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ORIGINAL ARTICLE

Oral morphine induces spinal 5-hydroxytryptamine (5-HT) release using an opioid receptor-independent mechanism

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Abstract

Morphine induces spinal 5-hydroxytryptamine (5-HT) release, but the role and mechanism of the spinal 5-HT release induced by morphine are not well understood. The purpose of this study was to define the role and mechanism of spinal 5-HT release induced by oral morphine. We also examined whether persistent pain affected the spinal 5-HT release induced by oral morphine. Spinal 5-HT release was measured using microdialysis of lumbar cerebrospinal fluid (CSF). Two opioids, morphine and oxycodone, were orally administered and 5-HT release was measured in awake rats. Naloxone and β-funaltrexamine (β-FNA) were used to determine whether the effect of morphine on 5-HT release was mediated by opioid receptor activation. To study persistent pain, a formalin test was used. At 45 min after oral morphine administration, the formalin test was started and spinal 5-HT release was measured. Oral morphine, but not oral oxycodone, increased 5-HT release at the spinal cord to approximately 4000% of the baseline value. This effect of morphine was not antagonized by either naloxone or β-FNA at a dose that antagonized the antinociceptive effect of morphine. Formalin-induced persistent pain itself had no effect on spinal 5-HT release but enhanced the oral morphine-induced spinal 5-HT release. Oral morphine-induced spinal 5-HT release was not mediated by opioid receptor activation. Spinal 5-HT induced by oral morphine did not play a major role in the antinociceptive effect of morphine in the hot plate test. Persistent pain increased oral morphine-induced spinal 5-HT release.

KEYWORDS
5-HT, antinociception, morphine, oxycodone, persistent pain, spinal cord

1 | INTRODUCTION

Morphine is a strong opioid which has been used as an analgesic for many years and exerts strong analgesic effects on various pain conditions, such as cancer pain and postsurgical pain. Morphine produces an analgesic/antinociceptive effect via activation of μ opioid receptor and this effect of morphine is antagonized by naloxone, a μ opioid receptor antagonist. Systemic morphine administration

Abbreviations: %, percentage; %MPE, percent maximum possible effect; 5-HT, 5-hydroxytryptamine; ANOVA, analysis of variance; CSF, lumbar cerebrospinal fluid; HPLC, high-performance liquid chromatography; ICV, intracerebroventricular; IP, intraperitoneally; M3G, morphine-3-glucuronide; M6G, morphine-6-glucuronide; SEM, standard error of the mean; TLR4, Toll-like receptor 4; β-FNA, β-funaltrexamine.

increases 5-hydroxytryptamine (5-HT) levels in the spinal cord.[1–3] Intracerebroventricular (ICV) injection of morphine also induces 5-HT release in the spinal cord,[4,5] and 5-HT itself modifies pain transmission in the spinal cord.[6] However, the role of the 5-HT induced by morphine is not yet fully understood. Kimura et al.[2] reported that the antinociceptive effect of morphine was partially mediated by spinal 5-HT released by systemic morphine in normal rats through activation of 5-HT3 receptors but spinal 5-HT reduced its antinociceptive effect in neuropathic pain model rats by activation of 5-HT3 receptors. Dogrul and Seyrek[7] found, using the heat tail flick test, that systemic morphine produced an antinociceptive effect that was mediated by spinal 5-HT7, but not 5HT1A and 5-HT2, receptors in the spinal cord.

How morphine triggers the release of spinal cord 5-HT has not been clarified. ICV injection of morphine, but not β-endorphin, induces 5-HT release into the lumbar cerebrospinal fluid (CSF).[8] This suggested that morphine-induced spinal 5-HT release depended on a morphine-specific, but not opioid receptor-mediated, mechanism.

In the present study, to define the role and mechanism of the spinal 5-HT release induced by systemic morphine, we used microdialysis to measure the level of 5-HT induced by oral morphine and oxycodone in L5-level CSF in awake rats. In addition, we examined whether persistent pain affected systemic morphine-induced spinal 5-HT release using a formalin test.

2 | METHODS

2.1 | Animals

This study was conducted according to a protocol approved by the Institutional Animal Care Committee of Kumamoto University, Kumamoto, Japan. In this study, we used male Sprague–Dawley rats (250–300 g; Japan SLC, Inc.). The animals were kept individually in a cage with soft bedding under a 12-h dark–light cycle in a temperature-controlled (21 ± 1°C) room and provided food and water ad libitum. Before use, the animals were housed for at least 7 days to acclimate to their new circumstances. Immediately after behavioral and microdialysis studies, the animals were sacrificed using high concentrations of isoflurane. All animals were quiet throughout the isoflurane euthanization. Each animal was used in only one experiment.

2.2 | Hot plate test

The hot plate test was carried out to assess the effect of oral morphine and oxycodone on the thermal nociceptive threshold. Rats were placed on a 52.5°C hot plate (35150, Ugo Basile) and the response latency to either a hindpaw lick or jump was recorded. In the absence of a response, the animals were removed from the hot plate at 60 s to avoid tissue injury, and a 60-s latency was assigned as the response. Two baseline measurements were recorded before the drug administration.

2.3 | Formalin test

To perform the formalin test, 50 μL of 5% formalin was injected subcutaneously, under light isoflurane anesthesia, into the dorsal surface of the right hind paw using a 26-gauge needle. Formalin injection resulted in spontaneous flinching of the injected paw. This behavior started within 1 min after formalin injection. Flinching was defined as rapid and brief withdrawal or flexion of the injected paw. This pain-related flinching was quantified by counting the number of flinches for 1-min periods at 5-min intervals from 0 to 60 min after injection. In the formalin test, animals showed two phases of spontaneous flinches: an initial acute phase (phase 1) and a prolonged tonic phase (phase 2). Phase 1 behavior was observed in the first 6 min after subcutaneous formalin injection and phase 2 behavior was observed between 10 and 60 min after formalin injection.[9]

2.4 | Intrathecal microdialysis

Under anesthesia with 2% isoflurane in 100% oxygen using a nose cone, the animals were placed in a stereotaxic apparatus (Model 900; David KOPF Instruments), and a microdialysis probe was implanted. An intrathecal microdialysis probe (exposed tip, 10 mm; cut-off of 50 kDa; EICOM) was passed 7.5 cm caudally from the atlanto-occipital membrane, and the tip of the probe was placed in the lumbar enlargement. After recovery from anesthesia, each rat was individually placed in a plastic box (29 × 29 × 34 cm) and allowed to move freely. The probe was perfused with artificial CSF overnight at a rate of 1 μL/min.

2.5 | Assay of 5-HT levels

Using reverse-phase high-performance liquid chromatography (HPLC) and electrochemical detection (ECD-300, EICOM), 5-HT levels in the microdialysis samples were measured. We used a reverse-phase column (EICOMPAK CAX, 2.0 × 200 mm, EICOM). The mobile phase comprised 0.1 M ammonium acetate buffer solution with 50 mg/mL EDTA-2Na and 0.05 M sodium sulfate in methanol in water (7:3, v/v) adjusted to pH 6.0. An HPLC pump system (EP-300, EICOM) was used, and the flow rate was set at 0.25 mL/min. The column temperature was set at 35°C, and the applied potential was set at +450 mV (ATC-300, EICOM). Quantification was performed using standard curves.

2.6 | Drugs and administration

The agents used in this study were morphine hydrochloride hydrate (Daiichi Sankyo), oxycodone hydrochloride (Daiichi Sankyo), naloxone (Daiichi Sankyo), and β-funaltrexamine (Axon Medchem). Morphine was diluted in saline to 10, 30, 60 and 100 mg/kg doses and oxycodone was diluted in saline to a 66 mg/kg dose. We chose

an oral oxycodone dose of 66 mg/kg because the ratio of the antinociceptive titer of morphine and oxycodone is 3:2 for its oral administration in humans.[10] A stainless steel tube was inserted through the esophagus to the stomach, through which 2 mL of morphine, oxycodone or saline solution was administered. Naloxone and β-FNA were dissolved in saline solution. Naloxone (1 mg/kg) was injected intraperitoneally (IP) 10 min prior to morphine, whereas β-FNA (20 mg/kg) was injected subcutaneously 24 h prior to morphine.

General status, such as sedative condition and motility, was carefully observed after drug administration.

2.7 | Experimental protocol

2.7.1 | Dose–response study

To determine the correct dose of morphine for the microdialysis study, a dose–response study was performed for hot plate and formalin tests. For comparison, saline was administered orally.

In the hot plate test, morphine, oxycodone or saline was administered orally and the hot plate latency was measured at 15, 30, 45, 60, 75, 90, 105, and 120 min after the drug administration. In the formalin test, formalin was injected 45 min after oral morphine or saline administration.

2.7.2 | Microdialysis study

Microdialysis was performed after overnight perfusion of artificial CSF via an intrathecal probe (1 μL/min) into conscious and freely moving rats. After 60 min of constant perfusion at a rate of 2 μL/min, dialysate sampling was started. Before oral administration studies were begun, three consecutive 15-min baseline fractions were collected. After oral administration, dialysate was collected at 0–15, 15–30, 30–45, 45–60, 60–75, 75–90, 90–105, and 105–120 min. Throughout the microdialysis study, perfusate fractions were collected into an autoinjector (EAS-20; EICOM). Samples (10 μL) were automatically injected and analyzed to determine the 5-HT concentration using HPLC with electrochemical detection by an ECD-300 analyzing system (EICOM). To determine whether morphine-induced 5-HT release at the spinal cord was mediated by opioid receptor activation, naloxone 1 mg/kg was IP administered 10 min before morphine administration (morphine + naloxone group) and β-FNA (20 mg/kg) was subcutaneously administered 24 h before morphine administration (morphine + β-FNA group). The doses and timings of the naloxone injections were based on our previous report[11] while those of β-FNA were based on Hayes et al.[12]

To determine whether persistent pain itself affects morphine-induced 5-HT release at the spinal cord, 50 μL of 5% formalin was injected into the rat hind paw 45 min after the morphine administration under light isoflurane anesthesia (formalin study). For comparison, saline was administered orally.

2.8 | Statistical analysis

2.8.1 | Hot plate test

To analyze the effects of morphine and oxycodone in the hot plate test, the percent maximum possible effect (%MPE) was calculated, where %MPE = ([postdrug maximum response latency − predrug response latency]/[cut-off time (60 s) − predrug response latency]) × 100. The predrug response latency was defined as the mean of two baseline measurements. The postdrug maximum response latency was defined as the single longest response latency in the 120 min after the oral drug administration. To analyze the drug effects, one-way analysis of variance (ANOVA) was used. When significant differences were observed, Dunnett's multiple comparisons test was used. To analyze antagonist effects, Student's *t* test was used.

2.8.2 | Formalin study

In the formalin test, we present the mean number of flinches (±standard error of the mean [SEM]) per minute in time–response graphs. The periods between 1–2 and 5–6 min after formalin treatment were the phase 1 response, and the period between 10 and 60 min was the phase 2 response. Phase 1 and phase 2 data were analyzed separately. The sum of the formalin-evoked flinches during phases 1 and 2 were calculated for each rat to perform dose–response analysis. For dose–response analysis of the phase 1 and phase 2 data, one-way ANOVA was used. For the multiple comparison, Dunnett's test was used.

2.8.3 | Microdialysis study

All data were not corrected for "recovery" of the dialysis procedure. The percentage of the control value was used to present the microdialysis data. The control 5-HT concentration in the dialysates was calculated as the mean 5-HT concentration of the three baseline fractions collected before oral administration. The 5-HT concentration at each time point was divided by the control 5-HT concentration, and the percentage (%) of the control value was 100 times the quotient. This value was used as the microdialysis data. The mean and standard errors were calculated for each treatment group. In the formalin study, as formalin was injected 45 min after morphine administration, the effect of formalin-induced persistent pain on 5-HT release was analyzed between 45 and 120 min after morphine administration.

Time course data were analyzed using two-way ANOVA. When significant differences were observed between the mean values of each treatment, the Holm-Sidak method was used.

All data are presented as the mean ± SEM. Statistical significance was set at $p < .05$. All statistical procedures were carried out with SigmaPlot 14.0 (Systat Software Inc.).

3 | RESULTS

3.1 | General status of animals

All rats were sedated with 100 mg/kg morphine and remained quiet but in a normal position. In the oxycodone study, 1 of the 5 rats was sedated and remained quiet.

3.2 | Dose–response study

3.2.1 | Hot plate test

Baseline values in the hot plate test were 14.5 ± 1.9 s in 10 mg/kg morphine rats ($n = 5$), 14.1 ± 1.0 s in 30 mg/kg morphine rats ($n = 5$), 13.5 ± 0.8 s in 60 mg/kg morphine rats ($n = 5$), 14.7 ± 0.9 s in 100 mg/kg morphine rats ($n = 5$), 13.2 ± 1.7 s in 66 mg/kg oxycodone rats ($n = 5$), 9.5 ± 1.2 s in 100 mg/kg + naloxone rats ($n = 5$), 9.9 ± 1.8 s in 100 mg/kg + β-FNA rats ($n = 5$), 12.2 ± 1.5 s in oxycodone + naloxone rats ($n = 5$) and 11.3 ± 2.1 s in saline-treated rats ($n = 5$). There were no significant differences between the groups ($F = 1.67$; $p = .14$).

Oral morphine administration produced a significant antinociceptive effect in a dose-dependent manner at a dose between 10 and 100 mg/kg ($F = 31.3$; $p < .0001$; Figures 1 and 2). The antinociceptive effect of 100 mg/kg morphine (%MPE = 95.7 ± 5.78) was antagonized by naloxone ($n = 5$, %MPE = 36.1 ± 8.19, $p < .001$, Figures 1 and 2) and β-FNA ($n = 5$, %MPE = 21.7 ± 8.19, $p < .001$, Figure 1). Oral oxycodone 66 mg/kg produced a significant antinociceptive effect compared with saline (oxycodone: $n = 5$, %MPE = 88.0 ± 12.0; saline: $n = 5$, %MPE: 9.80 ± 3.72, $p < .0005$; Figure 3) and the antinociceptive effect of oxycodone was antagonized by naloxone ($n = 5$, %MPE = 42.8 ± 5.90, $p < .01$) (Figure 3). There was no significant difference in the %MPE between morphine 100 mg/kg (%MPE = 95.7 ± 5.78) and oxycodone 66 mg/kg (%MPE = 88.0 ± 12.0) ($p = .75$). Although all animals showed sedation, 2 of the 5 rats administered 100 mg/kg morphine did not reach the 60-s cut-off. In the 66 mg/kg oxycodone study, one of the 5 rats was sedated and remained quiet, and 4 of the 5 rats reached the 60-s cut-off. These data suggested that 100 mg/kg morphine and 66 mg/kg oxycodone are adequate doses for examining an antinociceptive effect, but not a sedative effect. Based on these results, we selected a morphine dose of 100 mg/kg and oxycodone dose of 66 mg/kg for the microdialysis experiments.

3.2.2 | Formalin study

Oral morphine administration produced a significant antinociceptive effect in a dose-dependent manner at a dose between 10 and 100 mg/kg (phase 1: $F = 19.4$; $p < .001$; phase 2: $F = 49.5$, $p < .001$; Figures 4 and 5). This antinociceptive effect was antagonized by IP naloxone (phase 1, $p < .01$; phase 2, $p < .001$; Figure 4). Based on these results, we selected a morphine dose of 100 mg/kg for the subsequent experiments.

3.3 | Microdialysis study

3.3.1 | Morphine study

The baseline intrathecal 5-HT concentration was not significantly different between the groups (saline, 0.842 ± 0.14 pg/10 μL; morphine, 1.5 ± 0.81 pg/10 μL; oxycodone, 1.96 ± 1.07 pg/10 μL; morphine + naloxone, 1.48 ± 0.67 pg/10 μL; morphine + β-FNA, 2.18 ± 0.51 pg/10 μL; saline + formalin, 0.97 ± 0.19 pg/10 μL; morphine + formalin, 1.09 ± 0.38 pg/10 μL; $p = .686$). Administration of morphine 100 mg/kg ($n = 5$) led to a significant increase in the 5-HT concentration compared with the saline group ($n = 5$), reaching approximately 4000% of the baseline value ($p < .001$, Figure 6). In both the morphine + naloxone group ($n = 5$) and morphine + β-FNA group ($n = 5$), oral morphine also led to an increase in the 5-HT concentration compared with the saline group (both $p < .001$, Figure 6). There were no significant differences in the 5-HT concentration between the morphine group and

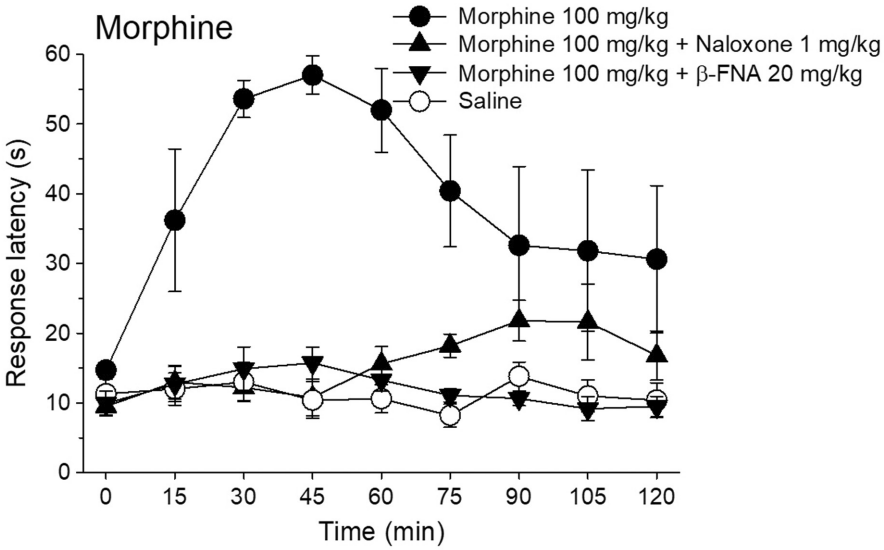

FIGURE 1 Time-effect curves of orally administered 100 mg/kg morphine, 100 mg/kg morphine + 1 mg/kg naloxone and saline on the thermal nociceptive threshold in a 52.5°C hot plate test. A significant antinociceptive effect was induced by 100 mg/kg morphine ($p < .0001$). Both naloxone and β-FNA alone significantly antagonized the effects of morphine ($p < .001$). Ordinate: response latency (s); abscissa: time after drug administration (min). Each line represents the group mean and SEM of 5 rats.

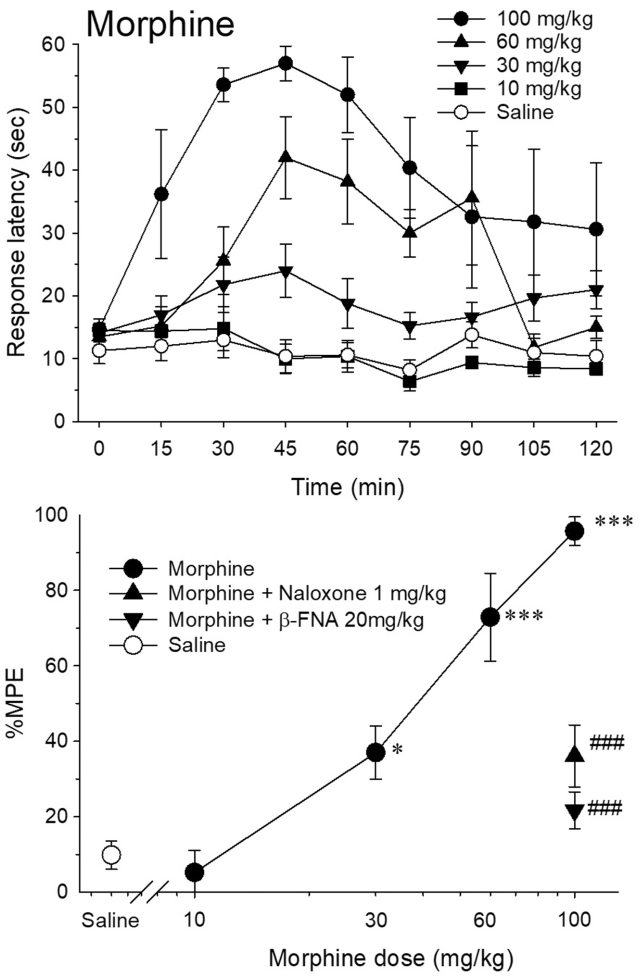

FIGURE 2 Dose–response effects of orally administered morphine on the thermal nociceptive threshold in a 52.5°C hot plate test. Upper panel: Time courses of the full dose–response curves. Ordinate: response latency (s); abscissa: time after drug administration (min). Lower panel: Dose–response curve of oral morphine in the hot plate test. Morphine increased the %MPE level in a dose-dependent manner. Ordinate: percent maximum possible effect (%MPE); abscissa: morphine dose (mg/kg). Each line represents the group mean and SEM of 5 rats. $^{*}p<.05$, $^{***}p<.001$ compared with saline-treated rats. $^{\#\#\#}p<.001$ compared with 100 mg/kg morphine-treated rats.

the morphine+naloxone group ($p=.779$, Figure 6) or between the morphine group and the morphine+β-FNA group ($p=.061$, Figure 6). Administration of oxycodone 66 mg/kg ($n=5$) did not increase the 5-HT concentration compared with the saline group ($p=.938$, Figure 7).

3.3.2 | Formalin study

When formalin was injected into the rat hind paw 45 min after the oral saline administration ($n=5$), formalin injection did not affect spinal 5-HT release compared with saline-administered rats without formalin injection ($n=5$) ($p=.162$, Figure 8). When formalin was injected 45 min after the morphine administration ($n=6$), formalin injection increased oral morphine-induced spinal 5-HT release

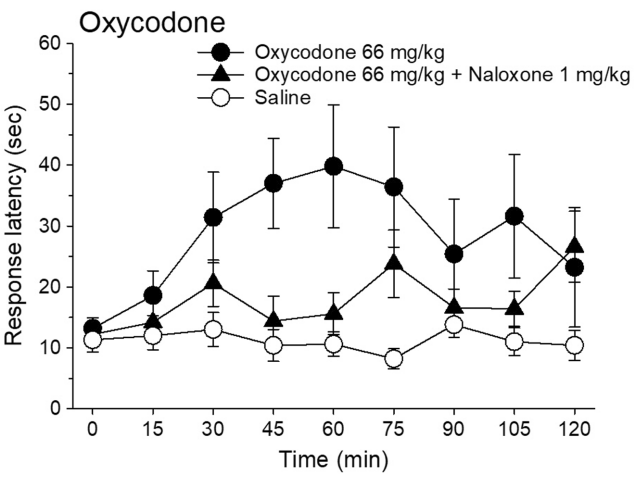

FIGURE 3 Time-effect curves of orally administered 66 mg/kg oxycodone, 66 mg/kg oxycodone + 1 mg/kg naloxone and saline on the thermal nociceptive threshold in a 52.5°C hot plate test. A significant antinociceptive effect was induced by 66 mg/kg oxycodone ($p<.0005$). Naloxone significantly antagonized the effects of morphine ($p<.01$). Ordinate: response latency (s); abscissa: time after drug administration (min). Each line represents the group mean and SEM of 5 rats.

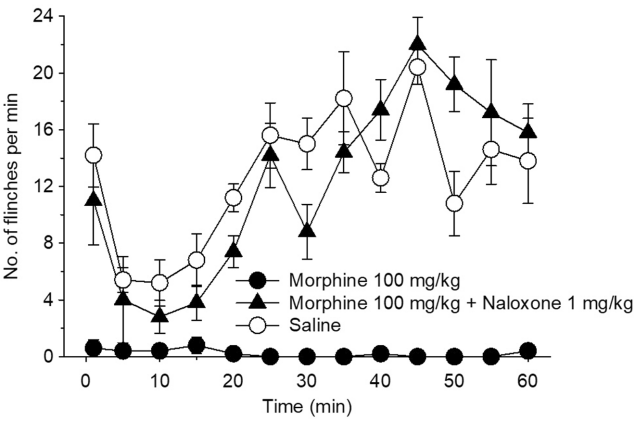

FIGURE 4 Time courses of the effects of orally administered 100 mg/kg morphine, 100 mg/kg morphine + 1 mg/kg naloxone and saline in the formalin test. In the saline group, animals showed the two typical phases of flinching behaviors: phase 1 (initial acute phase) and phase 2 (prolonged tonic phase). The number of flinching responses was decreased by 100 mg/kg morphine in both phase 1 ($p<.001$) and phase 2 ($p<.001$) and this effect of morphine was antagonized by naloxone (phase 1, $p<.01$; phase 2, $p<.001$). Each group contained 5 rats. Each bar represents the mean ± SEM.

compared with morphine-administered rats without formalin injection ($p<.05$, Figure 8).

4 | DISCUSSION

Oral administration of either 100 mg/kg morphine or 66 mg/kg oxycodone produced an antinociceptive effect in rats and the

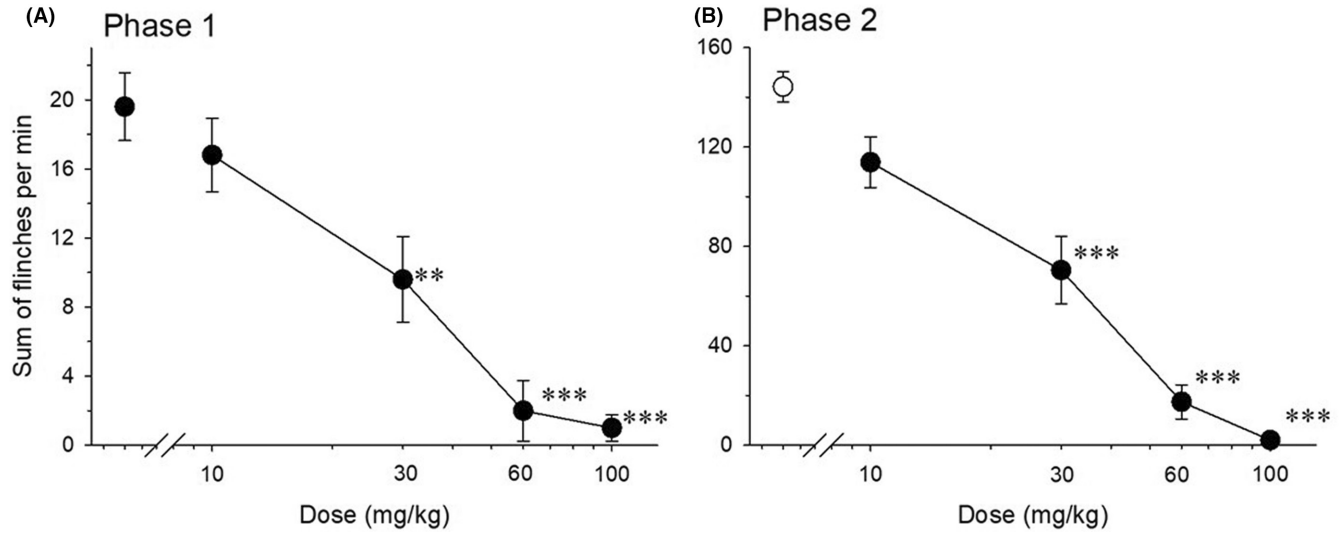

FIGURE 5 Dose–response effect of oral morphine on phase 1 (A) and phase 2 (B) responses in the formalin test. Abscissa: morphine dose (mg/kg); ordinate: sum of flinches per min. Each point represents the mean ± SEM of 5 rats. $^{**}p < .005$ and $^{***}p < .001$ compared with saline-treated rats.

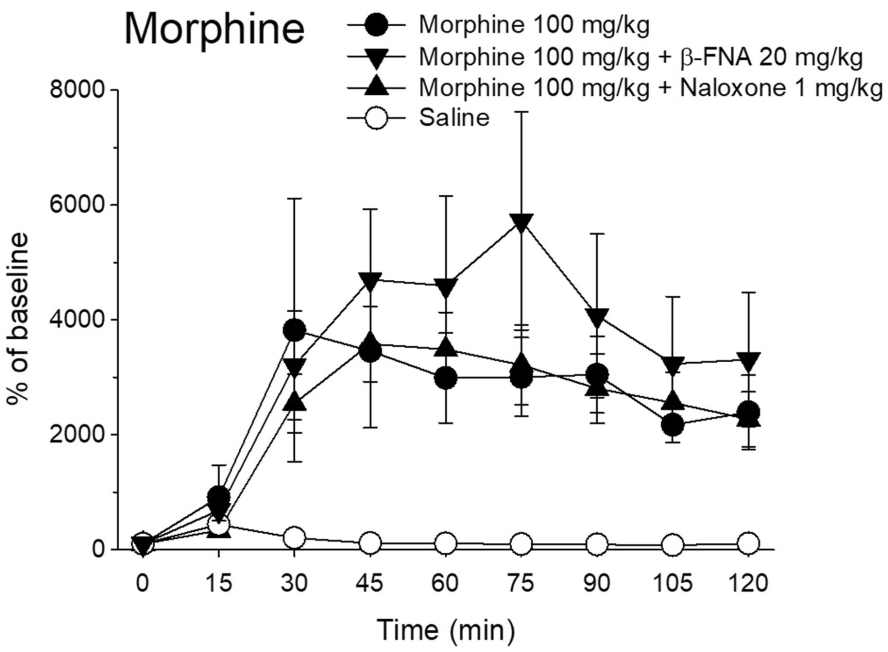

FIGURE 6 Spinal 5-HT release after oral administration of 100 mg/kg morphine, 100 mg/kg morphine + 1 mg/kg naloxone, 100 mg/kg morphine + 20 mg/kg β-FNA and saline. Oral morphine significantly increased 5-HT release ($p < .001$) and this effect was not antagonized by naloxone ($p = .779$) or β-FNA ($p = .061$). Ordinate: 5-HT release as a percentage of control; abscissa: time from drug administration in 15-min intervals. Each group contained 5 rats. Each point represents the mean ± SEM.

%MPE level in the morphine-treated rats was comparable to that in the oxycodone-treated rats. Figure 3 shows that the response latency in 66 mg/kg oxycodone-treated rats reached around 40 s, but not 60 s. As described in the Results section, 4 of the 5 rats treated with 66 mg/kg oxycodone reached the 60-s cut-off. The timing at which the response latency reached the 60-s cut-off was highly variable and the duration of action of oxycodone was shorter than that of morphine. Although the highest response latency in the time course graph was around 40 s, there was no difference between the %MPE in the 100 mg morphine and 66 mg/kg oxycodone groups. These antinociceptive effects of morphine and oxycodone alone were antagonized by IP naloxone. Moreover, the antinociceptive effect of morphine in the hot plate test was antagonized by subcutaneous β-FNA. The duration of action of naloxone was short[13] and it is possible that the antagonistic effect of naloxone weakened during the experiment. β-FNA is a long-lasting μ opioid antagonist[12,14] and β-FNA antagonized the effect of morphine throughout the experiment. This suggested that an opioid receptor-dependent mechanism plays an important role in producing the antinociceptive effects of morphine and oxycodone in the hot plate test. In a microdialysis study, we found that oral morphine induced spinal 5-HT release even with naloxone or β-FNA pretreatment, unlike oral oxycodone. These results suggested that spinal 5-HT release is particular to oral morphine, not to oral oxycodone, and that opioid receptor does not directly participate in the morphine-induced spinal 5-HT release. As mentioned in the

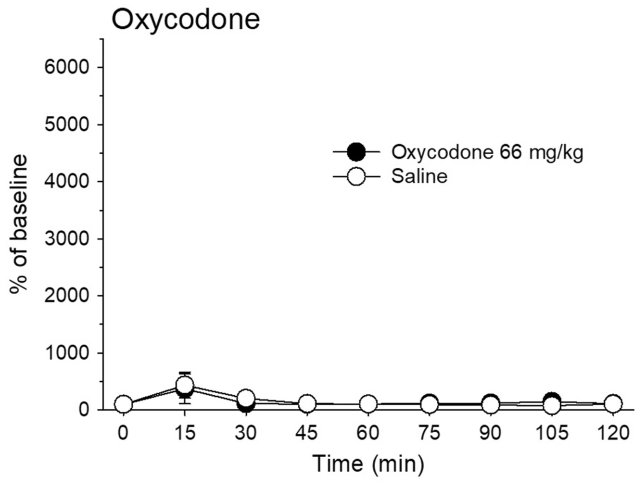

FIGURE 7 Spinal 5-HT release after oral administration of 66 mg/kg oxycodone and saline. Oral oxycodone did not increase 5-HT release ($p = .938$). Ordinate: 5-HT release as a percentage of control; abscissa: time from drug administration in 15-min intervals. Each group contained 5 rats. Each point represents the mean ± SEM.

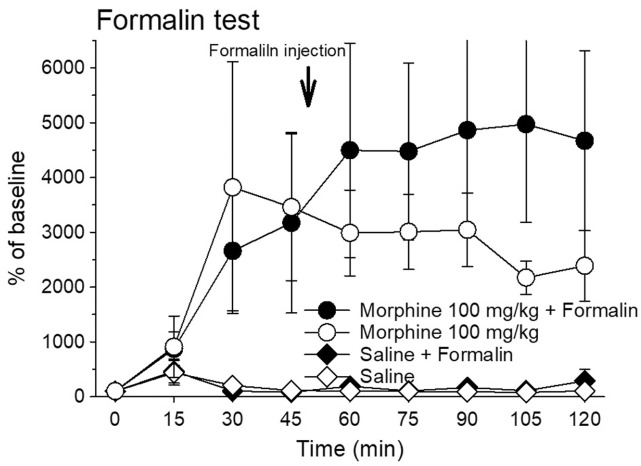

FIGURE 8 Spinal 5-HT release after 100 mg/kg morphine + formalin injection, 100 mg/kg morphine, saline + formalin injection and saline. Formalin injection was performed 45 min after drug administration. Formalin injection significantly enhanced oral morphine-induced 5-HT release ($p < .05$). Formalin injection itself had no effect on spinal 5-HT release ($p = .162$). Ordinate: 5-HT release as a percentage of control; abscissa: time from drug administration in 15-min intervals. Each group contained 5 rats. Each point represents the mean ± SEM.

introduction, the role of spinal 5-HT induced by morphine is not clear. Our data suggested that spinal 5-HT does not play a major role in the antinociceptive effects of oral morphine in the hot plate test.

We do not know the precise mechanisms underlying how oral morphine induces spinal 5-HT release. Yaksh and Tyce[15] reported that morphine (5 μg) microinjection into the periaqueductal gray increased spinal 5-HT release into CSF by 478%; this increase was antagonized by naloxone. This suggested that the mechanism of the spinal 5-HT release after oral morphine was different from that after periaqueductal gray morphine microinjection and that spinal 5-HT release after morphine microinjection into the periaqueductal gray, but not after oral morphine, was mediated by opioid receptor activation. Jung et al.[8] reported that ICV injection of morphine, but not β-endorphin, increased spinal 5-HT release and suggested that the spinopetal serotonergic descending pathway was activated by a morphine-specific mechanism when morphine was ICV injected.

Another possible mechanism is mediated by a morphine metabolite, morphine-3-glucuronide (M3G). Morphine is metabolized to two main metabolites, M3G and morphine-6-glucuronide (M6G). M6G has agonistic activity at the μ opioid receptor and, while M3G has no such agonistic activity, it has the ability to activate Toll-like receptor 4 (TLR4).[16] It is possible that morphine-induced spinal 5-HT release is mediated by activation of TLR4. Although the precise mechanism has not been determined, these data suggested that, in association with ICV injection, oral morphine-induced spinal 5-HT release may be mediated by an opioid receptor-independent and morphine-specific mechanism. Further work is required to reveal the mechanisms.

Formalin injection into a hind paw induces persistent inflammatory pain; this reaction is exploited as part of the formalin test.[9] In this study, formalin injection itself did not induce spinal 5-HT release, but it did increase oral morphine-induced spinal 5-HT release. This suggests that persistent pain does not affect spinal 5-HT release but that oral morphine-induced spinal 5-HT release is affected by the existence of persistent pain. The role of spinal 5-HT after formalin injection is complicated. Oyama et al.[17] reported that the 5-HT1A receptor plays an antinociceptive role and that 5-HT3 receptor plays a pronociceptive role. Kimura et al.[2] found that in a rat study, spinal 5-HT induced by systemic morphine played an antinociceptive role in the normal state but in a neuropathic pain model, the spinal 5-HT induced by systemic morphine attenuated morphine-induced antinociception. Kimura et al.[2] also showed that the spinal 5-HT release level induced by systemic morphine in a neuropathic pain model was not different from that in the normal rat. This suggested that formalin-induced persistent pain differs from neuropathic pain in its impact on systemic morphine-induced spinal 5-HT release. It is possible that the oral morphine-induced spinal 5-HT during the formalin test may have a specific effect, either nociceptive or antinociceptive.

In this study, we reported that oral morphine increased 5-HT release into CSF at the lumbar spinal level to approximately 4000% of the baseline value in awake rats. Kimura et al.[2] showed that IP injection of 10 mg/kg morphine increased spinal dorsal horn 5-HT release at the L3–L6 level to approximately 500% of the baseline value by using microdialysis in 0.5% isoflurane-anesthetized rat. In this study, we administered morphine at an oral dose of 100 mg/kg. The metabolism of orally administered morphine is reportedly affected by the first-pass effect and the area under plasma concentration versus time course for oral morphine is only 18% of that observed after intravenous administration in the rat.[18] This suggests that 100 mg/kg oral morphine may be equivalent to 18 mg/kg intravenous morphine. Thus, 100 mg/kg oral morphine is not that different from 10 mg/

kg IP morphine. Tzschentke et al.[19] reported that using isoflurane-anesthetized rats, IP morphine at between 1 and 10 mg/kg decreased the spinal 5-HT level in a non-dose-dependent and statistically non-significant manner. Kimura et al.[2] and Tzschentke et al.[19] measured 5-HT at the spinal dorsal horn in isoflurane-anesthetized rats while we measured 5-HT in CSF in awake rats. Tzschentke et al.[19] suggested that anesthesia decreases drug-induced transmitter release in the spinal cord. This may explain the difference between the present study and the previous studies.

Although 100 mg/kg morphine induced sedative effects in all rats, 100 mg/kg morphine did not reach full antinociceptive effect because 2 of the 5 rats administered 100 mg/kg morphine did not reach the 60-s cut-off. In this study, we focused on antinociceptive effect of morphine, but not sedative effect of morphine and found there is no relationship between an antinociceptive effect of morphine and spinal 5-HT released by oral morphine.

In this study, only male rats were used. Because estrous cyclicity may affect the antinociceptive effects of morphine and spinal release of 5-HT, different results might be obtained in female rats.

5 | CONCLUSIONS

Morphine and oxycodone have antinociceptive effects in the hot plate test, and these antinociceptive effects are mediated by opioid receptor activation. Oral administration of morphine, but not oxycodone, induces spinal 5-HT release and this effect is not antagonized by either naloxone or β-FNA. Thus, oral morphine-induced spinal 5-HT does not play an important role in the opioid receptor-mediated antinociceptive effect of morphine in the hot plate test. Persistent pain induced by formalin injection increases oral morphine-induced spinal 5-HT release.

AUTHOR CONTRIBUTIONS

SN, and Tatsuo Yamamoto participated in research design. SK, Toshihiko Yamada, HK and SN conducted experiments, and Tatsuo Yamamoto performed data analysis. All authors contributed to drafting the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors report no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

This study was conducted according to a protocol approved by the Institutional Animal Care Committee of kumamoto University, Kumamoto, Japan.

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