Inhibition by O-desmethyltramadol of glutamatergic excitatory transmission in adult rat spinal substantia gelatinosa neurons

Akiko Koga¹,², Tsugumi Fujita¹, Lian-Hua Piao¹, Terumasa Nakatsuka¹, and Eiichi Kumamoto¹

Abstract
To reveal cellular mechanisms for antinociception produced by clinically used tramadol, we investigated the effect of its metabolite O-desmethyltramadol (M1) on glutamatergic excitatory transmission in spinal dorsal horn lamina II (substantia gelatinosa; SG) neurons. The whole-cell patch-clamp technique was applied at a holding potential of –70 mV to SG neurons of an adult rat spinal cord slice with an attached dorsal root. Under the condition where a postsynaptic action of M1 was inhibited, M1 superfused for 2 min reduced the frequency of spontaneous excitatory postsynaptic current in a manner sensitive to a μ-opioid receptor antagonist CTAP; its amplitude and also a response of SG neurons to bath-applied AMPA were hardly affected. The presynaptic effect of M1 was different from that of noradrenaline or serotonin which was examined in the same neuron. M1 also reduced by almost the same extent the peak amplitudes of monosynaptic primary-afferent Aδ-fiber and C-fiber excitatory postsynaptic currents evoked by stimulating the dorsal root. These actions of M1 persisted for >10 min after its washout. These results indicate that M1 inhibits the quantal release of L-glutamate from nerve terminals by activating μ-opioid but not noradrenaline and serotonin receptors; this inhibition is comparable in extent between monosynaptic primary-afferent Aδ-fiber and C-fiber transmissions. Considering that the SG plays a pivotal role in regulating nociceptive transmission, the present findings could contribute to at least a part of the inhibitory action of tramadol on nociceptive transmission together with its hyperpolarizing effect as reported previously.

Keywords
O-Desmethyltramadol, μ-opioid receptor, glutamatergic synaptic transmission, spinal substantia gelatinosa, patch-clamp, antinociception

Introduction
Tramadol, (1RS; 2RS)-2-[(dimethylamino)methyl]-1-(3-methoxyphenyl)cyclohexanol hydrochloride, is a clinically used drug which acts as an analgesic in the central nervous system.¹ Behavioral studies have demonstrated that the administration of tramadol inhibits nociceptive responses in rats.²⁻⁵ Among cellular mechanisms for this antinociception, there are (1) μ-opioid receptor activation²⁻⁴ and (2) inhibition of the reuptake of noradrenaline and serotonin, neurotransmitters involved in the descending inhibitory pathway to the spinal dorsal horn from brainstem.⁴⁻⁶⁻⁷ The former mechanism has been revealed from the results of a μ-opioid receptor binding of tramadol⁸ and its [³⁵S]GTP-γ-S binding stimulation.⁹ Consistent with the latter mechanism, Kimura et al.³ have demonstrated in in vivo microdialysis experiments that intraperitoneally applied tramadol increases the levels of noradrenaline and serotonin in the rat spinal cord.

¹Department of Physiology, Saga Medical School, Saga, Japan
²Department of Anesthesiology & Critical Care Medicine, Saga Medical School, Saga, Japan

Corresponding Author:
Eiichi Kumamoto, Department of Physiology, Saga Medical School, 5-1-1 Nabeshima, Saga 849-8501, Japan.
Email: kumamoto@cc.saga-u.ac.jp

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The substantia gelatinosa (SG; lamina II) of the spinal dorsal horn plays an important role in regulating nociceptive transmission through primary-afferent Aδ-fiber and C-fiber contained in the dorsal root. Antinociception produced by opioids, noradrenaline, and serotonin has been attributed to their modulatory actions on synaptic transmission in SG neurons. Thus, it is possible that at least a part of the antinociception produced by tramadol is due to a modulation of synaptic transmission in SG neurons.

Tramadol is metabolized to various compounds including O-desmethyltramadol (M1) via N- and O-dimethylation in humans and animals; M1 appears in blood immediately after a single intravenous administration of tramadol in rats. M1 has the highest affinity for the cloned human μ-opioid receptors among the metabolites and is more effective in antinociception than tramadol according to a behavioral study in rats. M1 has an ability to inhibit the reuptake of noradrenaline and serotonin. It is, therefore, possible that the antinociceptive action of tramadol is partly due to a modulatory action of M1 on excitatory transmission in SG neurons through the activation of μ-opioid, noradrenaline, and serotonin receptors. It has not been fully examined yet how M1 affects excitatory transmission in SG neurons. This study examined the effect of M1 on glutamatergic spontaneous excitatory transmission in SG neurons in adult rat spinal cord slices with a focus on an involvement of their receptors. Since primary-afferent Aδ-fiber and C-fiber transfer different types of nociceptive information, we also investigated the effect of M1 on monosynaptic excitatory transmission evoked in SG neurons by stimulating each of the fibers. These experiments were performed by using the whole-cell patch-clamp technique under the condition where a previously reported hyperpolarized effect of M1 was inhibited.

**Materials and methods**

This study was approved by the Animal Care and Use Committee of Saga University and was conducted in accordance with the Guiding Principles for the Care and Use of Animals in the Field of Physiological Science of the Physiological Society of Japan. All efforts were made to minimize animal suffering and the number of animals used.

**Preparation of spinal cord slices**

The methods used for obtaining slice preparations of the adult rat spinal cord were similar to those described elsewhere. Briefly, male rats (7–8 weeks old) were deeply anesthetized with urethane (1.2 g/kg, i.p.), and then a lumbar sacral laminectomy was performed.

The lumbar sacral segment (L1-S3) of the spinal cord with the ventral and dorsal roots attached was removed and placed in ice-cold (1–4°C) Krebs solution preequilibrated with 95% O₂ and 5% CO₂; the rats were then immediately sacrificed by exsanguination. After cutting all ventral and dorsal roots near the root entry zone, except for the L4 or L5 dorsal root on one side, the arachnoid membrane was removed. The spinal cord was mounted on a microslicer (DTK-1000, Dousaka, Kyoto, Japan), and then a 650-μm-thick transverse slice with a dorsal root attached was cut. The slice was placed on a nylon mesh in the recording chamber and was completely submerged and superfused at a drip rate of 15–18 ml/min with Krebs solution equilibrated with 95% O₂ and 5% CO₂ at 36 ± 1°C. Flow of solution was induced by gravity through a polyethylene tube from the reservoir. The composition of Krebs solution used was (in mM): NaCl 117, KCl 3.6, CaCl₂ 2.5, MgCl₂ 1.2, NaH₂PO₄ 1.2, NaHCO₃ 25, and glucose 11 (pH = 7.4).

**Patch-clamp recordings from SG neurons**

Blind whole-cell recordings were made from SG neurons with patch-pipettes fabricated from a thin-walled fiberglass (1.5 mm OD) using a single-stage horizontal puller (P-97/PC; Sutter Instrument, Novato, CA), as reported previously. The patch-pipettes had a tip resistance of 8–15 MΩ when filled with a solution of the following composition (in mM): Cs₂SO₄ 110, CaCl₂ 0.5, MgCl₂ 2, EGTA 5, HEPES 5, Mg-ATP 5, tetraethylammonium (TEA) 5, and guanosine 5'-O-(2-thiodiphosphate) (GDP-β-S) 1 (pH = 7.2); where GDP-β-S and K⁺-channel blockers (Cs⁺ and TEA) were used to inhibit a postsynaptic effect of M1 through the action of G-proteins and to block an activation of K⁺ channels which results from the postsynaptic effect, respectively.

Signals were gained with a patch-clamp amplifier (Axopatch 200B; Axon Instruments, Foster City, CA). Data were low-pass filtered at 5 kHz, digitized at 333 kHz with an A/D converter, stored, and analyzed with a personal computer using pCLAMP data acquisition program (Version 6.0, Axon Instruments). The program (AxoGraph 4.0, Axon Instruments) used to analyze spontaneous excitatory postsynaptic currents (EPSCs) detects spontaneous events if the difference between the baseline and a following current value exceeds a given threshold of 5 pA, and the peak is separated from an adjacent peak by an intervening valley that is deeper than 50% of the adjacent peak. A validity of whether spontaneous EPSCs (sEPSCs) were accurately detected by the program was confirmed by measuring visually individual sEPSCs in a fast time scale on a computer screen in several cases. Orthodromic stimulation of the dorsal root to elicit EPSCs was performed with a suction electrode with a constant current source of...
pulse at a frequency of 0.1 Hz unless otherwise mentioned. The strength of stimuli (duration: 0.1 ms) used was 1.2 times the threshold to elicit EPSCs. The conduction velocities (CVs) of primary-afferent fibers involved in the production of the EPSCs were calculated from the latency of monosynaptic EPSC from a stimulus artifact and the length of dorsal root. Under the voltage-clamp condition, the holding potential \( V_{\text{H}} \) used was \(-70\) mV at which GABA \(_A\) and glycine receptor-mediated synaptic currents were invisible. \(^{12}\) Neurons from which recordings were made were identified as SG neurons under a binocular microscope, where the SG could be easily distinguished as a colorless band located in the superficial dorsal horn.

**Application of drugs**

Drugs were dissolved in Krebs solution and applied by perfusing via a three-way stopcock Krebs solution containing drugs of a known concentration without an alteration in the perfusion rate and temperature; a change in solutions in the recording chamber completed within 20 s. The drugs used in this work were (±)-M1 (M1), (±)-noradrenaline, serotonin creatine sulfate, \( \alpha\)-Phe-Cys-Tyr-\( \alpha\)-Trp-Arg-Thr-Pen-Thr-NH\(_2\) (CTAP; Sigma-Aldrich Chemical Co., St. Louis, MO), \( \alpha\)-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (Tocris Neuramin, Bristol, England). The concentration of M1 used was 1 mM, at which concentration M1 produced a maximal outward current in SG neurons. \(^{18}\) Data are presented as mean \( \pm \) SEM. Statistical significance was determined as \( P < 0.05 \) using either the paired or unpaired Student’s \( t \) test. In all cases, \( n \) refers to the number of neurons studied.

**Results**

Whole-cell patch-clamp recordings were made from a total of 92 SG neurons. Stable recordings could be obtained from neurons in spinal cord slices maintained in vitro for more than 6 h, and recordings were made from single neurons for up to 2 h. All experiments were performed at least 10 min later after the establishment of whole-cell configuration; this time was enough for GDP-\( \beta\)-S and K\(^+\)-channel blockers in patch-pipette solutions to diffuse into SG neurons. M1 (1 mM) hardly changed holding currents at \(-70\) mV.

**Effect of M1 on spontaneous excitatory transmission in SG neurons**

In all SG neurons tested, sEPSCs were downward at \(-70\) mV and were blocked by CNQX (10 \( \mu \)M; \( n = 4 \)), as reported previously, \(^ {13,19} \) indicating the activation of non-\( N\)-methyl-D-aspartate (non-NMDA) receptors. In all SG neurons examined (\( n = 31 \)), the occurrence of the sEPSCs was depressed by M1 (1 mM) superfused for 2 min and this action persisted after washout of M1, as shown in Figure 1(a). When quantitatively estimated in some neurons, sEPSC frequency was 68\% \( (P < 0.05) \) of that before the application of M1 (control; \( 12.4 \pm 0.9 \) Hz; amplitude, \( 20.3 \pm 2.7 \) pA; \( n = 7 \)), which are plotted against time. Each point with vertical bars indicates the mean values and SEM, calculated from sEPSCs measured for 30 s. (c) Current responses induced in a SG neuron by superfusing AMPA (5 \( \mu \)M) in the absence (a) and presence (b) of M1. Holding potential \( V_{\text{H}} = -70\) mV. AMPA: \( \alpha\)-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid.

![Figure 1](image-url)  

**Figure 1.** \( \alpha\)-Desmethyltramadol (M1; 1 mM) presynaptically inhibits glutamatergic spontaneous excitatory transmission in rat substantia gelatinosa (SG) neurons. (a) Chart recording of spontaneous excitatory postsynaptic currents (EPSCs) in the absence and presence of M1. In this and subsequent figures, the duration of drug superfusion is shown by a horizontal bar above the chart recording; four consecutive traces of spontaneous EPSCs (sEPSCs) for a period indicated by a short bar designated as (a) to (d) below the chart recording are shown in an expanded scale in time. (b) Averages of the frequency (closed circles) and amplitude (open circles) of sEPSCs under the action of M1, relative to those before its application (control: frequency, \( 12.4 \pm 0.9 \) Hz; amplitude, \( 20.3 \pm 2.7 \) pA; \( n = 7 \)), which are plotted against time. Each point with vertical bars indicates the mean values and SEM, calculated from sEPSCs measured for 30 s. (c) Current responses induced in a SG neuron by superfusing AMPA (5 \( \mu \)M) in the absence (a) and presence (b) of M1. Holding potential \( V_{\text{H}} = -70\) mV. AMPA: \( \alpha\)-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid.
of control (12.4 ± 0.9 pA, n = 15), although this amplitude had a tendency to be reduced. Figure 1(b) demonstrates averages of the time courses of M1-induced changes in sEPSC frequency and amplitude that were measured from seven neurons. Such a reduction in sEPSC frequency persisted for at least more than 20 min after washout of M1 (n = 5). Consistent with insignificant change in sEPSC amplitude, M1 (1 mM) hardly affected the peak amplitude of the response of SG neurons to AMPA (5 μM) (85 ± 6% (P > 0.05) of control (42.3 ± 11.4 pA, n = 3)), as seen in Figure 1(c).

Figure 2(a) demonstrates how a μ-opioid receptor antagonist CTAP (1 μM) affects the effect of M1 (1 mM) on spontaneous excitatory transmission in SG neurons. Superfusing M1 with pretreatment with CTAP for 2 min had no effect on sEPSC frequency [96 ± 6% (P > 0.05) of control (12.4 ± 1.8 Hz, n = 7) around 4 min after the beginning of M1 superfusion]. Since the antinociceptive effect of M1 is possibly due to not only μ-opioid receptor activation but also inhibition of the reuptake of monoamines such as noradrenaline and serotonin, we next examined the effects of M1 (1 mM) on spontaneous excitatory transmission in single SG neurons where noradrenaline (50 μM) or serotonin (40 μM) was tested. As seen in Figure 2(b), noradrenaline did not affect sEPSC frequency, whereas this frequency was reduced by M1. This was confirmed in four neurons; sEPSC frequencies around 4 min after the onset of noradrenaline and M1 superfusion were, respectively, 96 ± 6% (n = 4; P > 0.05) and 71 ± 8% (n = 4; P < 0.05) of control (9.4 ± 2.5 Hz). The effect of serotonin on sEPSC frequency was also different from M1’s one (Figure 2(c)). sEPSC frequencies around 4 min and 6 min after the onset of serotonin application were, respectively, 82 ± 6% (n = 8; P > 0.05) and 525 ± 101% (n = 8; P < 0.05) of control (13.8 ± 1.9 Hz), as reported previously. On the other hand, M1 reduced sEPSC frequency around 4 min and 6 min after the beginning of its superfusion; they were, respectively, 68 ± 7% (n = 8; P < 0.05) and 48 ± 6% (n = 8; P < 0.05) of control.

Effects of M1 on monosynthetically evoked Aδ-fiber and C-fiber excitatory transmissions in SG neurons

Stimulating the dorsal root with a strength of more than 26 μA (sufficient to recruit Aδ-fibers) elicited in some neurons monosynaptic EPSCs which displayed no failure and no change in latency when examined at 20 Hz. CV values estimated from the latency of EPSC averaged to be 7.5 ± 1.0 m/s (5.1–12.0 m/s; n = 8); this was within the range of those of Aδ-fibers, as reported previously.13,19,20 Monosynaptic Aδ-fiber EPSCs evoked at 0.1 Hz had a mean amplitude of 278 ± 96 pA (range: 59–902 pA; n = 8). On the other hand, stimuli with a strength larger than 270 μA (enough to activate C-fibers) evoked in some neurons monosynaptic EPSCs which had no failures albeit there was a variety in the latency when stimulated at 1 Hz. The monosynaptic EPSCs had an average CV of 0.53 ± 0.05 m/s (0.4–0.8 m/s; n = 8), values comparable to those of C-fibers.13,19,20 Monosynaptic C-fiber EPSCs evoked at 0.1 Hz had a mean amplitude of 180 ± 95 pA (range: 38–345 pA; n = 8). Some SG neurons exhibited both monosynaptic Aδ-fiber and C-fiber EPSCs. These Aδ-fiber and C-fiber EPSCs were largely inhibited in peak amplitude by CNQX (10 μM; n = 4), indicating the activation of non-NMDA receptors.12

Figure 2. The sEPSC frequency reduction produced by M1 (1 mM) is due to μ-opioid receptor activation but not inhibition of the reuptake of noradrenaline (NA) or serotonin (5-HT) in the SG. (a) Recordings of sEPSCs in the absence and presence of M1 together with a μ-opioid receptor antagonist CTAP (1 μM). (b) and (c) Recordings of sEPSCs in the absence of NA (50 μM; b) or 5-HT (40 μM; c) in a neuron where the effect of M1 on sEPSC frequency was examined. $V_m = -70$ mV. CTAP: D-Phe-Cys-Tyr-O-Trp-Arg-Thr-Pen-Thr-NH$_2$. $\Delta$-fiber and C-fiber excitatory transmissions in SG neurons.
As seen in Figure 3(a) and (b), each of monosynaptic Aδ-fiber and C-fiber EPSCs was reduced in peak amplitude by M1 (1 mM). Figure 3(c) demonstrates the time courses of changes in the peak amplitudes of Aδ-fiber and C-fiber EPSCs, relative to control, following M1 (1 mM) superfusion. These inhibitory actions of M1 did not disappear at least 15 min after its washout, as seen in sEPSC frequency reduction produced by M1. When examined in many neurons, Aδ-fiber and C-fiber EPSC peak amplitudes were, respectively, 51 ± 7% (P < 0.05; n = 8) and 57 ± 7% (P < 0.05; n = 8) of control (278 ± 96 pA and 180 ± 95 pA, respectively) around 4 min after the beginning of M1 superfusion. These percentage values were not significantly different from each other (P > 0.05; Figure 3(d)). No difference in extent between Aδ-fiber and C-fiber EPSC peak amplitude reductions produced by M1 was observed in a single neuron where both monosynaptic Aδ-fiber and C-fiber EPSCs were observed; their extents were 47% and 40%, respectively (Figure 3(e)).

**Discussion**

This study demonstrated that bath-applied M1 (1 mM) irreversibly reduces the frequency but not amplitude of sEPSC in SG neurons, albeit its amplitude has a tendency to be reduced. This irreversibility was also seen in the production of an outward current by M1 in SG neurons.\(^1\) Consistent with this change in sEPSC amplitude, a response of SG neurons to AMPA also had a tendency to be inhibited by M1. The change in sEPSC amplitude and AMPA response appeared to be unlikely due to a rundown of non-NMDA receptors in the recording neurons, because sEPSC amplitude remained to be constant 60 min after the whole-cell configuration\(^2\) under the same condition as that in this study. Such a postsynaptic action of M1 may be due to its inhibitory effect on non-NMDA receptors, albeit insignificantly, because M1 is reported to inhibit various types of neurotransmitter receptor-coupled ion channel.\(^3\) Similar sEPSC frequency and amplitude changes produced by M1 in rat SG neurons have been demonstrated by using the in vivo patch-clamp technique.\(^4\)

The sEPSC frequency decrease was observed under the condition where a postsynaptic hyperpolarizing effect of M1 was inhibited in a recording SG neuron by using GDP-β,S- and K\(^+\)-channel blockers-containing patch-pipette solution. It is possible that bath-applied M1 hyperpolarizes membranes of neurons, which are synaptically connected to the recording neuron, resulting in a decrease in the spontaneous release of L-glutamate from terminals of the neurons and thus in sEPSC frequency decrease. This possibility is, however, unlikely, because sEPSCs are unaffected in amplitude and frequency by the voltage-gated Na\(^+\)-channel blocker tetrodotoxin and therefore are not mediated by neuronal activities.\(^4\) M1 inhibited the reuptake of noradrenaline and serotonin in rat frontal cortex or hypothalamic synaptosomes.\(^5\) Bloms-Funke et al.\(^6\) have demonstrated in in
vivo microdialysis experiments that tramadol increases extracellular levels of noradrenaline and serotonin in the rat ventral hippocampus. Since noradrenergic and serotonergic descending pathways from the brainstem such as the locus coeruleus and raphe complex are involved in an inhibition of nociceptive transmission in the spinal dorsal horn, it is possible that an inhibition by M1 of the reuptake of noradrenaline and serotonin increases their concentrations in the spinal dorsal horn, resulting in a modulation of spontaneous excitatory transmission in SG neurons. In support of this idea, a selective serotonin reuptake inhibitor fluvoxamine modulated spontaneous excitatory transmission in superficial dorsal horn neurons of mouse spinal cord slices. Antinociception produced by the intrathecal administration of tramadol has been attributed to an activation of α2-adrenoceptors in the rat spinal cord. In this study, in SG neurons where M1 decreased sEPSC frequency, noradrenaline did not change sEPSC frequency and serotonin increased sEPSC frequency, as reported previously in SG neurons. This observation that M1 affects sEPSC frequency in a manner different from noradrenaline and serotonin indicates that the presynaptic effect of M1 is not mediated by noradrenaline and serotonin, as different from the ideas proposed previously. In fact, M1 was much less active in inhibiting the reuptake of serotonin than tramadol in rat frontal cortex synaptosomes and in rat dorsal raphe nucleus slices. We previously reported that M1-induced outward current is not mediated by noradrenaline. To ascertain more reliably our idea that M1 activity is not mediated by serotonin, further experiments using serotonin-related drugs will be required.

The sEPSC frequency decrease produced by M1 in this study disappeared in the presence of a µ-opioid receptor antagonist CTAP, indicating an involvement of µ-opioid receptors. This result is consistent with the observations that µ-opioid receptor activation in the SG results in a decrease in the spontaneous release of L-glutamate from nerve terminals and that µ-opioid receptors exist in superficial layers of the spinal cord, particularly the SG in rats. M1-induced sEPSC frequency decrease in rat SG neurons in vivo was abolished in the presence of a nonselective opioid-receptor antagonist naltrexone.

M1 reduced not only sEPSC frequency but also the peak amplitudes of monosynaptic primary-afferent Aδ-fiber and C-fiber EPSCs evoked in SG neurons by stimulating the dorsal root. This EPSC amplitude reduction was irreversible, as seen in the sEPSC frequency reduction produced by M1. Since M1 hardly affects sEPSC amplitude, the EPSC amplitude reduction is mainly presynaptic in origin. This result was the same as that of a µ-opioid receptor agonist [d-Ala2, N-Me-Phe4, Gly5-ol]-enkephalin (DAMGO). The Aδ fiber is myelinated and thus transfers fast, sharp, and more-localized pain, while the C fiber is unmyelinated and thus transfers slow, dull, and less-localized pain. The EPSC peak amplitude reduction produced by M1 was almost comparable in extent between monosynaptic Aδ-fiber and C-fiber transmissions, as seen in the action of adenosine in SG neurons. On the other hand, monosynaptic Aδ-fiber and C-fiber excitatory transmissions were depressed by nociceptin, noradrenaline, and galanin in a different manner from each other. Noradrenaline and galanin inhibited more effectively Aδ-fiber than C-fiber transmission, while C-fiber transmission was more sensitive to nociceptin than Aδ-fiber one. M1 is suggested to suppress fast- and slow-conducting pain transmissions with a comparable efficacy. Although M1 exhibits a high affinity to µ-opioid receptors, this result is different from that of DAMGO. DAMGO reduced monosynaptic C-fiber evoked EPSC peak amplitude more effectively than monosynaptic Aδ-fiber one. Minami et al. have demonstrated by using a Ca2+-activated Cl− channel current assay method that M1 and DAMGO activate µ-opioid receptors in Xenopus oocytes expressing cloned human µ-opioid receptors with a less efficacy of M1 than DAMGO. A Cl− current produced by M1 appeared to be somewhat different from that of DAMGO. M1 is known to act on various types of voltage-gated and neurotransmitter receptor-coupled ion channels, and thus to affect not only µ-opioid but also other G protein-coupled receptors located in primary-afferent central terminals. Therefore, M1 may exhibit an action different from that of DAMGO. The difference in the reduction of evoked EPSC amplitude between M1 and DAMGO remains to be examined. This study revealed for the first time that M1 inhibits primary-afferent Aδ-fiber and C-fiber excitatory transmissions in SG neurons. The concentration (1 mM) of M1 used in this study was much higher than 2 μM, a concentration estimated to be clinically relevant. Although M1 at a low concentration such as 2 μM was reported to decrease sEPSC frequency in rat SG neurons in vivo, this action appeared to be reversible, as different from our observation. The irreversibility of the action of M1 at the high concentration used in this study may be due to a slow rate of diffusion of M1 out of spinal cord slices.

Summary

M1 inhibited the release of l-glutamate onto SG neurons from nerve terminals by activating µ-opioid receptors; this inhibition was comparable in extent between monosynaptic primary-afferent Aδ-fiber and C-fiber transmissions. This presynaptic inhibitory action of M1 could contribute to at least a part of the inhibitory action of...
tramadol on pain transmission together with its hyperpolarizing effect as reported previously. 18

Authors’ Note
AK, TF, TN, and L-HP are currently affiliated with Koga Clinic, Fukuoka Dental University, Kansai University of Health Sciences, and Jilin University, respectively.

Author Contributions
AK, TF, L-HP, TN, and EK designed research; AK, TF, L-HP, and TN performed experiments; AK, TF, L-HP, and TN analyzed data; AK, TF, L-HP, TN, and EK wrote the paper. All authors read, edited, and approved the final manuscript.

Declaration of Conflicting Interest
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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