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Oral acetaminophen-induced spinal 5-hydroxytriyptamine release produces analgesic effects in the rat formalin test

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ABSTRACT

The mechanism by which acetaminophen produces its analgesic effects is not fully understood. One possible mechanism is the activation of the spinal 5-hydroxytryptamine (5-HT) receptor, although direct evidence of spinal 5-HT release has not yet been reported. N-arachidonoylphenolamine (AM404), a metabolite of acetaminophen, is believed to be the key substance that contributes to the analgesic effects of acetaminophen. In this study, we examined whether acetaminophen and AM404 induce spinal 5-HT release and the mechanism through which spinal 5-HT receptor activation exerts analgesic effects in a rat formalin test in an inflammatory pain model. Spinal 5-HT release was examined by intrathecal microdialysis in conscious and freely moving rats. Acetaminophen was administered orally, and AM404 was administered intracerebroventricularly. In rat formalin tests, oral acetaminophen and intracerebroventricular AM404 induced significant spinal 5-HT release and produced analgesic effects. The analgesic effect of oral acetaminophen was partially antagonized by intrathecal administration of WAY100135 (a 5-HT_{1A} receptor antagonist) and SB269970 (a 5-HT_{7} receptor antagonist). In contrast, the analgesic effect of intracerebroventricular AM404 was completely antagonized by WAY100135, while SB269970 had no effect. Our data suggest that while oral acetaminophen and intracerebroventricular AM404 activate the spinal 5-HT system, the role of the spinal 5-HT system activated by oral acetaminophen differs from that activated by intracerebroventricular AM404.

1. Introduction

Acetaminophen is prescribed worldwide as an analgesic and antipyretic drug [1]. Non-steroidal anti-inflammatory drugs (NSAIDs) are also used as nonopioid analgesic drugs; however, they can cause severe adverse effects, such as gastrointestinal problems and nephrotoxicity. Although the safety of chronic use of acetaminophen has been questioned [8], the adverse effects caused by acetaminophen are less severe than those caused by NSAIDs. Therefore, acetaminophen is more commonly prescribed to geriatric patients 3JR: American Geriatrics Society Panel on Pharmacological Management of Persistent Pain in Older Persons, Pharmacological management of persistent pain in older persons J. Am. Geriatr. Soc. 57 2009 1331 1346 https://doi.10.1111/j.1532-5415.2009.02376.xAmerican Geriatrics Society Panel on Pharmacological Management of Persistent Pain in Older Persons, Pharmacological management of persistent pain in older persons, J. Am. Geriatr. Soc. 57 1331–1346. https://doi.10.1111/j.1532–5415.2009.02376.x.[11].

The mechanism by which acetaminophen produces its analgesic effect is not fully understood. A recent study showed that acetaminophen is metabolized to the primary amine, p-aminophenol, in the liver. The P-aminophenol then moves to the brain, where it conjugates with arachidonic acid to form a potent transient receptor potential vanilloid 1 (TRPV1) agonist, N-arachidonoylphenolamine (AM404) [6,20]. AM404 also acts as an anandamide transport inhibitor and has been reported to increase endogenous anandamide levels in the rat brain [4]. AM404 is believed to be a key substance in the production of acetaminophen-induced analgesic effects [12]. However, the site of action and the precise mechanisms underlying the AM404 function are not well understood.

Intraperitoneal injection of acetaminophen has been reported to induce a significant increase in 5-hydroxytryptamine (5-HT) concentration in the pontine and cortical areas [17]. The analgesic effect of acetaminophen is reported to be mediated, at least in part, by the activation of the spinal 5-HT receptor [14-16,2,23,3,5,7]. These data suggest that the analgesic effect of acetaminophen may involve the spinal 5-HT system. Unfortunately, direct evidence of spinal 5-HT release after oral acetaminophen has not yet been reported, and little is known

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regarding how systemically administered acetaminophen and brain AM404 affect the spinal 5-HT system. In this study, we first investigated whether the peroral (PO) administration of acetaminophen and intracerebroventricular (ICV)-injected AM404 induced spinal 5-HT release by intrathecal microdialysis in the rat formalin test, an inflammatory pain model.

In the rat formalin test, 5-HT_7, but not 5-HT_{1A} receptor, is reported to play a pronociceptive role in the spinal cord [19]. Therefore, we also examined the roles of spinal 5-HT_{1A} and 5-HT_7 in the analgesic effects exerted by PO acetaminophen and ICV AM404 using 5-HT_{1A} and 5-HT_7 antagonists.

2. Methods

2.1. Animal care

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., Shizuoka, Japan). The animals were kept under a 12-h dark-light cycle and provided food and water ad libitum. Before use, the animals were kept for at least 3 days. Immediately after microdialysis and behavioral studies, the animals were sacrificed using high concentrations of isoflurane.

2.2. Intrathecal microdialysis

Under isoflurane anesthesia, the animals were placed in a stereotaxic apparatus (Model 900; David KOPF Instruments, CA), and a microdialysis probe was implanted. Intrathecal microdialysis probe (exposed tip, 10 mm; cut-off of 50 kDa; EICOM, Kyoto, Japan) 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 box and allowed to move freely. The probe was perfused with artificial cerebrospinal fluid overnight at a rate of 1 μL/min.

2.3. Assay of 5-HT levels

Reverse phase high-performance liquid chromatography (HPLC) and electrochemical detection (ECD-300, EICOM) measured 5-HT levels in the microdialysis samples. We used a reverse phase column (EICOMPAK CAX, 2.0 × 200 mm, EICOM). The mobile phase was composed of 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.

2.4. Formalin test

To perform the formalin test, 50 μL of 5% formalin was injected subcutaneously, under light isoflurane anesthesia, onto 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 of formalin injection. Flinching was defined as rapid and brief withdrawal or flexion of the injected paw and was quantified by counting the number of flinches for 1-min at 5 min intervals from 0 to 60 min after injection. In the formalin test, the animals showed two phases of spontaneous flinching: an initial acute phase (phase 1) and a prolonged tonic phase (phase 2). Phase 1 behavior was observed in the first 6 min after formalin subcutaneous injection, and phase 2 behavior was observed between 10 and 60 min after formalin injection. The phase 1 response is mediated by direct nociceptor stimulation induced by formalin, and the phase 2 response is mediated by the formalin-induced inflammatory response [25].

2.5. ICV injection cannula

The animals were placed in a stereotaxic apparatus, and a stainless steel guide cannula was implanted for ICV injection (24-gauge, 0.64 mm outer diameter, 15 mm long) into the right lateral ventricle through a burr hole (0.5 mm caudal to the coronal suture and 1 mm lateral to the sagittal suture; 3 mm deep in the dura) under isoflurane anesthesia. The cannulae were fixed to the skull using stainless steel screws and cranioplastic cement. In our experience, drug injection through the cannula is optimal approximately 4 days after implantation because the cannula will be free from any plug of cells until then. Some cannulae were plugged 7 days after implantation. Therefore, drug injection through the implanted cannula was performed 4 days after cannula implantation. Only animals that showed normal postoperative feeding and drinking behaviors were used for subsequent experiments. Animals were not examined for neurological deficits.

2.6. Drugs and injection

The agents used in this study were acetaminophen (Wako, Osaka, Japan), carboxymethylcellulose sodium salt (CMC; Wako), 5-HT_{1A} receptor antagonist WAY100135 (Sigma-Aldrich, St. Louis, MO, USA), 5-HT_7 receptor antagonist SB269970 (Sigma-Aldrich), AM404 (Abcam, Cambridge, UK), and dimethyl sulfoxide (DMSO; Fujifilm, Osaka, Japan).

For PO administration, acetaminophen was suspended in 0.5% CMC solution. A stainless steel tube was inserted through the esophagus to the stomach, through which 2 mL of acetaminophen was administered.

For ICV injection, AM404 was dissolved in DMSO. A total volume of 2 μL of AM404 was administered by ICV injection.

In an antagonist study, WAY100135 and SB269970 were dissolved in 10 μL of saline. The drugs were injected into the intrathecal space via the L5–L6 intervertebral space under light isoflurane anesthesia with a 30-gauge needle.

2.7. Experimental protocol

2.7.1. Dose-response study

To determine the correct dosages for the microdialysis study, a dose-response study was performed.

2.7.1.1. Oral administration study. In a preliminary study to determine the optimal timing for the oral administration of acetaminophen, 1000 mg/kg of acetaminophen was administered orally 30, 45, and 60 min before formalin injection. The dose of acetaminophen was determined according to our previous study [21]. For comparison, 0.5% CMC was administered orally. Acetaminophen produced an analgesic effect when administered at 45 min (phase 1: $p = 0.0000229$; $t = 7.97$; phase 2: $p = 0.000000522$; degrees of freedom [DF], 9; $t = 12.6$) or 60 min (phase 1: $p = 0.000361$; $t = 5.54$; phase 2: $p = 0.000111$; DF: 9; $t = 6.50$) before formalin administration compared to 0.5% CMC-administered rats. When acetaminophen was administered 30 min before formalin injection, it produced an analgesic effect in phase 2 ($p = 0.0000271$; $t = 7.80$), but not in phase 1 ($p = 0.0714$; $t = 2.04$), in response to 0.5% CMC. We found that acetaminophen administered 45 min before formalin injection produced a more profound analgesic effect than acetaminophen administered 30 min or 60 min before formalin injection (phase 1: 30 min; $p = 0.032$; $t = 2.98$; 60 min; $p = 0.170$; $t = 1.45$; phase 2: 30 min; $p = 0.047$; $t = 2.56$; 60 min; $p = 0.036$; $t = 0.291$, by one-way analysis of variance (ANOVA) ($p = 0.034$; DF, 2; $F = 4.44$)) (Fig. 1). Therefore, we determined that oral administration of acetaminophen 45 min before formalin injection was the optimal time to examine its effects. After determining the timing of

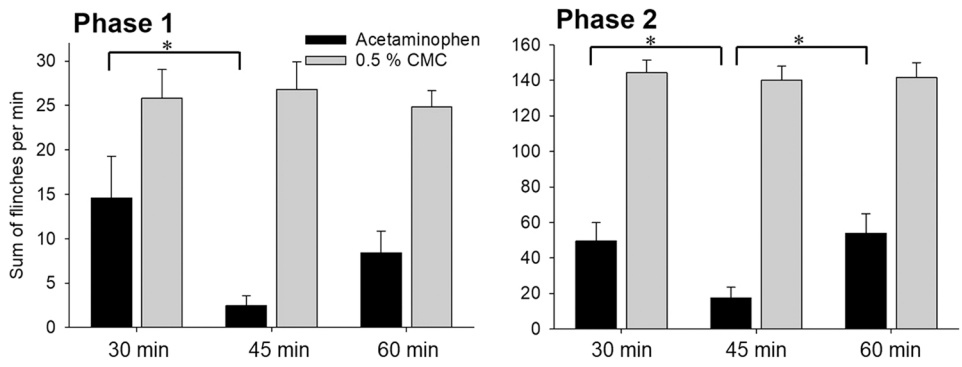

Fig. 1. Effect of 1000 mg/kg acetaminophen when administered orally 30, 45, and 60 min before formalin injection. For comparison, the effect of 0.5% CMC is investigated. Acetaminophen administered 45 min before formalin injection produced a more profound analgesic effect than when administered 30 min or 60 min before formalin injection (phase 1: 30 min, $p = 0.032$; 60 min, $p = 0.170$; phase 2: 30 min, $p = 0.047$; 60 min, $p = 0.036$, by one-way ANOVA)., Each group contained five rats. Each bar represents the mean ± SEM., Ordinate: Sum of flinches during phases 1 (left panel) and 2 (right panel)., $^{*}p < 0.05$.

oral administration, a dose-response study was performed. To obtain control data, 0.5% CMC was administered orally.

2.7.1.2. ICV injection study. To better understand the dose response to AM404, AM404 was injected intracerebroventricularly 10 min before formalin injection.

2.7.2. Microdialysis study

Microdialysis was performed after overnight perfusion of the intrathecal probe (1 μL/min) in conscious and freely moving rats. The probe was perfused with artificial cerebrospinal fluid at a rate of 2 μL/min during the experiment. Before commencing PO administration and ICV injection studies, three baseline fractions were collected. Forty-five min after PO administration and 15 min after ICV injection, 5% formalin (50 μL) was injected into the dorsal side of the right hind paw under light isoflurane anesthesia. Throughout the microdialysis study, dialysate samples were collected every 15 min. To examine whether PO acetaminophen alone or ICV AM404 alone affected 5-HT release in the spinal cord, dialysate samples were collected 105 min after PO administration or 75 min after ICV injection. The samples were analyzed for 5-HT levels.

2.7.3. Antagonist study

2.7.3.1. Oral administration study. To determine whether acetaminophen uses spinal 5-HT_{1A} and/or 5-HT_7 receptor activation to produce an analgesic effect, WAY100135 (46 μg/10 μL) and/or SB269970 (10 μg/10 μL) was administered intrathecally (IT) 10 min before formalin injection. The doses and timing of the WAY100135 and SB268870 injections were based on a previous report [10].

2.7.3.2. ICV injection study. To determine whether AM404 uses spinal 5-HT_{1A} and/or 5-HT_7 receptor activation to produce an analgesic effect, WAY100135 (46 μg/10 μL) and/or SB269970 (10 μg/10 μL) were administered IT 10 min before AM404 injection.

2.8. Statistical analysis

2.8.1. Microdialysis study

The basal 5-HT concentration in dialysates, uncorrected for the "recovery," was 0.401 nM. All data were not corrected for "recovery" of the dialysis procedure. The percentage of control values was used to present the microdialysis data. The control 5-HT concentrations in the dialysates were calculated as the mean 5-HT concentration of the three basal fractions collected before PO administration or ICV injection. 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. The mean and standard errors were calculated for each treatment group. All data are presented as the mean ± standard error of the mean (SEM). A two-way ANOVA was used to compare the % of control values between treatments (acetaminophen vs. 0.5% CMC with or without formalin and AM404 vs. DMSO with or without formalin). For multiple comparisons, the Holm−Sidak method was used. A Student's *t*-test was used to compare acetaminophen with 0.5% CMC and AM404 with DMSO at each time point.

2.8.2. Behavioral study

In the time-response graph, we presented the mean number of flinches (± SEM) per minute. Periods between 1 and 2 min and 5–6 min after formalin treatment were considered phase 1 responses, while periods between 10 and 60 min were regarded as phase 2 responses. Phase 1 and phase 2 data were analyzed separately. The sum of formalin-evoked flinches during phases 1 and 2 was calculated for each rat to perform a dose-response analysis. For the dose-response analysis of phase 1 and phase 2 data, one-way ANOVA with Dunnett's test was used. In the antagonist study, one-way ANOVA with the Holm−Sidak method for multiple comparisons was used. Statistical significance was set at $p < 0.05$.

3. Results

Formalin injection elicited a highly reliable biphasic flinching behavior in 0.5% CMC-treated rats, and this behavior was comparable to that in our previous report [25].

3.1. Dose-response study

3.1.1. Oral administration study

Oral administration of acetaminophen depressed formalin-induced phase 1 and phase 2 flinching behaviors significantly at doses between 10 and 1000 mg/kg (phase 1: $p < 0.001$; DF, 3; $F = 16.7$; phase 2: $p < 0.001$; DF, 3; $F = 22.9$, by one-way ANOVA; Fig. 2).

3.1.2. ICV injection study

ICV injection of AM404 suppressed formalin-induced phase 2, but not phase 1, flinching behavior significantly at doses between 300 and 3000 μg (phase 1: $p = 0.154$; DF, 3; $F = 1.99$; phase 2: $p < 0.001$; DF, 3; $F = 12.825$, by one-way ANOVA) (Fig. 3).

3.2. Microdialysis study

The dose-response study showed that the optimal dose for microdialysis investigations was 1000 mg/kg of acetaminophen for the oral administration study and 3000 μg of AM404 for the ICV injection study.

3.2.1. Oral administration study

In rats that received formalin injections and those that did not, oral administration of 1000 mg/kg acetaminophen increased spinal 5-HT release compared to 0.5% CMC-treated rats (with formalin injection: $p < 0.001$; t=5.95; without formalin injection: $p = 0.014$; t=2.78, by

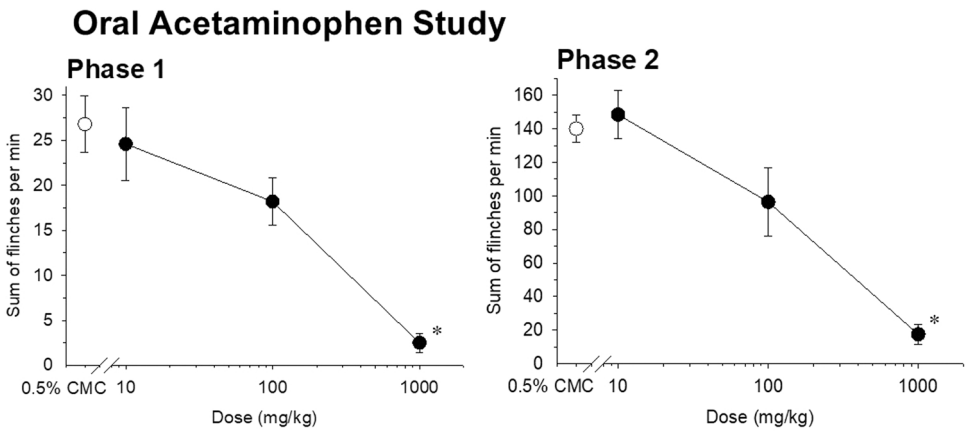

Fig. 2. Dose-response effect of oral acetaminophen on phase 1 and phase 2 responses. Each point represents the mean ± SEM of five rats except for the 1000 mg/kg group (n = 6)., Abscissa: acetaminophen dose (mg/kg); Ordinate: sum of flinches per min., $*p < 0.001$ compared to 0.5% CMC-treated rats.

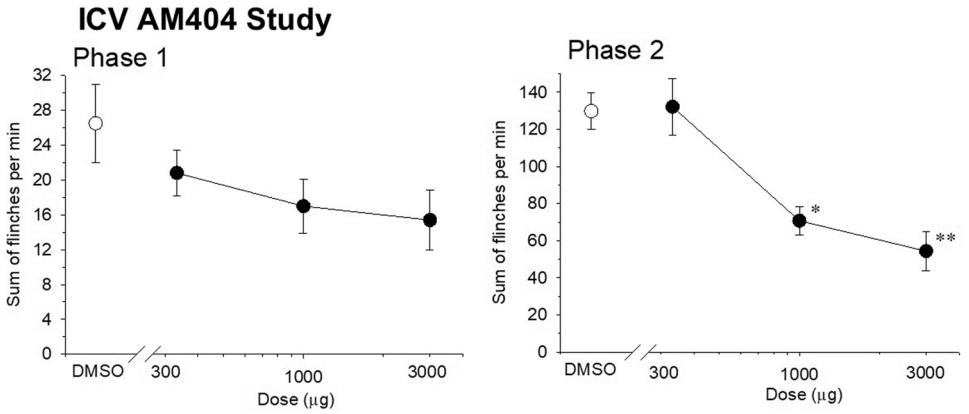

Fig. 3. Dose-response effect of ICV AM404 on phase 1 and phase 2 responses. The DMSO group contained six rats, and the other groups each contained five rats. Each point represents the mean ± SEM., Abscissa: AM404 dose (μg); Ordinate: sum of flinches per min., $*p < 0.005$ compared to DMSO-treated rats. $**p < 0.001$ compared to DMSO-treated rats.

two-way ANOVA [$p < 0.001$; DF, 3; $F = 15.6$]) (Fig. 4). The magnitude of spinal 5-HT release elicited by acetaminophen was significantly greater in the group that received formalin injections than in the group that did not ($p = 0.014$; t=2.94). In the group without acetaminophen administration, the level of spinal 5-HT release in rats that received formalin injections was similar to that in rats that did not receive formalin ($p = 0.827$; t=0.219). During the formalin test, a significant increase in 5-HT release induced by acetaminophen was observed from 15 min before formalin injection to 60 min after formalin injection (time 0: $p = 0.0348$; t=2.72; time 15: $p = 0.0328$; t=2.76; time 30: $p = 0.0286$; t=2.87; time 45: $p = 0.0218$; t=3.08; time 60: $p = 0.0190$; t=3.18, Student's t-test) (Fig. 4).

During the formalin test, there was no difference in 5-HT levels at any time point in acetaminophen-treated rats (DF, 4; F = 0.151;

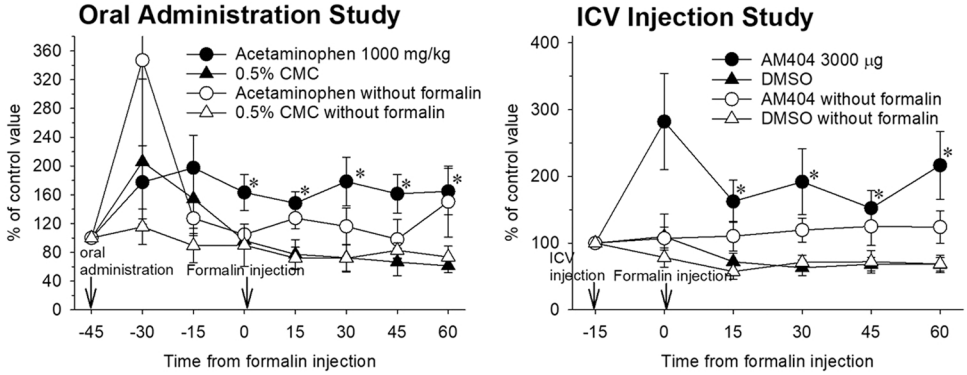

Fig. 4. Spinal 5-HT release after oral administration of acetaminophen (left) or ICV AM404 (right)., Left: Oral administration of acetaminophen (1000 mg/kg) induced significant spinal 5-HT release compared to 0.5% CMC-treated rats ($p < 0.001$ by two-way ANOVA). A significant increase is observed between times 0 and 60 (i.e., between 15 min before formalin injection and 60 min after formalin injection). Each group consisted of four rats. Each point represents the mean ± SEM., Right: ICV injection of 3000 μg AM404 induced significant spinal 5-HT release compared to ICV DMSO-treated rats ($p < 0.001$ by two-way ANOVA). A significant increase is observed between times 15 and 60 (i.e., between formalin injection and 60 min after formalin injection)., The formalin-injected group consisted of five rats each. Four rats were not included in the formalin group. Each point represents the mean ± SEM., Ordinate: 5-HT release as a percentage of the control abscissa: time from formalin injection, at 15 min intervals., $*p < 0.05$, Student's t test compared with the 0.5% CMC group (right) or DMSO group (left).

p = 0.959 by one-way ANOVA) (Fig. 5).

3.2.2. ICV injection study

Similarly, in rats that received formalin injections and those that did not, ICV injection of 3000 μg of AM404 increased spinal 5-HT release compared with DMSO-treated rats (with formalin injection: $p < 0.001$; t=7.33; without formalin injection: $p = 0.032$; t=2.61; two-way ANOVA ($p < 0.001$; DF, 3; $F = 24.3$)) (Fig. 4). The magnitude of spinal 5-HT release elicited by AM404 was significantly greater in the group that received formalin injections than in the group that did not ($p < 0.001$; t=4.56). In the group without AM404, the level of spinal 5-HT release in rats injected with formalin was similar to that of rats that did not receive formalin ($p = 0.690$; t=0.400). A significant increase in 5-HT release induced by AM404 was observed between the time of formalin injection and 60 min after injection (time 15, $p = 0.0295$; t=2.65; time 30: $p = 0.0346$, t=2.54; time 45: $p = 0.0174$, t=2.99, time 60: $p = 0.0210$, t=2.86, by Student's t-test) (Fig. 4).

During the formalin test, there was no difference in 5-HT levels at any time point in AM404 treated rats (DF, 4; $F = 1.128$; $p = 0.371$ by one-way ANOVA) (Fig. 6).

3.3. Antagonist study

3.3.1. Oral administration study

The antagonistic effect of pretreatment with WAY100135 or SB269970 on the analgesic effect of acetaminophen was observed in both phase 1 (WAY100135: $p = 0.016$; t=2.78; SB269970: $p = 0.039$; t=2.30) and phase 2 (WAY100135: $p = 0.026$; t=2.51; SB269970: $p = 0.007$; t=3.19) compared to those of rats not pretreated with antagonists (Fig. 5). However, even in the presence of antagonists,

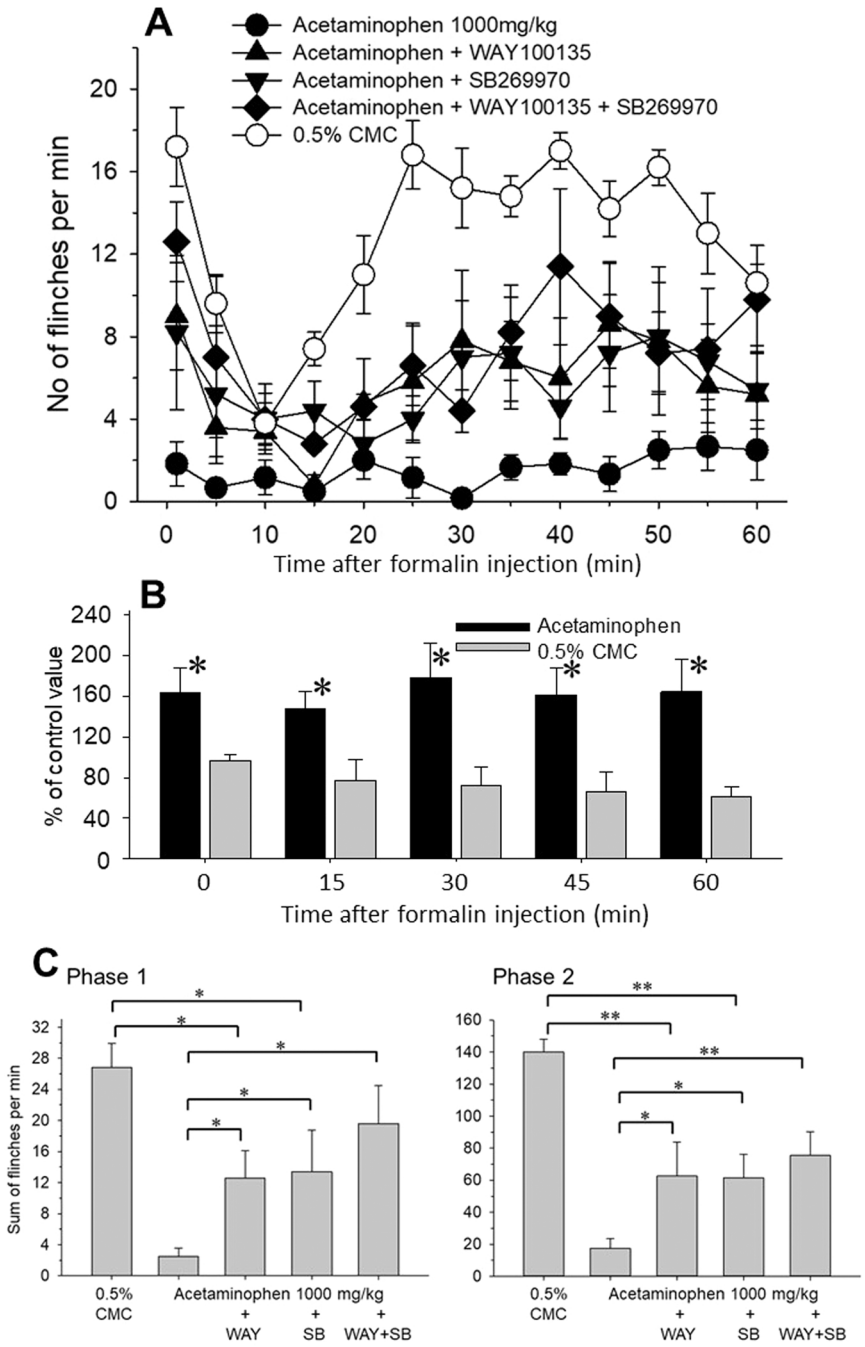

Fig. 5. Effect of 1000 mg/kg oral acetaminophen and 0.5% CMC in the rat formalin test., [A]: Time course of the effect of 1000 mg/kg of acetaminophen and 0.5% CMC. Time courses of the effects of IT WAY100135, SB269970, and WAY100135 + SB269970 on the analgesic effect of 1000 mg/kg of acetaminophen are also shown., [B] For comparison, 5-HT levels during the formalin test are shown in rats treated with 1000 mg/kg of acetaminophen and those treated with 0.5% CMC. At all time points, 5-HT levels in acetaminophen-treated rats were higher than those in 0.5% CMC-treated rats ($p < 0.05$). There was no difference in 5-HT levels at any time point in the acetaminophen-treated rats ($p = 0.959$)., [C]: Effects of IT WAY100135, SB269970, and WAY100135 + SB269970 on phase 1 and phase 2 responses in rats treated with 1000 mg/kg of acetaminophen., Each group contained five rats, except for the acetaminophen group (n = 6). Each point represents the mean±SEM., Abscissa: time after formalin injection; ordinate: upper panel: number of flinches per minute; lower panel: sum of flinches per minute., *$p < 0.005$.

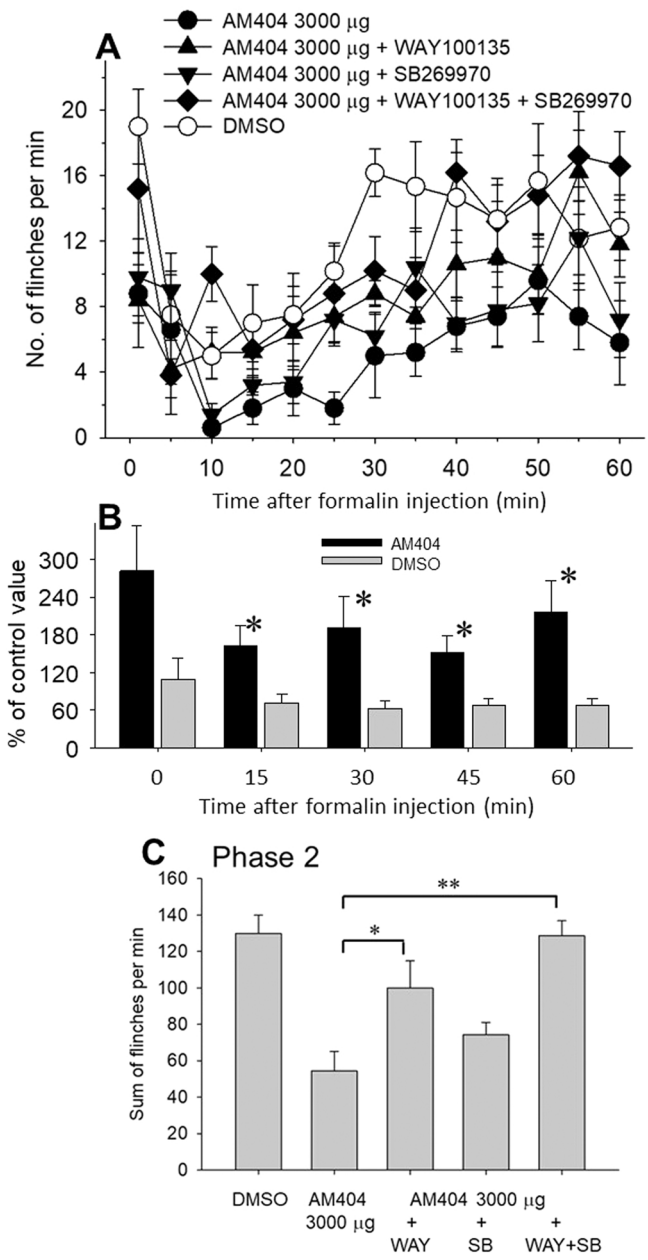

Fig. 6. The effect of 3000 μg of ICV AM404 and DMSO in the rat formalin test., [A]: Time course of the effect of 3000 μg ICV AM404 and DMSO. Time courses of the effects of IT WAY100135, SB269970, and WAY100135 + SB269970 on the analgesic action of 3000 μg of ICV AM404 are also shown., [B] For comparison, 5-HT levels were observed in rats treated with 3000 μg ICV AM404 and those treated with DMSO. At all time points, 5-HT levels in acetaminophen-treated rats were higher than those in 0.5% CMC-treated rats ($p < 0.05$). There was no difference in 5-HT levels at any time point in AM404 treated rats ($p = 0.371$)., [C]: Effects of IT WAY100135, SB269970, and WAY100135 + SB269970 on phase 2 response in rats treated with 3000 μg of ICV AM404. As 3000 μg of ICV AM404 produced an analgesic effect on the response to phase 2, but not phase 1 response, only the data from phase 2 are presented., Each group contained five rats, except for the DMSO group ($n = 6$). Each point represents the mean ± SEM., *$p < 0.005$.

acetaminophen elicited significant analgesic effects in both phase 1 (WAY100135: $p = 0.005$; t=3.74; SB269970: $p = 0.035$; t=2.71) and phase 2 (WAY100135: $p = 0.002$; t=4.13; SB269970: $p < 0.001$; t=5.46) compared to 0.5% CMC-treated rats (Fig. 5). Co-administration of WAY100135 and SB269970 antagonized the analgesic effect of acetaminophen on the responses of phase 1 ($p = 0.004$, t=3.83) and phase 2 ($p = 0.001$, t=4.19) compared to rats not pretreated with antagonists (Fig. 5). Furthermore, the magnitude of the antagonistic effect produced by co-administration of WAY100135 and SB269970 was the same as that of WAY100135 alone (phase 1: $p = 0.276$; t=1.17; phase 2: $p = 0.636$; t=0.492) or SB269970 (phase 1: $p = 0.416$; t=0.858; phase 2: $p = 0.524$; t=0.666) (Fig. 5).

3.3.2. ICV injection study

Pretreatment with WAY100135, but not SB269970, antagonized the analgesic effect of AM404 on the phase 2 response (WAY100135: $p = 0.040$; t=2.64; SB269970: $p = 0.176$; t=1.43; Fig. 6). In contrast to the oral administration study, when WAY100135 was pretreated, AM404 did not produce an analgesic effect ($p = 0.094$; t=1.81) compared to DMSO-treated rats (Fig. 6). Co-administration of WAY100135 and SB269970 antagonized the analgesic effect of AM404 in phase 2 ($p < 0.001$; t=5.21) compared with rats not pretreated with the antagonists (Fig. 6). The magnitude of the antagonistic effect produced by the co-administration of WAY100135 and SB269970 did not differ from that of WAY100135 alone ($p = 0.106$; t=1.75) (Fig. 6).

4. Discussion

In this study, we found that both PO acetaminophen and ICV AM404 induced spinal 5-HT release, regardless of whether formalin was injected. However, the magnitude of spinal 5-HT release was greater with formalin injection than without formalin injection. Although we do not know the precise mechanisms by which PO acetaminophen and ICV AM404 induce spinal 5-HT release, our data suggest that nociceptive inputs, such as injection of formalin, augment PO acetaminophen- and ICV AM404-induced spinal 5-HT release.

In this study, the formalin test was used as an inflammatory pain model. In the formalin test, 50 μL of 0.5% formalin was injected subcutaneously into the dorsal surface of the hind paw. Formalin injection has been reported to produce a concentration-dependent increase in nociceptive responses, such as flinching behavior at concentrations between 1% and 5%, and the analgesic effect of mechanically distinct compounds varied with the concentration of formalin injected [9,24]. Therefore, we used 5% formalin, based on our previous study [25].

An intrathecal microdialysis probe was implanted under isoflurane anesthesia the day before the microdialysis experiment, and the probe was perfused with artificial cerebrospinal fluid overnight. Formalin was injected with light isoflurane. In this study, intrathecal 5-HT levels were measured in freely moving rats. Furthermore, during the formalin test, there were no differences in 5-HT levels at any time point. We believe that isoflurane anesthesia did not affect intrathecal 5-HT concentration.

In both oral administration and ICV injection studies, antagonists, such as WAY100135 and SB269970, were administered IT, but not orally and ICV. The purpose of this study was to examine whether PO acetaminophen or ICV AM404 induced spinal 5-HT release and whether spinal 5-HT induced by PO acetaminophen or ICV AM404 played an important role in producing an analgesic effect. Therefore, WAY100135 and SB269970 were administered with IT.

4.1. Oral administration study

A significant increase in spinal 5-HT release was observed from 15 min before formalin injection to 60 min after formalin injection, and the analgesic effect of acetaminophen was found to act in phases 1 and 2. Furthermore, during the entire formalin test, the level of spinal 5-HT release was constant. The time course of the analgesic effect of PO acetaminophen was consistent with that of the release of spinal 5-HT.

The analgesic effect of PO acetaminophen in phase 1 and phase 2 was partially and equally antagonized by both 5-HT_{1A} and 5-HT_7 receptor antagonists. This suggests that the analgesic effect of acetaminophen observed during the formalin test was partly mediated by the activation of the 5-HT_{1A} and 5-HT_7 receptors. The magnitude of the antagonistic

effect produced by the co-administration of WAY100135 and SB269970 on the analgesic effect of PO acetaminophen was the same as that of the sole administration of WAY100135 or SB269970. The 5-HT_1 receptor is a Gi/Go protein-coupled receptor whose activation decreases intracellular concentrations of cAMP [18]. Meanwhile, the 5-HT_7 receptor is a G protein-coupled receptor that increases intracellular concentrations of cAMP when activated [18]. Although activation of the 5-HT_1 and 5-HT_7 receptors elicits opposing inhibitory and excitatory effects, the analgesic output of activation of the 5-HT_{1A} receptor was the same as that of 5-HT_7 receptor activation. Furthermore, there was no interaction between the 5-HT_{1A} receptor and 5-HT_7 receptor activation. Our data suggested that in the oral administration study, released spinal 5-HT activates both the 5-HT_{1A} and 5-HT_7 receptors and that the mechanisms by which the 5-HT_{1A} receptor helps produce an analgesic effect are the same as those of the 5-HT_7 receptor.

4.2. ICV AM404 study

A significant increase in spinal 5-HT release was observed between the time of formalin injection and 60 min after formalin injection. Furthermore, during the entire formalin test, the level of spinal 5-HT release was constant. This indicated that significant 5-HT release commenced during the first 15 min after formalin injection. ICV AM404 elicited an analgesic effect in the response of phase 2, but not in phase 1. It is possible that an insufficient amount of 5-HT was released to produce an analgesic effect during the first 10 min after formalin injection.

The analgesic effect of ICV AM404 was completely antagonized by IT WAY100135, while IT SB269970 had no effect. This suggests that the role of spinally released 5-HT induced by ICV AM404 differs from that of PO acetaminophen-induced 5-HT. With ICV AM404, spinally released 5-HT activates only the 5-HT_{1A} receptor. This is the sole mechanism by which the ICV AM404-induced analgesic effect is produced, and the 5-HT_7 receptor is not required for the activity of ICV AM404.

4.3. Role of 5-HT induced by supraspinal AM404 in the analgesic effects of PO acetaminophen during the formalin test

As described above, the role of ICV AM404-induced spinal 5-HT differs from that of PO acetaminophen-induced 5-HT. Our data suggest that spinal 5-HT induced by ICV AM404 may not contribute to the analgesic effect of oral acetaminophen.

IT AM404 has been reported to produce a thermal analgesic effect at doses between 0.04 and 0.4 μg [13]. Högestätt et al. [6] reported that 20 min after intraperitoneal injection of acetaminophen (300 mg/kg), AM404 was detected in both the brain and spinal cord and that the level of AM404 in the brain was lower than in the spinal cord. In this study, we found that ICV AM404 produced an analgesic effect at 1000 and 3000 μg. This further suggested a limited role for supraspinal AM404 in the analgesic effect of PO acetaminophen.

The rostroventromedial medulla (RVM) has been reported to be a key pain control center involved in descending pain modulation in the spinal cord through local release of 5-HT and plays a unique role in the balance of bidirectional control (i.e., inhibitory and facilitatory) from the brain to the spinal cord [22]. RVM may be one of the sources of spinal 5-HT induced by PO acetaminophen and ICV AM404.

4.4. Role of 5-HT_{1A} and 5-HT_7 receptors in the rat formalin test

Rocha-González et al. [19] reported that IT 5-HT produced an analgesic effect at doses between 25 and 200 nmol during the phase 2 response, and neither SB269970 nor WAY100635, a 5-HT_{1A} receptor antagonist, affected the analgesic effect of IT 5-HT. These results using exogenous 5-HT are not consistent with our PO acetaminophen and ICV AM404 data. Therefore, the role of exogenous 5-HT in producing an analgesic effect during the formalin test may be different from that of endogenous 5-HT. The authors also reported that spinal 5-HT_{1A} receptors are partially involved in the control of antinociception, while the spinal 5-HT_7 receptor is associated with nociception when using 5-carboxamidotryptamine, a 5-$HT_{7/1A}$ receptor agonist. In our PO acetaminophen study, activation of the spinal 5-HT_{1A} or spinal 5-HT_7 receptors was sufficient to produce an analgesic effect. These data suggest that the role of spinal 5-HT_7 receptor activation induced by PO acetaminophen differs from that of 5-HT_7 receptor activation induced by IT 5-carboxamidotryptamine.

5. Conclusions

In conclusion, oral acetaminophen and ICV AM404 produced analgesic effects and induced spinal 5-HT release in the rat formalin test. However, the role of 5-HT induced by oral acetaminophen in producing an analgesic effect differs from that of 5-HT induced by ICV AM404. Further studies are needed to clarify the correlation of analgesic effects with spinal 5-HT release and administration of acetaminophen.

CRediT authorship contribution statement

Shingo Nakamura: Conceptualization, Methodology, Investigation. **Takahiro Nonaka:** Methodology, Investigation. **Shuji Komatsu:** Data curation. **Toshihiro Yamada:** Data curation. **Tatsuo Yamamoto:** Conceptualization, Data curation, Writing – review & editing.

Conflict of interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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