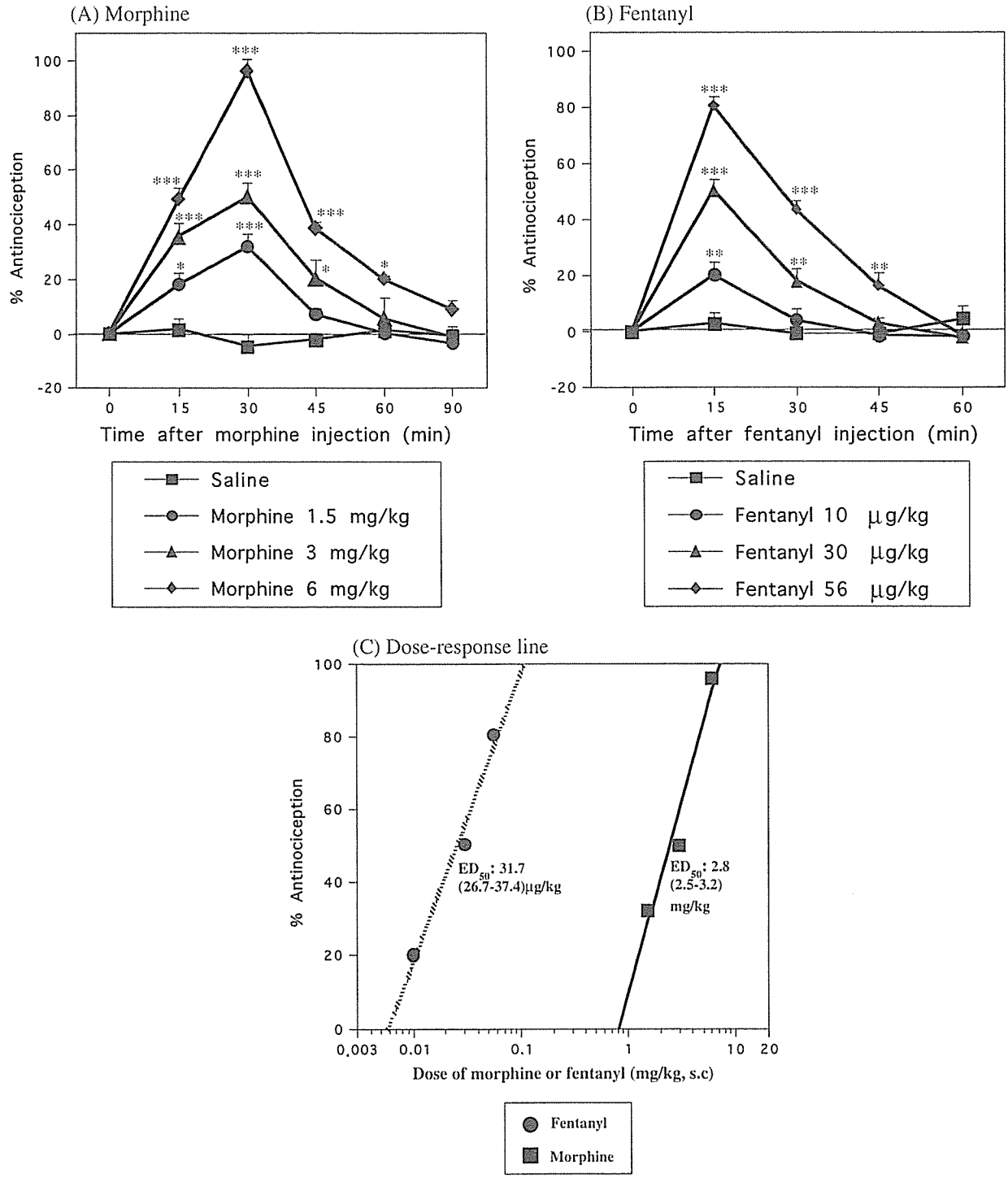

Fig. 1. Antinociceptive effect of μ-opioid receptor agonist following s.c. administration in the ferret by Randall–Selitto test. Time courses (A–B) and dose–response line (C) for antinociception induced by morphine or fentanyl in ferrets. The dose–response line data represent the observation of 30 or 15 min after morphine or fentanyl injection, respectively. Antinociception was expressed as %Antinociception. Each data represents the mean ± S.E.M. of 4–6 ferrets. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. saline group.

antinociception induced by morphine or fentanyl was 2.8 (2.5–3.2) mg/kg or 31.7 (26.7–37.4) μg/kg, respectively (Fig. 1C). The ED_{50} value for fentanyl-induced antinociception was approximately 90 times lower than that of morphine.

3.2. *Effect of baclofen on* μ-opioid receptor agonist-induced antinociception in ferret

Groups of ferrets were pretreated s.c. with vehicle or baclofen (0.3 mg/kg) at 30 min before the animals were

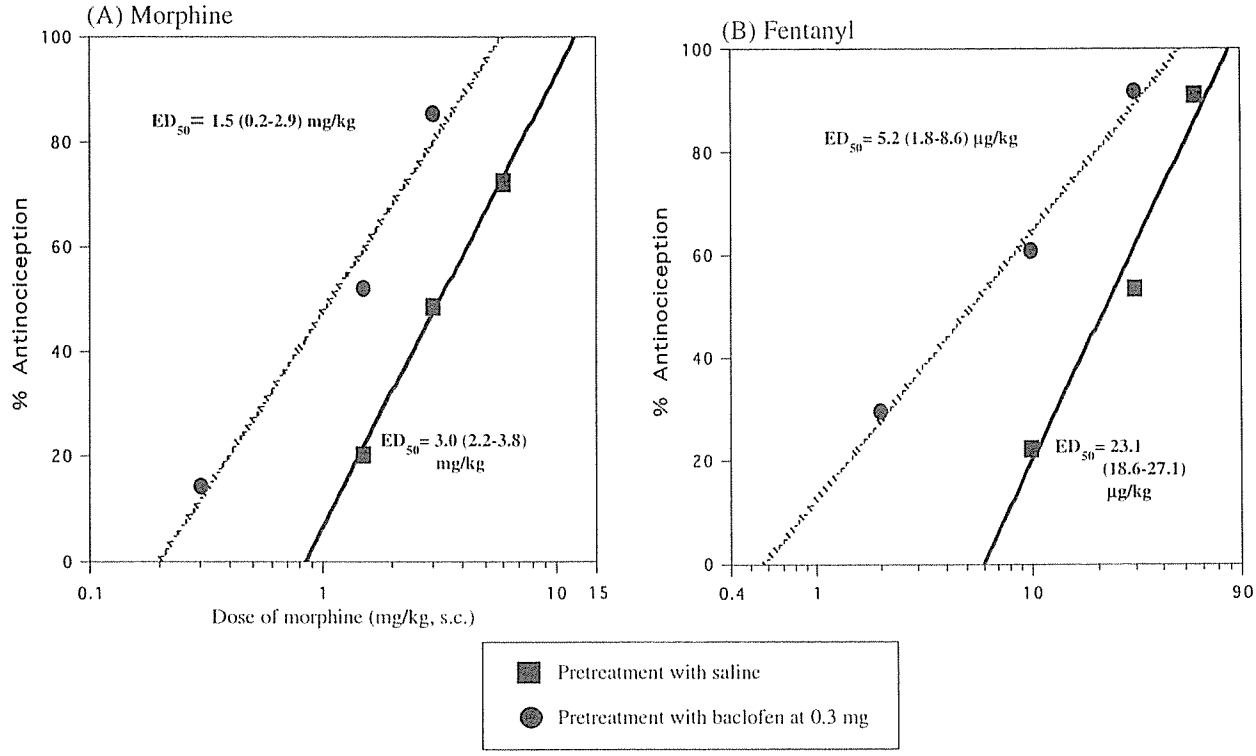

Fig. 2. Effects of baclofen on the μ-opioid receptor agonist-induced antinociception in ferrets. Groups of ferrets were pretreated s.c. with baclofen (0.3 mg/kg) at 30 min before morphine or fentanyl injection. The antinociceptive response was measured at 30 min or 15 min after morphine or fentanyl injection, respectively. Antinociception was expressed as %Antinociception. Each data represents the mean ± S.E.M. of 4–6 ferrets. The ED_{50} values for antinociception induced by morphine or fentanyl with or without baclofen are given in the figure. Values in parentheses indicate the 95% confidence range.

challenged s.c. with various doses of μ-opioid receptor agonist. Pretreatment with baclofen significantly enhanced the antinociceptive effect induced by either morphine or fentanyl relative to pretreatment with saline (Fig. 2). The ED_{50} values of morphine and fentanyl in ferrets pretreated with baclofen were significantly reduced from 3.0 (2.2–3.8) mg/kg and 23.1 (18.6–27.1) μg/kg to 1.5 (0.2–2.9) mg/kg and 5.2 (1.8–8.6) μg/kg ($p < 0.05$ vs. saline-pretreated group), respectively.

3.3. *Effect of baclofen on the* μ-opioid receptor agonist-induced antinociception in mice

In the mouse tail-flick test, either morphine or fentanyl produced a marked antinociception. The maximal antinociceptive response induced by morphine and fentanyl reached at 30 min and 15 min after the injection, respectively (data not shown). The ED_{50} values for the antinociception induced by morphine or fentanyl were 1.9 (1.1–3.1) mg/kg or 25.9 (17.3–38.8) μg/kg, respectively (Table 1). Pretreatment with baclofen enhanced the antinociceptive effect induced by morphine ($p < 0.01$ vs. saline-pretreated group) and the ED_{50} value (1.9 (1.1–3.1) mg/kg, s.c.) was significantly decreased (ED_{50} = 0.8 (0.5–1.3) mg/kg, Table 1). Like morphine, pretreatment with baclofen potentiated the antinociceptive effect induced by fentanyl ($p < 0.01$ vs. saline-pretreated group) and the ED_{50} value (25.9 (17.3–38.8) μg/kg, s.c.) was significantly decreased (ED_{50} = 13.8 (9.33–20.5) μg/kg, Table 1).

3.4. *Effect of i.t. or i.c.v. administration of CGP 35348 on the baclofen-induced augmentation of antinociception of the* μ-opioid receptor agonist in mice

As shown in Fig. 3A and B, the enhancement of morphine-induced antinociception caused by s.c.-administered baclofen (3 mg/kg, s.c.), which had no

Table 1
The ED_{50} values for antinociception induced by morphine or fentanyl with or without baclofen in mice

	ED_{50} values for antinociception	
	Without baclofen	With baclofen
Morphine (mg/kg)	1.9 (1.1–3.1)	0.8 (0.5–1.3)
Fentanyl (μg/kg)	25.9 (17.3–38.8)	13.8 (9.3–20.5)

Groups of mice were pretreated with baclofen (3 mg/kg, s.c.) 30 min before morphine or fentanyl injection. The data represent the result of 30 min or 15 min after morphine or fentanyl injection, respectively. Values in parentheses indicate the 95% confidence range.

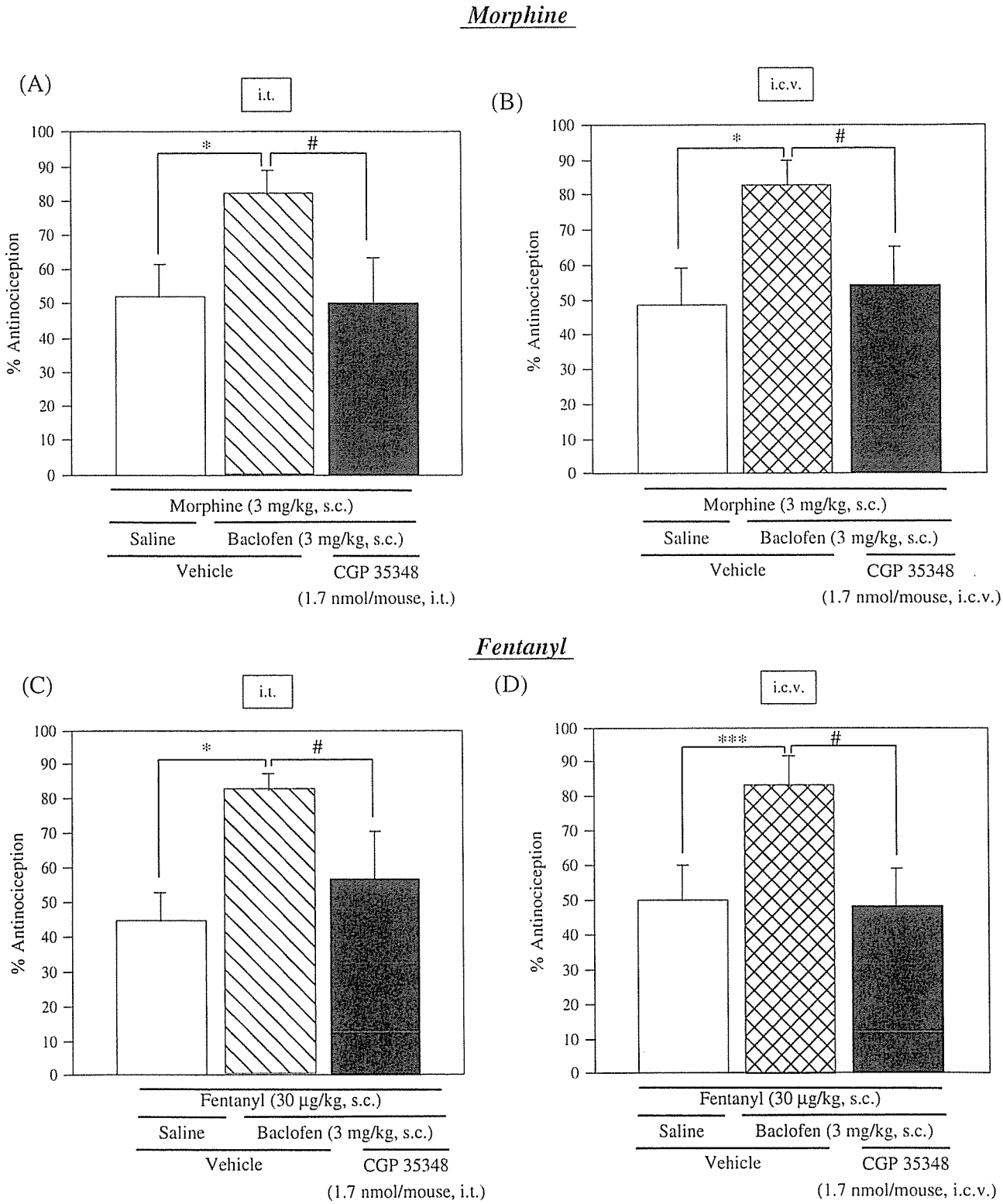

Fig. 3. Effect of pretreatment intrathecally (i.t.) or intracerebroventricularly (i.c.v.) with the selective $GABA_B$ receptor antagonist CGP 35348 on the inhibition of the morphine (A, B) and fentanyl (C, D)-induced antinociception induced by baclofen in mice. Groups of mice were pretreated i.t. (A, C) or i.c.v. (B, D) with CGP 35348 (1.7 nmol/mouse) 10 min prior to baclofen (3 mg/kg, s.c.) injection. The morphine (3 mg/kg, s.c.) or fentanyl (30 μg/kg, s.c.) injection was performed 30 min after baclofen injection. The data represent the result of 30 min or 15 min after morphine or fentanyl injection, respectively. Antinociception was expressed as a percentage of maximum possible effect (%Antinociception). Each bar represents the mean ± S.E.M. of 8–17 mice. $^{**}p < 0.01$ vs. Vehicle + Saline + Saline group, $^{\#\#}p < 0.01$ vs. Vehicle – saline + morphine or fentanyl group, $^{\dagger\dagger}p < 0.01$ vs. Vehicle + baclofen ± morphine or fentanyl group.

antinociceptive effect when given alone (11.8 ± 8.4 %Antinociception), was completely abolished by either i.t. or i.c.v. pretreatment with the selective $GABA_B$ antagonist CGP 35348 (1.7 nmol/mouse). Similarly, the augmentation of the fentanyl-induced antinociception caused by s.c. injection of baclofen (3 mg/kg, s.c.), which had no antinociceptive effect when given alone (9.0 ± 6.0 %Antinociception), was completely abolished by either i.t. or i.c.v. pretreatment with CGP 35348 in mice (Fig. 4C and D). The dose of CGP 35348 (1.7 nmol/mouse) for either i.t. or i.c.v. injection failed to cause the nociceptive effect in mice (i.t., 4.8 ± 5.0 %Antinociception: i.c.v., 2.5 ± 2.0 %Antinociception).

3.5. Motor incoordination produced by baclofen in mouse rota-rod assay

In the rota-rod test, baclofen (10 mg/kg) when given s.c. produced a significant motor impairment in mice at 30 min after the injection compared to saline-treated mice, whereas baclofen at doses of 1, 3 and 5.6 mg/kg did not produce motor dysfunction (Fig. 4).

3.6. The emetic response after s.c. injection of μ-opioid receptor agonists in ferrets

Morphine (0.1–3.0 mg/kg, s.c.) induced an increase in the number of either retching or vomiting and showed the bell-shaped dose–response curve for the expression of retching and vomiting in ferrets (Fig. 5B, C). In contrast, fentanyl (1.0–56.0 μg/kg, s.c.) failed to induce either retching or vomiting in ferrets (Fig. 5A). The peak time of morphine-induced vomiting and retching was achieved at 6–8 min after the s.c. injection (0.6 mg/kg). This effect disappeared 15 min after the injection. The optimal dose of retching and vomiting responses induced by morphine was achieved at 0.6 mg/kg (s.c.) ($^{**}p < 0.01$, $^{***}p < 0.001$ vs. saline group, Fig. 5). The number of retching and vomiting was decreased at 1.2 mg/kg (s.c.) of morphine and completely abolished at 3 mg/kg (s.c.) of morphine.

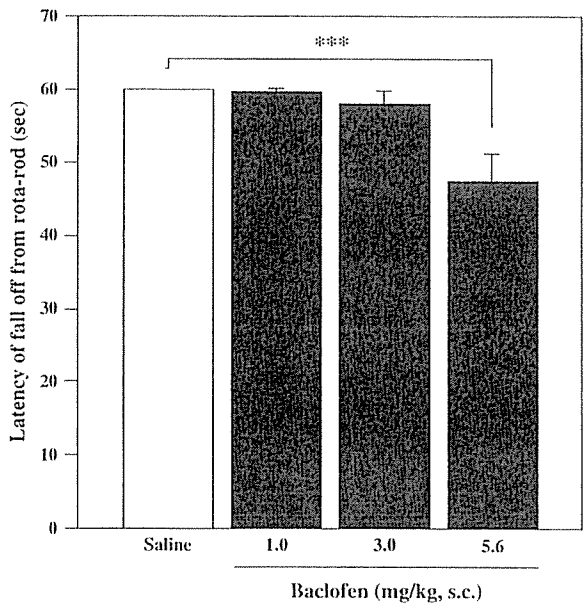

Fig. 4. Motor incoordination produced by baclofen in mice. The motor incoordination produced by baclofen was evaluated by the time until fall-off from a rota-rod at 8 rpm. Next day of the final training for walking on the rota-rod, the time until fall-off from the rota-rod was measured for 60 s at 30 min after the baclofen (1, 3 and 5.6 mg/kg, s.c.) injection. Each bar represents the mean ± S.E.M. of 10–12 mice. $^{***}p < 0.001$ vs. saline group.

3.7. Effect of baclofen on the morphine-induced retching and vomiting in ferrets

The next study was to investigate the effect of baclofen on the morphine-induced emesis in ferrets. As shown in Fig. 6, all doses of baclofen (0.1, 0.3 and 1 mg/kg) when given s.c. inhibited the morphine-induced emetic response, and the doses of 0.3 and 1 mg/kg of baclofen (s.c.) completely abolished these responses ($^{***}p < 0.001$ vs. saline group). The s.c. administration of baclofen did not induce the muscle relaxation in ferrets (data not shown).

3.8. Effect of baclofen on the μ-opioid receptor agonist-induced rewarding effect

The rewarding effect of μ-opioid receptor agonist was assessed by counter balance methods in rats as described in Section 2. Either morphine (8 mg/kg, s.c.) or fentanyl (56 μg/kg, s.c.) produced a significant place preference in rats. Under these conditions, as shown in Fig. 7, pretreatment with baclofen (1.5 and 3 mg/kg, s.c.) significantly suppressed both morphine- and fentanyl-induced rewarding effects in rats ($^{*}p < 0.05$, $^{**}p < 0.01$ vs. saline-morphine or saline-fentanyl group), whereas baclofen alone failed to show the place preference or aversion.

4. Discussion

In the present study, we found that the antinociceptive effect of fentanyl observed in ferrets or mice was much potent than that induced by morphine. These findings are consistent with both clinical experiences (Mather, 1983) and our previous study (Narita et al., 2002). We also demonstrated that co-administration of baclofen potentiated the antinociception induced by either morphine or fentanyl in both mice and ferret. In addition, the augmentation of antinociception induced by systemic administration of baclofen at the dose

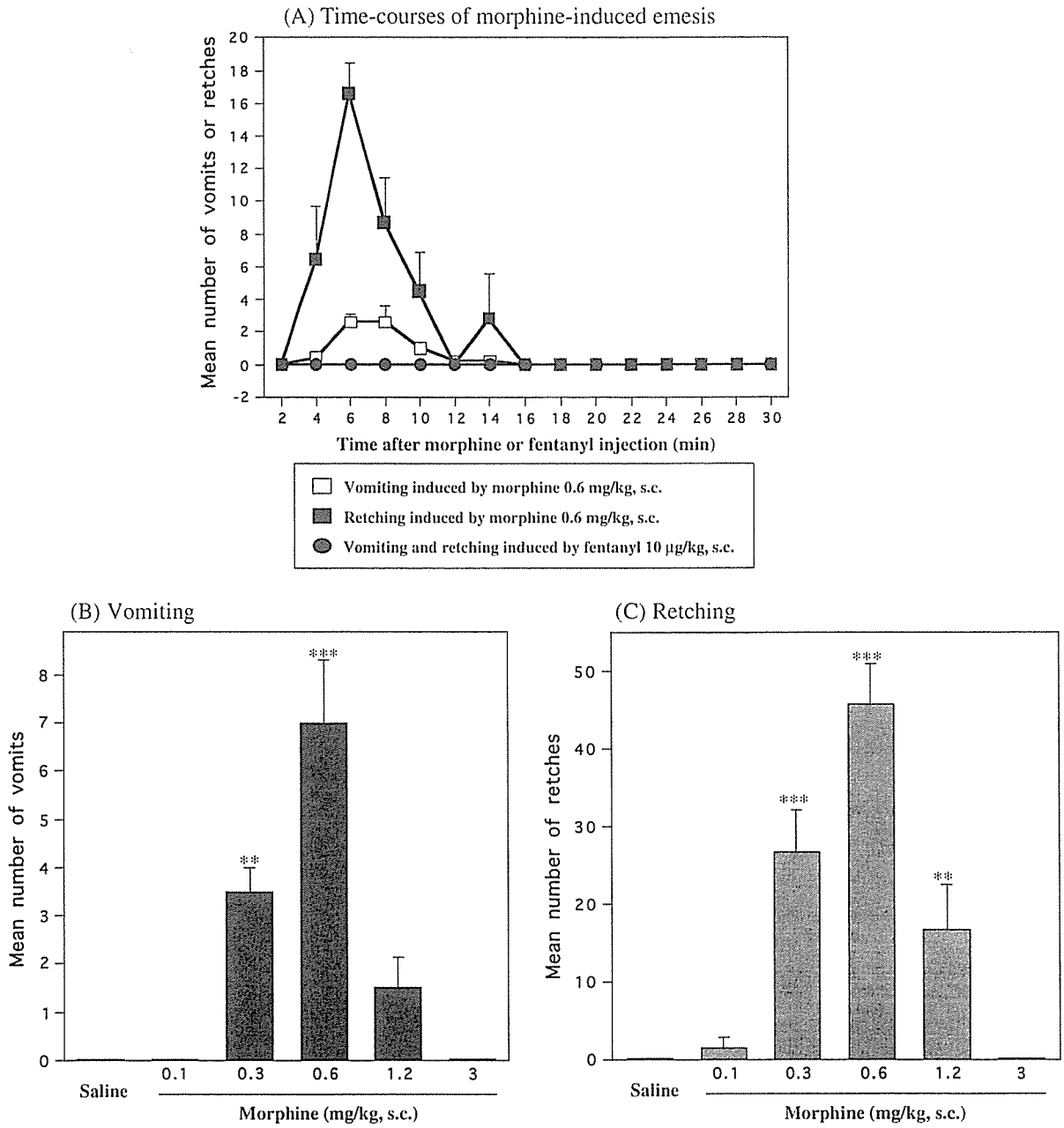

Fig. 5. (A) Time course changes in emetic response induced by s.c. injection of μ-opioid receptor agonists in ferrets. Each point represents the mean number of vomits or retches in 2 min interval time ± S.E.M. of 4–6 ferrets. Animals were observed for 30 min after s.c. injection of morphine (0.6 mg/kg) or fentanyl (10 μg/kg). (B) and (C) Dose relationship for the morphine-induced emetic response in ferrets. Each column represents the mean number of vomiting (B) or retching (C) ± S.E.M. of 4–6 ferrets. Animals were observed for 30 min after s.c. injection of morphine (0.1–3.0 mg/kg).

(3 mg/kg, s.c.) that did not cause motor dysfunction, muscle relaxation or sedation with morphine or fentanyl was completely abolished by either i.c.v. or i.t. pretreatment with the selective $GABA_B$ receptor antagonist CGP 35348 in mice. It has been widely accepted that the nociceptive signals to the CNS are transmitted primarily by sensory afferent fibers to the substantia gelatinosa in the dorsal horn, with further rostral spread to the ventral-posterior nucleus of the thalamus (Craig et al., 1994; Han et al., 1998). Furthermore, it is considered that nociceptive information is transmitted by neuronal pathway projecting from thalamus to the various brain regions such as cortex, which is involved in identifying the source of the pain. Thus, pain processing is inhibited at the spinal and supraspinal levels. In the spinal cord, opioid receptors are located pre- and post-synaptically at interneurons of the substantia gelatinosa of the dorsal horn of the spinal cord. The opioid interneurons inhibit the release of excitatory transmitters and reduce the transmission of the pain signal from the primary

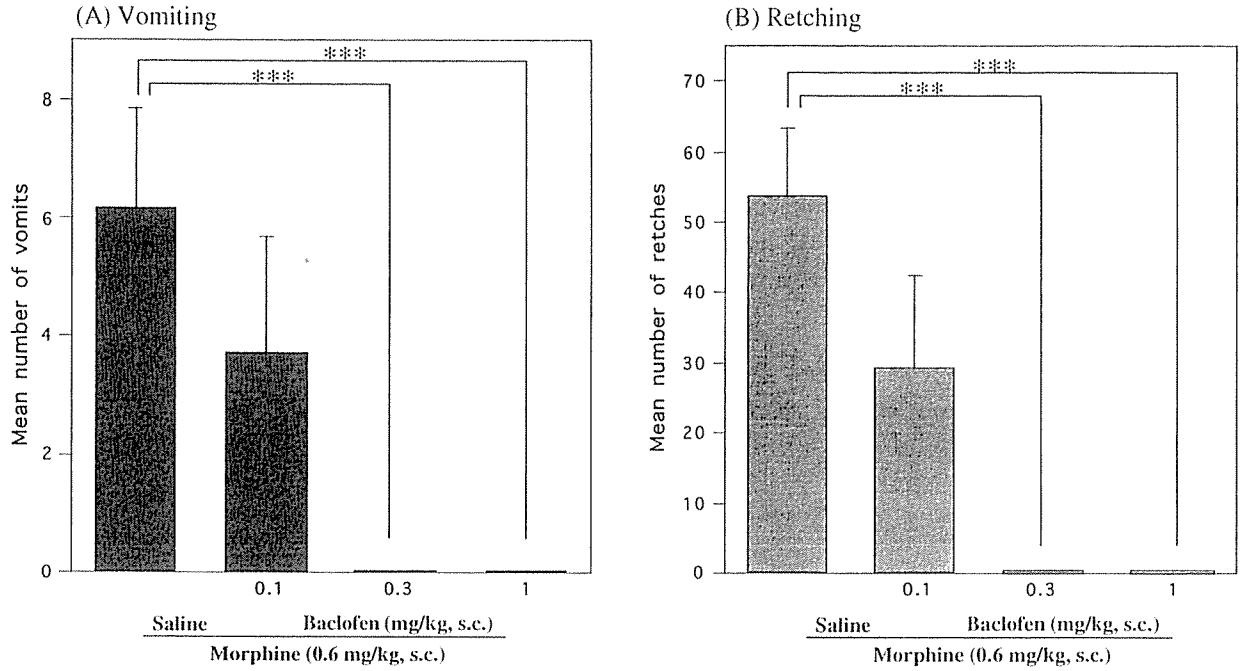

Fig. 6. Effects of baclofen on the morphine-induced vomiting (A) or retching (B) in ferrets. Groups of ferrets were pretreated with baclofen (0.1, 0.3 and 1 mg/kg, s.c.) 30 min before morphine (0.6 mg/kg, s.c.) injection. Animals were observed for 30 min after s.c. injection of morphine. Each data represents the mean ± S.E.M. of 4–6 ferrets. ***$p < 0.001$ vs. saline group.

afferents to the secondary neurons of the ascending spinal pain pathway (Hohmann et al., 1999; Jodie et al., 2000; Abbadie et al., 2001). Supraspinally, opioids are located in different pain-related regions such as the brainstem, periaqueductal gray matter, limbic system, thalamic nuclei, basal ganglia and cortex (Abbadie et al., 2000). In addition to the inhibitory effect at the spinal site and at the ascending pain transmission, opioids

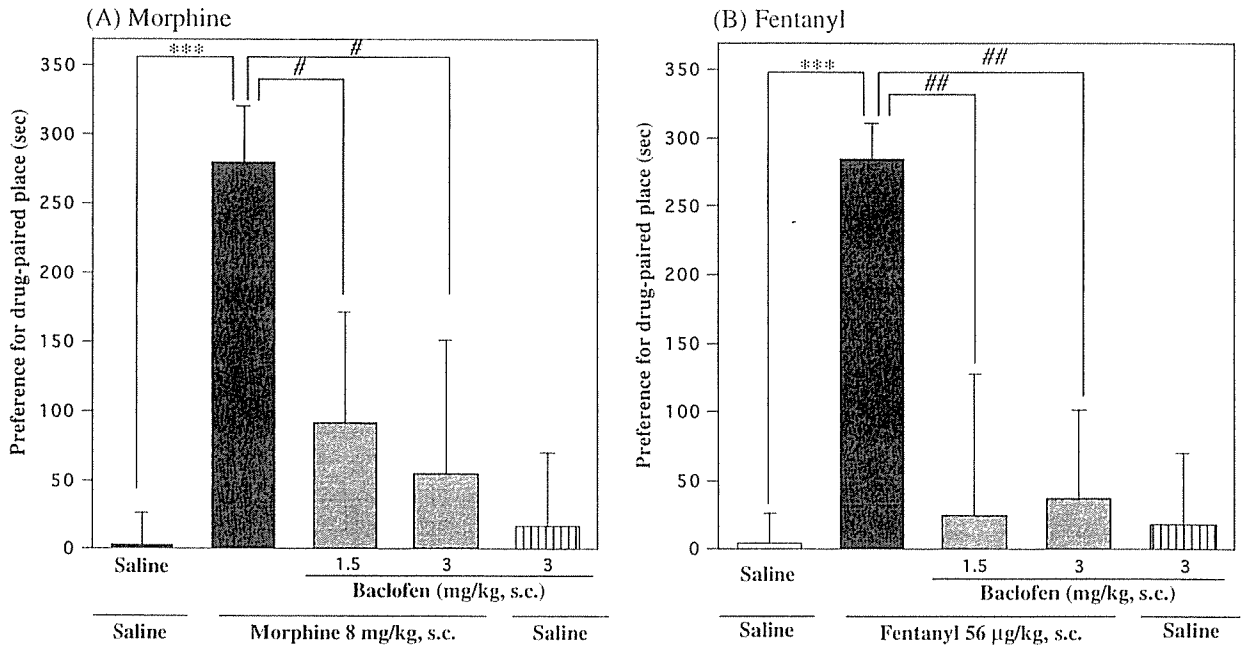

Fig. 7. Effect of baclofen on the morphine (A)- or fentanyl (B)-induced place preference in rats using conditioned place preference test. Groups of rats were pretreated with baclofen (1.5 and 3 mg/kg) at 30 min before morphine (8 mg/kg, s.c.) or fentanyl (56 μg/kg, s.c.) injection, respectively. Ordinate: mean difference(s) between times spent in the drug- and saline-paired sides of the test box (preference for drug-paired place). Each data represents the mean ± S.E.M. of 6–12 rats. ***$p < 0.001$ vs. saline – saline group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. saline – μ-opioid receptor agonist group.

activate a descending pain inhibitory system, which originates from different centers of the pons and medulla. High affinity binding sites for $GABA_B$ receptors are widely located through the central nervous system including the pain-related regions such as the spinal cord, thalamus and frontal cortex (Bowery et al., 1987; Towers et al., 2000). In addition, $GABA_B$ receptors are also located in the neuron terminals of the primary afferent Aδ and C fibers (Towers et al., 2000). Taken together, the present findings support the idea that the augmentation of antinociception induced by systemic administration of baclofen with morphine or fentanyl may result from the potentiation of μ-opioid receptor-mediated inhibition of pain-like stimulation through both the spinal and supraspinal $GABA_B$ receptors.

In the management of pain, nausea and vomiting are one of the most distressing adverse effects. The quality of life has been shown to be significantly reduced in patients who experience opioid-induced nausea and vomiting. Considering the clinical experiences, we next investigated the emetic response induced by μ-opioids in ferrets. As a result, morphine with lower doses than that used for antinociceptive assay produced an increase in the number of either retching or vomiting. The bell-shaped dose–response curve for emesis induced by morphine in the present study was consistent with previous reports using ferrets (Barnes et al., 1991; Thompson et al., 1992; Wynn et al., 1993). We also found that morphine produced a rapid onset and short duration of emetic response in ferrets. The maximal effect of emetic response induced by morphine was approximately achieved at 6–8 min after the injection. It was quite different from the antinociceptive effect that was achieved at 30 min after the injection. These findings indicate the different mechanism and site of action between emesis and antinociception induced by morphine. Regarding the clinical experiences, fentanyl is believed to produce nausea and vomiting in some cases, whereas we demonstrated here that fentanyl failed to produce retching or vomiting in ferrets. Although the specific reason for these discrepancies between the clinical experiences and our results remains unclear at this point, it could result from the different physiology or metabolism between the ferret and human. Another possibility results from the situation that some cancer patients are received with anticancer drugs and radiotherapy as well as fentanyl treatment. Both treatments are able to induce nausea and vomiting in the clinical (Morrow et al., 1998; Oettle and Riess, 2001).

The key finding in the present study is that baclofen at doses used in the present study significantly suppressed the morphine-induced retching or vomiting in ferrets, indicating the involvement of $GABA_B$ receptors in emetic control pathway. The emetic reflex is regulated by the dorsal vagal complex (DVC) in the brainstem (Miller, 1999). The DVC includes the area postrema, the nucleus of the solitary tract, and the dorsal motor nucleus of the vagus (DMN) (Leslie, 1985). The DVC is also the major site of termination of vagal afferent fibers from the gut (Andrews et al., 1990). The whole cell voltage-clamp analysis demonstrated that GABA mediates the inhibitory synaptic currents in these areas (Travagli et al., 1991). Thus, the inhibitory effect of baclofen on the morphine-induced emesis may result from direct inhibitory action of baclofen in DVC neurons.

A number of clinical studies have suggested that undue anxiety about psychological dependence on morphine in cancer patients has caused physicians and patients to use inadequate doses of opioids (WHO, 1996). In the present study, we found that baclofen inhibited either morphine- or fentanyl-induced place preference in rats. Various studies have pointed out that the mesolimbic dopamine system is a critical pathway for the initiation of opioid-induced reinforcement (Funada et al., 1995; Koob et al., 1998; Narita et al., 2001). It is widely accepted that the dopamine neuronal activity in the ventral tegmental area (VTA), which is a critical area for the initiation of the μ-opioid-induced reinforcement, is modulated by either GABA-containing inhibitory interneurons or excitability glutamatergic neurons originated from the medial prefrontal cortex (mPfc) (Kalivas et al., 1990; Koob, 1992). Intra-VTA microinjection of [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]enkephalin (DAMGO) produced a decrease in the extracellular GABA level in the VTA (Narita et al., 2001), resulting in the expression of rewarding effect. We previously demonstrated that microinjection of the baclofen into the VTA significantly suppressed the morphine-induced place preference (Tsuji et al., 1996). Furthermore, it has been reported that the stimulation of $GABA_B$ receptors in the mPfc causes the inhibition of glutamate release in the VTA (Harte and O'Connor, 2005). Taken together, the inhibitory effect of baclofen on the morphine-induced rewarding effect appears to result from either direct inhibitory action of baclofen in the VTA neurons or cortical $GABA_B$ receptor-mediated inhibition of glutamate transmission in the VTA. Thus, the present study provides further evidence for a critical role of $GABA_B$ receptors in the μ-opioid-regulated rewarding system.

Finally, we conclude that fentanyl produces potent antinociception with much less emesis compared to morphine. Furthermore, the key finding of the present study was that the highly selective $GABA_B$ receptor agonist baclofen at the doses that significantly potentiated the μ-opioid-induced antinociception without apparent adverse effects such as motor dysfunction, sedation and muscle relaxation markedly suppressed the morphine-induced rewarding effects, retching and vomiting. We, therefore, propose here that co-administration

of baclofen with opioids may pave the way for the new strategy for the control of pain.

Acknowledgments

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Involvement of spinal metabotropic glutamate receptor 5 in the development of tolerance to morphine-induced antinociception

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Abstract

It is well known that prolonged exposure to morphine results in tolerance to morphine-induced antinociception. In the present study, we found that either intrathecal (i.t.) or subcutaneous (s.c.) injection of the selective metabotropic glutamate receptor 5 (mGluR5) antagonist, methyl-6-(phenylethynyl)-pyridine hydrochloride (MPEP), attenuated the development of tolerance to morphine-induced antinociception. Using the receptor binding assay, we found here that the number of mGluR5 in the mouse spinal cord was significantly increased by repeated treatment with morphine. Furthermore, repeated treatment with morphine produced a significant increase in the level of mGluR5 immunoreactivity in the dorsal horn of the mouse spinal cord. Double-labeling experiments showed that the increased mGluR5 was predominantly expressed in the neurons and sparsely expressed in the processes of astrocytes following repeated treatment with morphine. Consistent with these results, the response of Ca^{2+} to the selective group I mGluR agonist, 3,5-dihydroxyphenylglycine (DHPG), in cultured spinal cord neurons was potently enhanced by 3 days of *in vitro* treatment with morphine. These findings support the idea that the increased mGluR5 following repeated treatment with morphine leads to enhanced neuronal excitability and synaptic transmission in the dorsal horn of the spinal cord and, in turn, suppresses the morphine-induced antinociception in mice.

Keywords: antinociception, metabotropic glutamate receptor 5, morphine, spinal cord, synaptic plasticity, tolerance.

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Glutamate is the major excitatory neurotransmitter in the mammalian CNS, the actions of which are regulated by ionotropic glutamate receptors (iGluRs) and metabotropic glutamate receptors (mGluRs). mGluRs have been identified and classified into three groups according to their sequence homology, signal transduction pathways and pharmacological selectivity: group I (mGluR1 and mGluR5), group II (mGluR2 and mGluR3) and group III (mGluR4, mGluR6, mGluR7 and mGluR8). Of these, group I mGluRs are predominately located in postsynaptic neurons where they couple to G_q proteins to activate phospholipase C (PLC). PLC catalyzes the production of diacylglycerol (DAG), which activates protein kinase C (PKC), and inositol (1,4,5)-triphosphate (IP_3), which activates IP_3 receptor to release Ca^{2+} from intracellular stores (Conn and Pin 1997; Schoepp 2001).

In the spinal cord, glutamate mediates the transmission of sensory information. Recent behavioral and electrophysiological evidence has shown that administration of selective mGluR1 and mGluR5 agonists enhances behavioral responses to noxious stimulation and induces activity in dorsal horn neurons (Fisher and Coderre 1996; Neugebauer *et al.* 1999). Expression of mGluR5 is predominantly found in the soma and dendrites of superficial dorsal horn neurons and sparsely found in the astrocytes (Alvarez *et al.* 2000; Gallo and Ghiani 2000; Tao *et al.* 2000). Recent observations have revealed

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Abbreviations used: BCA, bicinchoninic acid; DAG, diacylglycerol; DHPG, 3,5-dihydroxyphenylglycine; F_0, baseline fluorescence; GFAP, glial fibrillary acidic protein; i.c.v., intracerebroventricular; IP_3, inositol (1,4,5)-triphosphate; IR, immunoreactivity; i.t., intrathecal; LSN, lateral spinal nucleus; MAP2a/b, microtubule-associated protein 2a/b; mGluR5, metabotropic glutamate receptor 5; MPEP, methyl-6-(phenylethynyl)-pyridine hydrochloride; NeuN, neuronal nuclei; NGS, normal goat serum; PBS, phosphate-buffered saline; PI, phosphoinositide; PKC, protein kinase C; PLC, phospholipase C; SDS, sodium dodecyl sulfate; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; TBS, Tris-buffered saline.

that glial mGluRs can be involved in the interaction between glia and neurons in physiological as well as pathological conditions (Aronica *et al.* 2000; Peavy *et al.* 2002).

The administration of morphine into the spinal cord produces a powerful antinociception/analgesia (Besse *et al.* 1990). It is well known that prolonged exposure to morphine results in tolerance to morphine-induced antinociception (Narita *et al.* 1994, 2002; Smith *et al.* 2003). We previously reported that repeated *in vivo* treatment with morphine induced astroglial hypertrophy associated with activating neuronal PKC in the mouse spinal cord (Narita *et al.* 2004). It has been documented that systemic and brain injections of mGluR5 antagonists significantly attenuate the development of tolerance to morphine-induced antinociception (Kozela *et al.* 2003; Smith *et al.* 2004). However, the specific contribution of mGluR5 in the spinal cord to the suppression of tolerance to morphine-induced antinociceotion remains unclear. In the present study, we therefore investigated whether the spinal mGluR5 could contribute to the development of tolerance to morphine-induced antinociception in mice.

Materials and methods

Animals

The present study was conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, Hoshi University, as adopted by the Committee on Animal Research of Hoshi University, which is accredited by the Ministry of Education, Culture, Sports, Science and Technology of Japan. We used male ICR mice (Tokyo Laboratory Animals Science Co., Ltd, Tokyo, Japan). Animals were kept in a room with an ambient temperature of 23 ± 1°C and a 12 h light–dark cycle (lights on 08.00–20.00 hours). Food and water were available *ad libitum*.

Drugs and intrathecal injection procedure

Morphine hydrochloride (Sankyo, Tokyo, Japan) and methyl-6-(phenylethynyl)-pyridine hydrochloride (MPEP; Sigma-Aldrich, St Louis, MO, USA) were dissolved in saline. 3,5-Dihydroxyphenylglycine (DHPG) was purchased from Tocris (Ellisville, MO, USA). Intrathecal (i.t.) injection was performed according to the method described by Hylden and Wilcox (1980) using a 25 μL Hamilton syringe with a 30 gauge 1/2 inch needle. The volume for i.t. injection was 4 μL per mouse.

Assessment of antinociception

The morphine-induced antinociceptive response was evaluated by recording the latency to paw licking or tapping in the hot-plate test (55 ± 0.5°C; Muromachi Kikai Co., Ltd, Tokyo, Japan). To prevent tissue damage, we established a 30 s cut-off time. The test was performed 30 min after morphine treatment. Each animal served as its own control, and the latency to response was measured both before and after drug administration. To investigate the development of antinociceptive tolerance following repeated treatment with morphine, mice were repeatedly injected with morphine (10 mg/kg s.c.) or saline (0.1 mL/kg s.c.) once a day for 7 consecutive days. In the combination study, MPEP (10 mg/kg, s.c. or 1 nM/mouse, i.t.) was administered 30 min before s.c. treatment with morphine. Antinociception was calculated as percentage of the maximum possible effect (% antinociception) according to the following formula: % antinociception = (test latency − pre-drug latency)/(cut-off time − pre-drug latency) × 100. The antinociceptive response represents the mean ± SEM of percentage antinociception.

Western blotting

For membrane preparation, the spinal cords of mice were rapidly removed following decapitation 24 h after repeated morphine injection, and homogenized in ice-cold buffer containing 20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 0.5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 25 μg/mL leupeptin and 0.1 mg/mL aprotinin. Protein concentration in the samples was assayed by the method of Bradford (1976). An aliquot of tissue sample was diluted with an equal volume of 2 × electrophoresis sample buffer (Protein Gel Loading Dye-2 x; Amresco, Solon, OH, USA) containing 2% sodium dodecyl sulfate (SDS) and 10% glycerol with 0.2 M dithiothreitol. Proteins (10 μg/lane) were separated by size on 4–20% SDS-polyacrylamide gradient gel using the buffer system, then transferred to nitrocellulose membranes in Tris-glycine buffer containing 25 mM Tris and 192 mM glycine. For immunoblot detection, membranes were blocked in Tris-buffered saline (TBS) containing 5% non-fat dried milk (Bio-Rad Laboratories, Hercules, CA, USA) for 1 h at room temperature with agitation. The membrane was incubated with primary antibody diluted in TBS [1 : 50 000 mGluR5 (Upstate, Virginia, VA, USA)] containing 5% non-fat dried milk, overnight, at 4°C. The membrane was washed in TBS containing 0.05% Tween 20 (TTBS), followed by a 2 h incubation at room temperature with horseradish peroxidase-conjugated goat anti-rabbit IgG (Southern Biotechnology Associates, Inc., Birmingham, AL, USA) diluted 1 : 10 000 in TBS containing 5% non-fat dried milk. The antigen-antibody peroxidase complex was finally detected by enhanced chemiluminescence (Pierce, Rockford, IL, USA) according to the manufacture's instructions and visualized by exposure to Amersham Hyperfilm (Amersham Life Sciences, Arlington Heights, IL, USA).

Immunohistochemistry

Mice were repeatedly injected with morphine (10 or 20 mg/kg, s.c.) or saline (0.1 mL/kg, s.c.) once a day for 7 days. Twenty-four hours after the last injection, mice were deeply anesthetized with sodium pentobarbital (70 mg/kg, i.p.) and perfusion-fixed with 4% paraformaldehyde (pH 7.4). The spinal cords were quickly removed and post-fixed in 4% paraformaldehyde for 2 h. Spinal cord sections were prepared as described previously (Narita *et al.* 2004). Sections were cut transversely at a thickness of 10 μm on a cryostat (Leica CM1510, Leica Microsystems, Heidelberg, Germany). The spinal cord sections were blocked in 10% normal goat serum (NGS) in 0.01 M phosphate-buffered saline (PBS) for 1 h at room temperature. Each primary antibody was diluted in 0.01 M PBS containing 10% NGS [1 : 3000 mGluR5 (Upstate), 1 : 320 microtubule-associated protein 2a/b (MAP2a/b, Chemicon International, Inc., Temecula, CA, USA), 1 : 500 neuronal nuclei (NeuN, Chemicon International, Inc.), 1 : 400 glial fibrillary acidic protein (GFAP, Chemicon International, Inc.) and 1 : 800 S100β (Sigma-Aldrich)] and incubated for 2 days at 4°C. The antibodies were then rinsed

and incubated with an appropriate secondary antibodies conjugated Alexa 488 and Alexa 546 for 2 h at room temperature. In the cases of mGluR5/NeuN, mGluR5/MAP2ab and mGluR5/GFAP double-labelings, at first Alexa 488-conjugated goat anti-rabbit IgG was used (for mGluR5), and after washing out, and challenging the next 1 st antibody to NeuN, MAP2a/b or GFAP, Alexa546-conjugated goat anti-mouse IgG was used (for NeuN, MAP2a/b or GFAP, respectively). In view of mGluR5/S100β double-labeling, Alexa 488-conjugated goat anti-mouse IgG was used for S100β and Alexa546-conjugated goat anti-rabbit IgG was used for mGluR5. The slides were then coverslipped with PermaFluor Aqueous mounting medium (Immunon, Pittsburgh, PA, USA). Fluorescence of immunolabeling was detected using a light microscope (Olympus AX-70; Olympus, Tokyo, Japan) or a confocal microscope (Radiance 2000, Bio-Rad Laboratories). Digitized images of the superficial laminae of spinal dorsal horn sections were captured at a resolution of 1316 × 1035 pixels with a digital camera (Polaroid PDMCII/OL; Olympus). The density of mGluR5 labeling was measured with a computer-assisted imaging analysis system (NIH Image program, developed at the National Institutes of Health available at http://rsb.info.nih.gov/nih.image). The upper and lower threshold density ranges were adjusted to encompass and match the immunoreactivity; this provided an image with immunoreactive material appearing in black pixels and non-immunoreactive material in white pixels. A standardized rectangle was positioned over the superficial laminae of the dorsal horn of the spinal cord area from saline-treated mice. The area and density of pixels within the threshold value representing immunoreactivity were calculated and the integrated density was the product of the area and density. The same box was then 'dragged' to the corresponding position on the superficial laminae of the dorsal horn in the spinal cord area from morphine-treated mice, and the integrated density of pixels within the same threshold was again calculated.

In vitro receptor binding assay

For membrane preparation, the spinal cords of mice were quickly removed 24 h after repeated morphine (10 mg/kg) injection and rapidly transferred to a tube filled with ice-cold buffer. The membrane homogenate was prepared as described previously (Aoki *et al.* 2004). Saturation binding experiments were performed in triplicate with increasing concentrations of [^{3}H]MPEP (0.2–100 nM). The binding assay was carried out by incubation for 2 h, and non-specific binding was determined in the presence of 10 μM MPEP. The binding was terminated by rapid filtration through glass filters (Unifilter-96 GF/C plate; Perkin-Elmer Life Sciences, Emeryville, MA, USA) pre-soaked with 0.3% polyethyleneimine using a 96-well plate cell harvester. Filters were washed three times with ice-cold assay buffer. After the addition of scintillant, radioactivity was determined by liquid scintillation spectrometry (TopCount; Packard Instruments, Meriden, CT, USA). Protein concentrations were measured using the bicinchoninic acid (BCA) compatible protein assay kit (Pierce) with bovine serum albumin as the standard. The binding curves were fitted using the GraphPad prism 4.0 program (Graphpad Software, San Diego, CA, USA).

Confocal Ca^{2+} imaging

Spinal cord neuron/glia co-cultures were grown as follows. Spinal cord was obtained from newborn ICR mice (Tokyo Laboratory Animals Science), minced, and treated with papain (9 U/mL; Worthington Biochemical, Lakewood, NJ, USA) dissolved in PBS solution containing 0.02% L-cysteine monohydrate, 0.5% glucose and 0.02% bovine serum albumin. After enzyme treatment at 37°C for 15 min, cells were seeded at a density of 2×10^6 cells/cm^2. The cells were maintained for 7 days in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Grand Island, NY, USA) supplemented with 10% precolostrum newborn calf serum, 10 U/mL penicillin and 10 μg/mL streptomycin. On day 8, the cells were treated with morphine (10 μM) and MPEP (10 μM) for 3 days. Cells were loaded with 10 μM fluo-3 acetoxymethyl ester (Wako Pure Chemical, MD, USA) for 90 min at room temperature. After a further 20–30 min of de-esterification with the acetoxymethyl ester, the coverslips were mounted on a microscope equipped with a confocal Ca^{2+} imaging system (Radiance 2000, Bio-Rad Laboratories). Fluo-3 was excited with the 488 nm line of an argon-ion laser and the emitted fluorescence was collected at wavelengths >515 nm; average baseline fluorescence (F_0) of each cell preparation was calculated. To compensate for the uneven distribution of fluo-3, self-ratios were calculated (Ratio: $R_s = F/F_0$). DHPG (10–100 μM) was perfused for 30 s at 5 mL/min at room temperature in cultured spinal cord neurons, followed by superfusion of balanced salt saline (BSS, pH 7.4) containing 150 mM NaCl, 5.0 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM $MgCl_2$, 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid and 10 mM D-glucose.

Statistical analysis

The data are presented as the mean ± SEM. The statistical significance of differences between the groups was assessed with Student's *t*-test or one-way ANOVA followed by the Bonferroni/Dunnett test.

Results

Suppression of the development of tolerance to morphine-induced antinociception by pretreatment with MPEP

We first examined the effect of pre-treatment s.c. and i.t. with the selective mGluR5 antagonist, MPEP, on the development of tolerance to morphine-induced antinociception. In both s.c. and i.t. saline-pretreated mice, the s.c. injection of morphine produced about a 70% antinociceptive effect on the first day. However, the antinociception was significantly decreased during consecutive exposure to morphine and was clearly reduced over 7 days, indicating the development of tolerance to morphine-induced antinociception (**$p < 0.01$ and ***$p < 0.001$ vs. the first day of saline-pre-treated morphine group, Figs 1a and b). The development of tolerance to morphine-induced antinociception was significantly suppressed by both s.c. and i.t. pre-injection with MPEP (Fig. 1a: s.c. MPEP-pre-treated morphine group vs. s.c. saline-pre-treated morphine group, $F_{1,18} = 6.67$, $p < 0.05$; Fig. 1b: i.t. MPEP-pre-treated morphine group vs. i.t. saline-pre-treated morphine group, $F_{1,15} = 16.23$, $p < 0.01$).

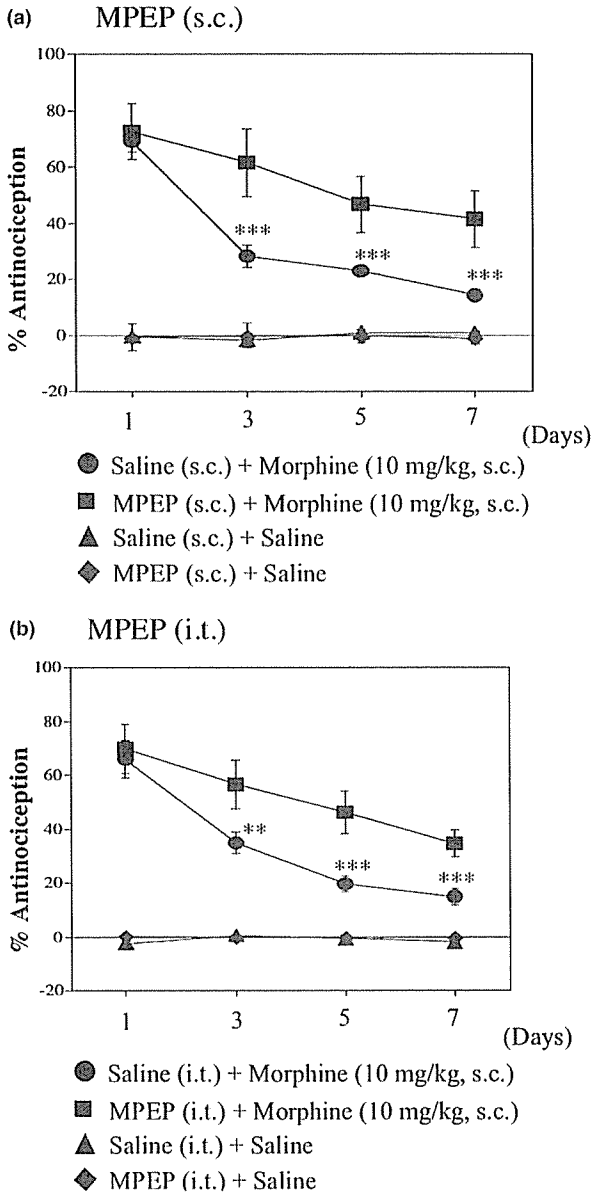

Fig. 1 Effect of pre-treatment with MPEP on the development of tolerance to morphine-induced antinociception. Mice were repeatedly injected with morphine (10 mg/kg s.c.) or saline once a day for 7 consecutive days. MPEP (a) at 10 mg/kg, s.c. or (b) 1 nM/mouse, i.t. was administered 30 min before every morphine treatment. Each point represents percentage antinociception at 30 min after saline or morphine injection. In both s.c. and i.t. saline-pre-treated mice, the antinociception induced by morphine was significantly decreased during consecutive exposure to morphine (**$p < 0.01$ and ***$p < 0.001$ vs. the first day of saline-pre-treated morphine group, a and b). The development of tolerance to morphine-induced antinociception was significantly inhibited by both s.c. and i.t. injection with MPEP (a, s.c. MPEP-pre-treated morphine group vs. s.c. saline-pre-treated morphine group, $F_{1,18} = 6.67$, $p < 0.05$; b, i.t. MPEP-pre-treated morphine group vs. i.t. saline-pre-treated morphine group, $F_{1,15} = 16.23$, $p < 0.01$). Each group used 10–12 mice.

Increase in level of mGluR5 by repeated treatment with morphine in the superficial dorsal horn of the mouse spinal cord

We next examined the change in levels of mGluR5 by monitoring the binding of [^{3}H]MPEP to membranes of the mouse spinal cord following repeated treatment with morphine. Figures 2(a) and 2(b) show the saturation curves and Scatchard plots of [^{3}H]MPEP binding in spinal cord membranes from morphine- and saline-treated mice. The B_{max} value of [^{3}H]MPEP in the membrane preparation from the spinal cord was significantly increased in morphine-treated mice compared with saline-treated mice (*$p < 0.05$). There was no significant different in the K_d values for [^{3}H]MPEP between saline-treated and morphine-treated mice (Fig. 2b). Furthermore, we assessed the effect of repeated treatment with morphine on membranes levels of mGluR5 in

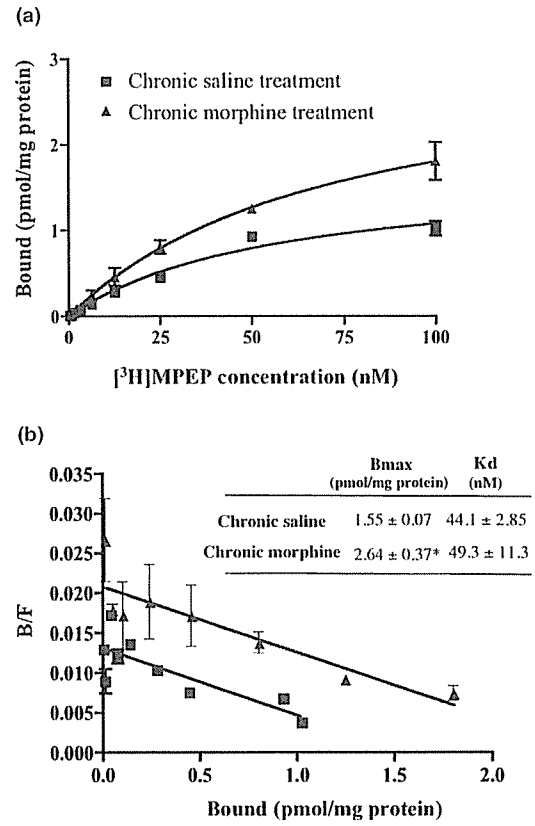

Fig. 2 Saturation curves (a) and Scatchard plots (b) for the specific binding of [^{3}H]MPEP to spinal cord membranes from chronic saline- and morphine-treated mice. Groups of mice were treated with morphine (10 mg/kg s.c.) and saline once a day for 7 consecutive days. Twenty-four hours after the last injection, membrane fractions were prepared. [^{3}H]MPEP binding assay was carried out in a range from 0.2 to 100 nM. The specific binding was defined as the difference in binding observed in the absence and presence of 10 μM unlabeled MPEP. The data represent the mean ± SEM from three separate experiments performed in triplicate. *$p < 0.05$ vs. chronic saline-treated mice.

the mouse spinal cord, as detected by western blotting. The spinal cord membrane was prepared 24 h after the last injection of saline and morphine. Repeated s.c. treatment with morphine produced a significant increase in levels of mGluR5-IR in membranes of mouse spinal cord compared with those found in saline-treated mice (159.9 ± 4.5% of increase, *** $p < 0.001$ vs. saline-treated mice, Fig. 3).

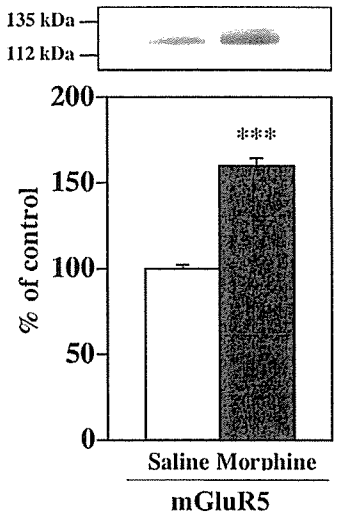

Fig. 3 Influence of levels of mGluR5 in membranes of the mouse spinal cord after repeated morphine treatment. (a) Representative western blot of mGluR5. (b) Changes in immunoreactivity for the mGluR5 in membranes of spinal cords obtained from saline- or morphine-treated mice. Mice were repeatedly injected with saline or morphine (10 mg/kg, s.c.) once a day for 7 consecutive days. The membrane fraction was prepared at 24 h after the last injection. Each column represents the mean ± SEM of three independent samples. *** $p < 0.001$ vs. saline-treated mice.

Twenty-four hours after the last repeated injection of morphine, immunoreactivity (IR) for the mGluR5 in the spinal cord was observed by immunohistochemical analysis. In saline-treated mice, mGluR5-IR was strongly distributed in laminae I and II, progressively decreasing in density in laminae III, IV and V (Fig. 4a). Furthermore, mGluR5-IR was found in the network of lateral spinal nucleus (LSN) neurites located in the dorsolateral funiculus. The most intense mGluR5-IR appeared to be concentrated in the inner part of lamina II (lamina IIi). In the high magnification image of the heavily immunoreactive lamina IIi, it was apparent that the neuropil contained granular immunolabelings were observed surrounding neuronal somata (Fig. 4b). Using semi-quantitative analysis, repeated s.c. treatment with morphine produced a significant increase in the level of mGluR5-IR in the dorsal horn of the spinal cord in a dose-dependent manner (10 mg/kg: 168.9 ± 10.6% of control ***$p < 0.001$ vs. saline-treated mice; 20 mg/kg: 192.9 ± 1.8% of control ***$p < 0.001$ vs. saline-treated mice, Figs 4c–f). It should be mentioned that the increase in mGluR5-IR produced by morphine extended to the outer part of laminae II and I, as well as lamina IIi. Furthermore, double-labeling experiments showed that the neuron-specific nuclear protein maker NeuN-IR (red) in the dorsal horn of the spinal cord was surrounded by mGluR5-IR (green, Figs 5a and b). mGluR5-IR (green) was apparent co-localizing with the dendritic protein maker MAP2a/b-IR (red) in the spinal cord of morphine-treated mice (Fig. 5d). Astrocytes in the dorsal horn of the spinal cord were stained with GFAP. In mice treated chronically with morphine, GFAP-IR (red) in the dorsal horn of the mouse spinal cord was increased with morphological differentiation (Fig. 6a), which was sparsely colocalized with mGluR5-IR (green,

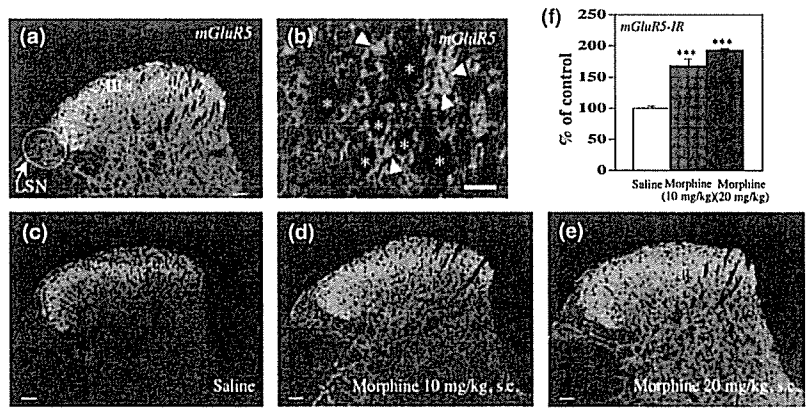

Fig. 4 (a) Distribution of mGluR5-IR in mouse spinal cord. mGluR5-IR was distributed in the inner part of lamina II (IIi) and in the lateral spinal nucleus (LSN; arrow). (b) High magnification of the heavily immunoreactive lamina II. The neuropil contained granular-like mGluR5-IR (arrowheads) and many neuronal somata (astarisks). (c, d, e) Increase in levels of mGluR5-IR in the dorsal horn of the spinal cord following repeated treatment with morphine in a dose-dependent manner. (f) Semi-quantitative analysis of mGluR5-IR was performed using NIH image (10 mg/kg: 168.9 ± 10.6% of control ***$p < 0.001$ vs. saline-treated mice; 20 mg/kg: 192.9 ± 1.8% of control ***$p < 0.001$ vs. saline-treated mice). Each column represents the mean ± SEM of three independent samples. Scale bars: 50 μm.

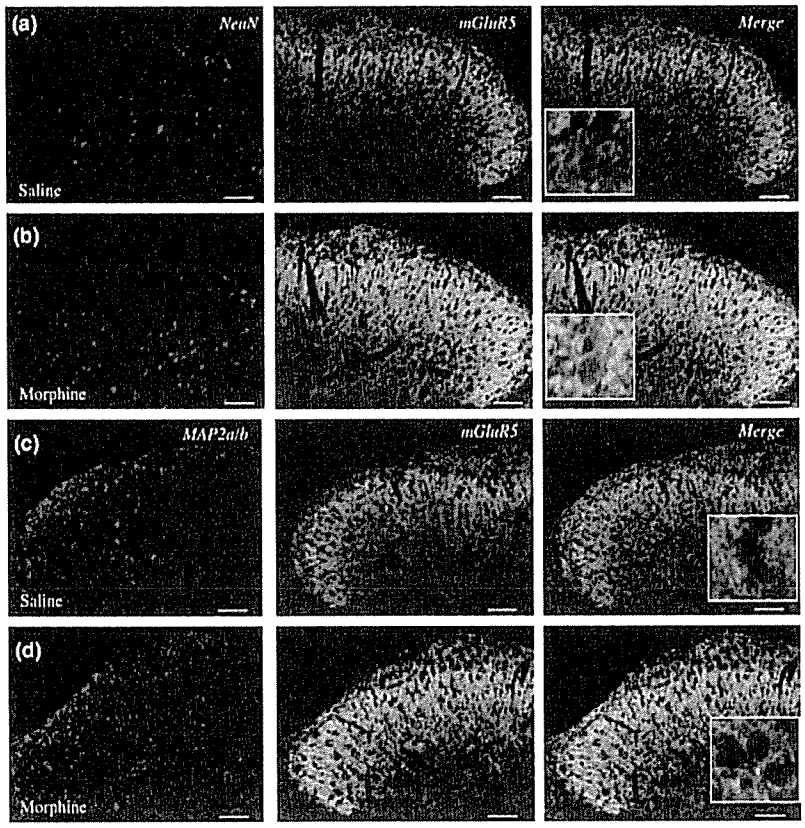

Fig. 5 Localization of the increased mGluR5-IR in the dorsal horn of the spinal cord following repeated treatment with morphine. (a, b) NeuN-IR (red) in the dorsal horn of the spinal cord was surrounded by mGluR5-IR (green). (c, d) The increased mGluR5-IR (green) was more apparent co-localizing with MAP2a/b-IR (red) in the spinal cord of morphine-treated mice compared with saline-treated mice. Scale bars: 50 μm.

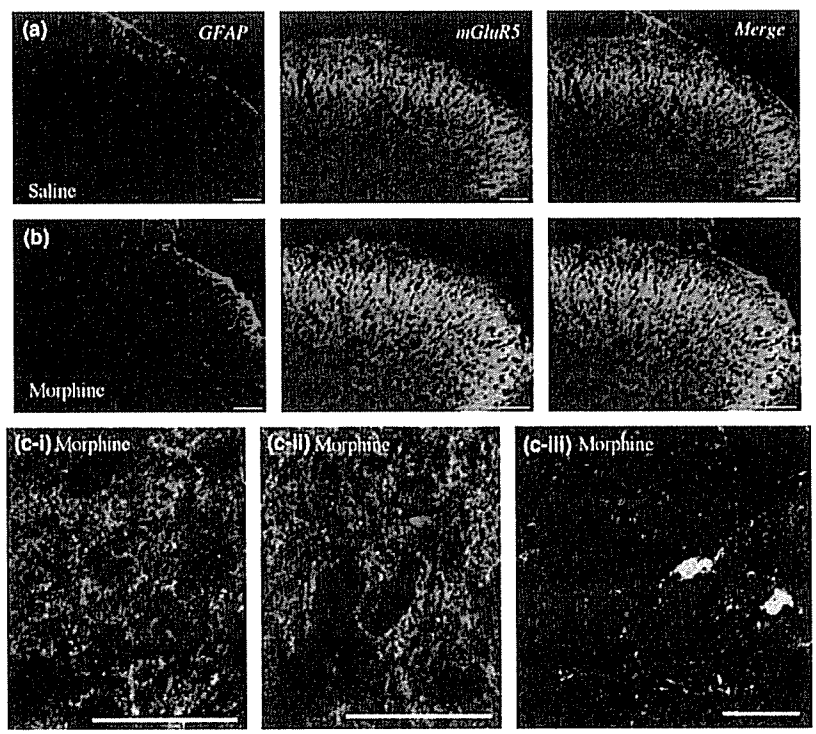

Fig. 6 Localization of the increased mGluR5-IR in the dorsal horn of the spinal cord following repeated treatment with morphine. GFAP-IR (red) in the dorsal horn of mouse spinal cord increased with morphological differentiation after repeated morphine treatment (b), which was sparsely co-localized with the increased mGluR5-IR (green). High magnification image of the superficial laminae of the dorsal horn analyzed by confocal microscope. The red labeled for GFAP and the green labeled for mGluR5 (c-i, c-ii) or the green labeled for S100β and the red labeled for mGluR5 (c-iii) are no apparent co-localization in the superficial layers of the spinal cord of morphine-treated mice. Scale bars: (a, b) = 50 μm; (c) = 10 μm.

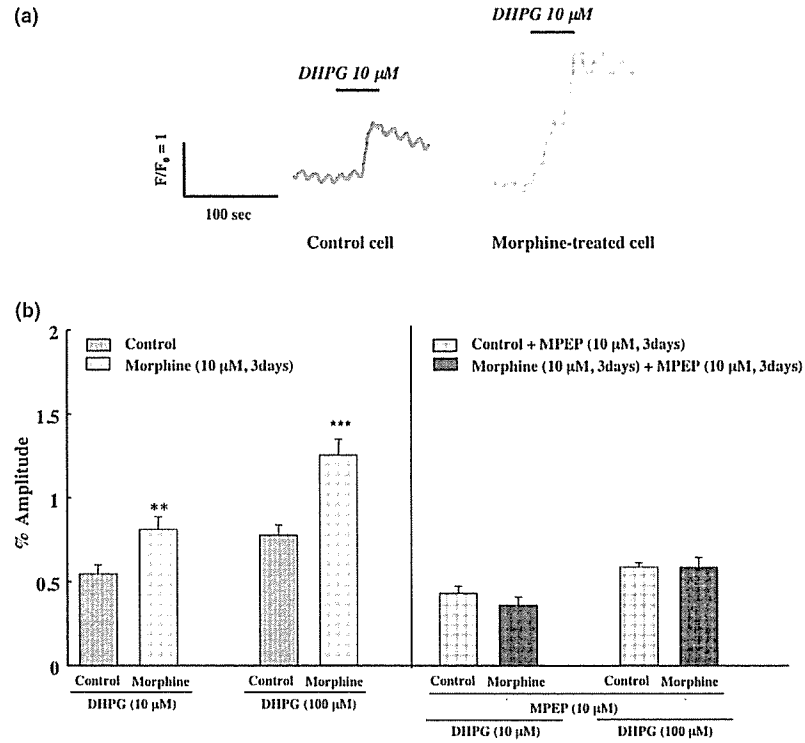

Fig. 7 Changes in Ca^{2+} response to DHPG in the spinal cord neuron following repeated treatment with morphine. (a) Traces show the DHPG (10 μM)-evoked increase in intracellular Ca^{2+} concentration in control and morphine (10 μM)-treated spinal cord neurons. (b) The response of Ca^{2+} to DHPG in control and morphine-treated spinal cord neurons is summarized. The response of Ca^{2+} to DHPG (10–100 μM) in spinal cord neurons was dose-dependently enhanced by 3 days of treatment with morphine (10 μM: $^{**}p < 0.01$ vs. control cells, 100 μM: $^{***}p < 0.001$ vs. control cells). These effects were blocked by 3 days of treatment with MPEP (10 μM). Data represent the mean ± SEM of 35–40 cells from three separate observations.

Fig. 6b). In order to investigate further whether mGluR5 could be expressed on the reactive astrocytes more clearly, mGluR5-IR was analyzed at high magnification using the optical-sectioning capabilities of confocal microscopy. As a result, the increase in mGluR5-IR produced by morphine showed hardly any co-localization with either GFAP-IR (Figs 6c-i and c-ii) or the marker of the cell body for astrocytes, S100β-IR (Fig. 6c-iii).

Enhancement of DHPG-evoked increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) in morphine-treated spinal cord neuron/glia co-cultures

Finally, we evaluated the change in neuronal activity by monitoring the $[Ca^{2+}]i$ evoked by DHPG in morphine-treated neuron/glia co-cultures. DHPG (10–100 μM) produced a transient increase in the $[Ca^{2+}]i$ in cultured spinal cord neurons (Fig. 7). The responses of Ca^{2+} to DHPG in neurons were dose-dependently enhanced by 3 days of treatment with morphine (10 μM: $^{**}p < 0.01$ vs. control cells, 100 μM: $^{***}p < 0.001$ vs. control cells, Fig. 7). These effects were blocked by 3 days of treatment with MPEP (10 μM).

Discussion

The key finding in the present study was that repeated *in vivo* treatment with morphine produces a dose-dependent increase in mGluR5-IR in the superficial dorsal horn of the spinal cord. Double-immunofluorescence analysis revealed that the increased mGluR5 was predominantly localized in the neuropil, and the surface of the neural membrane of laminae I–III neurons, following repeated treatment with morphine. Furthermore, using the receptor binding assay, we found that mice tolerant to morphine exhibited a marked increase in the B_{max} value of [^{3}H]MPEP, a selective radioligand for mGluR5, without changing the K_d value in the spinal cord. Although it has been reported that s.c. or intracerebroventricular (i.c.v.) administration of the selective mGluR5 antagonist MPEP prevents the development of the antinociceptive tolerance to morphine (Kozela *et al.* 2003; Smith *et al.* 2004), we found here, for the first time, that i.t. pretreatment with MPEP significantly inhibits the development of tolerance to morphine-induced antinociception.

Activation of mGluR5 results in PLC-catalyzed phosphoinositide (PI) hydrolysis, which leads to the release of Ca^{2+} from intracellular sources and stimulation of PKC (Kawabata *et al.* 1998). We previously reported that PKC inhibitors attenuate the development of tolerance to the actions of morphine (Narita *et al.* 1995) and that repeated *in vivo* treatment with morphine produces a significant increase in neuron-specific PKCγ-IR located in the lamina IIi with its expanding distribution in the dorsal horn of the spinal cord (Narita *et al.* 2004). Taken together, these findings support the idea that the increased number of membrane-bound mGluR5 following repeated treatment with morphine may lead to a long-lasting activation of neuronal PKC in the dorsal horn of the spinal cord, which is responsible for the

development of tolerance to morphine-induced antinociception.

The increase in mGluR5 following repeated morphine treatment led us to wonder whether this increase was due to the increased expression of functional receptors located on the membrane. Therefore, we next investigated whether the function of mGluR5 in cultured spinal cord neurons could be enhanced following repeated treatment with morphine as evaluated by monitoring the response of Ca^{2+} to the group I mGluR agonist DHPG. In the present study, the response of Ca^{2+} to DHPG in cultured spinal cord neurons was potently enhanced by chronic *in vitro* exposure to morphine. This effect was blocked by treatment with MPEP. It is therefore likely that chronic treatment with morphine leads to the functional up-regulation of group I mGluR including mGluR5, which may result in the enhancement of neuronal activity and synaptic transmission in the spinal cord.

It has recently been reported that quantitative changes in levels of GFAP can be observed in the rat spinal cord after chronic administration of morphine (Song and Zhao 2001; Raghavendra *et al.* 2002). We have previously demonstrated that mice tolerant to morphine exhibit astroglial hypertrophy and proliferation associated with activating neuronal PKC in the dorsal horn of the spinal cord (Narita *et al.* 2004). Accumulating evidence indicates that activation of glial mGluR5 leads to the release of glutamate through a soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)-dependent exocytotic mechanism to modulate neuronal excitability and synaptic functions (Bezzi *et al.* 2004; Montana *et al.* 2004). In the present study, the increased IR for mGluR5 was expressed in non-glial cells in the dorsal horn of the spinal cord of morphine-treated mice, as shown by no apparent co-localization with either GFAP-IR or S100β-IR. These findings suggest that the increased mGluR5 located in the spinal cord neuron is implicated in the development of tolerance to morphine-induced antinociception.

Here, we found that repeated treatment with morphine produces a dose-dependent increase in mGluR5-IR with its expanding distribution in the dorsal horn of the spinal cord. Although the data suggest that the increased expression of neuronal mGluR5 following repeated treatment with morphine may be responsible for the development of tolerance to morphine-induced antinociception, the molecular mechanism underlying this phenomenon is unclear. It has been documented that most mGluR5-IR is observed in the dendritic shafts, spike-like structures and cell body of the postsynaptic regions (Alvarez *et al.* 2000). One speculation related to the present results is that repeated stimulation of μ-opioid receptor promotes the new protein synthesis of mGluR5, or suppresses the protein degradation of mGluR5 associated with receptor internalization to increase the excitatory synaptic transmission in opposition to excessive activation of inhibitory neurons in the dorsal horn. It should be noted in our preliminary data that MPEP-treated mice failed to exhibit the increased level of mGluR5 in the spinal dorsal horn following repeated morphine treatment (data not shown). These data support the idea that repeated stimulation of μ-opioid receptors may initially cause the activation of membrane-bound mGluR5 and, in turn, increase the number of functional mGluR5 associated with the new production of mGluR5 or the suppression of the degradation of mGluR5 through its related intracellular signaling pathway. This phenomenon would be directly responsible for the development of tolerance to spinal antinociception induced by morphine.

In conclusion, the present study indicates that repeated *in vivo* treatment with morphine induces an increase in functional mGluR5s in the mouse spinal cord, which contributes to the development of tolerance to morphine-induced antinociception.

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Direct evidence for the involvement of brain-derived neurotrophic factor in the development of a neuropathic pain-like state in mice

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Abstract

Thermal hyperalgesia and tactile allodynia induced by sciatic nerve ligation were completely suppressed by repeated intrathecal (i.t.) injection of a TrkB/Fc chimera protein, which sequesters endogenous brain-derived neurotrophic factor (BDNF). In addition, BDNF heterozygous (+/–) knockout mice exhibited a significant suppression of nerve ligation-induced thermal hyperalgesia and tactile allodynia compared with wild-type mice. After nerve ligation, BDNF-like immunoreactivity on the superficial laminae of the ipsilateral side of the spinal dorsal horn was clearly increased compared with that of the contralateral side. It should be noted that a single i.t. injection of BDNF produced a long-lasting thermal hyperalgesia and tactile allodynia in normal mice, and these responses were abolished by i.t. pre-treatment with either a Trk-dependent tyrosine kinase inhibitor K-252a or a selective protein kinase C (PKC) inhibitor Ro-32-0432. Supporting these findings, we demonstrated here for the first time that the increase in intracellular Ca^{2+} concentration by application of BDNF in cultured mouse spinal neurons was abolished by pre-treatment with either K-252a or Ro-32-0432. Taken together, these findings suggest that the binding of spinally released BDNF to TrkB by nerve ligation may activate PKC within the spinal cord, resulting in the development of a neuropathic pain-like state in mice.

Keywords: brain-derived neurotrophic factor, brain-derived neurotrophic factor heterozygous (+/–) knockout mouse, neuropathic pain, protein kinase C, spinal cord, TrkB.
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Neuropathic pain is well characterized by spontaneous burning pain, hyperalgesia (an exaggerated pain in response to painful stimuli) and allodynia (a pain evoked by normally innocuous stimuli). A growing body of evidence suggests that sensory nociceptive processing in the spinal dorsal horn appears to undergo significant plastic changes following peripheral nerve injury, leading to the development of neuropathic pain (Ji and Woolf 2001). In addition, it has been demonstrated that the functional changes in the ascending pain pathway from the dorsal horn to brain areas such as thalamus and cortex and the descending inhibitory pathway from the brainstem to the spinal cord may also contribute to the development of neuropathic pain (Lin 1996; Sun *et al.* 2001; Back *et al.* 2003). However, the mechanisms underlying neuropathic pain remain largely unclear.

Brain-derived neurotrophic factor (BDNF), a neurotrophin, presents in a subpopulation of small- to medium-diameter sensory dorsal root ganglia (DRG) neurons (Zhou *et al.* 1999; Obata *et al.* 2003). A synthesized BDNF in the DRGs is anterogradely transported to the primary afferent terminals in the dorsal horn where it is involved in the modulation of painful stimuli (Zhou and Rush 1996; Michael *et al.* 1997). In laminae I and II of the spinal cord, BDNF is localized in synaptic vesicles of sensory nerve terminals, but

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Abbreviations used: BSS, balanced salt saline; bp, base pairs; BDNF, brain-derived neurotrophic factor; DMSO, dimethylsulfoxide; DRG, dorsal root ganglion; GFAP, glial fiblillary acidic protein; IR, immunoreactivity; i.t., intrathecal; MAP2a/b, microtubule-associated protein 2a/b; NGS, normal goat serum; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PGK, phosphoglycerate kinase; PKC, protein kinase C; PLC, phospholipase C; Ro-32-0432, 2-{8-[(dimethylamino)methyl]-6,7,8,9-tetrahydropyrido[1,2-a]indol-3-yl}-3-(1-metyl-1H-indol-3-yl)maleimide; TrkB/Fc, recombinant human TrkB/Fc chimera protein.

not in postsynaptic neurons (Luo *et al.* 2001). There is considerable evidence demonstrating that BDNF is released from primary afferent neurons as an endogenous neurotransmitter/neuromodulator (Kafitz *et al.* 1999; Kerr *et al.* 1999; Lever *et al.* 2001; Poo 2001; Pezet *et al.* 2002), and the release of BDNF within the dorsal horn of the spinal cord is dependent on the pattern of primary afferent activity (Lever *et al.* 2001). Recently, it has been documented that the expression of BDNF is increased in the ipsilateral DRGs following nerve injury (Shen *et al.* 1999; Zhou *et al.* 1999), and the intensity of BDNF-like immunoreactive neurons is increased in the ipsilateral DRGs and the superficial dorsal horn of the spinal cord in nerve-injured animals (Zhou *et al.* 1999; Ha *et al.* 2001; Obata *et al.* 2003). Further investigation has been reported that rats exhibiting thermal hyperalgesia after loose ligation of the sciatic nerve revealed a significant increase in the concentration of BDNF in the lumbar spinal dorsal horn (Miletic and Miletic 2002). These results are consistent with our finding that repeated intrathecal (i.t.) injection of specific antibody to BDNF produced a marked suppression of thermal hyperalgesia associated with an increase in protein levels of membrane-located TrkB receptor following sciatic nerve ligation in mice (Yajima *et al.* 2002). These findings raise the fascinating possibility that the increased release of endogenous BDNF induced by nerve injury may cause a facilitation of the excitability of dorsal horn neurons through the activation of TrkB receptor, resulting in a neuropathic pain-like state.

In the present study, we investigated whether BDNF heterozygous (+/–) mutant mice could exhibit any neuropathic pain-like behaviours induced by sciatic nerve ligation. Furthermore, to clarify a substantial role of the spinal BDNF/TrkB receptor-mediated signalling pathway in the development of a neuropathic pain-like state, we demonstrated whether a sequestration of endogenous BDNF within the spinal cord by repeated i.t. treatment with TrkB receptor chimera protein could affect neuropathic pain-like behaviours in mice with nerve ligation.

Materials and methods

The present study was conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, as adopted by the Committee on Animal Research of Hoshi University, which is accredited by the Ministry of Education, Culture, Sports, Science and Technology of Japan. Every effort was made to minimize the numbers and any suffering of animals used in the following experiments. Animals were used only once in the present study. All behavioural experiments were conducted in a single-blind fashion in order to avoid the effect of subjectivity.

Animals

Breeding pairs of mice (inbred mixed BALB/c-B6-129/Sv background) heterozygous for a null mutation in the BDNF gene (STOCK $Bdnf^{tm1Jae}$) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). The animals used in the present study were litter-mate wild-type controls (+/+) and BDNF heterozygous (+/–) knockout [BDNF (+/–) knockout] mice raised within our colony. In addition, male ICR mice (Tokyo Laboratory Animals Science, Tokyo, Japan) were also used in the present study. Animals were housed in a room maintained at 22 ± 1°C with an alternating 12-h light-dark cycle. Food and water were available ad libitum.

Genotyping

The genotype of offspring from the BDNF (+/–) knockout mice was determined by polymerase chain reaction (PCR) using tail DNA obtained from pentobarbital (60 mg/kg, i.p.) anaesthetized mice.

PCR analysis was performed using BD Advantage^{TM}-GC 2 PCR kit (BD Biosciences Clontech, Palo Alto, CA, USA) according to the manufacture's manual with the extracted DNA template (10 ng/μL) and synthesized BDNF primers as described by Ward and Hagg (2000): a sense primer of BDNF (5′-ATGAAAGAAGTAAACGTC-CAC-3′), an antisense primer of BDNF (5′-CCAGCAG-AAAGAGTAGAGGAG-3′) and an antisense primer of phosphoglycerate kinase (PGK) (5′-GGGAACTTCCTGACTAGGGG-3′), which includes a neo expression cassette inserted into a deleted exon 5 of the BDNF gene (Ernfors *et al.* 1994).

After denaturation at 94°C for 5 min, 30 cycles of amplification were performed using the following conditions: 94°C, 1 min; 55°C, 30 s; 68°C, 1 min. The PCR products were separated by electrophoresis on 2% agarose gels and were visualized by ethidium bromide staining.

PCR products generated using the primers of antisense PGK and sense BDNF revealed a single band of 340 base pairs (bp) (targeted deficient allele), and those generated using the primers of sense BDNF and antisense BDNF revealed a single band of 277 bp (wild-type allele). Therefore, BDNF (+/–) knockout mice yielded both two amplification products (Ward and Hagg 2000).

Measurement of body temperature, basal hot-plate latency, basal tail-flick latency and rota-rod performance in BDNF (+/–) knockout mice

Body temperature in BDNF (+/–) knockout mice was evaluated by the measurement of rectal temperature using a monitoring thermometer (Thermalert TH-5, Physitemp, Clifton, NJ, USA). Basal nociceptive responses were assessed by either hot-plate (55 ± 0.5°C; Analgesia Meter Model hot-plate MK350B, Muromachi Kikai, Tokyo, Japan) or tail-flick analgesic apparatus (Analgesia Meter Model Tail-Flick MK330B, Muromachi Kikai). Either hot-plate or tail-flick latency was determined as the average of two measurements. The motor coordination was evaluated by the measurement of latency until fall-off from a rota-rod (3 cm in diameter) at 8 r.p.m. (KN-95; Natsume Seisakusyo, Tokyo, Japan). Initially, mice were trained five times to walk on the rota-rod. The time until fall-off from the rota-rod was measured at 1 h after the training.

Neuropathic pain model

The mice were anaesthetized with sodium pentobarbital (60 mg/kg, i.p.). We produced a partial sciatic nerve injury by tying a tight ligature with 7-0 or 8-0 silk suture around approximately one-third to one-half the diameter of the sciatic nerve located on the right side (ipsilateral side) under light microscope (SD30, Olympus, Tokyo,

Japan) as described previously (Malmberg and Basbaum 1998). In sham-operated mice, the nerve was exposed without ligation.

Measurement of paw withdrawal latency to a thermal stimulus

To assess the sensitivity to thermal stimulation, each of the hind paws of the mice were tested individually using a thermal stimulus apparatus (model 33 Analgesia Meter; IITC/Life Science Instruments, Woodland Hills, CA, USA). The intensity of the thermal stimulus was adjusted to achieve an average baseline paw withdrawal latency of approximately 8 to 10 s in naive mice. Only quick hind paw movements (with or without licking of hind paws) away from the stimulus were considered to be a withdrawal response. Paw movements associated with locomotion or weight shifting were not counted as a response. The paws were measured, alternating between left and right with an interval of more than 3 min between the measurements. The latency of paw withdrawal after the thermal stimulus was determined as the average of three measurements per paw. Before testing of the behavioural responses to the thermal stimulus, mice were habituated at least 1 h in an acrylic cylinder (15 cm high and 8 cm in diameter). Under these conditions, the latency of paw withdrawal in response to the thermal stimulus was tested. The measurement of latency of paw withdrawal to the thermal stimulus was performed before the surgery and 1, 3, 5 and 7 days after the surgery.

In the experiment of a single i.t. treatment with BDNF, the measurement of thermal paw withdrawal latency was performed before and after the injection until the latency returned to the baseline. The latency of paw withdrawal to the thermal stimulus was determined as the average of both paws.

Measurement of paw withdrawal response to a tactile stimulus

To quantify the sensitivity to tactile stimulus, paw withdrawal response to tactile stimulus was measured using two different bending forces (0.02 g for BDNF (+/–) knockout mice or 0.02 and 0.16 g for ICR mice) of von Frey filaments (North Coast Medical, Morgan Hill, CA, USA). Each von Frey filament was applied to the plantar surface of hind paw for 3 s and repeated three times at a minimum of 5-s intervals. Each of the hind paws of the mice were tested individually. The paw withdrawal responses to the tactile stimulus were evaluated by the scoring as follows: 0, no response; 1, a slow and/or slight response to the stimulus; 2, a quick withdrawal response away from the stimulus without flinching and licking; 3, a intense withdrawal response away from the stimulus with brisk flinching and/or licking. The paw withdrawal response to each filament was determined as the average of two scores per paw. Paw movements associated with locomotion or weight shifting were not counted as a response. The paws were measured, alternating between left and right with an interval of more than 3 min between the measurements. Before testing of the behavioural responses to the tactile stimulus, mice were habituated at least 1 h on an elevated nylon mesh floor. Under these conditions, the paw withdrawal response to the tactile stimulus was tested. The measurement of paw withdrawal threshold to the tactile stimulus was performed before the surgery and next day after the measurement of thermal threshold (day 2, 4, 6 and 8).

In the experiment of a single i.t. treatment with BDNF, the measurement of paw withdrawal responses to the tactile stimulus was performed before and after the injection until the response returned to the baseline. The paw withdrawal score to the tactile stimulus was determined as the average of both paws.

Intrathecal injection

Intrathecal injection was performed as described by Hylden and Wilcox (1980) using a 25-μL Hamilton syringe with a 30 1/2-gauge needle. The needle was inserted into the intervertebral space between the L5 and L6 level of the spinal cord. A reflexive flick of the tail was considered to be a sign of the accuracy of each injection. The injection volume was 4 μL for i.t. injection.

Groups of mice were repeatedly i.t. treated with a recombinant human TrkB/Fc chimera protein (10 ng/mouse; TrkB/Fc) 1 h before the surgery and once a day for 8 consecutive days after the surgery. A single i.t. injection of a recombinant human BDNF (BDNF; 50 ng/mouse) was performed in naive mice. Either i.t. pre-treatment with a Trk-dependent tyrosine kinase inhibitor K-252a or a selective protein kinase C (PKC) inhibitor 2-{8-[(dimethylamino)methyl]-6,7,8,9-tetrahydropyrido[1,2-a]indol-3-yl}-3-(1-metyl-1H-indol-3-yl)maleimide (Ro-32-0432) was performed 30 min before a single i.t. injection of BDNF (50 ng/mouse).

Immunohistochemistry

Sample preparation

Seven days after nerve ligation, mice were deeply anaesthetized with sodium pentobarbital (70 mg/kg, i.p.) and intracardially perfusion-fixed with freshly prepared 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.4). After perfusion, the lumbar spinal cord was quickly removed and post-fixed in 4% paraformaldehyde for 2 h, and permeated with 20% sucrose in 0.1 M PBS for 1 day and 30% sucrose in 0.1 M PBS for 2 days with agitation. Then, the L5 lumbar spinal cord segments were frozen in an embedding compound (Sakura Finetechnical, Tokyo, Japan) on isopentane using liquid nitrogen and stored at −30°C until used. Frozen spinal cord segments were cut with a freezing cryostat (Leica CM 1510, Leica Microsystems AG, Wetzlar, Germany) at a 10-μm thickness and thaw-mounted on a poly-L-lysine-coated glass slides.

The spinal cord sections were blocked in 10% normal goat serum (NGS) in 0.01 M PBS for 1 h at 23°C. The primary antibody was diluted in 0.01 M PBS containing 10% NGS 1 : 1500 chicken anti BDNF (R & D Systems, Minneapolis, MN, USA) and incubated twice overnight at 4°C. The antibody was then rinsed and incubated with secondary antibody for 2 h at room temperature. For BDNF single staining assay, Alexa 488-conjugated goat anti-chicken IgG (Molecular Probes, Eugene, OR, USA) was diluted 1 : 400 in PBS containing 10% NGS. Because staining intensity might vary between experiments, control sections were included in each run of staining.

The slides were then cover-slipped with PermaFluor Aqeous mounting medium (Immunon™; ThermoShandon, Pittsburgh, PA, USA). Fluorescence immunolabelling was detected using an U-MNIBA filter cube (Olympus) for Alexa 488. All sections were observed with the aid of light microscope (Olympus BX-80; Olympus) and photographed with a digital camera (CoolSNAP HQ; Olympus). Digitized images of superficial laminae of the spinal dorsal horn sections were captured at a resolution of 1316 × 1035 pixels by camera. The density of BDNF labelling was measured with a computer-assisted imaging analysis system (NIH Image). The