Acetaminophen Metabolite *N*-Acylphenolamine Induces Analgesia *via* Transient Receptor Potential Vanilloid 1 Receptors Expressed on the Primary Afferent Terminals of C-fibers in the Spinal Dorsal Horn

Nobuko Ohashi, M.D., Ph.D., Daisuke Uta, Ph.D., Mika Sasaki, Ph.D., Masayuki Ohashi, M.D., Ph.D., Yoshinori Kamiya, M.D., Ph.D., Tatsuro Kohno, M.D., Ph.D.

ABSTRACT

Background: The widely used analgesic acetaminophen is metabolized to *N*-acylphenolamine, which induces analgesia by acting directly on transient receptor potential vanilloid 1 or cannabinoid 1 receptors in the brain. Although these receptors are also abundant in the spinal cord, no previous studies have reported analgesic effects of acetaminophen or *N*-acylphenolamine mediated by the spinal cord dorsal horn. We hypothesized that clinical doses of acetaminophen induce analgesia *via* these spinal mechanisms.

Methods: We assessed our hypothesis in a rat model using behavioral measures. We also used *in vivo* and *in vitro* whole cell patch-clamp recordings of dorsal horn neurons to assess excitatory synaptic transmission.

Results: Intravenous acetaminophen decreased peripheral pinch-induced excitatory responses in the dorsal horn ($53.1 \pm 20.7\%$ of control; n = 10; $P < 0.01$), while direct application of acetaminophen to the dorsal horn did not reduce these responses. Direct application of *N*-acylphenolamine decreased the amplitudes of monosynaptic excitatory postsynaptic currents evoked by C-fiber stimulation (control, 462.5 ± 197.5 pA; *N*-acylphenolamine, 272.5 ± 134.5 pA; n = 10; $P = 0.022$) but not those evoked by stimulation of Aδ-fibers. These phenomena were mediated by transient receptor potential vanilloid 1 receptors, but not cannabinoid 1 receptors. The analgesic effects of acetaminophen and *N*-acylphenolamine were stronger in rats experiencing an inflammatory pain model compared to naïve rats.

Conclusions: Our results suggest that the acetaminophen metabolite *N*-acylphenolamine induces analgesia directly *via* transient receptor potential vanilloid 1 receptors expressed on central terminals of C-fibers in the spinal dorsal horn and leads to conduction block, shunt currents, and desensitization of these fibers. (**Anesthesiology** 2017; 127:355-71)

ACETAMINOPHEN is one of the most widely used analgesic agents. However, its metabolism is complex, and its analgesic mechanisms have not been elucidated. Several studies suggest that acetaminophen is metabolized to *p*-aminophenol, which then crosses the blood–brain barrier and is metabolized by fatty acid amide hydrolase (FAAH) to yield *N*-acylphenolamine (AM404). AM404 then induces analgesia in the central nervous system.[1,2] In addition to AM404, acetaminophen is metabolized to compounds, such as *N*-acetyl-*p*-benzoquinoneimine (NAPQI) and *p*-benzoquinone (*p*-BQ), both of which also appear to produce analgesia.[3,4] However, AM404 is widely known to be one of the most important mediators of acetaminophen-induced analgesia. AM404 has been thought to be an anandamide analog[5,6] but it was recently shown that AM404 also acts on the transient receptor potential vanilloid 1 (TRPV1)

What We Already Know about This Topic

- Despite widespread use as an analgesic drug, the molecular mechanisms of acetaminophen remain incompletely understood
- Metabolites of acetaminophen such as *N*-acylphenolamine act directly on transient receptor potential vanilloid 1 receptors in the brain, but their roles in spinal analgesia are unknown

What This Article Tells Us That Is New

- Direct application of *N*-acylphenolamine but not of acetaminophen reduced excitatory transmission in the spinal cord dorsal horn by direct activation of transient receptor potential vanilloid 1 receptors on presynaptic C-fiber terminals
- Increased activity of transient receptor potential vanilloid 1 in inflammatory pain provides a mechanism by which acetaminophen may reduce inflammatory pain through its metabolite *N*-acylphenolamine

<zdoi;10.1097/ALN.0000000000001700>

and cannabinoid 1 (CB1) receptors of the midbrain and medulla,[7–9] which are colocalized modulators of pain.[10–12] Therefore, these receptors are widely believed to be mediators of acetaminophen-induced analgesia.

The spinal cord is also critical to pain perception. In fact, a previous study reported that systemic administration of acetaminophen attenuates hyperalgesia induced by intrathecal administration of substance P.[13] Other studies have reported that AM404 decreases neuronal c-*fos*-positive immunoreactivity induced by nonnoxious stimulation of the spinal cord in a rat model of neuropathic pain.[14] In addition, AM404 inhibits calcium currents in cultured dorsal root ganglion (DRG) neurons.[15] It is also known that both TRPV1 and CB1 receptors are abundant in the spinal cord dorsal horn. Indeed, TRPV1 is only present at central terminals of sensory neurons, and CB1 is present at central terminals of sensory neurons and intrinsic spinal cord neurons. Furthermore, it is widely known that primary afferent Aδ- and C-fibers terminate in the substantia gelatinosa (SG; Rexed lamina II) of the spinal dorsal horn and modulate nociceptive transmission. TRPV1 receptors are especially highly expressed on primary afferent C-fibers, but not Aδ-fibers.[16–19] The above observations suggest that direct action of acetaminophen at the dorsal horn may contribute to its analgesic effects. However, the precise mechanisms of acetaminophen action *via* its AM404 metabolite are unknown. We hypothesized that in addition to its actions in the brain, acetaminophen also induces analgesia *via* direct activation of TRPV1 and/or CB1 receptors in the spinal cord dorsal horn. We investigated whether application of clinical doses of acetaminophen or AM404 to the dorsal horn induce analgesia. We also assessed the underlying mechanisms of synaptic modulation using behavioral measures, as well as *in vivo* and *in vitro* whole cell patch-clamp recordings.

Materials and Methods

Study Approval

All animal experiments were conducted in accordance with international guidelines on the ethical use of animals, and efforts were made to minimize pain or discomfort experienced by the animals. Animal housing and surgical procedures were approved by the Institutional Animal Care and Use Committee of Niigata University Graduate School of Medical and Dental Science (Niigata City, Japan; approval No. 456-6).

Animals

Male Wistar rats (200 to 250 g) were used in all experiments. Animals were housed under a 12-h light/dark cycle with *ad libitum* access to food and water.

Implantation of Intrathecal Catheters

For intrathecal drug administration, rats were implanted with a polyethylene PE-10 catheter using a modification of a previously described technique.[20] The rats were anesthetized using 2 to 3% isoflurane, and PE-10 polyethylene catheters were inserted rostrally into the lumbar enlargement *via* a mini-laminectomy at the L5 vertebra. The animals were allowed to recover for 3 to 6 days before the start of the experiments. Only animals without evidence of neurologic dysfunction completed the studies.

von Frey Test

The rats were acclimated to the experimental room for at least 30 min before drug administration. We investigated sensitivity to mechanical stimulation to assess drug-induced analgesia. The force threshold for paw withdrawal in response to probing with a series of calibrated von Frey filaments was determined. Each filament was applied perpendicularly to the plantar surface of the paws while the rats remained in wire-mesh cages.[21] The withdrawal threshold was defined as the lowest force that evoked a clear withdrawal response at least twice in 10 applications. Testing occurred every 10 min, beginning at the time of drug administration.

Radiant Heat Test

The rats were acclimated to the experimental room for at least 30 min before drug administration. Thermal hyperalgesia was measured using the plantar test (Ugo Basile, Italy) for paw withdrawal latency according to the method described by Hargreaves *et al.*[22] The rats were placed in a transparent acrylic enclosure with a glass plate floor in a temperature-controlled and quiet room. They were then allowed to acclimate to their environment for 1 h before testing. A high-intensity movable radiant heat source was placed under the glass and focused onto the plantar surface of the left hind paw. The nociceptive endpoint in the radiant heat test was characteristic lifting or licking of the hind paw. The time from onset of radiant heat to endpoint was defined as the paw withdrawal latency. The radiant heat intensity was adjusted at the beginning of the experiment to obtain basal paw withdrawal latencies of 8 to 10 s and was kept constant thereafter. An automatic 25-s cutoff was used to prevent tissue damage. Each animal was tested every 15 min after the intraperitoneal or intrathecal injections.

Inflammatory Pain Model

A 29-gauge needle attached to a 100-μl microsyringe (Hamilton, USA) was inserted into the posterior aspect of the plantar surface and advanced subcutaneously to the approximate center of the hind paw, where 100 μl of complete Freund's adjuvant (CFA) was deposited. The rats were then returned to their cages, and experiments were conducted 2 days after the CFA injection, as this time point corresponds to the peak of the inflammatory response. The rats that had force thresholds of 1.4 to 2.0 g for paw withdrawal using von Frey filaments after the CFA injection were used as the inflammatory pain model. The individuals who injected the CFA into the hind paw did not perform the behavioral tests.

However, inflammatory responses at the hind paw, such as swelling and redness due to the injection of CFA, could be observed visually. Therefore, the experimenters were not blinded. There is a possibility that rats subjected to pain due to the CFA injection have mirror-image pain on the contralateral side. To strictly exclude this bias, we did not use the contralateral side as control.

In Vivo *Patch-Clamp Recording from SG Neurons*

The methods used for the *in vivo* patch-clamp recording of SG neurons were similar to those described previously.[23,24] In brief, the rats were anesthetized using urethane (1.5 g/kg intraperitoneally). Thoracolumbar laminectomy was performed at the level of L3 to L5 to expose the lumbar enlargement of the spinal cord. The rats were then placed in a stereotaxic apparatus. After the dura mater was opened, the pia-arachnoid membrane was cut to make a window to allow the patch electrode to enter into the SG. The surface of the spinal cord was equilibrated with Krebs solution (10 to 15 ml/min) and irrigated with a 95% O_2/5% CO_2 gas mixture at 36 ± 1°C. The Krebs solution contained the following: 117 mM NaCl, 3.6 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 25 mM $NaHCO_3$, and 11.5 mM D-glucose. Patch electrodes were fabricated from thin-walled borosilicate glass capillary tubes using a puller (Sutter Instrument, USA) and had resistances of 8 to 12 MΩ when filled with a potassium-gluconate-based intracellular solution of 5 mM KCl, 0.5 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM EGTA, 5 mM HEPES, 5 mM tetraethylammonium, and 5 mM adenosine triphosphate-Mg. Series resistance was assessed based on the response to a 5-mV hyperpolarizing step. This value was monitored during the recording session, and the data were rejected if values changed by more than 15%. The patch electrode was advanced into the spinal cord using a micromanipulator. Blinded whole cell recordings were obtained from SG neurons at a depth of 30 to 150 μm from the surface,[24] and voltage-clamped neurons were held at –70 mV for the recording of excitatory postsynaptic currents (EPSCs). Area under the curve, which is the area surrounded by the baseline and border of the EPSCs, was measured. Signals were amplified using an Axopatch 200B amplifier (Molecular Devices, USA), filtered at 2 kHz and digitized at 5 kHz. The data were collected and analyzed using the pCLAMP 10.2 software suite (Molecular Devices).

Acetaminophen was administered systemically by intravenous injection *via* a femoral vein catheter using a 1-ml microsyringe for 1 min during the *in vivo* patch-clamp recordings. We determined the skin area wherein a noxious pinch stimulus produced the desired neural response. The noxious mechanical stimulus consisted of pinching skin folds using toothed forceps at the ipsilateral hind limb at the maximal response point of the receptive area. To maintain constant stimulus strength, a weight was placed on the toothed forceps after fixation to a stand.[24] Pinch stimuli were administered for 10 s every 2 min at identical points during the recordings.

In Vitro *Patch-Clamp Recordings from SG Neurons*

The rats were anesthetized using urethane (1.5 g/kg intraperitoneally). A dorsal laminectomy was performed, and the lumbosacral segment of the spinal cord was removed. The rats were then immediately euthanized by exsanguination. The isolated spinal cords were placed in preoxygenated ice-cold Krebs solution. After severing all ventral and dorsal roots except for the L5 dorsal root, the arachnoid membrane was removed. Each spinal cord was mounted on the metal stage of a microslicer (Linear Slicer PRO 7; Dosaka, Japan) and cut into 650-μm transverse slices with the L5 dorsal root attached. These slices were transferred to a recording chamber and perfused continuously with Krebs solution (10 to 15 ml/min) equilibrated with a 95% O_2/5% CO_2 gas mixture at 36 ± 1°C. Patch electrodes were fabricated from thin-walled borosilicate glass capillary tubes using a puller (Sutter Instrument) and had resistances of 5 to 10 MΩ when filled with a cesium-based intracellular solution of 110 mM Cs_2SO_4, 0.5 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM EGTA, 5 mM HEPES, 5 mM tetraethylammonium, and 5 mM adenosine triphosphate-Mg. Under a dissecting microscope with transmission illumination, lamina II of the dorsal horn was discernible as a relatively translucent band across the dorsal horn. Whole cell patch-clamp recordings were performed, and the data were analyzed as described (see "*In Vivo* Patch-Clamp Recording from SG Neurons").

Excitatory synaptic currents were evoked by dorsal root stimulation. The L5 dorsal root was stimulated using a suction electrode. Stimulation was performed at 100 μA for 0.05 ms for Aδ-fibers and at 1,000 μA for 0.5 ms for C-fibers. Aδ-fiber-evoked EPSCs were judged monosynaptically, based on both their short and constant latencies, as well as the absence of failures with repetitive stimulation at 20 Hz.[25] C-fiber-evoked EPSCs were judged monosynaptically, based on the absence of failures with low-frequency (1 Hz) repetitive stimulation. In contrast, polysynaptic EPSCs were recognized by their unreliable, variable latencies under these stimulation protocols.

Drug Administration

Acetaminophen (Acelio; Terumo, Japan) was administered by intraperitoneal injection (10, 20, or 40 mg/kg) using a 26-gauge needle for the behavioral experiments and by intravenous injection (20 mg/kg) through a femoral vein catheter using a 1-ml microsyringe for *in vivo* patch-clamp recordings. AM404 (Sigma–Aldrich, USA) was administered by intrathecal injection (0.1, 0.3, or 1 nmol) for behavioral experiments using a 10-μl bolus delivered by catheter. The bolus was created by first dissolving the drug in dimethyl sulfoxide (DMSO), followed by dilution in normal saline to a final concentration of 5% DMSO. Capsazepine was administered by intraperitoneal injection (20 mg/kg) using a 26-gauge needle and by intrathecal injection (0.3 nmol) using a 10-μl bolus delivered by catheter. The bolus was created by first dissolving the drug in DMSO, followed by

dilution in normal saline to a final concentration of 5% DMSO for behavioral experiments.

AM404, capsazepine, and cannabinoid 1 receptor antagonist AM251 (Sigma–Aldrich) were first dissolved in DMSO at 1,000 times the final concentration for storage, while tetrodotoxin (Wako, Japan) was first dissolved in distilled water at 1,000 times the final concentration (AM404, 30 μM; capsazepine, 10 μM; and AM251, 3 μM) for storage. These stock solutions were diluted to their final concentrations in Krebs solution immediately before use.

We chose a dose of 20 mg/kg for acetaminophen because it is a clinical dose and a concentration of 30 μM for AM404 because it is the concentration of AM404 in cerebrospinal fluid in humans. Clinically, we often administer 1,000 mg (20 mg/kg) acetaminophen orally or intravenously to patients. It is reported that 1,000 mg oral acetaminophen produces a C_{max} of 24 to 45 μg/ml in plasma, which corresponds to 160 to 300 μM.[26,27] A number of animal studies have measured cerebrospinal fluid concentrations of acetaminophen.[28,29] One study measured spinal acetaminophen in patients with lumbosacral nerve-root compression pain.[1] Patients in this study received a single 2,000-mg intravenous infusion of acetaminophen prodrug, which corresponds to 1,000 mg acetaminophen and is equivalent to the clinical dose. The resulting cerebrospinal fluid concentration of acetaminophen was 1.5 to 9.0 μg/ml.[1] This concentration range is equivalent to the concentration of AM404 (10 to 30 μM) in the *in vitro* whole cell patch-clamp experiment. Furthermore, another study using whole cell patch-clamp recordings of cultured DRG neurons reported the EC_{50} value of AM404 to be more than 10 μM, which corresponds to the concentration used in our study.[15]

Statistical Analysis

The data are expressed as means ± SD. Randomization methods were not used to assign animals to the experimental conditions. In addition, we did not use blinding during the procedures. We did not perform power analyses, as the sample sizes were based on previous studies. Statistical comparisons between data were performed using Student's paired two-tailed *t* tests. Behavioral studies were analyzed using within-group data. The mechanical threshold and withdrawal latency after vehicle, 10, 20, or 40 mg/kg of acetaminophen, or vehicle, 0.1, 0.3, or 1 nmol of AM404 were analyzed using two-way repeated-measures ANOVAs (time and drug dose as variables). The ANOVAs were followed by Bonferroni multiple *post hoc* comparisons. Statistical significance was defined as *P* < 0.05. The StatView 5 (SAS Institute, USA) program was used to conduct the statistical analysis.

Results

Systemic Administration of Acetaminophen Induces Analgesia to Thermal Stimulation

To examine whether systemic administration of acetaminophen induces behavioral analgesia in naïve rats, we first investigated the effects of acetaminophen using the von Frey and radiant heat tests. The mechanical threshold for paw withdrawal during von Frey stimulation, which measures resistance to evoked pain, was not affected by intraperitoneal injection of acetaminophen (10, 20, or 40 mg/kg; n = 6; *P* > 0.05, two-way repeated-measures ANOVA followed by Bonferroni *post hoc* comparisons; fig. 1A). However, withdrawal latency during the radiant heat test, which measures resistance to thermal stimulation, was significantly and concentration-dependently prolonged by acetaminophen (n = 6; *P* < 0.01, two-way repeated-measures ANOVA followed by Bonferroni *post hoc* comparisons; fig. 1A). The maximal effect was observed 30 to 45 min after intraperitoneal injections of acetaminophen. This was followed by a gradual decline to baseline (fig. 1A). These results suggest that systemic administration of acetaminophen induces analgesia to thermal stimulation in naïve rats.

Systemic Administration of Acetaminophen Suppresses the Spinal Response Induced by Peripheral Pinch Stimuli

We investigated whether systemic administration of acetaminophen acts on spinal dorsal horn neurons to suppress spinal responses induced by peripheral pinch stimuli. We used *in vivo* whole cell patch-clamp recordings of SG neurons in the spinal cord dorsal horn, while a noxious pinch stimulus was applied to the ipsilateral hind limbs naïve rats. The areas under the curve after the peripheral pinch stimuli were significantly reduced 10 min after intravenous injection of acetaminophen (20 mg/kg) when compared to control conditions (53.1 ± 20.7% of control; n = 10; *P* < 0.01, paired *t* test; fig. 1B). The maximal inhibitory effect was observed 10 min after intravenous injection of acetaminophen and gradually recovered to baseline by 60 min after the injection (fig. 1B). These results suggest that systemic administration of acetaminophen induces analgesia by inhibiting excitatory activity in spinal cord dorsal horn neurons.

Direct Application of Acetaminophen to the Spinal Cord Does Not Change the Amplitudes or Frequencies of Miniature EPSCs in SG Neurons

We investigated whether direct application of acetaminophen to the spinal cord affects excitatory synaptic transmission in SG neurons using *in vitro* whole cell patch-clamp recordings from spinal cord slice preparations. Miniature EPSCs (mEPSCs) were isolated by adding tetrodotoxin (1 μM) to the perfusate. One value was excluded because series resistances were changed by more than 15% during the recording. Bath-applied acetaminophen (30 μM, 2 min) did not affect the mean mEPSC amplitude (control, 12.9 ± 2.3 pA; acetaminophen, 12.3 ± 2.6 pA; 95.5 ± 7.5% of control; range, 81.5 to 103.5%; n = 7; *P* = 0.67, paired Student's *t* test; fig. 1C) or frequency (control, 8.8 ± 2.8 Hz; acetaminophen, 8.6 ± 2.8 Hz; 96.9 ± 4.3% of control; range, 91.6 to 101.5%; n = 7; *P* = 0.86, paired Student's *t* test; fig. 1C). Figure 1C shows the effects of acetaminophen on the cumulative distribution of mEPSC amplitudes and interevent
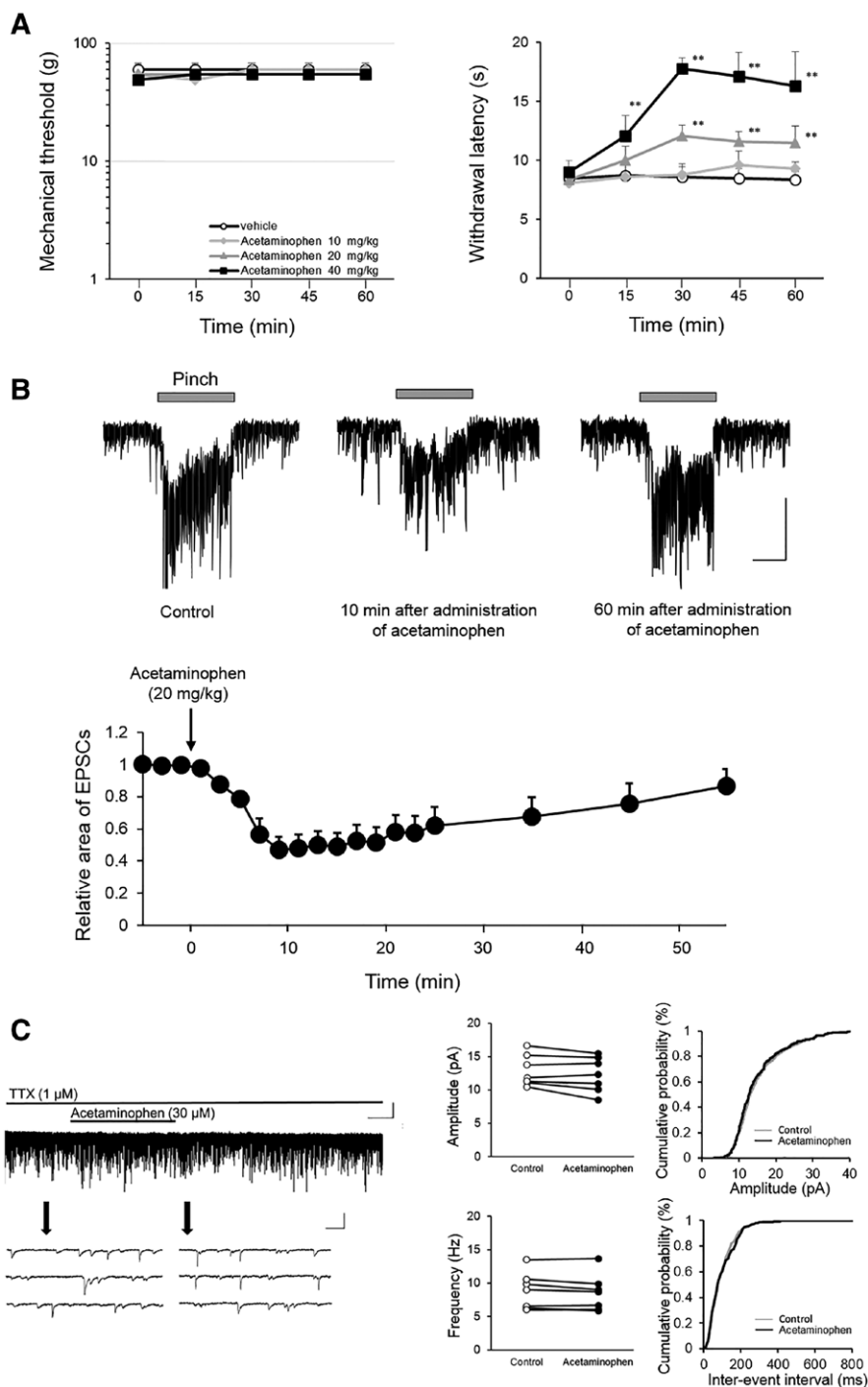
Fig. 1. Systemic administration of acetaminophen induces analgesia in behavioral and *in vivo* whole cell patch-clamp experiments, although direct application of acetaminophen to the spinal cord does not affect substantia gelatinosa (SG) neurons in *in vitro* whole cell patch-clamp recordings. (*A*) Mechanical thresholds for paw withdrawal during von Frey stimulation were not affected by acetaminophen, although withdrawal latencies during the radiant heat test were significantly and concentration-dependently prolonged by acetaminophen. Acetaminophen (10, 20, or 40 mg/kg) was injected intraperitoneally (n = 6; **$P < 0.01$, two-way repeated-measures ANOVA with time and drug dose as variables followed by Bonferroni *post hoc* comparisons). (*B*) Using *in vivo* whole cell patch-clamp recordings of SG neurons in the spinal cord dorsal horn, the area under the curve, surrounded by the baseline and border of excitatory postsynaptic currents (EPSCs), induced by peripheral pinch stimuli was significantly reduced 10 min after intravenous acetaminophen (20 mg/kg; n = 10). The *horizontal scale bar* represents 5 s, and the *vertical bar* represents 50 pA. (*C*) Using *in vitro* whole cell patch-clamp recordings, administration of acetaminophen (30 μM, 2 min) to the spinal cord did not change the amplitude or frequency of miniature EPSCs in SG neurons (n = 7). The *top horizontal scale bar* represents 30 s, and the *top vertical bar* represents 10 pA. The *bottom horizontal scale bar* represents 500 ms, and the *bottom vertical bar* represents 10 pA. The data are given as means ± SDs. TTX = tetrodotoxin.
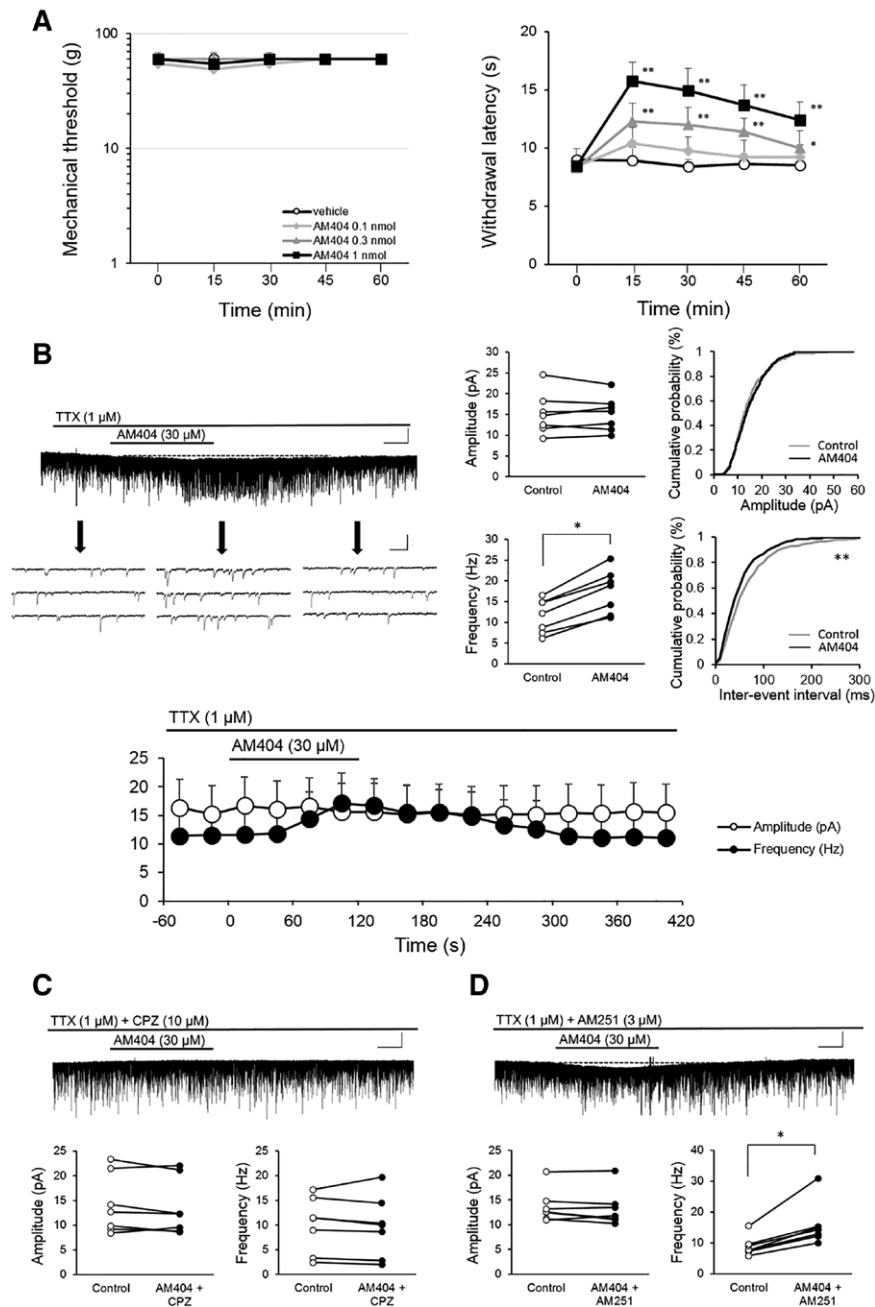
Fig. 2. Intrathecal administration of the acetaminophen-derived metabolite, *N*-acylphenolamine (AM404), induces analgesia in behavioral experiments, and direct application of AM404 to the spinal cord increases the frequency of miniature excitatory postsynaptic currents (mEPSCs) sensitive to presynaptic transient receptor potential vanilloid 1, but not cannabinoid 1, receptors on substantia gelatinosa neurons. (*A*) Mechanical thresholds for paw withdrawal during von Frey stimulation were not affected by AM404, although withdrawal latencies during the radiant heat test were significantly and concentration-dependently prolonged by AM404. AM404 was injected intrathecally at 0.1, 0.3, and 1 nmol (n = 6; *$P < 0.05$, **$P < 0.01$, two-way repeated-measures ANOVA with time and drug dose as variables followed by Bonferroni *post hoc* comparisons). (*B*) Using *in vitro* whole cell patch-clamp recordings from substantia gelatinosa neurons of the spinal cord dorsal horn, direct administration of AM404 (30 μM, 2 min) to the spinal cord did not change the mean mEPSC amplitude and had no effect on the cumulative distribution of mEPSC amplitudes. In contrast, AM404 significantly increased the mean mEPSC frequency and induced a significant leftward shift in the cumulative interevent interval distribution of the mEPSCs (n = 7; *$P < 0.05$, **$P < 0.01$, paired Student's *t* test). The *top horizontal scale bar* represents 30 s, and the *top vertical bar* represents 10 pA. The *bottom horizontal scale bar* represents 500 ms, and the *bottom vertical bar* represents 10 pA. (*C*) In the presence of the transient receptor potential vanilloid 1 receptor antagonist capsazepine (CPZ; 10 μM), the AM404-induced increase in mEPSC frequency was not observed (n = 7). The *horizontal scale bar* represents 30 s, and the *vertical bar* represents 10 pA. (*D*) In the presence of the cannabinoid 1 receptor antagonist AM251 (3 μM), a significant AM404-induced increase in mEPSC frequency was observed (n = 7; *$P < 0.05$, paired Student's *t* test). The *horizontal scale bar* represents 30 s, and the *vertical bar* represents 10 pA. The data are given as means ± SDs. TTX = tetrodotoxin.

intervals. Acetaminophen had no effect on the cumulative distribution of mEPSC amplitudes and interevent intervals over the recording period (Kolmogorov–Smirnov test $P >$ 0.05; fig. 1C). These results suggest that direct application of acetaminophen to spinal cord slices does not affect excitatory synaptic transmission in SG neurons of the dorsal horn.

Intrathecal Administration of the Acetaminophen-derived Metabolite AM404 Induces Thermal Analgesia

Several previous studies have reported that the acetaminophen-derived metabolite AM404 induces analgesia by direct action at TRPV1 or CB1 receptors in the brain.[1,2] We therefore investigated the analgesic effects of intrathecal AM404 in naïve rats using the von Frey and radiant heat tests.

The mechanical threshold for paw withdrawal during von Frey stimulation was not affected by AM404 (0.1, 0.3, or 1 nmol; n = 6; $P > 0.05$, two-way repeated-measures ANOVA followed by Bonferroni *post hoc* comparisons; fig. 2A). However, heat withdrawal latencies were significantly and concentration-dependently prolonged by AM404 (n = 6; $P < 0.05$, two-way repeated-measures ANOVA followed by Bonferroni *post hoc* comparisons; fig. 2A). After intrathecal injection of AM404, withdrawal latencies peaked at 15 min before gradually returning to baseline by 60 min after the injection (fig. 2A). These results suggest that intrathecal administration of AM404 produces thermal analgesia in naïve rats.

Direct Application of AM404 to the Spinal Cord Increases the Frequency of mEPSCs in SG Neurons without Changing Their Amplitude

Intrathecal administration of AM404 induced analgesia in the behavioral study. Therefore, we examined whether direct application of AM404 to the spinal cord affects excitatory synaptic transmission in SG neurons. Although bath-applied AM404 (30 μM, 2 min) did not affect the mean mEPSC amplitude (control, 15.2 ± 5.0 pA; AM404, 15.2 ± 4.2 pA; $101.3 \pm 9.1\%$ of control; range, 90.6 to 113.6%; n = 7; $P =$ 0.99, paired Student's t test; fig. 2B), it did increase the mean mEPSC frequency from 11.5 ± 4.1 Hz in the control group to 17.5 ± 5.3 Hz in the AM404 group (155.6% of control; range, 133.3 to 191.3%; n = 7; $P = 0.036$, paired Student's t test; fig. 2B). Figure 2B (*right*) shows the effect of AM404 on the cumulative distribution of mEPSC amplitudes and interevent intervals. Although AM404 had no effect on the cumulative distribution of mEPSC amplitudes over the recording period, it induced a significant leftward shift of the cumulative interevent interval distribution for mEPSCs when compared to the control condition (Kolmogorov–Smirnov test, $P < 0.01$; fig. 2B). The mean mEPSC frequency, but not amplitude, was increased after AM404 superfusion but declined to baseline within 5 min after the end of the perfusion (fig. 2B). Given that the frequency of miniature synaptic events correlates with presynaptic glutamate release, these findings indicate that AM404 increases presynaptic glutamate release in SG neurons.

AM404 Increases the Frequency of mEPSCs Sensitive to Presynaptic TRPV1, but Not CB1, Receptors on SG Neurons of the Dorsal Horn

We next investigated the mechanism underlying the AM404-induced increase in mEPSC frequencies. Previous studies have reported that AM404 acts on TRPV1 or CB1 receptors in the brain; therefore, we investigated whether AM404 acts on presynaptic TRPV1 and/or CB1 receptors in SG neurons. The AM404-induced increase in mEPSC frequencies was not observed in the presence of the TRPV1 receptor antagonist capsazepine (10 μM) (control, 10.1 ± 5.6 Hz; AM404, 9.7 ± 6.2 Hz; $93.3 \pm 10.3\%$ of control; range, 83.3 to 114.5%; n = 7; $P = 0.92$, paired Student's t test; fig. 2C), although the effect was still observed in the presence of the CB1 receptor antagonist AM251 (3 μM) (control, 9.0 ± 3.1 Hz; AM404, 15.9 ± 6.9 Hz; $167.3 \pm 16.4\%$ of control; range, 146.9 to 199.4%; n = 7, paired Student's t test; $P = 0.033$; fig. 2D). These results suggest that AM404 increases mEPSC frequencies by acting on presynaptic TRPV1, but not CB1, receptors on SG neurons of the dorsal horn.

AM404 Reduces Glutamatergic Evoked EPSC Amplitudes *via* TRPV1 Receptors Expressed on C-fibers

Several studies have reported that primary afferent Aδ- and C-fibers terminate in the SG.[30,31] TRPV1 is especially highly expressed at the presynaptic terminals of C, but not Aδ, afferents.[16–19] We therefore examined the effects of AM404 on primary afferent-evoked EPSCs by stimulating the dorsal root in naïve rats.

Although bath-applied AM404 (30 μM, 2 min) did not change the amplitudes of monosynaptic EPSCs evoked by Aδ-fiber stimulation (control, 458.8 ± 206.7 pA; AM404, 435.2 ± 178.2 pA; $96.2 \pm 5.7\%$ of control; range, 87.2 to 104.2%; n = 7; $P = 0.82$, paired Student's t test; fig. 3A), it decreased the amplitudes of EPSCs evoked by C-fiber stimulation (control, 462.5 ± 197.5 pA; AM404, 272.5 ± 134.5 pA; $58.3 \pm 10.3\%$ of control; range, 45.8 to 79.8%; n = 10; $P = 0.022$, paired Student's t test; fig. 3A). The decreased amplitudes of EPSCs evoked by C-fiber stimulation gradually returned to baseline after stoppage of perfusion (fig. 3A). This AM404-induced decrease in the amplitudes of EPSCs evoked by C-fiber stimulation was not observed in the presence of the TRPV1 receptor antagonist, capsazepine (10 μM) (control, 345.4 ± 196.4 pA; AM404, 333.4 ± 187.9 pA; $97.6 \pm 8.8\%$ of control; range, 90.0 to 116.1%; n = 7; $P = 0.91$, paired Student's t test; fig. 3B), although the effect was observed in the presence of the CB1 receptor antagonist AM251 (3 μM) (control, 473.5 ± 228.6 pA; AM404, 256.3 ± 97.8 pA; $58.2 \pm 14.7\%$ of control; range, 34.1 to 76.4%; n = 7; $P = 0.039$, paired Student's t test; fig. 3C). These results suggest that AM404 acts *via* TRPV1 receptors

expressed on C-fibers to reduce glutamate-evoked EPSCs in SG neurons of the dorsal horn.

Acetaminophen and AM404 Have Stronger Analgesic Effects in an Inflammatory Pain Model than in Naïve Rats

Our results indicate that the acetaminophen-derived metabolite AM404 induces analgesia in naïve rats *via* TRPV1

receptors expressed on C-fibers of the spinal cord dorsal horn. However, acetaminophen is clinically used to treat patients with pain. Therefore, we replicated our experiments in an inflammatory pain model, which was achieved by administering CFA to the hind paws of rats. One rat was excluded because its thresholds for paw withdrawal from von Frey filaments after the CFA injection were outside the
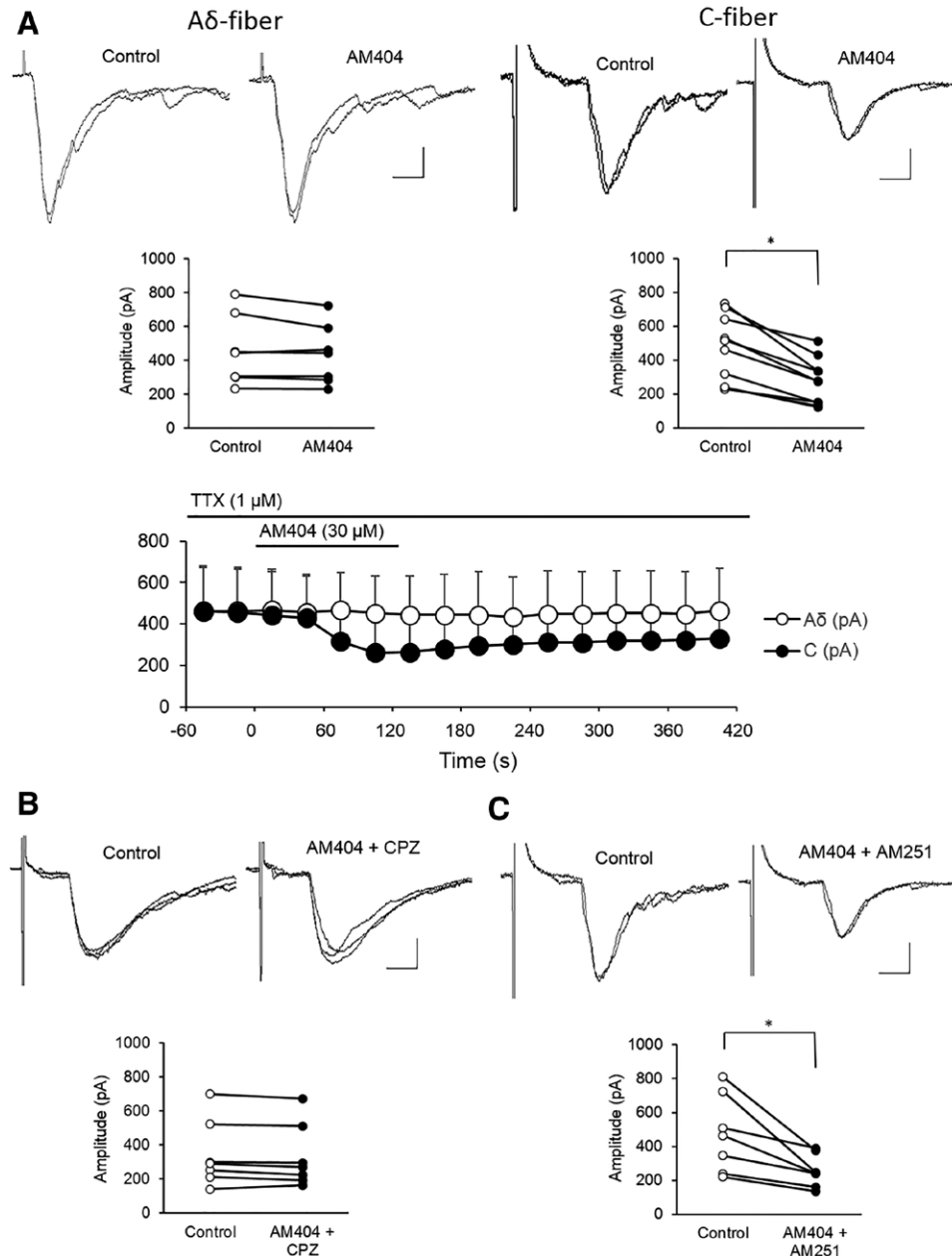

Fig. 3. *N*-acylphenolamine (AM404) reduces glutamate-evoked excitatory postsynaptic currents (EPSCs) *via* transient receptor potential vanilloid 1 receptors expressed on C-fibers. (*A*) AM404 (30 μM, 2 min) did not change the amplitude of monosynaptic EPSCs evoked by Aδ-fiber stimulation (n = 7). In contrast, AM404 decreased the amplitude of EPSCs evoked by C-fiber stimulation (n = 10; *P < 0.05, paired Student's *t* test). (*B*) In the presence of transient receptor potential vanilloid 1 receptor antagonist capsazepine (CPZ; 10 μM), the AM404-induced decrease in amplitude of EPSCs evoked by C-fiber stimulation was not observed (n = 7). (*C*) In the presence of cannabinoid 1 receptor antagonist AM251 (3 μM), the AM404-induced decrease in amplitude of EPSCs evoked by C-fiber stimulation was statistically significant (n = 7; *P < 0.05, paired Student's *t* test). The *horizontal scale bar* represents 50 ms, and the *vertical bar* represents 100 pA for all recordings. The data are given as means ± SDs. TTX = tetrodotoxin.
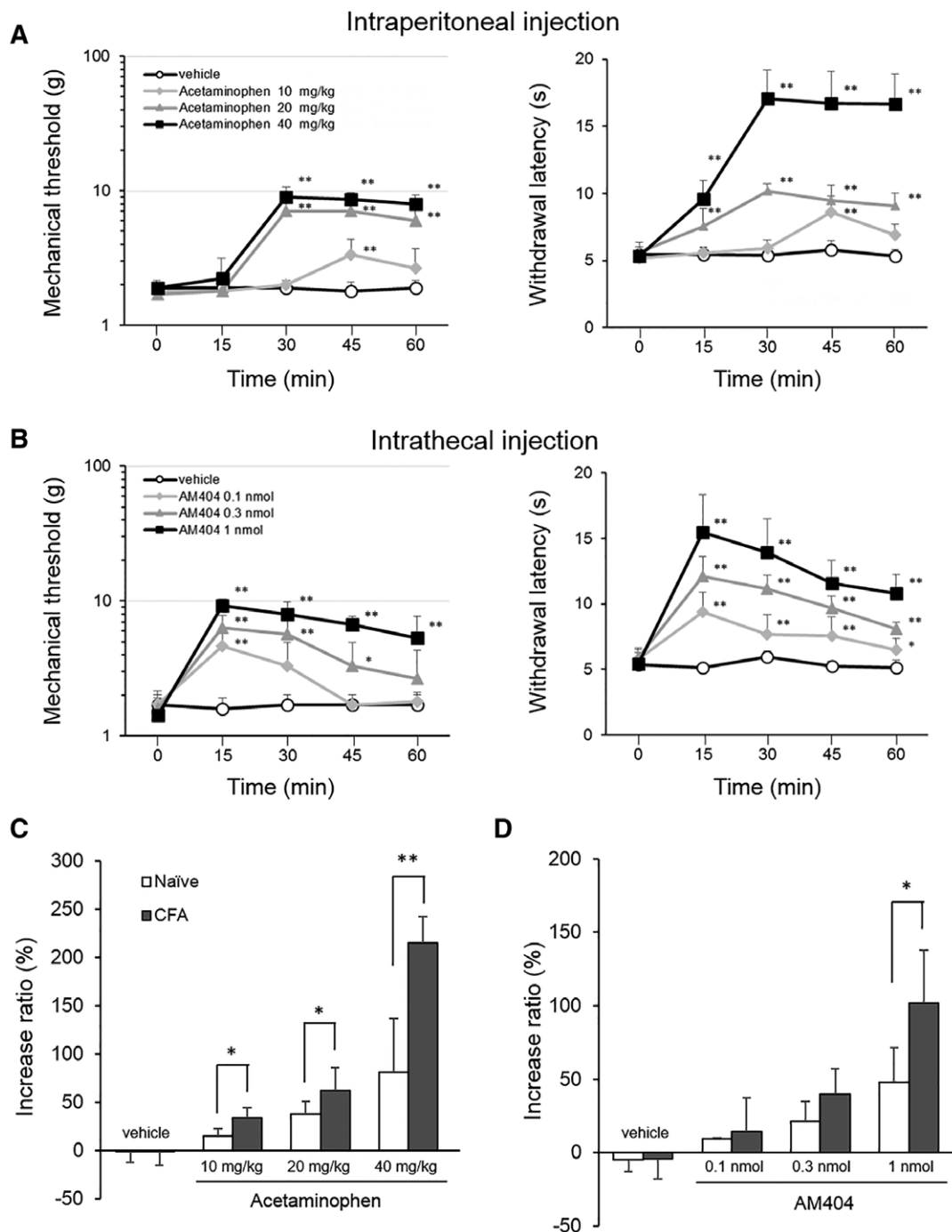
Fig. 4. Acetaminophen and *N*-acylphenolamine (AM404) induce stronger analgesia in an inflammatory pain model than in naïve rats. (*A*) Mechanical thresholds to von Frey stimulation were significantly increased, and withdrawal latencies to radiant heat stimulation were significantly and concentration-dependently prolonged by acetaminophen in inflammatory pain model rats. Acetaminophen was injected intraperitoneally at doses of 10, 20, and 40 mg/kg (n = 6; **$P < 0.01$, two-way repeated-measures ANOVA with time and drug dose as variables followed by Bonferroni *post hoc* comparisons). (*B*) Mechanical thresholds to von Frey stimulation were significantly and concentration-dependently increased, and withdrawal latencies to radiant heat stimulation were prolonged by AM404 in inflammatory pain model rats. AM404 was injected intrathecally at doses of 0.1, 0.3, and 1 nmol (n = 6; *$P < 0.05$, **$P < 0.01$, two-way repeated-measures ANOVA with time and drug dose as variables followed by Bonferroni *post hoc* comparisons). (*C*) Withdrawal latency ratios increased at 60 min after intraperitoneal injection of acetaminophen; acetaminophen induced more analgesia in the inflammatory pain model than in naïve rats (n = 6; 10 and 20 mg/kg, *$P < 0.05$; 40 mg/kg, **$P < 0.01$, paired Student's *t* test). (*D*) The increased withdrawal latency ratios at 60 min after intrathecal injection of AM404 indicate that AM404 induced stronger analgesia in inflammatory pain model rats than in naïve rats (n = 6; 1 nmol, *$P < 0.05$, paired Student's *t* test). The data are given as means ± SDs. CFA = complete Freund's adjuvant.

range of 1.4 to 2.0 g, which indicated that the animal was not appropriate for use as an inflammatory pain model.

We first demonstrated that intraperitoneal injection of CFA resulted in a significant concentration-dependent increase in the mechanical threshold to von Frey stimulation and prolonged withdrawal latencies to radiant heat stimulation (n = 6; $P < 0.05$, two-way repeated-measures ANOVA followed by Bonferroni *post hoc* comparisons; fig. 4A). The maximal effect was observed 30 min after administration and lasted for more than 60 min (fig. 4A). Coadministration of acetaminophen with capsazepine suppressed these increased mechanical thresholds at 30 min after administration when compared to single administration of acetaminophen (20 mg/kg acetaminophen, 7.0 ± 1.1 g; 20 mg/kg acetaminophen with 20 mg/kg capsazepine, 3.7 ± 0.8 g; n = 6; $P < 0.01$). Coadministration of acetaminophen with capsazepine also suppressed these prolonged withdrawal latencies 30 min after administration when compared to single administration of acetaminophen (20 mg/kg acetaminophen, 10.2 ± 0.5 s; 20 mg/kg acetaminophen with 20 mg/kg capsazepine, 6.7 ± 0.7 s; n = 6; $P < 0.01$). Mechanical thresholds to von Frey stimulation and withdrawal latencies to radiant heat were significantly and concentration-dependently increased by intrathecal injection of AM404 (n = 6; $P < 0.05$, two-way repeated-measures ANOVA followed by Bonferroni *post hoc* comparisons; fig. 4B). However, the maximal effect was observed 15 min after the injection and lasted for more than 60 min (fig. 4B). Coadministration of AM404 with capsazepine suppressed these increased mechanical thresholds 15 min after administration when compared to single administration of AM404 (0.3 nmol AM404, 6.3 ± 1.5 g; 0.3 nmol AM404 with 0.3 nmol capsazepine, 3.3 ± 1.0 g; n = 6; $P < 0.01$). Coadministration of AM404 with capsazepine also suppressed these prolonged withdrawal latencies 15 min after administration when compared to single administration of AM404 (0.3 nmol AM404, 12.1 ± 1.5 s; 0.3 nmol AM404 with 0.3 nmol capsazepine, 7.4 ± 0.9 s; n = 6; $P < 0.01$).

Comparison of the withdrawal latency ratio 60 min after intraperitoneal acetaminophen between naïve rats and inflammatory pain model rats indicated that acetaminophen induces greater analgesia in the latter group (10 or 20 mg/kg, $P < 0.05$; 40 mg/kg, $P < 0.01$; n = 6; fig. 4C). Similarly, comparison of the withdrawal latency ratio 60 min after intrathecal injection of AM404 indicated that AM404 induces greater analgesia in inflammatory pain model rats than in naïve rats (1 nmol; $P < 0.05$; n = 6; fig. 4D).

We also examined whether systemic intravenous administration of acetaminophen decreases peripheral pinch stimulus-induced responses in inflammatory pain model rats *via* spinal mechanisms. We found that the area under the curve induced by peripheral pinch stimuli was significantly reduced 10 min after intravenous acetaminophen (20 mg/kg) when compared to the control condition (inhibition ratio, 71.3 ± 17.7% of control; n = 10; $P < 0.01$, paired

Student's *t* test; fig. 5A). The maximal inhibitory effect was observed 10 min after intravenous acetaminophen and endured for more than 60 min (fig. 5A). The inhibition ratios of responses 10 min after intravenous acetaminophen were larger in the inflammatory pain model rats than in the naïve rats (naïve, 53.1 ± 20.7%; inflammatory pain model, 71.3 ± 17.7%; n = 10; $P = 0.048$; fig. 5B).

We examined whether direct application of acetaminophen to the spinal cord affects excitatory synaptic transmission in inflammatory pain model rats. Similar to the results obtained in naïve rats, bath-applied acetaminophen (30 μM, 2 min) did not affect the mean mEPSC amplitude (control, 13.1 ± 2.7 pA; acetaminophen, 13.2 ± 3.1 pA; 100.4 ± 6.9% of control; range, 90.1 to 110.0%; n = 7; $P = 0.96$, paired Student's *t* test; fig. 6A) or frequency (control, 13.0 ± 4.5 Hz; acetaminophen, 13.4 ± 4.8 Hz; 102.6 ± 6.4% of control; range, 93.8 to 110.7%; n = 7; $P = 0.89$, paired Student's *t* test; fig. 6A). Acetaminophen had no effect on the cumulative distribution of the mEPSC amplitudes or interevent intervals over the recording period (Kolmogorov–Smirnov test, $P > 0.05$; fig. 6A). However, bath-applied AM404 (30 μM, 2 min) significantly increased the mean mEPSC frequency from 13.9 ± 5.1 Hz in the control group to 21.5 ± 6.7 Hz in the AM404 group (159.0 ± 24.3% of control; range, 134.2 to 185.0%; n = 7; $P = 0.032$, paired Student's *t* test; fig. 6B), although it did not significantly affect mEPSC amplitudes (control, 13.6 ± 5.2 pA; AM404, 14.2 ± 4.6 pA; 109.3 ± 30.1% of control; range, 85.2 to 154.2%; n = 7; $P = 0.81$, paired Student's *t* test; fig. 6B). Figure 6B (*right*) shows the effects of AM404 on the cumulative distribution of mEPSC amplitudes and interevent intervals. AM404 had no effect on the cumulative distribution of mEPSC amplitudes over the recording period, although it induced a significant leftward shift in the cumulative interevent interval distribution of the mEPSCs when compared to the control conditions (Kolmogorov–Smirnov test, $P < 0.01$; fig. 6B). Similar to the results obtained in naïve rats, this AM404-induced increase in mEPSC frequencies was not observed in the presence of the TRPV1 receptor antagonist capsazepine (10 μM) (control, 15.1 ± 6.6 Hz; AM404, 14.6 ± 6.5 Hz; 97.4 ± 10.2% of control; range, 81.6 to 108.0%; n = 7; $P = 0.89$, paired Student's *t* test; fig. 6C).

Finally, we examined the effects of AM404 on primary afferent terminals by stimulating the dorsal root in inflammatory pain model rats. Although bath-applied AM404 (30 μM, 2 min) did not change the amplitude of monosynaptic EPSCs evoked by Aδ-fiber stimulation (control, 446.7 ± 241.0 pA; AM404, 449.63 ± 238.4 pA; 104.8 ± 14.8% of control; range, 91.8 to 110.4%; n = 7; $P = 0.41$, paired Student's *t* test; fig. 7A), AM404 decreased the amplitudes of EPSCs evoked by C-fiber stimulation (control, 561.2 ± 277.5 pA; AM404, 277.1 ± 175.8 pA; 44.6 ± 17.3% of control; range, 9.1 to 66.9%; n = 10; $P = 0.013$, paired Student's *t* test; fig. 7A). This AM404-induced decrease in the amplitude of EPSCs evoked by C-fiber stimulation was not observed in
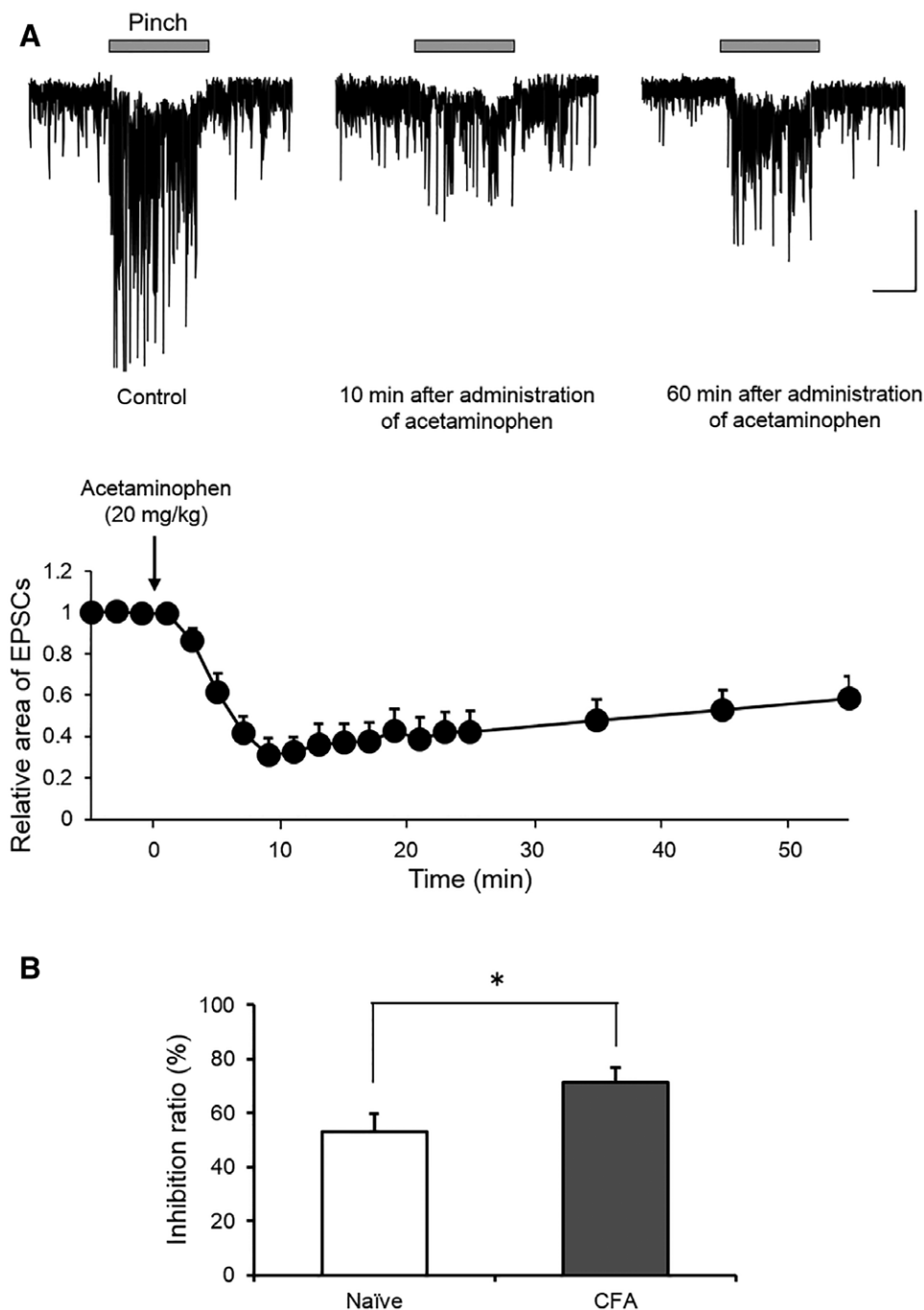
Fig. 5. The area under the curve induced by peripheral pinch stimuli was significantly reduced after intravenous injection of acetaminophen in inflammatory pain model rats compared to naïve rats. (A) Using *in vivo* whole cell patch-clamp recordings from substantia gelatinosa neurons in the spinal cord dorsal horn, the area under the curve surrounded by the baseline and border of excitatory postsynaptic currents (EPSCs) induced by peripheral pinch stimuli in inflammatory pain model rats was significantly reduced 10 min after intravenous injection of acetaminophen (20 mg/kg; n = 10). The *horizontal scale bar* represents 5 s, and the *vertical bar* represents 50 pA. (B) The inhibition ratio of response 10 min after intravenous injection of acetaminophen to inflammatory pain model rats was larger than in naïve rats (20 mg/kg; n = 10; *P < 0.05, paired Student's *t* test). The data are given as means ± SDs. CFA = complete Freund's adjuvant.

the presence of the TRPV1 receptor antagonist capsazepine (10 μM) (control, 394.5 ± 290.8 pA; AM404, 385.4 ± 279.6 pA; 100.3 ± 6.1% of control; range, 95.8 to 113.4%; n = 7; P = 0.95, paired Student's *t* test; fig. 7B). Furthermore, the

inhibition ratio for the amplitude of monosynaptic EPSCs evoked by C-fiber stimulation with AM404 superfusion in inflammatory pain model rats was larger than in naïve rats (naïve rats, 41.7 ± 10.3%; inflammatory pain model rats,
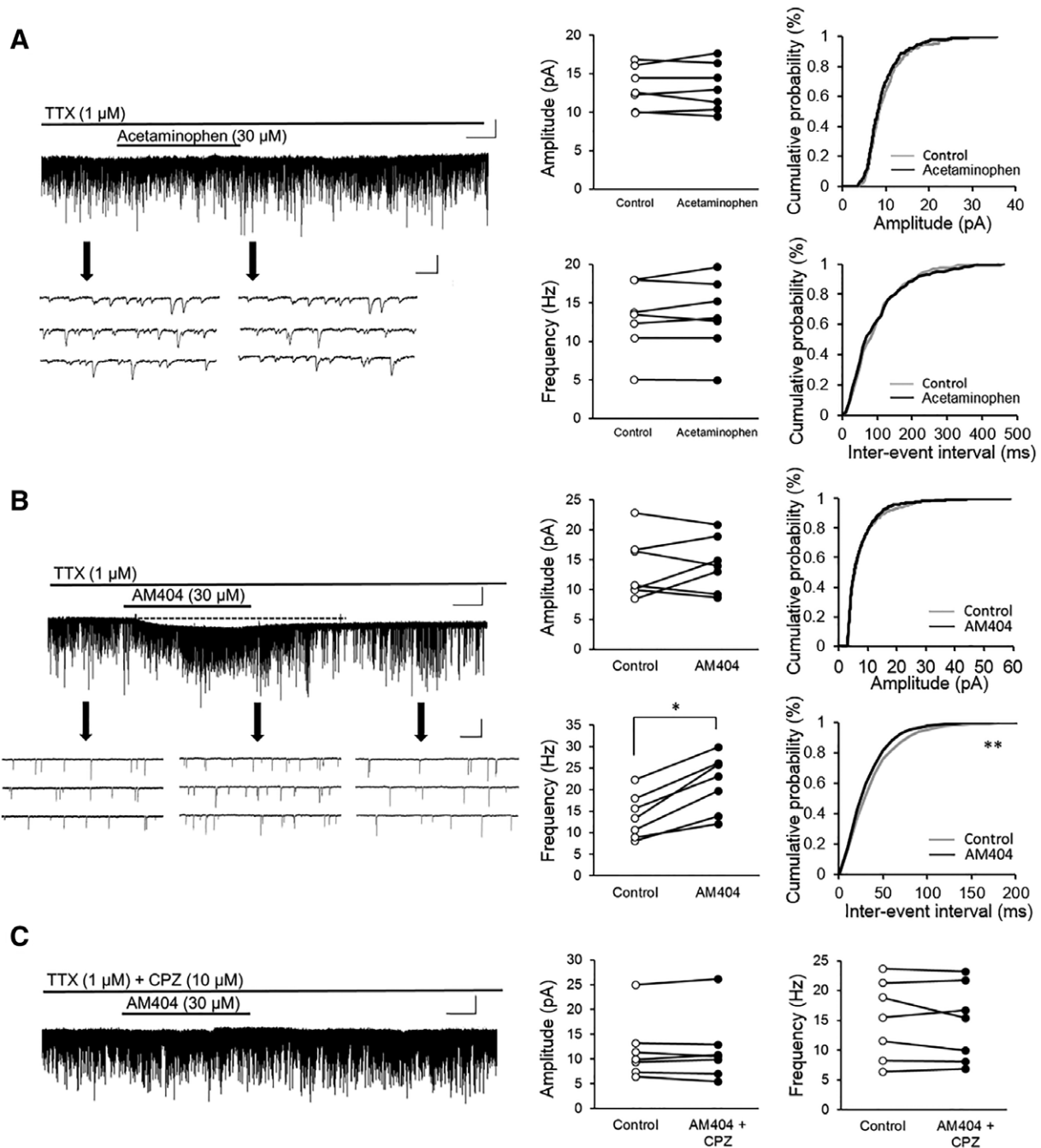
365

Ohashi *et al.*

Fig. 6. Direct application of *N*-acylphenolamine (AM404), but not acetaminophen, to the spinal cord of inflammatory pain model rats increases the frequency of miniature excitatory postsynaptic currents (mEPSCs) sensitive to presynaptic transient receptor potential vanilloid 1 on substantia gelatinosa neurons. (*A*) Direct administration of acetaminophen (30 μM, 2 min) to the spinal cord of inflammatory pain model rats did not change the amplitude or frequency of mEPSCs in substantia gelatinosa neurons (n = 7). The *top horizontal scale bar* represents 30 s, and the *top vertical bar* represents 10 pA. The *bottom horizontal scale bar* represents 500 ms, and the *bottom vertical bar* represents 10 pA. (*B*) Direct administration of AM404 (30 μM, 2 min) to the spinal cord did not change the mean mEPSC amplitude and had no effect on the cumulative distribution of mEPSC amplitudes. In contrast, AM404 significantly increased the mean mEPSC frequency and induced a significant leftward shift in the cumulative interevent interval distribution of mEPSCs (n = 7; *$P < 0.05$, **$P < 0.01$, paired Student's *t* test). The *top horizontal scale bar* represents 30 s, and the *top vertical bar* represents 10 pA. The *bottom horizontal scale bar* represents 500 ms, and the *bottom vertical bar* represents 10 pA. (*C*) In the presence of the transient receptor potential vanilloid 1 receptor antagonist, capsazepine (CPZ; 10 μM), the AM404-induced increase in mEPSC frequency was not observed (n = 7). Miniature EPSCs were measured using *in vitro* whole cell patch-clamp recordings. The *horizontal scale bar* represents 30 s, and the *vertical bar* represents 10 pA. The data are given as means ± SDs. TTX = tetrodotoxin.
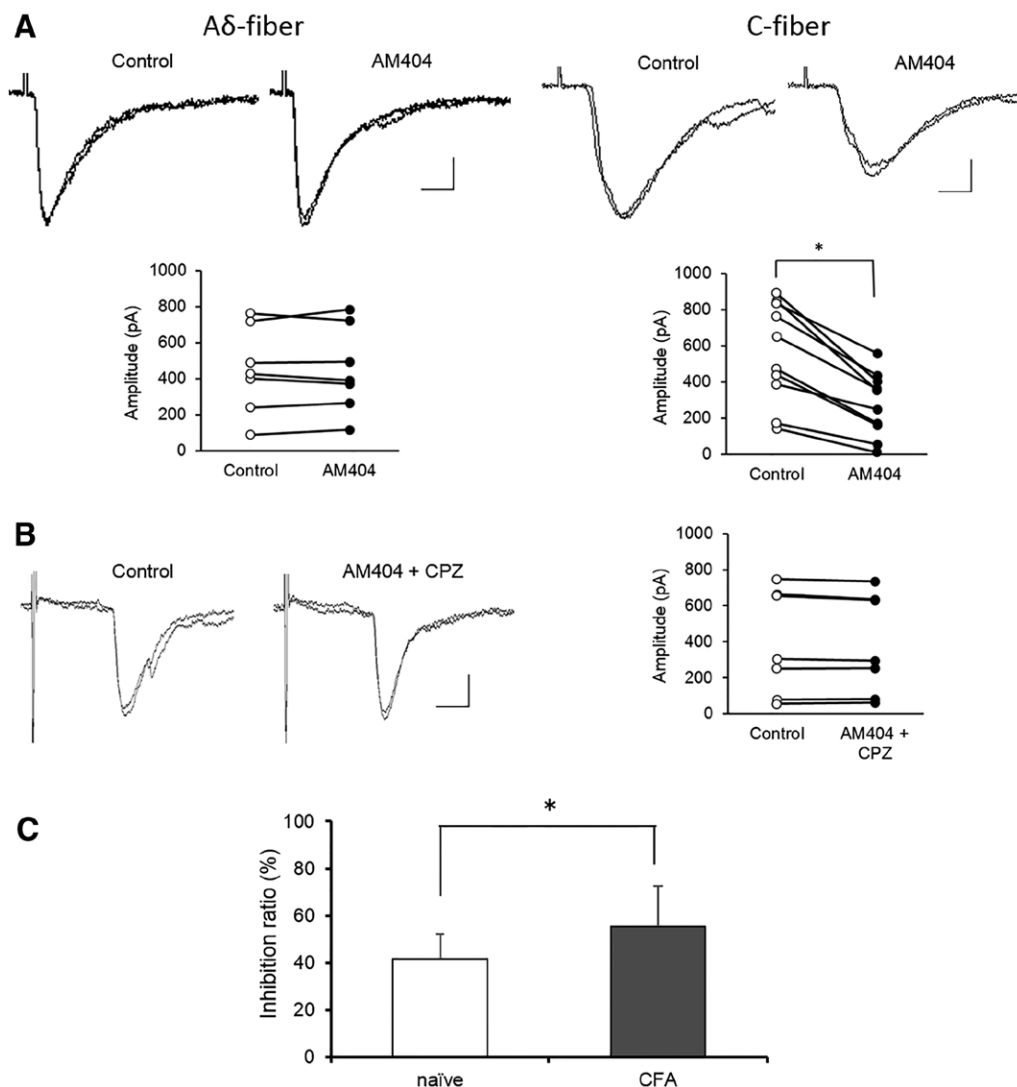
Fig. 7. Glutamate-evoked excitatory postsynaptic current (EPSC) amplitudes *via* transient receptor potential vanilloid 1 receptors expressed on C-fibers were significantly reduced by *N*-acylphenolamine (AM404) in inflammatory pain model rats compared to naïve rats. (*A*) AM404 (30 μM, 2 min) did not change the amplitude of monosynaptic EPSCs evoked by Aδ-fiber stimulation in inflammatory pain model rats (n = 7). In contrast, AM404 decreased the amplitude of EPSCs evoked by C-fiber stimulation (n = 10; *$P < 0.05$, paired Student's *t* test). (*B*) In the presence of the transient receptor potential vanilloid 1 receptor antagonist, capsazepine (CPZ; 10 μM), the AM404-induced decrease in amplitude of EPSCs evoked by C-fiber stimulation was not observed (n = 7). (*C*) The inhibition ratio of the amplitude of monosynaptic EPSCs evoked by C-fiber stimulation with AM404 superfusion in inflammatory pain model rats was larger than in naïve rats (n = 10; *$P < 0.05$, paired Student's *t* test). The *horizontal scale bar* represents 50 ms, and the *vertical bar* represents 100 pA for all recordings. The data are given as means ± SDs. CFA = complete Freund's adjuvant.

55.4 ± 17.3%; n = 10; $P = 0.046$; fig. 7C). These results suggest that AM404 also acts *via* TRPV1 receptors expressed on C-fibers of the spinal cord dorsal horn in inflammatory pain model rats. Furthermore, AM404 induces a greater degree of analgesia in inflammatory pain model rats than in naïve rats.

Discussion

The primary aims of our study were to investigate whether acetaminophen induces analgesia *via* spinal cord dorsal horn neurons and to determine the underlying mechanisms of this process

using behavioral tests and whole cell patch-clamp recordings. Although NAPQI and *p*-BQ also produce TRPA1-dependent analgesia, AM404 is widely known to be the most important metabolite for acetaminophen-induced analgesia. Furthermore, AM404 acts as an anandamide analog, although it was recently reported that AM404 also acts at TRPV1 and CB1 receptors in the midbrain and medulla,[7–9] which are colocalized mediators of pain modulation.[10–12] This is the first report that the acetaminophen metabolite AM404 induces analgesia *via* direct action on TRPV1 receptors on C-fibers in the spinal cord dorsal horn.

AM404, but Not Acetaminophen, Induces Analgesia in the Spinal Cord Dorsal Horn

Intraperitoneal or intrathecal injections of acetaminophen induced analgesia to noxious thermal stimulation. Systemic administration of acetaminophen suppresses spinal responses induced by peripheral pinch stimuli using *in vivo* whole cell patch-clamp recordings. However, *in vitro* whole cell patch-clamp recordings revealed that direct application of acetaminophen to the spinal cord does not affect excitatory synaptic transmission, whereas acetaminophen-derived metabolite AM404 does. Therefore, AM404, but not acetaminophen, induces analgesia *via* direct action on spinal dorsal horn neurons. We believe that this phenomenon is due to the metabolic processing of acetaminophen. Acetaminophen has very high oral bioavailability of 88%, has low affinity for plasma proteins,[32,33] and metabolizes to *p*-aminophenol, which easily crosses the blood–brain barrier and converts to AM404. Only 3% of the acetaminophen is excreted unchanged in the urine.[26,34–36] We therefore believe that systemic administration of acetaminophen acted on the spinal response because acetaminophen was metabolized to AM404 and acted on the spinal cord directly. In contrast, direct application of acetaminophen to spinal slices had no effects because acetaminophen was not metabolized to AM404. Indeed, Zygmunt and colleagues[2,5,6] have also demonstrated that the antinociceptive effects of acetaminophen are dependent of the effects of AM404 and that neither acetaminophen nor *p*-aminophenol interact with TRPV1.[37] It may also be the case that the enzymatic pathways required for acetaminophen metabolism in the slice are significantly impaired such that the effects of acetaminophen *in vitro* on mEPSC frequency are lost. Indeed, a previous study in mice lacking the gene coding for FAAH, which is necessary for conversion of acetaminophen to AM404, reported that FAAH may represent a target for the treatment of pain.[2,38] The mechanisms discussed above may thus explain our observations.

AM404 Acts *via* TRPV1, but Not CB1, Receptors Expressed on C-fibers in the Spinal Cord Dorsal Horn

Several studies have reported that AM404 induces analgesia in the brain *via* activation of TRPV1 or CB1 receptors.[8,9,39] However, we found that AM404 acts on TRPV1, but not CB1, receptors on spinal dorsal horn neurons. Based on previous studies,[25,40] we used male rats, although there is no evidence that these receptors are hormonally regulated. We therefore propose two explanations for the differences between our results and previous reports.

First, the concentration of AM404 was different. Previous research indicates that the response to AM404 (30 µM) is mediated by TRPV1, but not CB1, receptors on medullary neurons.[7] However, some previous studies have reported that CB1 receptors mediate the analgesic effects of AM404 when higher concentrations than those in this study (10 µg/rat, intracerebroventricular injection) were used.[41] Szallasi

and Di Marzo[42] compared the affinities of AM404 for brain TRPV1 and CB1 receptors and reported that the concentration of AM404 required to activate TRPV1 receptors is much lower than that required for CB1 receptors. Therefore, it is possible that the clinical concentration (30 µM) of AM404 used in the present study was insufficient to activate CB1 receptors in dorsal horn neurons. However, the clinically recommended dosage of acetaminophen is 20 mg/kg, which is equivalent to the present study; higher doses result in severe complications such as potentially fatal liver dysfunction. Therefore, we did not conduct additional experiments with higher concentrations of AM404. A second potential reason is the difference in AM404 distribution between tissues. The distribution of AM404 in the brain, as compared to the spinal cord, has previously been widely demonstrated with deuterium-labeled AM404.[2] These factors may thus explain the absence of an effect of AM404 on CB1 receptors.

In our study, C-fiber-evoked EPSCs were inhibited by AM404 in all neurons examined, and the same phenomenon was observed with capsaicin.[16] However, it is known that not all C-fibers express TRPV1 receptors. Several studies reported that some SG neurons (29%) are sensitive to both TRPV1 and TRPA1 receptors, and acetaminophen metabolites NAPQI and *p*-BQ produce TRPA1-dependent analgesia. Therefore, there is a possibility that C-fiber-evoked EPSCs are inhibited not only by TRPV1 receptor agonists but also by other receptor agonists, such as those acting on TRPA1 receptors. It is also known that TRPV1 receptors are not expressed in isolectin B4-positive/calcitonin gene-related peptide-negative neurons but are expressed in MAS-related G-protein-coupled receptor D-positive neurons of C-fibers, which terminate in lamina II (close to lamina III). Although we performed *in vitro* patch-clamp recordings within lamina II, as described in Materials and Methods, we may have recorded from neurons in lamina II that were close to lamina III. In this case, it is possible that AM404 has effects in all neurons, similar to capsaicin.

Analgesic Mechanism of AM404 in the Spinal Cord Dorsal Horn

AM404 has two contradictory effects on glutamatergic transmission in the SG *via* TRPV1 receptor activation. It increases mEPSC frequency and decreases the amplitudes of EPSCs evoked by C-fiber stimulation. Similar effects are observed in SG neurons with electrophysiologic experiments after capsaicin application.[16,43] It is known that capsaicin induces nociceptive responses but also has anti-nociceptive effects in the spinal cord dorsal horn.[11,44,45] Therefore, the analgesic mechanism of AM404 may be similar to that of capsaicin.

The analgesic mechanism of AM404 is attributed to the conduction block of C-fibers. AM404 activates TRPV1 receptors expressed on axons and nerve terminals of C-fibers and leads to depolarization.[46] This depolarizing action facilitates glutamate release from nerve terminals,

which is observed as increased mEPSC frequency in figure 2B. This depolarization also induces a conduction block of C-fibers *via* inactivation of voltage-gated Na⁺ channels, which is observed as decreased amplitude of C-fiber-evoked EPSCs in figure 3A. However, the increase in mEPSC frequency slightly precedes the decrease in the amplitude of C-fiber-evoked EPSCs in our study. Furthermore, our behavior studies suggest that acetaminophen and AM404 induce analgesia against the von Frey and radiant heat tests. Therefore, we believe that conduction block of C-fibers may be one of the mechanisms underlying the analgesic effects of AM404 (fig. 8).[16,40] AM404 may also shunt currents, as some lipids or cannabinoids cause Ca²⁺ influx *via* TRP channels, which then leads to shunting and produces the analgesic effects. However, investigating this mechanism is beyond the scope of our present study. We thus could not confirm these mechanisms. Finally, the analgesic mechanism of AM404 may also be the desensitization of TRPV1 receptors of C-fibers. Many reports indicate that capsaicin application causes persistent functional desensitization of polymodal primary nociceptors.[47,48] These mechanisms contribute to the analgesic effects of AM404 in the spinal cord dorsal horn.

Acetaminophen Induces Analgesia in an Inflammatory Pain Model

The mechanical threshold for paw withdrawal in naïve rats was not affected by intraperitoneal acetaminophen or intrathecal AM404, although these treatments had analgesic efficacy in CFA-treated rats. This finding may be due to a limitation of the von Frey test, as the mechanical threshold in naïve rats is already high at the start of the experiment, which would render the effects of acetaminophen or AM404

undetectable. Indeed, our behavioral studies suggest that acetaminophen and AM404 induce analgesia to thermal stimulation, and both *in vivo* and *in vitro* whole cell patch-clamp recordings have shown that these agents affect excitatory synaptic transmission in both naïve and inflammatory pain model rats.

It was previously believed that acetaminophen is not an appropriate treatment in inflammatory pain conditions because it is a very weak inhibitor of cyclooxygenase and does not inhibit neutrophil activation.[49] Acetaminophen also has minimal effects on a cyclooxygenase isoenzyme, which is induced in activated inflammatory cells.[50] However, it does reduce edema induced by intraplantar injection of carrageenan[51] and improves allodynia and ipsilateral paw volume with CFA-induced inflammation.[52] Therefore, our findings are consistent with previous research. Our study also suggested that acetaminophen and AM404 induce significantly greater analgesia in inflammatory model rats than in naïve rats. TRPV1 receptor expression in DRG neurons is upregulated during inflammation. Furthermore, 2 days after CFA, there is an increased proportion of TRPV1-protein-positive neurons in DRG and unmyelinated axons of the digital nerves.[53] Therefore, increased TRPV1 activity in inflammatory pain model rats is expected to lead to stronger analgesic effects after acetaminophen administration.

Conclusions

The acetaminophen metabolite AM404 induces analgesia *via* two opposing actions on glutamatergic synaptic transmission in the spinal cord dorsal horn: facilitation of spontaneous transmission and blockade of C-fiber-evoked transmission *via* direct activation of TRPV1 receptors. Our data further support a mechanism that acetaminophen also induces analgesia in inflammatory pain conditions. These findings are applicable to clinical pain management with acetaminophen.

Fig. 8. Analgesic mechanism of *N*-acylphenolamine (AM404) in the spinal cord dorsal horn. AM404 acts on transient receptor potential vanilloid 1 (TRPV1) receptors to depolarize the presynaptic terminals or axons of C-fibers and thus facilitates glutamate release, while inactivated axonal voltage-gated Na⁺ channels induce conduction blockade of C-fibers. SG = substantia gelatinosa.

Research Support

Competing Interests

The authors declare no competing interests.

Correspondence

Address correspondence Dr. Kohno: Division of Anesthesiology, Niigata University Graduate School of Medical and Dental Sciences, 1-757 Asahimachi Dori, Chuo-Ku, Niigata City 951-8510, Japan. kohno-t@umin.net. Information on purchasing reprints may be found at www.anesthesiology. org or on the masthead page at the beginning of this issue. ANESTHESIOLOGY's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.
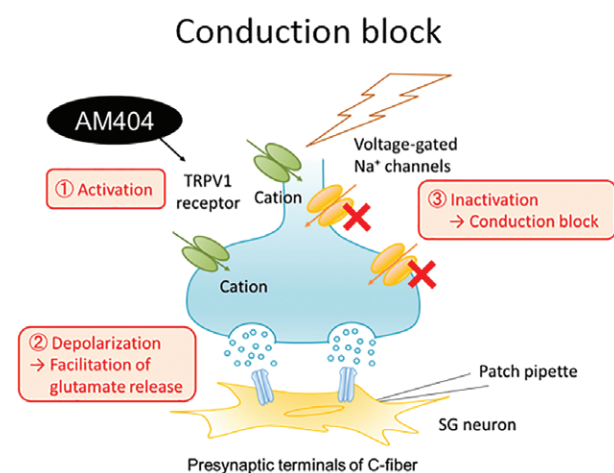
References

1. Bannwarth B, Netter P, Lapicque F, Gillet P, Péré P, Boccard E, Royer RJ, Gaucher A: Plasma and cerebrospinal fluid concentrations of paracetamol after a single intravenous dose of propacetamol. Br J Clin Pharmacol 1992; 34:79–81

2. Hogestatt ED, Jonsson BA, Ermund A, Andersson DA, Bjork H, Alexander JP, Cravatt BF, Basbaum AI, Zygmunt PM: Conversion of acetaminophen to the bioactive *N*-acylphenolamine AM404 via fatty acid amide hydrolase-dependent arachidonic acid conjugation in the nervous system. J Biol Chem 2005; 280:31405–12

3. Andersson DA, Gentry C, Alenmyr L, Killander D, Lewis SE, Andersson A, Bucher B, Galzi JL, Sterner O, Bevan S, Högestätt ED, Zygmunt PM: TRPA1 mediates spinal antinociception induced by acetaminophen and the cannabinoid Δ^9-tetrahydrocannabiorcol. Nat Commun 2011; 2:551

4. Gentry C, Andersson DA, Bevan S: TRPA1 mediates the hypothermic action of acetaminophen. Sci Rep 2015; 5:12771

5. Zygmunt PM, Chuang H, Movahed P, Julius D, Högestätt ED: The anandamide transport inhibitor AM404 activates vanilloid receptors. Eur J Pharmacol 2000; 396:39–42

6. Barrière DA, Mallet C, Blomgren A, Simonsen C, Daulhac L, Libert F, Chapuy E, Etienne M, Högestätt ED, Zygmunt PM, Eschalier A: Fatty acid amide hydrolase-dependent generation of antinociceptive drug metabolites acting on TRPV1 in the brain. PLoS One 2013; 8:e70690

7. Jennings EA, Vaughan CW, Roberts LA, Christie MJ: The actions of anandamide on rat superficial medullary dorsal horn neurons *in vitro*. J Physiol 2003; 548:121–9

8. Mallet C, Barriere DA, Ermund A, Jonsson BA, Eschalier A, Zygmunt PM, Hogestatt ED: TRPV1 in brain is involved in acetaminophen-induced antinociception. PLoS One 2010; 5:e12748

9. Roberts LA, Christie MJ, Connor M: Anandamide is a partial agonist at native vanilloid receptors in acutely isolated mouse trigeminal sensory neurons. Br J Pharmacol 2002; 137:421–8

10. De Petrocellis L, Bisogno T, Davis JB, Pertwee RG, Di Marzo V: Overlap between the ligand recognition properties of the anandamide transporter and the VR1 vanilloid receptor: Inhibitors of anandamide uptake with negligible capsaicin-like activity. FEBS Lett 2000; 483:52–6

11. Maione S, Bisogno T, de Novellis V, Palazzo E, Cristino L, Valenti M, Petrosino S, Guglielmotti V, Rossi F, Di Marzo V: Elevation of endocannabinoid levels in the ventrolateral periaqueductal grey through inhibition of fatty acid amide hydrolase affects descending nociceptive pathways via both cannabinoid receptor type 1 and transient receptor potential vanilloid type-1 receptors. J Pharmacol Exp Ther 2006; 316:969–82

12. Palazzo E, de Novellis V, Marabese I, Cuomo D, Rossi F, Berrino L, Rossi F, Maione S: Interaction between vanilloid and glutamate receptors in the central modulation of nociception. Eur J Pharmacol 2002; 439:69–75

13. Crawley B, Saito O, Malkmus S, Fitzsimmons B, Hua XY, Yaksh TL: Acetaminophen prevents hyperalgesia in central pain cascade. Neurosci Lett 2008; 442:50–3

14. Rodella LF, Borsani E, Rezzani R, Ricci F, Buffoli B, Bianchi R: AM404, an inhibitor of anandamide reuptake decreases Fos-immunoreactivity in the spinal cord of neuropathic rats after non-noxious stimulation. Eur J Pharmacol 2005; 508:139–46

15. Kerckhove N, Mallet C, François A, Boudes M, Chemin J, Voets T, Bourinet E, Alloui A, Eschalier A: $Ca_v3.2$ calcium channels: The key protagonist in the supraspinal effect of paracetamol. Pain 2014; 155:764–72

16. Yang K, Kumamoto E, Furue H, Li YQ, Yoshimura M: Action of capsaicin on dorsal root-evoked synaptic transmission to substantia gelatinosa neurons in adult rat spinal cord slices. Brain Res 1999; 830:268–73

17. Usoskin D, Furlan A, Islam S, Abdo H, Lönnerberg P, Lou D, Hjerling-Leffler J, Haeggström J, Kharchenko O, Kharchenko PV, Linnarsson S, Ernfors P: Unbiased classification of sensory neuron types by large-scale single-cell RNA sequencing. Nat Neurosci 2015; 18:145–53

18. Andresen MC, Hofmann ME, Fawley JA: The unsilent majority-TRPV1 drives "spontaneous" transmission of unmyelinated primary afferents within cardiorespiratory NTS. Am J Physiol Regul Integr Comp Physiol 2012; 303:R1207–16

19. Radu BM, Iancu AD, Dumitrescu DI, Flonta ML, Radu M: TRPV1 properties in thoracic dorsal root ganglia neurons are modulated by intraperitoneal capsaicin administration in the late phase of type-1 autoimmune diabetes. Cell Mol Neurobiol 2013; 33:187–96

20. Pogatzki EM, Zahn PK, Brennan TJ: Lumbar catheterization of the subarachnoid space with a 32-gauge polyurethane catheter in the rat. Eur J Pain 2000; 4:111–3

21. Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL: Quantitative assessment of tactile allodynia in the rat paw. J Neurosci Methods 1994; 53:55–63

22. Hargreaves K, Dubner R, Brown F, Flores C, Joris J: A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. Pain 1988; 32:77–88

23. Sonohata M, Furue H, Katafuchi T, Yasaka T, Doi A, Kumamoto E, Yoshimura M: Actions of noradrenaline on substantia gelatinosa neurones in the rat spinal cord revealed by *in vivo* patch recording. J Physiol 2004; 555:515–26

24. Furue H, Narikawa K, Kumamoto E, Yoshimura M: Responsiveness of rat substantia gelatinosa neurones to mechanical but not thermal stimuli revealed by *in vivo* patch-clamp recording. J Physiol 1999; 521 Pt 2:529–35

25. Ohashi N, Sasaki M, Ohashi M, Kamiya Y, Baba H, Kohno T: Tranexamic acid evokes pain by modulating neuronal excitability in the spinal dorsal horn. Sci Rep 2015; 5:13458

26. Rawlins MD, Henderson DB, Hijab AR: Pharmacokinetics of paracetamol (acetaminophen) after intravenous and oral administration. Eur J Clin Pharmacol 1977; 11:283–6

27. Rayburn W, Shukla U, Stetson P, Piehl E: Acetaminophen pharmacokinetics: Comparison between pregnant and nonpregnant women. Am J Obstet Gynecol 1986; 155:1353–6

28. Cheney-Thamm J, Alianello EA, Freed CR, Reite M: *In vivo* electrochemical recording of acetaminophen in non human primate brain. Life Sci 1987; 40:375–9

29. Ochs HR, Greenblatt DJ, Abernethy DR, Arendt RM, Gerloff J, Eichelkraut W, Hahn N: Cerebrospinal fluid uptake and peripheral distribution of centrally acting drugs: Relation to lipid solubility. J Pharm Pharmacol 1985; 37:428–31

30. Kumazawa T, Perl ER: Excitation of marginal and substantia gelatinosa neurons in the primate spinal cord: Indications of their place in dorsal horn functional organization. J Comp Neurol 1978; 177:417–34

31. Yoshimura M, Jessell TM: Primary afferent-evoked synaptic responses and slow potential generation in rat substantia gelatinosa neurons *in vitro*. J Neurophysiol 1989; 62:96–108

32. Duggin GG, Mudge GH: Renal tubular transport of paracetamol and its conjugates in the dog. Br J Pharmacol 1975; 54:359–66

33. Gazzard BG, Ford-Hutchinson AW, Smith MJ, Williams R: The binding of paracetamol to plasma proteins of man and pig. J Pharm Pharmacol 1973; 25:964–7

34. Depré M, van Hecken A, Verbesselt R, Tjandra-Maga TB, Gerin M, de Schepper PJ: Tolerance and pharmacokinetics of propacetamol, a paracetamol formulation for intravenous use. Fundam Clin Pharmacol 1992; 6:259–62

35. Slattery JT, Wilson JM, Kalhorn TF, Nelson SD: Dose-dependent pharmacokinetics of acetaminophen: Evidence of glutathione depletion in humans. Clin Pharmacol Ther 1987; 41:413–8

36. Toussaint K, Yang XC, Zielinski MA, Reigle KL, Sacavage SD, Nagar S, Raffa RB: What do we (not) know about how paracetamol (acetaminophen) works? J Clin Pharm Ther 2010; 35:617–38

37. Janero DR, Makriyannis A: Terpenes and lipids of the endo-cannabinoid and transient-receptor-potential-channel biosignaling systems. ACS Chem Neurosci 2014; 5:1097–106

38. Cravatt BF, Demarest K, Patricelli MP, Bracey MH, Giang DK, Martin BR, Lichtman AH: Supersensitivity to anandamide and enhanced endogenous cannabinoid signaling in mice lacking fatty acid amide hydrolase. Proc Natl Acad Sci USA 2001; 98:9371–6

39. Ottani A, Leone S, Sandrini M, Ferrari A, Bertolini A: The analgesic activity of paracetamol is prevented by the blockade of cannabinoid CB1 receptors. Eur J Pharmacol 2006; 531:280–1

40. Uta D, Furue H, Pickering AE, Rashid MH, Mizuguchi-Takase H, Katafuchi T, Imoto K, Yoshimura M: TRPA1-expressing primary afferents synapse with a morphologically identified subclass of substantia gelatinosa neurons in the adult rat spinal cord. Eur J Neurosci 2010; 31:1960–73

41. Beltramo M, de Fonseca FR, Navarro M, Calignano A, Gorriti MA, Grammatikopoulos G, Sadile AG, Giuffrida A, Piomelli D: Reversal of dopamine D(2) receptor responses by an anandamide transport inhibitor. J Neurosci 2000; 20:3401–7

42. Szallasi A, Di Marzo V: New perspectives on enigmatic vanilloid receptors. Trends Neurosci 2000; 23:491–7

43. Yang K, Kumamoto E, Furue H, Yoshimura M: Capsaicin facilitates excitatory but not inhibitory synaptic transmission in substantia gelatinosa of the rat spinal cord. Neurosci Lett 1998; 255:135–8

44. Dray A, Bettaney J, Forster P: Actions of capsaicin on peripheral nociceptors of the neonatal rat spinal cord-tail *in vitro*: Dependence of extracellular ions and independence of second messengers. Br J Pharmacol 1990; 101:727–33

45. Holzer P: Capsaicin: Cellular targets, mechanisms of action, and selectivity for thin sensory neurons. Pharmacol Rev 1991; 43:143–201

46. Tominaga M, Caterina MJ, Malmberg AB, Rosen TA, Gilbert H, Skinner K, Raumann BE, Basbaum AI, Julius D: The cloned capsaicin receptor integrates multiple pain-producing stimuli. Neuron 1998; 21:531–43

47. Carpenter SE, Lynn B: Vascular and sensory responses of human skin to mild injury after topical treatment with capsaicin. Br J Pharmacol 1981; 73:755–8

48. Jancsó N, Jancsó-Gábor A, Szolcsányi J: Direct evidence for neurogenic inflammation and its prevention by denervation and by pretreatment with capsaicin. Br J Pharmacol Chemother 1967; 31:138–51

49. Hanel AM, Lands WE: Modification of anti-inflammatory drug effectiveness by ambient lipid peroxides. Biochem Pharmacol 1982; 31:3307–11

50. Meade EA, Smith WL, DeWitt DL: Differential inhibition of prostaglandin endoperoxide synthase (cyclooxygenase) isozymes by aspirin and other non-steroidal anti-inflammatory drugs. J Biol Chem 1993; 268:6610–4

51. Bhattacharya SK, Rao PJ, Das Gupta G: Effect of centrally administered prostaglandin D2 and some prostaglandin synthesis inhibitors on carrageenan-induced paw oedema in rats. J Pharm Pharmacol 1989; 41:569–71

52. Mitchell VA, Greenwood R, Jayamanne A, Vaughan CW: Actions of the endocannabinoid transport inhibitor AM404 in neuropathic and inflammatory pain models. Clin Exp Pharmacol Physiol 2007; 34:1186–90

53. Carlton SM, Coggeshall RE: Peripheral capsaicin receptors increase in the inflamed rat hindpaw: A possible mechanism for peripheral sensitization. Neurosci Lett 2001; 310:53–6