesimeter (KN-205E; Natsume, Tokyo, Japan). A cutoff time of 15.0 s was used to minimize damage to the skin of the tail. After determination of baseline tail-flick latencies, 40 nmol/3 μl of intracerebroventricular morphine was administered. The tail-flick latencies were measured at 30, 60, 120, 180, 240, 300, and 360 min after the intracerebroventricular injection. To examine the involvement of spinal 5-HT_3 receptors and $GABA_A$ receptors in morphine analgesia, either TICM or a $GABA_A$ receptor antagonist bicuculline (Sigma) was intrathecally administered 170 min after morphine administration, and the effect of the antagonist on tail-flick latencies was evaluated 10 min later. TICM and bicuculline were freshly dissolved in ACSF in concentrations that allowed intrathecal injections in 10-μl volumes. Intrathecal drug administration was accomplished by using a microinjection syringe (Hamilton, Reno, NV) connected to the intrathecal catheter in awake, briefly restrained rats. All intrathecal drugs were administered manually over 10 s.

Data Analysis

In the microdialysis study, the changes of GABA and 5-HT concentrations are presented as mean ± SD of percentage of basal concentrations. Data were compared with basal concentration using a one-way analysis of variance for repeated measures followed by Dunnett test within a single group, and were analyzed using a two-way analysis of variance for repeated measures followed by the Tukey Kramer test for between-group comparisons. In the behavioral study on morphine antinociception, the values of withdrawal latency were converted to percent maximum possible effect:

$$\%MPE = (\text{postdrug latency} - \text{baseline latency})/(\text{cutoff latency} - \text{baseline latency}) \times 100.$$

Changes in percent maximum possible effect were compared with baseline using a one-way analysis of variance for repeated measures followed by Dunnett test within a single group, and analyzed using a paired *t* test for the comparison between pretreatment and posttreatment in the behavioral study. $P < 0.05$ was considered statistically significant.

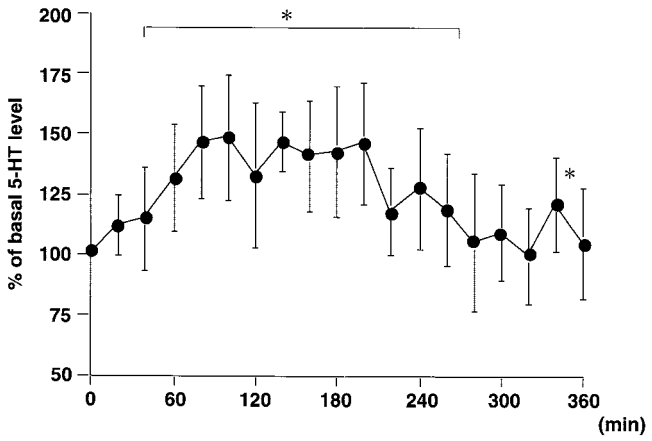

Fig. 1. Changes of 5-hydroxytryptamine (5-HT) concentration in the spinal cord after administration of 40 nmol intracerebroventricular morphine. Data are presented as mean ± SD of the percent of basal concentration, n = 8. *$P < 0.05$ compared with basal concentration.

Results

Microdialysis Study

Serotonin Analysis. The basal concentration of 5-HT was 2.57 ± 1.18 pg/20 μl. The coefficient of variation among the three consecutive samples for determination of basal concentrations was less than 7%. Intracerebroventricular morphine (40 nmol) evoked a significant increase in spinal 5-HT concentration ($P < 0.01$; fig. 1). Significant increases in 5-HT concentration were observed 40–240 min after morphine administration.

γ-Aminobutyric Acid Analysis. The basal concentration of GABA was 0.27 ± 0.14 pg/20 μl. The coefficient of variation among the three consecutive samples for determination of basal concentrations was less than 5%.

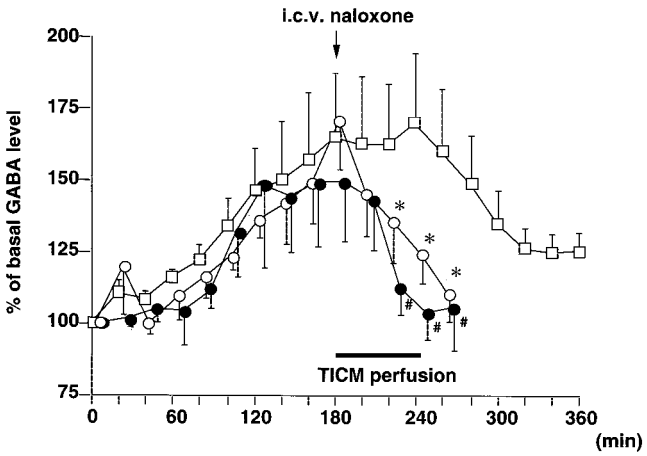

Fig. 2. Changes of γ-aminobutyric acid (GABA) concentration in the spinal cord. Open squares indicate GABA concentrations in the rats with administration of 40 nmol intracerebroventricular morphine (n = 8). Open circles indicate GABA concentrations in the rats with intracerebroventricular morphine followed by intracerebroventricular naloxone (n = 6). Intracerebroventricular naloxone was administered 180 min after morphine administration. Closed circles indicate GABA concentrations in the rats with intracerebroventricular morphine followed by spinal perfusion of 3-tropanyl-indole-3-carboxylate methiodide (TICM; n = 7). TICM was perfused 180–240 min after morphine administration. Data are presented as mean ± SD of the percent of basal concentration. #$P < 0.05$ between intracerebroventricular morphine and intracerebroventricular morphine followed by TICM. *$P < 0.05$ between intracerebroventricular morphine and intracerebroventricular morphine followed by intracerebroventricular naloxone. i.c.v. = intracerebroventricular.

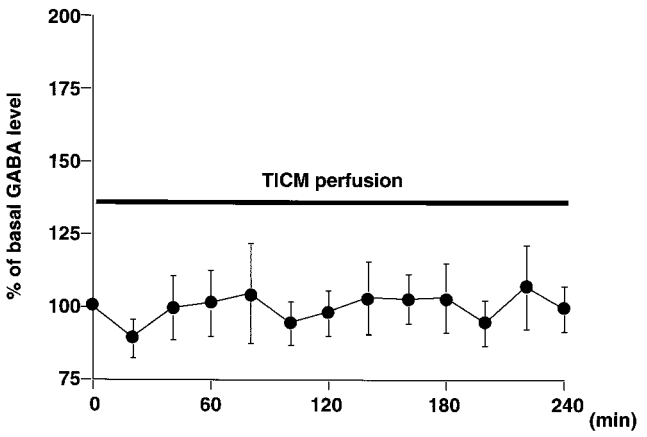

Fig. 3. Effect of spinal perfusion of 3-tropanyl-indole-3-carboxylate methiodide (TICM) on the basal γ-aminobutyric acid (GABA) concentration. TICM was perfused for 240 min. Data are presented as mean ± SD of the percent of basal concentration, n = 5.

Figure 2 shows the effect of 40 nmol intracerebroventricular morphine on spinal GABA concentration. GABA concentration was gradually increased after intracerebroventricular morphine administration and reached a plateau from 180 to 260 min after morphine administration. Significant increases in GABA concentration were observed from 80 up to 360 min after administration of morphine compared with the basal concentration ($P < 0.01$). Intracerebroventricular naloxone (40 nmol) administered at 180 min after morphine significantly reversed the morphine-induced increase in GABA concentration ($P < 0.01$). Spinal perfusion of 1 mM TICM for 60 min (from 180 to 240 min after morphine) also reversed morphine-induced increase in GABA concentration ($P < 0.01$). GABA concentrations 180 min after morphine administration were comparable among the three different treatments. TICM (1 mM) alone did not significantly affect the basal spinal GABA concentration for at least 240 min (fig. 3).

Behavioral Study

Effects of Intrathecal Bicuculline or TICM. Intrathecal administration of bicuculline at doses of greater than 0.02 μg shortened the tail-flick latency in the tail-flick test. For example, 0.2 μg bicuculline shortened the tail-flick latency, which was observed within 5 min after the administration and continued for 30 min (table 1). Doses of intrathecal TICM greater than 1.0 μg also shortened the tail-flick latency. For example, 10.0 μg TICM shortened the tail-flick latency that was observed within 5 min and continued for 30 min (table 2). Neither 0.02 μg intrathecal bicuculline nor 1.0 μg TICM affected the basal tail-flick latency (tables 1 and 2). Therefore, these doses were used in the behavioral study, and the antagonistic effects of these drugs were assessed at 10 min after intrathecal administration.

Antagonistic Effects of Intrathecal Bicuculline and TICM on the Intracerebroventricular Morphine–induced Antinociception

The mean baseline tail-flick latency in this experiment was 5.7 s (range, 5.1–6.2 s). Figure 4 shows the time course of the change in percent maximum possible effect after

Table 1. Change of Tail-flick Latency after Intrathecal Bicuculline

	Time after Intrathecal Administration (min)							
	0	5	10	15	20	30	45	60
0.02 μg Bicuculline	5.5 ± 0.2	5.4 ± 0.2	5.3 ± 0.2	5.4 ± 0.3	5.4 ± 0.2	5.5 ± 0.3	5.6 ± 0.1	5.4 ± 0.5 (s)
0.2 μg Bicuculline	5.6 ± 0.1	4.8 ± 0.4*	3.3 ± 0.5*	3.6 ± 0.4*	3.8 ± 0.5*	4.2 ± 0.6*	4.8 ± 0.6	5.4 ± 0.5 (s)

Values are mean ± SD of tail-flick latency (n = 6 in each dose).

*$P < 0.05$ compared with control (value at 0 min).

Table 2. Changes of Tail-flick Latency after Intrathecal TICM

	Time after Intrathecal Administration (min)							
	0	5	10	15	20	30	45	60
1.0 μg TICM	5.6 ± 0.1	5.5 ± 0.2	5.4 ± 0.2	5.4 ± 0.3	5.6 ± 0.2	5.5 ± 0.2	5.6 ± 0.4	5.5 ± 0.1 (s)
10.0 μg TICM	5.7 ± 0.2	3.8 ± 0.4*	3.6 ± 0.4*	3.9 ± 0.4*	4.5 ± 0.6*	4.7 ± 0.3*	5.4 ± 0.3	5.6 ± 0.1 (s)

Values are mean ± SD of tail-flick latency (n = 6 in each dose).

*$P < 0.05$ compared with control (value at 0 min).

TICM = 3-tropanyl-indole-3-carboxylate methiodide.

intracerebroventricular morphine administration. Intracerebroventricular morphine (40 nmol) produced significant antinociceptive effects from 30 min up to 360 min after its administration. The maximal antinociceptive effect was observed 30–180 min after morphine administration. Figure 5 shows the effects of intrathecal bicuculline and TICM on the intracerebroventricular morphine–induced antinociceptive effect. We evaluated the antagonistic effects of bicuculline and TICM 180 min after morphine administration, because at this time point, the maximal effect of morphine in GABA analysis was observed (fig. 2). Intrathecal bicuculline significantly reversed the morphine-induced antinociception ($P < 0.05$). Intrathecal TICM also significantly reversed the morphine-induced antinociception ($P < 0.05$). On the other hand, intrathecal ACSF alone did not produce any significant changes in the tail-flick latency. There were no significant differences in percent maximum possible effect before intrathecal administration among the three different treatments.

Discussion

Intracerebroventricular Morphine–induced Spinal Serotonin Release

The current study showed that intracerebroventricular morphine evoked spinal 5-HT release. Previous reports also found morphine–induced spinal 5-HT release.[9–11] The major source of serotonergic projections to the dorsal horn are neurons in the rostral ventromedial medulla, which contains both opioid peptides and opioid receptors.[12,13] Several investigators found that electrical or chemical stimulation of the dorsal lateral funiculus, bulbospinal neurons, or the nucleus raphe magnus evoked 5-HT release in the dorsal horn of the spinal cord.[14–16] Therefore, it would be considered that morphine evokes spinal 5-HT release through activation of rostral ventromedial medulla serotonergic neurons by the inhibition of inhibitory interneurons. However, this may not be always consistent. First, Matos *et al.*[17] concluded that intracerebroventricular morphine at an analgesic dose did not necessarily increase the extracellular concentration of 5-HT in the spinal dorsal horn using high-performance liquid chromatography with electrochemical detection, because morphine increased 5-HT

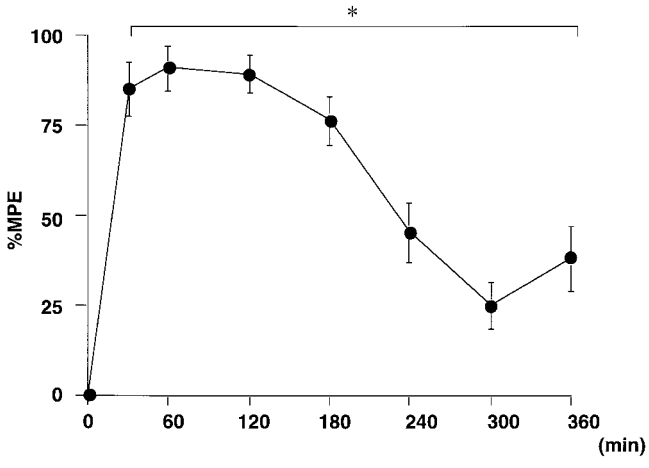

Fig. 4. Time course of percent maximum possible effect (%MPE) after administration of 40 nmol intracerebroventricular morphine in the tail-flick test. Data are presented as mean ± SD, n = 12.

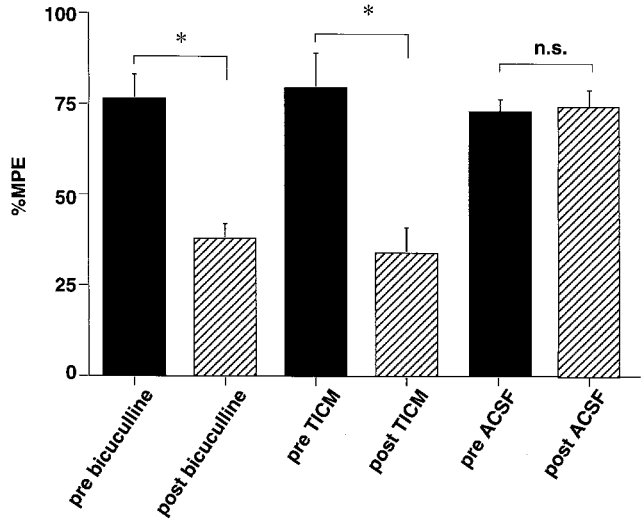

Fig. 5. Effects of intrathecal bicuculline, 3-tropanyl-indole-3-carboxylate methiodide (TICM), and artificial cerebrospinal fluid (ACSF) on the antinociceptive effect of intracerebroventricular morphine. Pre values indicate percent maximum possible effects (%MPE) before intrathecal drug administration (170 min after intracerebroventricular morphine). Post values indicate percent maximum possible effects 10 min after intrathecal drug administration (180 min after intracerebroventricular morphine). Data are presented as mean ± SD. *$P < 0.05$ between pre and post values. n.s. = no statistical significance. ACSF-treated rats, n = 6; TICM-treated rats, n = 8; bicuculline-treated rats, n = 8.

release in only two of four rats. The discrepancy between their results and ours may be caused by the sensitivity of assay system for 5-HT. Baseline 5-HT concentrations were close to the sensitivity limit in the high-performance liquid chromatography with electrochemical detection, and hence it is difficult to measure small changes reliably.[18] We used a smaller diameter (2.1 mm) detector column in the current study, compared with that used in the study by Matos *et al.*[17] (4.6 mm). This column enabled us to analyze 5-HT with high sensitivity. Using this assay system, morphine at the dose we used (40 nmol = 11.4 μg), which was almost comparable to the dose used by Matos *et al.* (10 μg), consistently increased spinal 5-HT concentration in all eight rats in the current study, and the degree of increase in 5-HT concentration was approximately 150% of the basal concentration. We performed the experiments 18–24 h after the implantation of the dialysis probe because of prevention of the decrease in recovery rate caused by gliosis. Therefore, there may be the possibility that the acute trauma of probe implantation modified the effect of morphine on 5-HT release. Second, electrophysiologic studies have demonstrated that most serotonergic rostral ventromedial medulla neurons are not affected by morphine.[19–21] However, those studies used the anesthetized animal. There is a possibility that anesthesia reduced serotonergic neuronal activity,[18,22] and serotonergic neuronal activity is lower during sleep than wakefulness.[23] We used the awake free-moving rats implanted with microdialysis probe in this study.

Intracerebroventricular Morphine–induced Activation of Spinal 5-HT$_3$ Receptors and Spinal γ-Aminobutyric Acid Release

Several lines of study have suggested that supraspinal morphine or PAG stimulation activates a spinal GABAergic system.[1,3,5,24–26] However, there has not been direct evidence that supraspinal morphine evokes spinal GABA release. In the current study, we clearly demonstrated that intracerebroventricular morphine evokes spinal GABA release concomitant with 5-HT release. There was the discrepancy in time course between intracerebroventricular morphine–induced increases in 5-HT and GABA concentrations in our results. Spinal microdialysis does not directly measure neurotransmitter concentrations at synaptic sites, but rather neurotransmitter concentrations that have diffused into the extracellular space. Diffusion into the extracellular space is likely to involve processes of uptake and metabolism, which can modify the amount of neurotransmitter in the dialysate. Therefore, it is possible that diffusion into the extracellular space would contribute to the discrepancy in time course between 5-HT and GABA concentration.

There are at least four subtypes of 5-HT receptors (5-HT$_1$, 5-HT$_2$, 5-HT$_3$, and 5-HT$_4$) in the spinal cord.[27,28] Among the subtypes of 5-HT receptors, 5-HT$_3$ receptors are unique because G proteins are not affected by agonist binding to 5-HT$_3$ receptors. 5-HT$_3$ receptors are directly linked to the opening of nonselective monovalent cation channels[4] and should mediate neuronal excitation. Alhaider *et al.*[3] demonstrated that the activation of the GABAergic system contributed to spinal 5-HT$_3$ receptor–mediated antinociception. Therefore, we focused on 5-HT$_3$ receptors. TICM used in the current study is a potent and selective 5-HT$_3$ receptor antagonist and has been commonly known as IC 205–930. Radioligands of IC 205–930 have been used to detect 5-HT$_3$ receptor binding site in the central nervous system.[29] In the current study, blockade of spinal 5-HT$_3$ receptors inhibited intracerebroventricular morphine–induced spinal GABA release. This indicates that morphine activates supraspinal serotonergic neurons projecting to the spinal cord, and then released 5-HT excites spinal GABAergic interneuron through 5-HT$_3$ receptors. Immunohistochemical and autoradiographic studies revealed that 5-HT$_3$ receptors are restricted to the superficial layers of the dorsal horn in the spinal cord.[29–31] Intense GABA immunoreactivities are also found within laminae I–III of the spinal cord.[32,33] It would be supposed that the GABAergic neuron with 5-HT$_3$ receptors are directly activated or activation of presynaptic 5-HT$_3$ receptors indirectly induce the activation of GABAergic neuron. There is a report that the 5-HT$_3$ receptors mainly were located in the primary afferent terminal in the dorsal horn.[34] In addition, Peng *et al.*[35] recently suggested that 5-HT$_3$ receptors located in central terminals of primary afferents might contribute to the generation of dorsal root reflex, leading to presynaptic inhibition. Therefore, further study is necessary to clarify the mechanism of 5-HT$_3$ receptor activation-induced GABA release.

Contributions of 5-HT$_3$ Receptor Activation and γ-Aminobutyric Acid Release to Supraspinal Morphine–induced Antinociceptive Effect

In a behavioral study, we examined whether spinal GABA release through the activation of 5-HT$_3$ receptors observed in our microdialysis study would be a component of supraspinal morphine–induced antinociception. GABA and 5-HT concentrations were measured within dorsal horn at the level of the L1 vertebra, and the tail-flick test was used in the behavioral study. The primary afferent neurons responsible for tail-flick response largely innervate in the dorsal horn of the sacrococcygeal spinal cord. In this study, GABA and 5-HT concentrations were measured within dorsal horn at the level of the L1 vertebra at which L4 nerve root mainly terminated. Therefore, results of the current microdialysis study may not directly reflect the changes of GABA and 5-HT in the sacral spinal cord. However, neurons originating in the nucleus raphe magnus, on which intracerebroventricular morphine acts, project to all levels of the spinal cord.[36–38] Therefore, intracerebroventricular

morphine–induced GABA and 5-HT release we observed should contribute to antinociception in the tail-flick test.

It has been generally considered that supraspinal morphine produces antinociceptive effects through the serotonergic and noradrenergic descending inhibitory systems. In respect to the serotonergic mechanism, the analgesic action of morphine is reduced by destruction of the serotonergic innervation of the spinal cord with selective neurotoxins[39] or lesions of the medullary raphe,[40,41] which contains the cell of the origin of the bulbospinal 5-HT pathway innervating the spinal dorsal horn. Intrathecal injection of a 5-HT receptor antagonist also attenuates the analgesia produced by microinjection of morphine into the PAG.[42] In addition, intrathecal 5-HT produces antinociceptive effects. However, the nature of the receptors involved in the 5-HT–induced modulation of pain in the spinal cord remains to be elucidated. To the best of our knowledge, there has been no report that examined the contribution of spinal 5-HT_3 receptors to intracerebroventricular morphine–induced antinociception in behavioral tests. The results of the current study have shown that spinal 5-HT_3 receptors are involved in supraspinal morphine–induced antinociceptive effects. In contrast, several studies found only a minor role of the serotonergic system in supraspinal morphine–induced antinociceptive effect.[43–45] However, because these studies used methysergide as the 5-HT receptor antagonist, which blocks 5-$HT_{1/2}$, not 5-HT_3 receptors, the role of a serotonergic mechanism may be underestimated. The current study showed that TICM did not completely reverse the morphine-induced antinociceptive effect. This suggests the involvement of the other subtypes of 5-HT receptors or noradrenergic system in intracerebroventricular morphine–induced analgesia. Indeed, it has been reported that $GABA_A$ receptor response is potentiated by the activation of 5-HT_2 receptor,[46] and that 5-HT_1 receptor activation inhibits the nociceptive transmission.[47] The contribution of noradrenergic system in intracerebroventricular morphine–induced analgesia was also well demonstrated.

Several investigators reported that the spinal GABA system contributes to nucleus raphe magnus or PAG stimulation– and supraspinal morphine–induced antinociceptive effects.[1,3,5,25,26,48,49] Although our microdialysis study demonstrated that intracerebroventricular morphine gradually increased spinal GABA release with a maximal effect at 180–240 min after administration, an antinociceptive effect was observed 30 min after the administration and thereafter. Because we did not assess the antagonistic effect of bicuculline at the early phase of morphine-induced antinociception, the involvement of GABA in morphine analgesia at the early phase is unclear. We speculate that another mechanism such as noradrenergic or direct serotonergic inhibitions, in addition to GABAergic inhibition, might be involved in the antinociceptive effect observed in the early phase.

In conclusion, the current study showed that intracerebroventricular morphine produced antinociceptive effect, in part through GABA release by the activation of 5-HT_3 receptors in the spinal cord.

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