Full Paper

Preventive and Alleviative Effect of Tramadol on Neuropathic Pain in Rats: Roles of α_2 -Adrenoceptors and Spinal AstrocytesMinoru Sakakiyama¹, Sanae Maeda¹, Kouichi Isami¹, Kayoko Asakura¹, Kanako So¹, Hisashi Shirakawa¹, Takayuki Nakagawa^{1,2,*}, and Shuji Kaneko¹¹Department of Molecular Pharmacology, Graduate School of Pharmaceutical Sciences, Kyoto University, 46-29 Yoshida-Shimoadachi-cho, Sakyo-ku, Kyoto 606-8501, Japan²Department of Clinical Pharmacology and Therapeutics, Kyoto University Hospital, 54 Shogoin-Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan

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Abstract. The acute analgesic effect of tramadol has been extensively investigated; however, its long-term effect on neuropathic pain has not been well clarified. In this study, we examined the effects of repeated administration of tramadol on partial sciatic nerve ligation–induced neuropathic pain in rats. Each drug was administered once daily from 0–6 days (preventive effect) or 7–14 days (alleviative effect) after the surgery. Mechanical allodynia was evaluated just before (preventive or alleviative effect) and 1 h after (analgesic effect) drug administration. Like morphine, first administration of tramadol (20 mg/kg) showed an acute analgesic effect on the developed mechanical allodynia, which was diminished by naloxone. Like amitriptyline, repeated administration of tramadol showed preventive and alleviative effects on the mechanical allodynia that was diminished by yohimbine, but not naloxone. The alleviative effects of tramadol lasted even after drug cessation or in the presence of yohimbine. Repeated administration of tramadol increased the dopamine β -hydroxylase immunoreactivity in the spinal cord. Furthermore, tramadol inhibited the nerve ligation–induced activation of spinal astrocytes, which was reduced by yohimbine. These results suggest that tramadol has both μ -opioid receptor–mediated acute analgesic and α_2 -adrenoceptor–mediated preventive and alleviative effects on neuropathic pain, and the latter is due to α_2 -adrenoceptor–mediated inhibition of astrocytic activation.

Keywords: tramadol, neuropathic pain, antidepressant, α_2 -adrenoceptor, astrocyte

Introduction

Neuropathic pain is associated with severe, chronic sensory disturbances characterized by spontaneous pain, increased responsiveness to painful stimuli (hyperalgesia), and pain perceived in response to normally non-noxious stimuli (allodynia). Neuropathic pain is based on a maladaptive plasticity caused by damage to peripheral or central nerves, and it is often resistant to current therapeutic approaches (1). Recent evidence suggests that neuropathic pain is associated with activation of spinal glial cells including microglia and astrocytes (2–5). Microglia in the spinal cord rapidly respond to peripheral

nerve injury and become in the active state (6), enabling them to send signals to astrocytes (7). Activated microglia and astrocytes secrete various biologically active pronociceptive mediators including proinflammatory cytokines and chemokines, which lead to hyperexcitability of spinal dorsal horn neurons (8).

Antidepressants, such as tricyclic antidepressants (TCAs) and serotonin and noradrenaline reuptake inhibitors (SNRIs), are widely used as first-choice drugs for the management of neuropathic pain (9–11). They inhibit neuropathic pain mainly by increasing extracellular concentrations of noradrenaline (NA) and serotonin (5-HT) in the spinal cord by blocking noradrenaline (NET) and serotonin transporter (SERT) (12). Specifically, the increase in NA elicited by blocking NET and the subsequent activation of presynaptic and postsynaptic α_2 -adrenoceptor (AR) in the spinal cord play

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a key role in the analgesic effects of antidepressants (13 – 15).

Tramadol {1*RS*, 2*RS*}-2-[(dimethylamino)-methyl]-1-(3-methoxyphenyl)-cyclohexanol} is a synthetic opioid from the aminocyclohexanol group, and it is used globally for the treatment of moderate to moderately severe pain, including post-operative pain, cancer pain, and chronic non-cancer-associated pain such as neuropathic pain (10, 11, 16). Tramadol and its metabolite, (+)-*O*-demethyltramadol (known as M1), exhibit dual analgesic mechanisms of action; namely, the latter is active as a μ -opioid receptor agonist, and both of them exhibit antidepressant-like enhancement of NA and 5-HT transmission in the descending inhibitory pathways by blocking monoamine reuptake (17 – 20). Thus, tramadol is a promising agent for the management of neuropathic pain, due to its complementary and synergistic pharmacological actions (21, 22). Furthermore, the lower analgesic tolerance and dependence found in clinical (23, 24) and pre-clinical studies (25) suggest that tramadol may be appropriate for long-term use such as for the treatment of neuropathic pain. However, although the acute analgesic effect of tramadol has been extensively investigated (26 – 29), the long-term effect of this drug on neuropathic pain has not been well clarified (30). In addition, it has not been determined whether repeated administration of tramadol could affect spinal glial cell activation in models of neuropathic pain.

The aim of the current study was to explore the efficacy of repeated administration of tramadol in a rat neuropathic pain model and determine the mechanism of action in tramadol-induced analgesia, using for comparison, a strong opioid, morphine, and a tricyclic antidepressant, amitriptyline. Here, we show that repeated administration of tramadol results in α_2 -AR-mediated preventive and alleviative effects, as well as a μ -opioid receptor-mediated analgesic effect, on neuropathic pain. Furthermore, we examined the roles of the descending noradrenergic pathway and spinal glial cells in tramadol-mediated preventive and ameliorated effects.

Materials and Methods

Animals

Male Sprague-Dawley rats initially weighing 180 – 220 g were used. Animals were purchased from Nihon SLC (Shizuoka). They were kept at a constant ambient temperature of 24°C $\pm$ 1°C under a 12-h light/dark cycle and were provided with free access to food and water. The rats were individually housed in plastic cages with woodchip bedding for at least 1 day before surgery. All animal care and experimental procedures were carried

out with the approval of the Kyoto University Animal Experimentation Committee and followed the policies issued by the Japanese Pharmacological Society and International Association for the Study of Pain. All efforts were made to minimize the number of animals used and limit experimentation to what was necessary to produce reliable scientific information.

Neuropathic pain model

For a well-characterized rat model of neuropathic pain, partial sciatic nerve ligation (pSNL) was performed as previously described (31) with slight modifications. Briefly, under isoflurane anesthesia, the left sciatic nerve was exposed at the upper-thigh level. The 1/3 – 1/2 dorsal section of the sciatic nerve was ligated tightly with a 7-0 silk suture (Natsume Seisakusyo, Tokyo), and the wound was closed by suturing the muscle and skin layers. In sham-operated rats, the nerve was exposed without ligation.

Drug administration

All drugs were administered systemically by intraperitoneal (i.p.) administration. Morphine hydrochloride (10 mg/kg; Takeda Chemical Industries, Osaka), amitriptyline hydrochloride (10 mg/kg; LKT Laboratories, Inc., St. Paul, MN, USA), and ($\pm$)-tramadol hydrochloride (20 mg/kg, a gift from Nippon Shinyaku Co., Kyoto) were freshly dissolved in sterile saline. To examine the preventive and analgesic effects on the induction of neuropathic pain, each drug or saline was administered 1 h before the pSNL surgery and then once daily for six consecutive days (day 0 – 6). To examine the alleviative and analgesic effect on the developed neuropathic pain, each drug or saline was administered once daily between 13:00 and 15:00 from day 7 to day 14 after the pSNL surgery. The μ -opioid receptor antagonist naloxone hydrochloride (3 mg/kg; Sigma, St. Louis, MO, USA) or the α_2 -adrenoceptor antagonist yohimbine hydrochloride (3 mg/kg, Sigma) was administered 10 min before drug administration. The volume of administration was 2 ml/kg.

Behavioral tests

Mechanical allodynia was measured by the modified up-down method as described previously (32) with slight modifications. Briefly, animals were individually placed on a wire mesh floor and acclimatized to the environment for at least 30 min. After acclimatization, the tactile stimulus was applied to the middle plantar surface of the paw by placing one of the series of von Frey filaments (1.0, 2.0, 4.0, 6.0, 8.0, 15.0 g) perpendicular to the surface of the paw. The testing was initiated at 2.0 g, and in the absence of a paw withdrawal response to the initially selected hair, a stronger stimulus was presented;

in the event of paw withdrawal, the next weaker stimulus was chosen. Four additional responses were observed after the first withdrawal response and the 50% paw withdrawal threshold (PWT) was calculated (33). In cases where continuous positive and negative responses were observed up to the exhaustion of the stimulus set, values of 15.0 g and 1.0 g were assigned respectively. PWT to mechanical stimulation was evaluated just before (pre-PWT) and 1 h after the drug administration (post-PWT).

Immunohistochemistry

One day after the last administration of drugs, the animals were deeply anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and perfused transcardially through the ascending aorta with 0.1 M phosphate-buffered saline (PBS). After perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer, the L4-L6 lumbar spinal cord was removed, post-fixed in the same fixative for 3 h, cryoprotected with 15% sucrose in 0.1 M phosphate buffer overnight at 4°C, and then frozen in liquid nitrogen. Fourteen coronal sections (30 μ m) were cut on a cryostat and thaw-mounted onto MAS-coated glass slides (Matsunami, Osaka).

For immunofluorescence imaging of marker proteins for astrocytes and microglia, sections were gently washed 3 times (10 min each) in PBS, and then permeabilized and blocked at room temperature for 1 h in 4% normal goat serum in PBS containing 0.1% Triton X-100. The sections were incubated overnight at 4°C with mouse anti-glial fibrillary acidic protein (GFAP) antibody (1:400; Sigma-Aldrich, St. Louis, MO, USA) or rabbit anti-ionized calcium binding adaptor molecule 1 antibody (Iba-1, 1:500; Wako Pure Chemical Industries, Ltd., Osaka) in PBS containing 0.1% Triton X-100 and 4% normal goat serum. The sections were washed 3 times in PBS and incubated with Alexa Fluor 594-labeled goat anti-mouse or anti-rabbit IgG antibody (1:200; Molecular Probes, Carlsbad, CA, USA) in PBS with 0.1% TritonX-100 and 4% normal goat serum for 1 h at room temperature. For dopamine β -hydroxylase (D β H) staining in the spinal cord, the sections were incubated with mouse anti-D β H monoclonal antibody (1:200; Chemicon International Inc., Temecula, CA, USA). Subsequently, the sections were incubated with Alexa Fluor 488-labeled donkey anti-mouse IgG antibody (1:200; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA).

For quantitative analysis of Iba-1, GFAP, and D β H staining intensity, 3 spinal cord sections immunostained with each marker were randomly selected from each rat. Confocal images in a square (400 $\times$ 400 μ m) on the medial two-third of the superficial dorsal horn (laminae

I–III) were captured under a 20 $\times$ objective using a Fluoview FV10i system (Olympus, Tokyo). The mean staining intensity following background correction was measured using Image J software (National Institute of Mental Health, Bethesda, MD, USA).

Statistical analyses

Data are expressed as means $\pm$ S.E.M. and were analyzed using Graphpad Prism version 5.0 (GraphPad, San Diego, CA, USA). Statistical analyses of the time course data for PWT were carried out using two-way repeated measures analysis of variance (ANOVA), followed by a *post-hoc* Bonferroni's comparison test, or one-way repeated measures ANOVA, followed by a *post-hoc* Dunnett's comparison test. Differences between the two groups (pre-PWT and post-PWT) were tested using a paired *t*-test at individual time points. The immunofluorescence intensities of Iba-1, GFAP, and D β H were analyzed by two-way ANOVA followed by a *post-hoc* Bonferroni's comparison test (side $\times$ drug) and one-way ANOVA, followed by the *post-hoc* Dunnett's comparison test at each paw. In all cases, differences with $P < 0.05$ were considered statistically significant.

Results

Effect of repeated administration of morphine, amitriptyline, or tramadol on the induction of neuropathic pain

Morphine, amitriptyline, tramadol, or saline was administered 1 h before pSNL surgery and then once daily for 6 consecutive days (day 0–6). PWT to mechanical stimulation was measured just before (pre-PWT) and 1 h after drug administration (post-PWT) on day 0 (only just before the first administration, basal PWT) and on days 1, 3, 5, and 7 (only one day after the last administration) (Fig. 1). In control animals administered with saline, pSNL surgery decreased pre- and post-PWTs of the ipsilateral hind paw (mechanical allodynia) to below the basal PWT. The decrease appeared on day 1 and lasted for at least 7 days. In the contralateral hind paw, pre- and post-PWTs were unchanged.

Figure 1A shows the effects of repeated administration of morphine (10 mg/kg) from day 0 to day 6 on PWTs after pSNL surgery in the ipsilateral and contralateral paws. Compared to the saline-treated group, repeated administration of morphine prevented the decrease in pre-PWT in the ipsilateral paw on day 1 partially, but significantly, indicating a partial preventive effect of morphine on the induction of neuropathic pain. This significant inhibitory effect disappeared by day 3. Each

A) Morphine **B) Amitriptyline** **C) Tramadol**

Fig. 1. Effect of repeated administration of morphine, amitriptyline, or tramadol on the induction of neuropathic pain. A) Morphine (10 mg/kg), B) amitriptyline (10 mg/kg), C) tramadol (20 mg/kg), or saline was administered 1 h before pSNL surgery, and then once daily for 6 consecutive days (day 0–6). PWTs in the ipsilateral (left panel) and contralateral (right panel) paws were measured just before (pre-PWT: open symbol) and 1 h after the drug administration (post-PWT: closed or gray symbol) on day 0 (only just before the first administration, basal PWT) and day 1, 3, 5, and 7 (only one day after the last administration). Naloxone (3 mg/kg) or yohimbine (3 mg/kg) was administered 10 min before each repeated administration of morphine, amitriptyline, or tramadol. Arrows indicate the timing of drug administration. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared with the corresponding PWT of saline-treated animals. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, compared with the corresponding PWT of drug-treated animals (two-way repeated measures ANOVA, followed by *post-hoc* Bonferroni's comparison test). $^{\$}P < 0.05$, $^{ss}P < 0.01$, compared with the corresponding pre-PWT (paired *t*-test). $n = 6-7$.

administration of morphine increased post-PWT in the ipsilateral paw, and a significant difference was observed on day 3, compared with the corresponding pre-PWT, indicating an analgesic effect of morphine. On day 5,

however, the analgesic effect was relatively weak, and there was a significant difference in post-PWTs between day 1 and day 5 ($P < 0.05$, paired *t*-test), suggesting the development of analgesic tolerance to

morphine. In the contralateral paw, repeated administration of morphine gradually decreased pre-PWT, and a significant difference with respect to the saline-treated group was observed on day 7, indicating the development of opioid-induced mechanical hypersensitivity. Each administration of morphine showed an analgesic effect against the decrease in PWTs. A significant difference was observed on day 3, compared with the corresponding pre-PWT. When an opioid receptor antagonist, naloxone (3 mg/kg), was given 10 min before morphine administration, both the analgesic and preventive effects of morphine in the ipsilateral paw were significantly diminished at all time points. Furthermore, naloxone significantly inhibited the analgesic effect of morphine on the repeated morphine-induced decrease in PWTs; rather, it facilitated it in the contralateral paw.

Repeated administration of amitriptyline (10 mg/kg) significantly blocked the decrease in pre-PWT in the ipsilateral paw at all time points, suggesting the preventive effect of amitriptyline. Since amitriptyline completely prevented mechanical allodynia, each administration of amitriptyline had no further effect on post-PWT. When an α_2 -AR antagonist, yohimbine (3 mg/kg), was given 10 min before amitriptyline administration, the preventive effect of amitriptyline in the ipsilateral paw was significantly diminished. Significant differences in pre- and post-PWTs were observed on day 3 and days 1, 3, and 5, respectively, compared with those in the amitriptyline-treated group. On the other hand, in the contralateral paw, repeated administration of amitriptyline or pretreatment with yohimbine had no effect on pre- and post-PWTs (Fig. 1B).

Like amitriptyline, repeated administration of tramadol (20 mg/kg) significantly prevented the decrease in pre-PWT in the ipsilateral paw at all time points, suggesting that tramadol had a preventative effect. Pretreatment with naloxone (3 mg/kg) had no effect on the preventive effect of tramadol. By contrast, pretreatment with yohimbine (3 mg/kg) reduced the preventive effect of tramadol in the ipsilateral paw, and significant effects were observed on day 5 and day 7. In the presence of yohimbine, significant analgesic effect of tramadol on the post-PWT was seen on day 1. On the other hand, in the contralateral paw, repeated administration of tramadol or pretreatment with naloxone or yohimbine had no effects on pre- and post-PWTs (Fig. 1C). Unlike morphine, tramadol showed no opioid-induced mechanical hypersensitivity even on day 7.

Effect of repeated administration of morphine, amitriptyline, or tramadol on developed neuropathic pain

Morphine, amitriptyline, tramadol, or saline was administered daily from day 7 to 14 after pSNL surgery.

Pre- and post-PWTs were measured on days 7, 10, and 14 (Fig. 2). In each group, pSNL surgery gradually decreased PWT in the ipsilateral paw on day 1 and 3, suggesting the development of mechanical allodynia, while it had no effect on PWT in the contralateral paw. Repeated administration of saline had no effect on the decrease in pre- and post-PWTs on any day.

Repeated administration of morphine (10 mg/kg) had no effect on pre-PWT, but each administration of morphine increased post-PWT in the ipsilateral paw, suggesting the analgesic effect of morphine. Significant analgesic effects were observed at all time points, compared with post-PWT in saline-treated groups and the corresponding pre-PWT. In the contralateral paw, repeated administration of morphine gradually decreased pre-PWT, although the effect was not statistically significant. Each administration of morphine elicited an analgesic effect against opioid-induced mechanical hypersensitivity, and significant differences with respect to the corresponding pre-PWT were observed on days 10 and 14 (Fig. 2A).

Repeated administration of amitriptyline (10 mg/kg) alleviated the decrease in pre-PWT in the ipsilateral paw, and the effect was significant on day 10, indicating alleviative effect of amitriptyline on the developed mechanical allodynia. The first administration of amitriptyline significantly increased post-PWT on day 7 compared to post-PWT in saline-treated groups and the corresponding pre-PWT, indicating an analgesic effect on the developed mechanical allodynia. Amitriptyline had no further analgesic effect on day 10, compared with the elevated pre-PWT. The alleviative and analgesic effects of amitriptyline disappeared by day 14. In the contralateral paw, repeated administration of amitriptyline had no effect on pre- and post-PWTs (Fig. 2B).

Like amitriptyline, repeated administration of tramadol (20 mg/kg) reversed the decrease in pre-PWT in the ipsilateral paw, and significant effects were observed on days 10 and 14, indicating that tramadol alleviated the developed mechanical allodynia. The first administration of tramadol significantly increased post-PWT on day 7, compared with the corresponding pre-PWT, indicating an analgesic effect on the developed mechanical allodynia. However, no further analgesic effects were observed on days 10 and 14. When naloxone (3 mg/kg) was given 10 min before tramadol administration, the analgesic effect of tramadol was significantly decreased on day 7. However, the alleviative effects on days 10 and 14 were not affected by naloxone pretreatment. In contrast, pretreatment with yohimbine (3 mg/kg) had no effect on the analgesic effect of tramadol on day 7, although it diminished the analgesic and alleviative effects on days 10 and 14. In the contralateral paw,

A) Morphine **B) Amitriptyline** **C) Tramadol**

Fig. 2. Effect of repeated administration of morphine, amitriptyline, or tramadol on the developed neuropathic pain. Morphine (A: 10 mg/kg), amitriptyline (B: 10 mg/kg), tramadol (C: 20 mg/kg), or saline was administered once daily from day 7 to 14 after the pSNL surgery. PWTs in the ipsilateral (left panel) and contralateral (right panel) paws were measured day 0, 1, 3, and then just before (pre-PWT: open symbol) and 1 h after the drug administration (post-PWT: closed or gray symbol) on day 7, 10, and 14. Naloxone (3 mg/kg) or yohimbine (3 mg/kg) was administered 10 min before each repeated administration of tramadol. Arrows indicate the timing of drug administration. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared with the corresponding PWT of saline-treated animals. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, compared with the corresponding PWT of drug-treated animals (two-way repeated measures ANOVA, followed by the *post-hoc* Bonferroni's comparison test). \$ $P < 0.05$, \$\$ $P < 0.01$, \$\$\$ $P < 0.001$, compared with the corresponding pre-PWT (paired *t*-test). $n = 4 - 12$.

repeated administration of tramadol or pretreatment with naloxone or yohimbine had no effects on pre- and post-PWTs (Fig. 2C).

Effect of drug cessation or an α_2 -AR antagonist on tramadol-induced amelioration of neuropathic pain

As noted above, when tramadol (20 mg/kg) was

administered daily from day 7 to 14 after pSNL surgery, significant analgesic (day 7) and alleviative (day 7 – 14) effects on the developed mechanical allodynia were observed. Following the repeated administration of tramadol, PWT was measured on days 16, 18, 20, 22, and 24 in the absence of drug. Even after the cessation of drug administration, the increase in PWTs persisted

Fig. 3. Effect of drug cessation or α_2 -AR antagonist on the tramadol-induced amelioration of neuropathic pain. A) Tramadol (20 mg/kg) was administered once daily from day 7 to day 14 after pSNL surgery. PWTs in the ipsilateral paw were measured on days 0, 1, and 3, and then just before (pre-PWT: open symbol) and 1 h after drug administration (post-PWT: closed symbol) on days 7, 10, and 14. Following the repeated administration of tramadol, PWTs were measured on days 16, 18, 20, 22, and 24 in the absence of drug. Arrows indicate the timing of drug administration. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared with the pre-PWT on day 7 (one-way repeated measures ANOVA, followed by the *post-hoc* Dunnett's comparison test). B) Tramadol (20 mg/kg) or saline was repeatedly administered once daily from day 7 to day 14 after pSNL surgery. PWTs in the ipsilateral paw were measured on days 0, 1, 3, and then just before (pre-PWT: open symbol) and 1 h after drug administration (post-PWT: closed symbol) on days 7, 10, and 14 (left panel). On day 15, the tramadol-treated animals were administered yohimbine (3 mg/kg), and pre- and post-PWTs were measured (right panel). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared with the corresponding PWT of saline-treated animals (two-way repeated measures ANOVA, followed by the *post-hoc* Bonferroni's comparison test). $^{\circ}P < 0.05$, compared with the corresponding pre-PWT (paired *t*-test). N.S., not significant. $n = 6$.

for 6 days, and then gradually decreased. Significant alleviative effects of tramadol were observed on days 16, 18, and 20, compared with pre-PWT on day 7 (Fig. 3A).

Next, we examined the effect of a single administration of yohimbine on the alleviative effect of tramadol on pSNL-evoked mechanical allodynia. Following repeated administration of tramadol (10 mg/kg) once daily from day 7 to day 14 after pSNL surgery, yohimbine (3 mg/kg) was administered, and pre- and post-PWTs were measured on day 15. There was no significant difference between pre- and post-PWTs 1 h after yohimbine administration (Fig. 3B).

Fig. 4. Effects of repeated administration of morphine, amitriptyline, or tramadol on the spinal D β H-immunoreactive fibers in neuropathic pain. Morphine (10 mg/kg), amitriptyline (10 mg/kg), tramadol (20 mg/kg), or saline was administered 1 h before pSNL surgery and then once daily for 6 consecutive days (day 0–6). On day 7, the spinal cord was dissected, sectioned, and immunostained with D β H antibody, a marker for NAergic neurons. Representative images of D β H immunofluorescence in the ipsilateral spinal dorsal horn of animals treated with saline (A), morphine (B), amitriptyline (C), or tramadol (D) on day 7. E) Quantification of D β H immunoreactivity in the contralateral (left) and ipsilateral (right) spinal dorsal horn. The value obtained for the contralateral spinal dorsal horn of sham-operated group served as the control (100%), and the results are presented as the mean of the percentage $\pm$ S.E.M. of 5–6 animals. Sham, sham-operated animal; Sal, saline; Mor, morphine; Ami, amitriptyline; Tram, tramadol. *** $P < 0.001$, compared with the saline-treated group at each side (one-way ANOVA, followed by the *post-hoc* Dunnett's comparison test). Scale bar = 100 μ m.

Effects of repeated administration of morphine, amitriptyline, or tramadol on spinal D β H-immunoreactive noradrenergic fibers

To determine whether repeated administration of drugs could affect the descending NAergic pathway, immunofluorescence of D β H, an enzyme that converts dopamine

Fig. 5. Effects of repeated administration of morphine, amitriptyline or tramadol on the activation of spinal microglia in neuropathic pain. Morphine (10 mg/kg), amitriptyline (10 mg/kg), tramadol (20 mg/kg), or saline was administered 1 h before pSNL surgery and then once daily for 6 consecutive days (day 0–6) in the absence or presence of pretreatment with naloxone (3 mg/kg) or yohimbine (3 mg/kg). On day 7, the spinal cord was dissected, and the sections were immunostained with an antibody against the microglial marker Iba-1. Representative images of Iba-1 immunofluorescence in the contralateral spinal dorsal horn of saline-treated animal (A) and in the ipsilateral spinal dorsal horn of animals treated with saline (B), morphine (C), naloxone + morphine (D), amitriptyline (E), yohimbine + amitriptyline (F), tramadol (G), naloxone + tramadol (H), or yohimbine + tramadol (I) on day 7. Scale bar = 100 μ m. Enlarged images of Iba-1-positive cells in ipsilateral and contralateral spinal dorsal horn of a saline-treated animal are shown in rectangles, respectively. J) Quantification of Iba-1 immunoreactivity in the contralateral (left) and ipsilateral (right) spinal dorsal horn. The value obtained for the contralateral side of the saline-treated group served as the control (100%), and the results are presented as the mean of the percentage $\pm$ S.E.M. of 4–9 animals. Sal, saline; Mor, morphine; Ami, amitriptyline; Tram, tramadol; Nlx, naloxone; Yoh, yohimbine. $^{###}P < 0.001$, compared with the corresponding contralateral spinal dorsal horn (two-way ANOVA, followed by the *post-hoc* Bonferroni's comparison test).

to noradrenaline, was investigated in the spinal dorsal horn 7 days after pSNL surgery (1 day after the last drug administration) as a marker for NAergic neurons (Fig. 4). D β H-immunoreactivity in saline-treated animals following the pSNL surgery was approximately 2-fold higher in both the ipsilateral and contralateral spinal dorsal horn, compared with the levels in sham-operated animals. When they were analyzed by two-way ANOVA, there was a significant difference between sham- and pSNL-operated animals ($F_{1,16} = 9.09$, $P < 0.01$), but no difference between contralateral and ipsilateral sides ($F_{1,16} = 0.16$, $P = 0.694$). Repeated administration of tramadol (20 mg/kg) increased the number of D β H-immunoreactive fibers and significantly increased D β H-immunoreactivity both in the ipsilateral and contralateral spinal dorsal horn, compared to that in the saline-

treated group. Morphine (10 mg/kg) and amitriptyline (10 mg/kg) had no effect. There was no difference in the D β H-immunoreactivity between contralateral and ipsilateral spinal dorsal horns.

Effects of repeated administration of morphine, amitriptyline, or tramadol on spinal glial activation

To determine whether repeated administration of morphine, amitriptyline, or tramadol (day 0–6) affected the activation of spinal glial cells observed in neuropathic pain, the activation of spinal microglia and astrocytes was evaluated by alterations in the immunofluorescence of the microglial marker Iba1 and the astrocytic marker GFAP, 7 days after pSNL surgery (1 day after the last drug administration) (Fig. 5 and Fig. 6, respectively). As indicated by the changes in the immunofluorescence

Fig. 6. Effects of repeated administration of morphine, amitriptyline, or tramadol on the activation of spinal astrocytes in neuropathic pain. Morphine (10 mg/kg), amitriptyline (10 mg/kg), tramadol (20 mg/kg), or saline was administered 1 h before pSNL surgery and then once daily for 6 consecutive days (day 0–6) in the absence or presence of pretreatment with naloxone (3 mg/kg) or yohimbine (3 mg/kg). On day 7, the spinal cord was dissected, and the sections were immunostained for GFAP, an astrocytic marker. Representative images of GFAP immunofluorescence in the contralateral spinal dorsal horn of saline-treated animals (A) and in the ipsilateral spinal dorsal horn of animals treated with saline (B), morphine (C), naloxone + morphine (D), amitriptyline (E), yohimbine + amitriptyline (F), tramadol (G), naloxone + tramadol (H), or yohimbine + tramadol (I) on day 7. Scale bar = 100 μ m. Enlarged images of GFAP-positive cells in the ipsilateral and contralateral spinal dorsal horn of a saline-treated animal are shown in rectangles, respectively. J) Quantification of GFAP immunoreactivity in the contralateral (left) and ipsilateral (right) spinal dorsal horn. The value obtained for the contralateral side of the saline-treated group served as the control (100%), and the results are presented as the mean of the percentage $\pm$ S.E.M. of 4–9 animals. Sal, saline; Mor, morphine; Ami, amitriptyline; Tram, tramadol; Nlx, naloxone; or Yoh, yohimbine. # P < 0.05, ## P < 0.01, ### P < 0.001, compared with the corresponding contralateral spinal dorsal horn (two-way ANOVA, followed by the *post-hoc* Bonferroni's comparison test). * P < 0.05, ** P < 0.01, compared with the saline-treated group in the ipsilateral spinal dorsal horn (one-way ANOVA, followed by the *post-hoc* Dunnett's comparison test).

intensities of Iba1 and GFAP and the altered morphology of the immunoreactive cells, pSNL surgery robustly activated spinal microglia and astrocytes in the ipsilateral spinal dorsal horn of saline-treated animals. Quantitative analyses revealed that both Iba-1 and GFAP immunoreactivities in the ipsilateral spinal dorsal horn were significantly increased, compared with those in the contralateral spinal dorsal horn of the saline-treated group.

Repeated administration of morphine (10 mg/kg), amitriptyline (10 mg/kg), or tramadol (20 mg/kg) once daily from day 0 to day 6 after pSNL surgery had no effect on the pSNL-induced increase in Iba-1 immunoreactivity in the ipsilateral spinal dorsal horn, as well as Iba-1 immunoreactivity in the contralateral spinal dorsal horn. Similarly, pretreatment with naloxone (3 mg/kg) or yohimbine (3 mg/kg) 10 min before each administration

of morphine, amitriptyline, or tramadol had no effect on Iba-1 immunoreactivity in the contralateral and ipsilateral spinal dorsal horns (Fig. 5).

Repeated administration of morphine with or without pretreatment with naloxone had no effect on the increase in GFAP immunoreactivity in the ipsilateral spinal dorsal horn induced by pSNL surgery. Repeated administration of amitriptyline or tramadol significantly reduced the increase in GFAP immunoreactivity in the ipsilateral spinal dorsal horn, compared with that in the saline-treated group. The significant inhibitory effects of amitriptyline and tramadol were removed by pretreatment with yohimbine, while the inhibitory effect of tramadol was not affected by pretreatment with naloxone. In the contralateral spinal dorsal horn, amitriptyline or tramadol tended to reduce GFAP immunoreactivity, and

pretreatment with yohimbine tended to block this effect of amitriptyline or tramadol, although this was not statistically significant (Fig. 6).

Discussion

The present study demonstrates for the first time that tramadol prevents and alleviates neuropathic pain via its effects on descending NAergic neurons and α_2 -AR-mediated inhibition of spinal astrocytic activation. An opioid-like acute analgesic effect, mediated via the stimulation of μ -opioid receptors, and antidepressant-like preventive and alleviative effects, mediated through stimulation of α_2 -ARs, were observed following repeated administration of tramadol after pSNL. This was accompanied by increased D β H-immunoreactivity in the spinal cord. In addition, preventive administration of tramadol and amitriptyline reduced pSNL-induced activation of spinal astrocytes, which was mediated through stimulation of α_2 -ARs, although they had no effect on the activation of microglia.

Consistent with previous reports, the acute analgesic effect of tramadol on pSNL-evoked mechanical allodynia was caused mainly by μ -opioid receptors, especially after the first administration (27, 28), and was visible in the presence of yohimbine. As with TCAs and SNRIs, acute treatment with tramadol increases the extracellular concentration of NA and 5-HT by blocking NET and SERT (34). The analgesic effect of tramadol on neuropathic pain is considered to be at least partly due to its interaction with presynaptic and postsynaptic α_2 -ARs located on the central terminals of primary nociceptive afferents and the spinal dorsal horn neurons, respectively (35–37). The analgesic potency of tramadol (26), as well as α_2 -AR agonists (38, 39), is enhanced in neuropathic pain, probably due to the increased activity of presynaptic and postsynaptic α_2 -ARs (40). Thus, the present results do not exclude the possibility that the analgesic effect of tramadol on neuropathic pain is partly mediated through α_2 -ARs.

Repeated administration of morphine had a mild preventive effect on neuropathic pain, which weakened with time. In general, repeated administration of morphine does not alleviate neuropathic pain. The effect of morphine on the induction of neuropathic pain observed in the present study may be due to its preemptive analgesia effect (41), since morphine was administered 1 h before pSNL surgery. On the other hand, repeated administration of morphine induced weak analgesic tolerance in the ipsilateral paw and opioid-induced mechanical hypersensitivity in the contralateral paw, consistent with previous clinical and experimental reports (42–44). Morphine also had an acute analgesic

effect against opioid-induced hypersensitivity in the contralateral paw. Unlike morphine, repeated administration of tramadol caused neither analgesic tolerance nor opioid-induced hypersensitivity. However, the acute analgesic effect of tramadol observed in the presence of yohimbine was weakened by repeated administration. These findings suggest that repeated administration of tramadol induces tolerance to the μ -opioid receptor-mediated analgesic effect, which is compensated by its preventive and alleviative effects on neuropathic pain. Furthermore, the present results suggest that tramadol cannot induce opioid-induced hypersensitivity, or otherwise tramadol-induced hypersensitivity may be blocked by its preventive effect. These findings further support the clinical efficacy of tramadol for the long-term treatment of chronic pain, including neuropathic pain.

Although the acute analgesic effect of a single administration of tramadol has been extensively investigated, its long-term effect on neuropathic pain has not been clarified. In the present study, we found that repeated administration of tramadol and amitriptyline prevented and alleviated neuropathic pain via the α_2 -AR. Consistent with our findings, several studies reported that repeated administration of TCAs, as well as SNRIs, alleviated peripheral nerve injury-induced neuropathic pain (45, 46), intermittent cold stress-induced fibromyalgia abnormal pain (47), and oxaliplatin-induced painful neuropathy (48). The preventive effects of tramadol and amitriptyline were diminished by pretreatment with yohimbine, suggesting that the effects involved stimulation of the α_2 -AR. This finding is supported by previous reports showing that repeated administration of α_2 -AR agonists alleviates neuropathic pain and other types of pain (49–51). The alleviative effect of tramadol is unlikely to be due to the cumulative effect of repeated administration. The elimination half-lives of tramadol and its active metabolites are 6 and 7.5 h, respectively, with hepatic metabolism and renal excretion (52). By contrast, the alleviative effect of tramadol persisted for about 1 week even after the cessation of drug administration. These findings suggest that the alleviation by tramadol is likely to be based on the long-term plasticity of pain signaling pathways induced by repeated administration of tramadol. Furthermore, the present findings that post-treatment with yohimbine had no effect on the ameliorative effect of tramadol the day after the last administration suggest that the prolonged effect of tramadol is not mediated by continuous stimulation of the α_2 -AR. In vivo microdialysis experiments reveal that the tramadol-induced increase in the extracellular NA level in the spinal dorsal horn is transient, as it peaks at 30 min and returns to the control level 3 h

after a single intraperitoneal administration (34). It is suggested that an increase in the extracellular NA level corresponds to the analgesic effect, rather than to the preventive and alleviative effect of tramadol. Consequently, it is conceivable that the mechanisms underlying the prolonged preventive and alleviative effects of tramadol involve other plastic changes triggered by stimulation of the α_2 -AR.

Peripheral nerve injury induces an increase in the number of D β H-immunoreactive fibers, i.e., the sprouting of descending NAergic fibers in both the ipsilateral and contralateral spinal dorsal horn, and this contributes to the increased analgesic efficacy of α_2 -AR agonists (53), which is consistent with the results of the present study. In addition, we found that repeated administration of tramadol further increased D β H-immunoreactivity in both the ipsilateral and contralateral spinal dorsal horns, thereby providing insights into the sprouting of NAergic fibers. These results suggest that repeated administration of tramadol could facilitate the descending NAergic pathway, which may lead to plasticity in pain signaling via the α_2 -AR and enhance the preventive and alleviative efficacy of tramadol. By contrast, neither morphine nor amitriptyline increased D β H immunoreactivity, suggesting that the increase in spinal D β H-immunoreactive fibers requires both stimulation of the μ -opioid receptor and inhibition of NA reuptake into the NAergic terminals. Although the affinities of tramadol and its metabolite M1 for the μ -opioid receptor and NET are much lower than those of morphine and amitriptyline, respectively (54), tramadol actually increases extracellular levels of NA (34, 55) and exerts both μ -opioid receptor- and reuptake inhibition-dependent analgesic effects. Consequently, it is conceivable that the extracellular increase in the NA level caused by tramadol-induced exocytotic NA release through the μ -opioid receptor-mediated activation of the descending NAergic neurons is potentiated by tramadol-mediated reuptake inhibition. The synergistic effect of weak μ -opioid receptor activity and reuptake inhibition of tramadol may cause the increase in D β H-immunoreactive fibers, although this was observed after neither morphine-induced exocytotic NA release nor amitriptyline-induced reuptake inhibition. Otherwise, the sprouting of spinal NAergic fibers induced by peripheral nerve injury is induced by brain-derived nerve growth factor (BDNF) in the spinal cord (53). Tramadol enhances the BDNF level upregulated by ketamine in the hippocampus, although tramadol alone had no effect (56). These findings suggest that tramadol may enhance the spinal BDNF level induced by peripheral nerve injury, which could facilitate the sprouting of spinal NAergic fibers.

In the present study, we provide the first evidence

that preventive administration of tramadol and amitriptyline inhibits the pSNL-induced activation of spinal astrocytes, but not that of microglia. Consistent with our findings, Cheng et al. recently reported that a combination of intrathecal pretreatment and post-injury intraperitoneal amitriptyline suppressed peripheral nerve injury-induced mechanical allodynia and activation of spinal astrocytes, although it suppressed also the activation of spinal microglia (57). Several reports indicate that TCAs can directly act on astrocytes. TCAs, including amitriptyline, increase glial cell line-derived neurotrophic factor production and activate fibroblast growth factor receptor signaling through a monoamine-independent pathway in cultured astrocytes (58, 59). However, the inhibition of astrocytic activation by repeated administration of tramadol was blocked by yohimbine, but not naloxone, suggesting that the inhibition is mediated through the stimulation of α_2 -ARs. Recent findings suggest that astrocytes, but not microglia, express α_{2A} -ARs (50). Furthermore, dexmedetomidine, a highly selective α_2 -AR agonist, inhibits the activation of spinal astrocytes and extracellular signal-regulated kinase (ERK) signaling, but not that of microglia, probably by activating α_2 -AR expressed on astrocytes, which suppresses inflammatory and neuropathic pain (50, 51). Taken together, it is suggested that the increase in NA induced by tramadol or amitriptyline acts on astrocytic α_2 -ARs and inhibits the pSNL-induced activation of spinal astrocytes, which may contribute to the preventive effect on neuropathic pain. It will be interesting to determine whether the prolonged alleviative effect of tramadol is accompanied with the inhibition of astrocytic activation. However, we cannot exclude the possibility that presynaptic and postsynaptic α_2 -ARs expressed on the terminals of primary nociceptive afferents and spinal dorsal horn neurons, respectively, indirectly contribute to the suppression of astrocytic activation.

Nevertheless, microglia also possess α_{1A} -, α_{2A} -, β_1 -, and β_2 -ARs, and the expression of α_{2A} -AR is strongly induced in activated microglia (60). Furthermore, intrathecal administration of clonidine, an α_2 -AR agonist, inhibits the activation of both astrocytes and microglia, as well as production of interleukin (IL)-1 and IL-6, and nuclear factor- κ B activation in the spinal cord of neuropathic pain model rats 1 h after the administration (61). Absence of the inhibitory effect of tramadol and amitriptyline on microglial activation may be due to quick recovery to the activated state under the neuropathic pain situation following once-daily administration, although the suppression of astrocytic activation can persist 1 day after the last drug administration. Further investigations will be needed to elucidate how tramadol

and amitriptyline preferentially suppressed pSNL-induced astrocytic activation, rather than microglial activation. On the other hand, the repeated administration of morphine induces activation of spinal microglia and astrocytes, which contributes to analgesic tolerance and opioid-induced hypersensitivity (43, 62). In our experiments, morphine tended to induce the activation of spinal microglia and astrocytes, although this did not reach statistical significance. Lower analgesic tolerance and opioid-induced hypersensitivity by tramadol may be due to the lack of its activating action on spinal microglia and astrocytes.

The analgesic effect of tramadol on the acute nociceptive pain is at least partly mediated through 5-HT receptors (17, 63–65). However, since excitatory 5-HT₃ receptor is potentiated in the neuropathic pain, the analgesic tone of descending inhibitory 5-HTergic neurons is weakened in the presence of peripheral nerve injury (65, 66). Indeed, the analgesic efficacy of SNRIs on neuropathic pain is greater than that of selective 5-HT reuptake inhibitors (15). Thus, it is considered that 5-HT receptors are less involved in the analgesic effect of tramadol on neuropathic pain, although we cannot ignore the possibility for the involvement in the preventive and alleviative effects.

In conclusion, the present study reveals that repeated administration of tramadol exerts an α_2 -AR-mediated preventive and ameliorative effect, as well as a μ -opioid receptor-mediated analgesic effect, on neuropathic pain, without showing the analgesic tolerance and opioid-induced hypersensitivity observed with morphine. These effects of tramadol are due, at least partly, to the facilitation of the descending noradrenergic pathway and α_2 -AR-mediated inhibition of spinal astrocytic activation. The efficacy of tramadol is comparable to that of amitriptyline, which is a first-choice drug for the treatment of neuropathic pain. Thus, tramadol is a viable and effective treatment for neuropathic pain with non-classical mechanisms of action.

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Conflicts of Interest

The authors indicated no potential conflicts of interest.

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