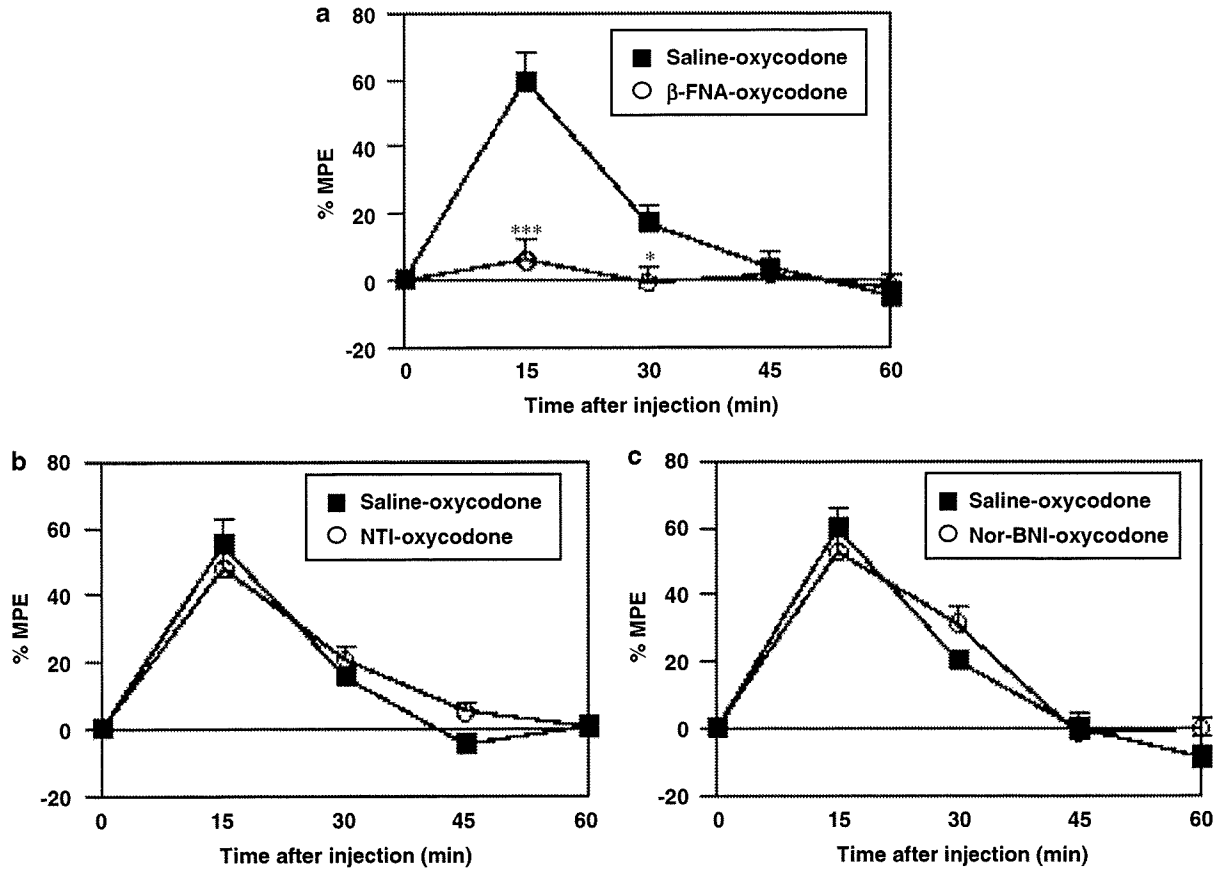

Figure 5 Effects of pretreatment with MOR antagonist β-FNA (a), DOR antagonist NTI (b), or KOR antagonist nor-BNI (c) on s.c. oxycodone-induced antinociception in mice. Antinociception was expressed as a % MPE. Groups of mice were pretreated with β-FNA (40 mg/kg), NTI (3 mg/kg), or nor-BNI (5 mg/kg) at 24 h, 30 min, or 24 h, respectively, before s.c. administration of oxycodone (1.7 mg/kg). Each point represents the mean ± SEM of 6–10 mice. $^{*}p<0.05$ and $^{***}p<0.001$ *vs* saline + oxycodone.

marked decrease in the latency of paw withdrawal against a thermal stimulus only on the ipsilateral side in mice ($^{***}p<0.001$ *vs* saline-saline group) (Figure 6c and d). The persistent painful state caused by intraplantar injection of CFA lasted for more than 14 days following CFA treatment in mice (data not shown). The s.c. injection of either morphine (1–10 mg/kg) or oxycodone (0.1–3 mg/kg) 7 days after sciatic nerve ligation or 1 day after CFA treatment recovered the decreased thermal threshold observed on the ipsilateral side in sciatic nerve-ligated or CFA-treated mice in a dose-dependent manner, and maximal anti-hyperalgesic responses were seen at 30 or 15 min after morphine or oxycodone injection, respectively (ligation-morphine 1 mg/kg, $^{***}p<0.001$; ligation-morphine 3 mg/kg, $^{**}p<0.01$ *vs* sham-saline group: Figure 6a, ligation-oxycodone 0.1 or 1 mg/kg, $^{**}p<0.01$; ligation-oxycodone 3 mg/kg, $^{***}p<0.001$ *vs* sham-saline group: Figure 6b, CFA-morphine 1, 5, or 10 mg/kg, $^{***}p<0.001$ *vs* saline-saline group: Figure 6c, CFA-oxycodone 0.1 or 3 mg/kg, $^{***}p<0.001$; CFA-oxycodone 1 mg/kg, $^{**}p<0.01$ *vs* saline-saline group: Figure 6d). At the dose of 5 mg/kg or 0.5 mg/kg, s.c. administration of morphine or oxycodone, respectively, almost completely reversed the decrease in the thermal threshold without excessive effects in sciatic nerve-ligated mice. In contrast, either 3 mg/kg (s.c.) of morphine or 0.5 mg/kg (s.c.) of oxycodone reversed the decreased thermal threshold in CFA-treated mice.

Characterization of the Antinociception Induced by Morphine or Oxycodone under a Neuropathic-Pain like State

We evaluated the antinociceptive effects induced by s.c. administration of morphine or oxycodone in sham-operated and sciatic nerve-ligated mice using the tail-flick assay. In the present study, s.c. administration of either morphine or oxycodone produced a dose-dependent antinociceptive effect in sham-operated mice, and maximal antinociceptive responses were seen at 30 and 15 min after injection, respectively. The antinociceptive effect of s.c.-injected morphine was significantly decreased in sciatic nerve-ligated mice (ligation-morphine 1.7 mg/kg, $^{*}p<0.05$; ligation-morphine 3 or 5.6 mg/kg, $^{**}p<0.01$; ligation-morphine 10 mg/kg, $^{***}p<0.001$ *vs* sham-morphine group) (Figure 7a). In contrast, s.c. injection of oxycodone produced profound antinociception in sciatic nerve-ligated mice at the same level as observed in sham-operated mice (Figure 7b).

We next examined the antinociceptive effects induced by either i.t.- or i.c.v.-administered morphine or oxycodone in

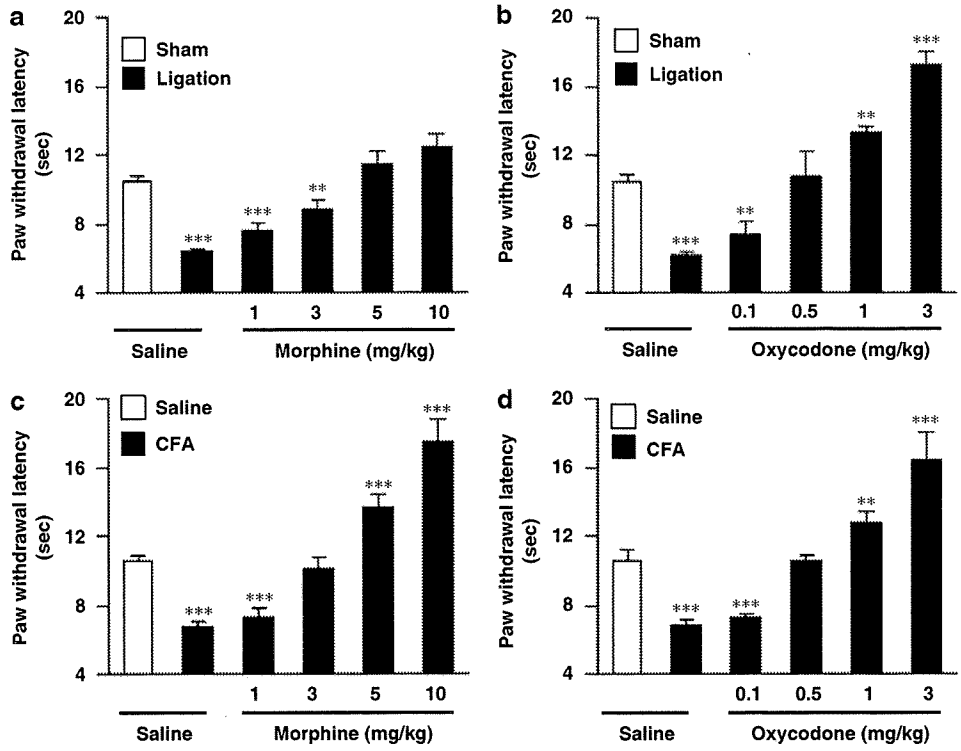

Figure 6 Effect of s.c. injection of morphine (a or c) or oxycodone (b or d) on the latency of paw withdrawal in response to thermal stimulus on the ipsilateral side in sham-operated and sciatic nerve-ligated mice (a or b) or saline- or CFA-injected mice (c or d). Measurement of thermal threshold was performed just before and 30 or 15 min after s.c. injection of morphine or oxycodone, respectively. Groups of mice were treated with morphine (1–10 mg/kg) or oxycodone (0.1–3 mg/kg) 7 days after sciatic nerve ligation or 1 day after CFA injection. Each column represents the mean ± SEM of 6–11 mice. ***$p<0.001$ and **$p<0.01$ *vs* sham-saline group or saline–saline group.

sham-operated and sciatic nerve-ligated mice. I.t. injected morphine or oxycodone each had a dose-dependent antinociceptive effect in sham-operated and sciatic nerve-ligated mice (Figure 7c and d). The maximal antinociceptive effect of i.t.-administered morphine or oxycodone was observed at 10 or 5 min, respectively, following each injection (data not shown). I.c.v.-administered morphine and oxycodone each produced significant antinociception in sciatic nerve-ligated mice to the same level as observed in sham-operated mice (Figure 7e and f). The maximal antinociceptive effect of i.c.v.-administered morphine or oxycodone was observed 10 or 5 min, respectively, following each injection (data not shown). As shown in Table 2, the ED_{50} values for the antinociceptive effects of morphine and oxycodone following each injection in sham-operated or sciatic nerve-ligated mice (Table 2).

Spinal and Supraspinal G-Protein Activation Induced by Morphine or Oxycodone in Nerve-Ligated Mice

We next investigated the ability of morphine (Figure 8a, c and e) or oxycodone (Figure 8b, d and f) to activate G proteins through the stimulation of MORs in membranes of the mouse spinal cord (Figure 8a and b), PAG (Figure 8c and d), and thalamus (Figure 8e and f) obtained from sham-operated and sciatic nerve-ligated mice. The activation of G proteins induced by morphine or oxycodone (10^{-8}–10^{-5} M) in these areas was examined by monitoring the binding of [^{35}S]GTPγS to membranes. Morphine and oxycodone each produced a concentration-dependent increase in [^{35}S]GTPγS binding to membranes of the mouse spinal cord, PAG and thalamus in sham-operated mice (Figure 8). In sciatic nerve-ligated mice, the levels of [^{35}S]GTPγS binding stimulated by morphine and oxycodone were similar to that found in sham-operated mice (Figure 8).

Suppression of the Antinociceptive Effect and G-Protein Activation Induced by M-6-G at Spinal or Supraspinal Levels under a Neuropathic Pain-like State

In the mouse tail-flick test, either s.c.-, i.t.- or i.c.v. administration of M-6-G induced marked antinociception in sham-operated mice as with morphine and oxycodone (Figure 9). The antinociceptive effects induced by all three injections of M-6-G were significantly decreased in nerve-ligated mice compared with those in sham-operated mice (s.c.: ligation-M-6-G 1 or 5.6 mg/kg *$p<0.05$; 1.7 or 3 mg/kg ***$p<0.001$ *vs* sham-M-6-G group; i.t.: ligation-M-6-G 0.01 or 0.03 nmol/mouse ***$p<0.001$; 0.056 or 0.1 nmol/mouse **$p<0.01$ *vs* sham-M-6-G group; i.c.v.: ligation-M-6-G 0.03 nmol/mouse *$p<0.01$; 0.3 or 0.56 nmol/mouse ***$p<0.001$ *vs* sham-M-6-G group) (Figure 9a–c).

We further investigated the changes in the ability of M-6-G to activate G-proteins in membranes of the spinal cord, PAG or thalamus obtained from sham-operated or sciatic nerve-ligated mice. The binding of [^{35}S]GTPγS stimulated by M-6-G was significantly decreased in nerve-ligated mice, whereas M-6-G (10^{-8}–10^{-5} M) produced a concentration-

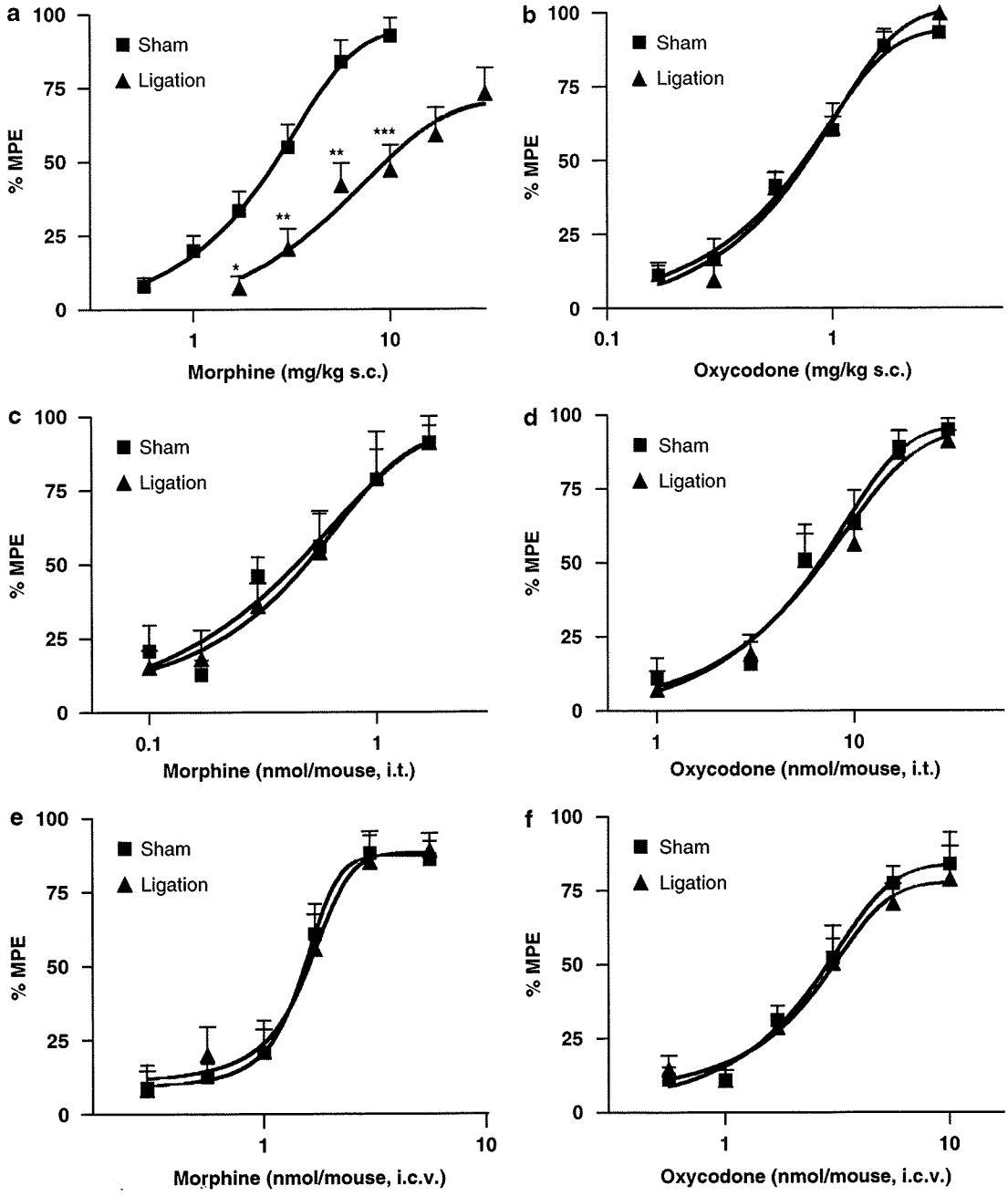

Figure 7 Dose–response curves for the antinociceptive effect induced by s.c., i.t., or i.c.v. morphine (a, c, or e) or oxycodone (b, d, or f) in sham-operated and sciatic nerve-ligated mice. Groups of mice were treated with morphine (0.56–30 mg/kg, s.c., 0.1–1.7 nmol/mouse, i.t., or 0.3–5.6 nmol/mouse, i.c.v.) or oxycodone (0.17–3 mg/kg, s.c., 1–30 nmol/mouse, i.t., or 0.56–10 nmol/mouse, i.c.v.) 7 days after nerve ligation. Antinociceptive effects were measured at 30 or 15 min after s.c., 10 or 5 min after i.t. or 10 or 5 min after i.c.v. injection of morphine or oxycodone as the peak time of morphine or oxycodone activity, respectively. Antinociception was expressed as a % MPE. Each value represents the mean ± SEM of 8–10 mice. $^{*}p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$ *vs* sham group.

dependent increase in the binding of [^{35}S]GTPγS to membranes of the spinal cord and PAG area in sham-operated mice (spinal cord: $F_{(1,42)}=28.80$, $^{**}p<0.01$ *vs* ligation-M-6-G group; PAG: $F_{(1,36)}=13.36$, $^{**}p<0.01$ *vs* ligation-M-6-G group) (Figure 10a and b). On the other hand, M-6-G produced a concentration-dependent increase in [^{35}S]GTPγS binding to membranes of the mouse thalamus in sham-operated mice. In sciatic nerve-ligated mice, the level of [^{35}S]GTPγS binding stimulated by M-6-G in the thalamus was similar to that found in sham-operated mice (Figure 10c).

Inhibition of Morphine- or Oxycodone-Induced Place Preference and G-Protein Activation in the Lower Midbrain with Sciatic Nerve-Ligated Mice

We next investigated whether morphine or oxycodone could produce rewarding effects in sciatic nerve-ligated

Table 2 ED_{50} Values and Shift-Ratio of the Antinociceptive Effects of Morphine, Oxycodone, or M-6-G in Sham-Operated and Sciatic Nerve Ligation

	ED_{50} values						Shift-ratio (ligation/sham)		
	Sham			Ligation					
Injection site	Morphine	Oxycodone	M-6-G	Morphine	Oxycodone	M-6-G	Morphine	Oxycodone	M-6-G
s.c. (mg/kg)	2.46 (2.08–2.92)	0.7 (0.59–0.83)	1.06 (0.89–1.27)	6.52 (4.81–8.83)***	0.71 (0.64–0.8)	4.84 (3.72–6.31)***	2.65	1.01	4.57
i.t. (nmol/mouse)	0.41 (0.31–0.53)	6.2 (4.90–7.84)	0.009 (0.0066–0.012)	0.44 (0.32–0.61)	6.53 (5.33–8.00)	0.046 (0.035–0.062)***	1.07	1.05	5.11
i.c.v. (nmol/mouse)	1.49 (1.23–1.80)	2.84 (2.35–3.44)	0.064 (0.046–0.089)	1.52 (1.21–1.90)	3.15 (2.46–4.04)	0.34 (0.18–0.63)***	1.02	1.11	5.31

ED_{50} values were determined using the analysis of variance and linear regression techniques. To calculate ED_{50} values, at least six drug doses were used and 6–11 mice were used for each dose. Values in parenthesis indicate the 95% confidence range. The shift-ratio was calculated as (ligation-group ED_{50} values/sham-group ED_{50} values). Groups of mice were treated with morphine, oxycodone, or M-6-G 7 days after the nerve ligation. ***$p<0.001$ *vs* sham-group.

mice using the CPP method. As shown in Figure 11, s.c.-administered morphine and oxycodone each produced a dose-dependent preference for the drug-associated place in sham-operated mice (sham-morphine 3 or 5.6 mg/kg, sham-oxycodone 1 or 3 mg/kg, $^{\#\#}p<0.01$; sham-morphine 10 mg/kg $^{\#\#\#}p<0.001$ *vs* sham-saline group). In contrast, neither morphine nor oxycodone induced a place preference under a neuropathic pain-like state (ligation-morphine 5.6 or 10 mg/kg $^{*}p<0.05$; ligation-oxycodone 1 or 3 mg/kg $^{*}p<0.05$ *vs* sham-morphine or -oxycodone group) (Figure 11a and b).

We assessed the morphine- or oxycodone-induced increase in [^{35}S]GTPγS binding in the lower midbrain including the VTA obtained from sham-operated or sciatic nerve-ligated mice. According to atlas described by Paxions and Franklin (2001), the coronal brain block of the lower midbrain area (box) was dissected obtained from 2.5 to 4.5 mm posterior to bregma (Figure 11c). Morphine and oxycodone (10^{-8}–10^{-5} M) each produced a concentration-dependent increase in the binding of [^{35}S]GTPγS to lower midbrain membranes in sham-operated mice. Conversely, the level of [^{35}S]GTPγS binding to this area stimulated by morphine or oxycodone in nerve-ligated mice was significantly lower than that observed in sham-operated mice ($F_{(1,36)}=13.43$, $^{*}p<0.05$ *vs* ligation-morphine group; $F_{(1,36)}=20.36$, $^{\#\#}p<0.01$ *vs* ligation-oxycodone group) (Figure 11d and e).

DISCUSSION

The present binding studies clearly suggest that oxycodone possesses high affinity for MORs in the mouse brain. In contrast, oxycodone showed no affinity for DORs or KORs. In the [^{35}S]GTPγS binding, the increased level of [^{35}S]GTPγS binding to membranes of the mouse thalamus induced by oxycodone was abolished by the MOR antagonist β-FNA. Furthermore, oxycodone failed to affect [^{35}S]GTPγS bindings to membranes of the guinea pig cerebellum, which is known to be a good source for the possible determination of KOR agonists. Consistent with these results, s.c. pretreatment with the β-FNA, but not NTI or nor-BNI, significantly attenuated the oxycodone-induced antinociception. Taken together, these findings suggest that the oxycodone-induced antinociception is mainly mediated through MORs in the CNS, and oxycodone can be classified as the preferential MOR agonist. However, our notion is inconsistent with the report by Ross and Smith (1997). In their report, the antinociception of oxycodone was antagonized by i.c.v. pretreatment of nor-BNI with a dose that failed to affect the antinociceptive effect induced by morphine, suggesting the implication of KOR in the oxycodone-induced antinociception. Although the specific reason for these discrepancies between previous studies by Ross and Smith and our studies remains unclear, one possibility is that the discrepancy may result from different experimental conditions. In the current study, we have carefully confirmed that neither KOR- nor DOR agonist-induced antinociceptive effect is attenuated by pretreatment with β-FNA at the present dose (data not shown). Additionally, it has been documented that oxycodone acts as the MOR agonist in mice and binds selectively to the MOR (Lemberg *et al*, 2006a). Thus, the present

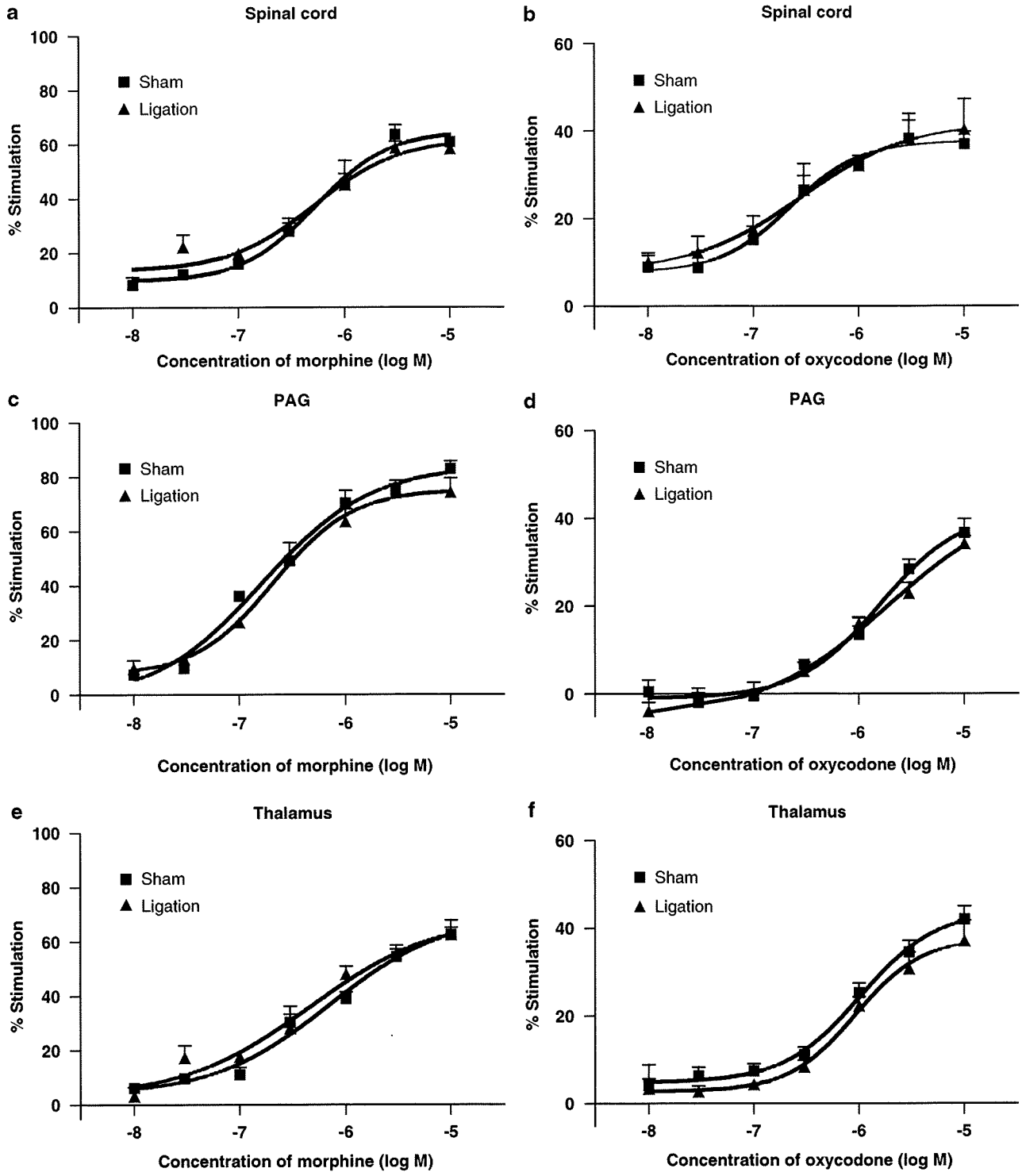

Figure 8 No difference was observed in the binding of [^{35}S]GTPγS stimulated by morphine (a, c, or e) or oxycodone (b, d, or f) in the spinal cord (a or b), the PAG (c or d) or thalamus (e or f) obtained from sham-operated and sciatic nerve-ligated mice. Membranes were prepared at 7 days after nerve ligation. Each value represents the mean ± SEM of six samples.

findings further suggest that MOR, but not KOR, is the primary site for the expression of antinociception induced by oxycodone.

In the present study, we also found that oxycodone showed about 10 times lower ligand-binding affinity than that of morphine. On the other hand, morphine and oxycodone were equipotent antinociceptive agents. It has been recently reported that oxycodone shows lower efficacy and potency to stimulate [^{35}S]GTPγS binding in the rat spinal cord and PAG than that of morphine or an oxycodone active metabolite, oxymorphone (Lemberg *et al*, 2006a). Although it is difficult to explain the reason

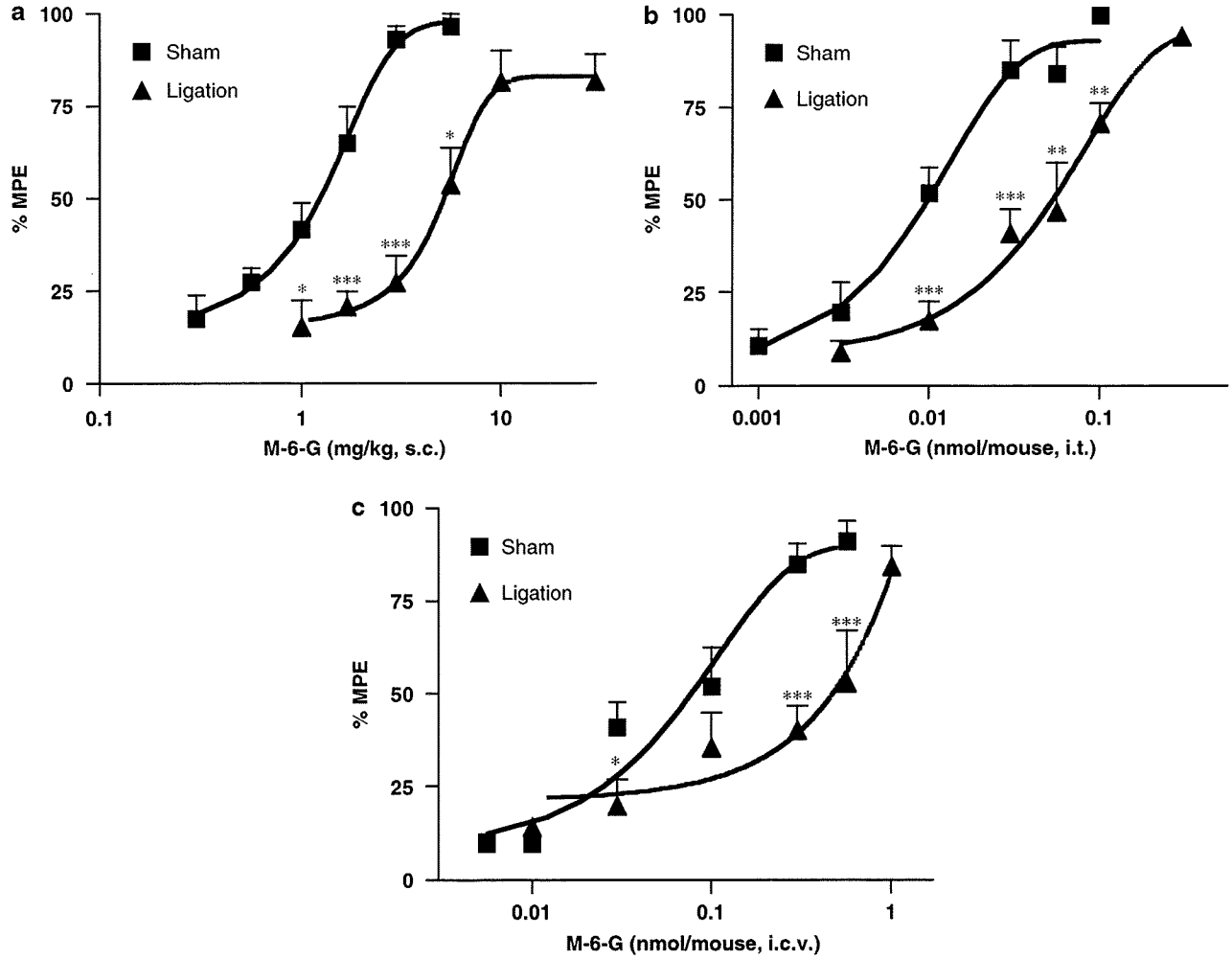

Figure 9 Dose–response curves for the antinociceptive effect induced by M-6-G in sham-operated and sciatic nerve-ligated mice. Groups of mice were treated s.c.(0.3–30 mg/kg, a), i.t.(0.001–0.1 nmol/mouse, b), or i.c.v.(0.0056–1 nmol/mouse, c) with M-6-G 7 days after the nerve ligation. Antinociceptive effects were measured at 45, 10, or 10 min after s.c., i.t., or i.c.v. injection of M-6-G as the peak time of M-6-G activity, respectively. Antinociception was expressed as a % MPE. Each value represents the mean ± SEM of 6–11 mice. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ *vs* sham group.

for the lower affinity to the MOR for oxycodone than for morphine, current evidence demonstrated that oxycodone is actively influxed across the BBB rather than morphine (Tunblad *et al*, 2003; Bostrom *et al*, 2006), which could, at least in part, result from this phenomenon.

In the present study, a neuropathic pain-like state and an inflammatory pain-like state were suppressed by s.c. treatment with either morphine or oxycodone in a dose-dependent manner. We proposed that the optimal doses for morphine- or oxycodone-induced anti-hyperalgesic effect in sciatic nerve-ligated and CFA-treated mice were 5 mg/kg or 0.5 μg/kg and 3 or 0.5 mg/kg, respectively. The optimal dose for morphine under a neuropathic pain-like state showed higher than that under an inflammatory pain-like state. Unlike morphine, the optimal dose for oxycodone was equipotent between a neuropathic pain-like state and an inflammatory pain-like state. These results suggest that neuropathic pain can be relieved by high dose of morphine, while the treatment with oxycodone produces a profound anti-hyperalgesic effect with the same degree under both a neuropathic pain-like state and an inflammatory pain-like state.

Using tail-flick assay, we confirmed that the antinociceptive effect induced by s.c. injection of morphine was significantly decreased in sciatic nerve-ligated mice, whereas sciatic nerve ligation did not alter the effect of oxycodone. However, i.t.- and i.c.v. administration of morphine each produced a significant antinociceptive effect in sciatic nerve-ligated mice similar to that observed in sham-operated mice. Consistent with these results, the increase in [^{35}S]GTPγS binding induced by morphine in membrane fractions of the spinal cord, PAG, and thalamus was not altered by sciatic nerve ligation. The present data are inconsistent with the previous findings that the effect of i.t.-administered morphine was attenuated under a neuropathic pain-like state in rodents (Siddall *et al*, 1994; Lee *et al*, 1995; Ossipov *et al*, 1995; Porreca *et al*, 1998). However, recent reports suggest the data that i.t. treatment with morphine produced greater dose-dependent inhibitions of neuronal nociception in rodents (Hao *et al*, 1998;

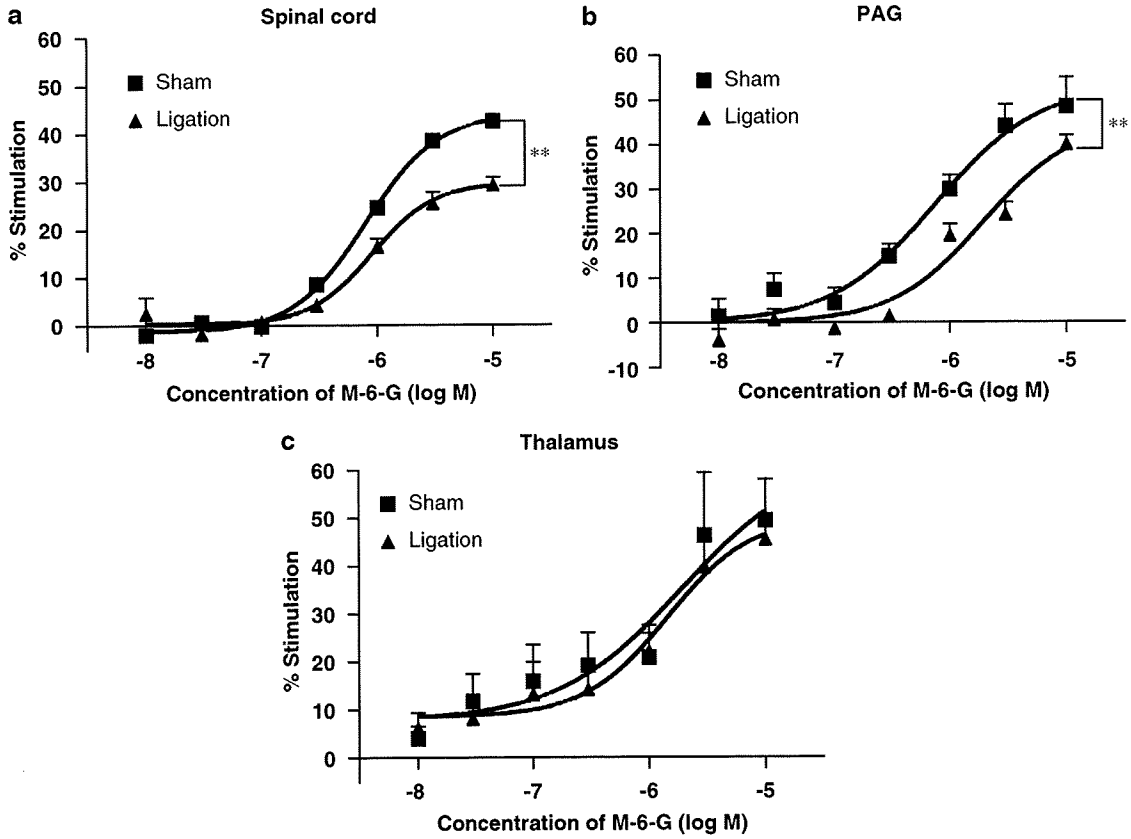

Figure 10 Concentration–response curve of M-6-G on the binding of [^{35}S]GTPγS to membranes of the spinal cord (a), PAG (b), or thalamus (c) obtained from sham-operated and sciatic nerve-ligated mice. Membranes were prepared at 7 days after nerve ligation. Each value represents the mean ± SEM of four to six samples. $^{**}p < 0.01$ *vs* sham group.

Suzuki *et al*, 1999; Zhao *et al*, 2004). Additionally, it has been reported that i.t. administration with morphine provides analgesia in patients with neuropathic pain syndromes (Krames 2002). Considering these reports, we propose here that the discrepancy and the effectiveness of morphine may be partly related to the timing of the treatment relative to the duration of the neuropathy.

It is well known that morphine is rapidly converted into two metabolites, M-3-G and M-6-G. Approximately, 10% of morphine is metabolized to M-6-G and 50% is metabolized to M-3-G. The fact that M-3-G has very low affinity for MOR and appears to lack significant analgesic activity suggests that M-6-G may be the only active metabolite of morphine. Several studies support the idea that M-6-G directly contributes to the analgesic effects of morphine (Sawe *et al*, 1985; Silva *et al*, 2000; Kilpatrick and Smith, 2005). Therefore, we next investigated the antinociceptive effect of M-6-G in nerve-ligated mice. S.c.-, i.t.-, and i.c.v. administration of M-6-G each induced a marked antinociceptive effect in sham-operated mice. Interestingly, M-6-G-induced antinociceptive effect following each injection was significantly attenuated in nerve-ligated mice compared with that in sham-operated mice. In addition, the concentration-dependent increases in the binding of [^{35}S]GTPγS to membranes obtained from the spinal cord and PAG, but not thalamus, stimulated by M-6-G were significantly decreased in nerve-ligated mice compared with those in sham-operated mice. It is considered that morphine in the brain and spinal cord cannot be converted into M-3-G or M-6-G. These findings suggest that the downregulation of M-6-G-sensitive MOR in the spinal cord or PAG area by sciatic nerve injury may result in the suppression of the antinociceptive effect induced by s.c. injection of morphine in nerve-ligated mice.

It has been reported that s.c.-administered oxycodone is metabolized in the liver, and a large proportion of oxycodone is metabolized to noroxycodone and oxymorphone by way of *N*-demethylation and *O*-demethylation in the first pass. Current evidence suggests that the metabolites of oxycodone including noroxycodone and oxymorphone do not significantly contribute to its pharmacological effects (Heiskanen and Kalso, 1997). It has been described that both oxymorphone and noroxycodone were more potent than oxycodone after i.t. administration (Lemberg *et al*, 2006a, b). In the present study, the increased [^{35}S]GTPγS binding induced by oxycodone in membrane fractions of the spinal cord, PAG, and thalamus and the antinociception induced by s.c.-, i.c.v.-, and i.t. administration of oxycodone were not changed by sciatic nerve ligation compared with those in sham-operated mice. Although it remains to be seen the discrepancy of the report regarding the antinociception of metabolites of oxycodone, the present data provide evidence that oxycodone may be a useful alternative to morphine in the treatment of neuropathic pain.

Another key finding in the present study is that the rewarding effects of s.c.-administered morphine or oxyco-

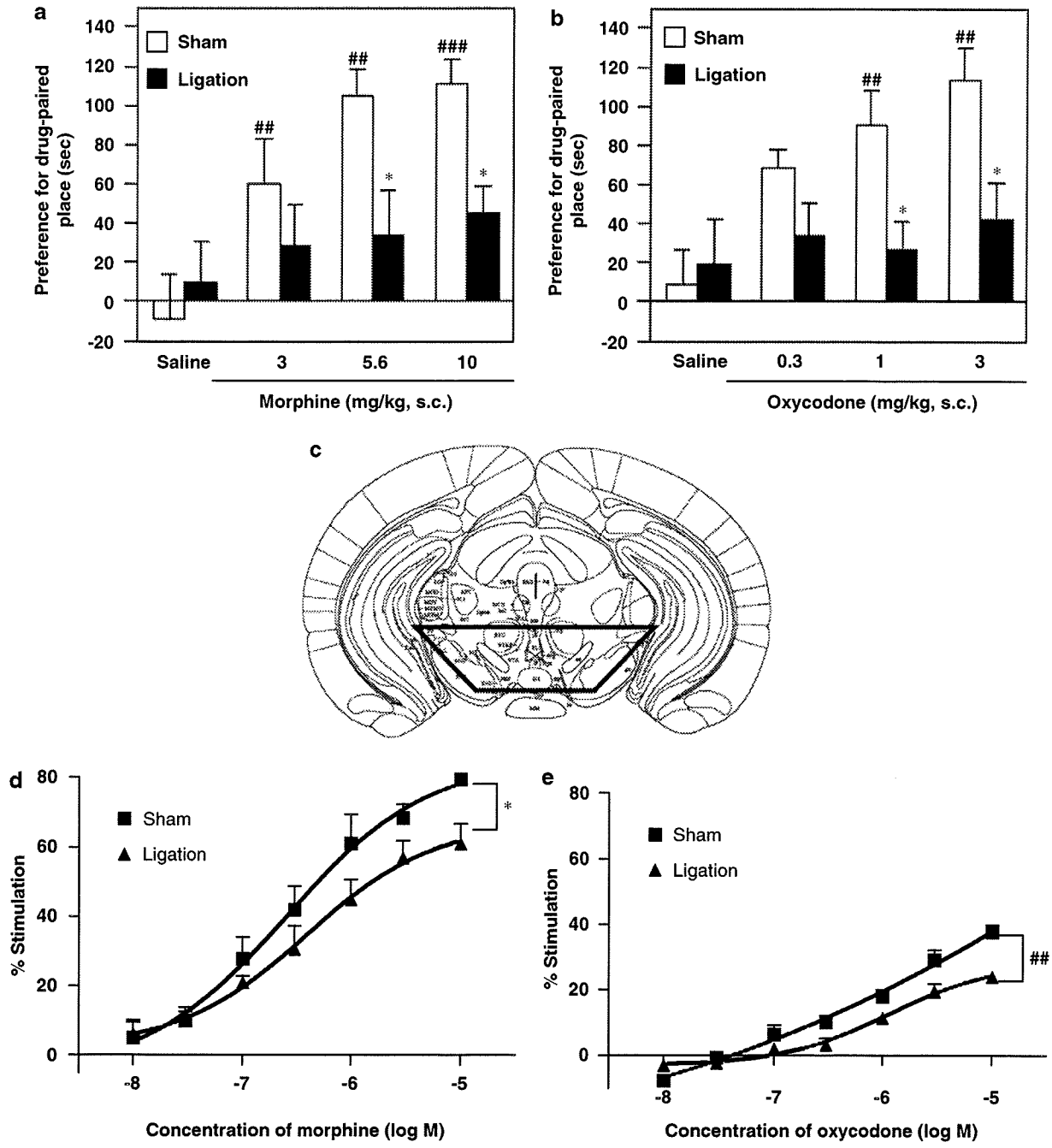

Figure 11 Conditioned place preference produced by s.c. administration of morphine (3–10 mg/kg, a) or oxycodone (0.3–3 mg/kg, b) in sham-operated and sciatic nerve-ligated mice using the conditioned place preference paradigm. Ordinate: mean difference between time spent in the post-conditioning test and pre-conditioning test. Immediately after s.c. injection of morphine or oxycodone, mice were placed and conditioned in either compartment for 1 h. Each column represents the mean ± SEM of seven to eight mice. $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ *vs* sham-saline group. $^{*}p<0.05$ *vs* sham-morphine or sham-oxycodone group. According to Paxions' atlas (2001), the coronal brain block of the lower midbrain area (box) was dissected obtained from 2.5 to 4.5 mm posterior to bregma (c). Effects of morphine (d) or oxycodone (e) on the binding of [^{35}S]GTPγS to membranes of the lower midbrain obtained from sham-operated and sciatic nerve-ligated mice. Membranes were prepared 7 days after nerve ligation. Each value represents the mean ± SEM of four samples. $^{*}p<0.05$ and $^{\#\#}p<0.01$ *vs* sham group.

done under a neuropathic pain-like state were suppressed following sciatic nerve ligation. The mesolimbic dopaminergic system, which projects from the VTA of the midbrain to the nucleus accumbens (N.Acc.), has been identified as the critical substrate of the reinforcing effects of opioids (Funada *et al*, 1995; Garzon and Pickel, 2001; Narita *et al*, 2001c). Considering these findings, we next assessed changes in the ability of morphine or oxycodone to activate G-proteins in the lower midbrain area including the VTA of sham-operated and sciatic nerve-ligated mice by monitoring the binding of [^{35}S]GTPγS to membranes. The increase in the binding of [^{35}S]GTPγS to lower midbrain membranes

in nerve-ligated mice stimulated by morphine or oxycodone was significantly lower than that observed in sham-operated mice. We previously reported that intra-VTA administration of DAMGO caused a dose-dependent preference for drug-associated place (Narita *et al*, 2001c). We also demonstrated that the enhancement of DA release in the N.Acc. stimulated by morphine was significantly suppressed by sciatic nerve ligation (Ozaki *et al*, 2002). Furthermore, we reported that sciatic nerve ligation caused a dramatic reduction in the activities of extracellular signal-regulated kinase in the tyrosine hydroxylase-positive dopaminergic neuron of the VTA (Ozaki *et al*, 2004). These findings suggest that the reduction in MOR-mediated G-protein activation in the lower midbrain area caused by sciatic nerve ligation may be responsible for inhibiting the rewarding effects induced by both morphine and oxycodone under a neuropathic pain-like state.

In conclusion, these findings provide further evidence that treatment with oxycodone produces a profound antinociceptive effect under a neuropathic pain-like state, with a less or less of a rewarding effect. Furthermore, the reduction in G-protein activation induced by M-6-G in the spinal cord and PAG may, at least in part, contribute to the suppression of the antinociceptive effect produced by morphine under a neuropathic pain-like state.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare that, expect for income received from our primary employer, no financial support or compensation has been received from any individual or corporate entity over the past 3 years for research or professional service and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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Direct evidence for the involvement of endogenous β-endorphin in the suppression of the morphine-induced rewarding effect under a neuropathic pain-like state

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Abstract

Recent clinical studies have demonstrated that when opioids are used to control pain, psychological dependence is not a major problem. In this study, we further investigated the mechanisms that underlie the suppression of opioid reward under neuropathic pain in rodents. Sciatic nerve ligation suppressed a place preference induced by the selective μ-opioid receptor agonist [D-Ala^2, *N*-MePhe^4, Gly-ol^5] enkephalin (DAMGO) and reduced both the increase in the level of extracellular dopamine by s.c. morphine in the nucleus accumbens and guanosine-5′-*o*-(3-[^{35}S]thio) triphosphate ([^{35}S]GTPγS) binding to membranes of the ventral tegmental area (VTA) induced by DAMGO. These effects were eliminated in mice that lacked the β-endorphin gene. Furthermore, intra-VTA injection of a specific antibody to the endogenous μ-opioid peptide β-endorphin reversed the suppression of the DAMGO-induced rewarding effect by sciatic nerve ligation in rats. These results provide molecular evidence that nerve injury results in the continuous release of endogenous β-endorphin to cause the dysfunction of μ-opioid receptors in the VTA. This phenomenon could explain the mechanism that underlies the suppression of opioid reward under a neuropathic pain-like state.

Keywords: Neuropathic pain; β-Endorphin; Ventral tegmental area

When a patient is an appropriate candidate for an opioid but the drug is not available in a sufficient dose to allow the patient to function adequately and maintain a reasonable lifestyle, it has often been proposed that opioid addiction does not arise as a consequence of the treatment of pain with opioid [1]. However, the scientific support for this clinical experience is still needed if physicians are to be confident in using adequate doses of opioids for the treatment of severe pain.

The mesolimbic dopamine (DA) system, which consists of DAergic neurons in the ventral tegmental area (VTA) and their projections to the nucleus accumbens (NAcc.), has been implicated in the rewarding properties of opioids [2]. We previously reported that morphine failed to induce rewarding effects in rodents under sciatic nerve ligation [6–8]. We also demonstrated that sciatic nerve ligation suppressed the morphine-induced dopamine release in the NAcc. associated with the inhibition of μ-opioid receptor-mediated G-protein activation in the VTA [6–8]. These findings suggest that the dysfunction of μ-opioid receptor in the VTA may be responsible for the suppression of morphine dependence under a chronic pain.

β-Endorphin is an endogenous opioid peptide that has a profound analgesic effect when administered to the central nervous system. The acute release of β-endorphin into ventricular CSF has been demonstrated during electrical stimulation of the periventricular grey matter in humans, and a relation has been found between analgesia and β-endorphin release [14]. Furthermore, it has been reported that pain induces the release of endogenous opioid peptides in the brain, including the mesolimbic area in humans [15]. Therefore, we proposed that sciatic nerve injury may increase the release of endogenous opioid peptides that interact with μ-opioid receptors in the mesolimbic area.

Transgenic β-endorphin knockout mice allow us to determine physiological functions mediated via central β-endorphin-containing fibers under a chronic pain-like state. The aim of the

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present study was to further investigate the molecular mechanisms that underlie the suppression of the opioid-induced rewarding effect under a neuropathic pain-like state using β-endorphin knockout mice.

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.

In the present study, we used the male and female β-endorphin derived from *proopiomelanocortin* (*Pomc*) gene-knockout mice (The Jackson Laboratory, Bar Harbor, ME, USA), which had a C57BL/6J and 129S2/SvPas mixed genetic background as described previously [12] and male Sprague–Dawley rats (Tokyo Laboratory Animals Science Co. Ltd., Tokyo, Japan). Animals were housed in a room maintained at 23 ± 1 °C with a 12 h light/dark cycle (light from 8:00 A.M. to 8:00 P.M.). Food and water were available *ad libitum*. The genotype of offspring from β-endorphin knockout (−/−) mice was determined as described previously [5].

For microinjection of rats, surgery was conducted as previously described [4]. Three days after surgery, the animals were injected with β-endorphin antibody (1:500 or 1:1000) (Peninsula Laboratories Inc., CA) diluted in saline or saline alone, which was delivered by a motorized syringe pump in a volume of 0.3 μl over 60 s, into the VTA 10 min before the start of nerve injury and once a day for three consecutive days after nerve injury.

The rats and mice were deeply anesthetized with isoflurane. We produced a partial sciatic nerve injury by tying a tight ligature with a 8–0 silk suture around approximately one-third to one-half the diameter of the sciatic nerve on the right side (ipsilateral side) under a light microscope (SD30, Olympus, Tokyo, Japan) as described previously [6]. In sham-operated animals, the nerve was exposed without ligation. To assess the sensitivity to thermal stimulation, each of the hind paws of rats were tested individually using a thermal stimulus apparatus (model 33 Analgesia meter; IITC Inc./Life Science Instruments, Woodland Hills, CA, USA) as described previously [7].

Place conditioning was performed following the methods described previously [4]. Conditioning sessions were started from three days after sciatic nerve surgery, and conducted once daily for six days. Immediately after the microinjection of DAMGO (Sigma Co., St Louis, MO, USA) (1 nmol) into the VTA (rats) or s.c. injection of morphine (Daiichi-Sankyo Co., Tokyo, Japan) (5 mg/kg) (mice), these animals were placed in the compartment opposite that in which they had spent the most time in the pre-conditioning test for 1 h. On alternative days, these animals received saline and were placed in the other compartment for 1 h. On the day after the final conditioning session, a post-conditioning test was performed.

To identify mesolimbic projecting neurons, the retrograde tracer fluoro-gold (FG) (4%, Fluorochrome, Englewood, CO) was injected into the NAcc. The anesthetized by isoflurane animals were placed in a stereotaxic apparatus. The skull was exposed and a small hole was drilled through the skull over the NAcc. (AP: +1.5 mm, ML: −0.5 mm, DV: −7.0 mm) according to the brain atlas [10]. A micropipette with a diameter of about 15–20 μm was filled with FG solution. FG was pressure-injected (200 nl) into the NAcc. of the right hemisphere. After the injection, the micropipette was left in place for 5 min to avoid leakage after removal of the injection cannula. Following surgery, the scalp was closed. Three days after FG injection, rats were re-anesthetized with isoflurane and subjected to surgery for partial nerve injury.

The procedure for the sample preparation and immunohistochemistry were performed following the methods described previously [3]. Ten days after nerve ligation, rats were deeply anesthetized with isoflurane and intracardially perfusion-fixed with freshly prepared 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.4). After perfusion, the brains were then quickly removed after perfusion, and thick coronal sections of the midbrain including the VTA was initially dissected using brain blocker. The VTA sections were blocked in 10% normal goat serum (NGS) in 0.01 M PBS for 1 h at room temperature. The primary antibody [1:400 phosphorylated–tyrosine hydroxylase (Ser31) (Chemicon International, Inc., CA, USA)] was diluted in 0.01 M PBS containing 10% NGS and incubated for two nights at 4 °C. The samples were then rinsed and incubated with an appropriate secondary antibody conjugated with Alexa 488 for 2 h at room temperature. Fluorescence of FG and immunolabeling were detected using a light microscope (Olympus BX-80; Olympus) and photographed with a digital camera (CoolSNAP HQ; Olympus).

For determination of μ-opioid receptor function, a section of the lower midbrain that included the VTA and limbic forebrain that included the NAcc., as described previously [6], were quickly removed after decapitation, and rapidly transferred to a tube filled with ice-cold buffer. The membrane homogenate was prepared and incubated at 25 °C for 2 h in 1 ml of assay buffer with various concentrations of each agonist, 30 μM guanosine-5′-diphosphate (GDP) and 50 pM guanosine-5′-*o*-(3-thio) triphosphate ([^{35}S]GTPγS) (specific activity, 1000 Ci/mmol; Amersham, Arlington Heights, IL, USA). The reaction was terminated by filtration using Whatman GF/B glass filters (Brandel, Gaithersburg, MD, USA). The filters were washed three times with 5 ml of ice-cold Tris–HCl buffer and then transferred to scintillation-counting vials containing 3 ml of Clear-sol II (Nacalai Tesque Inc., Kyoto, Japan) and equilibrated for 12 h. The radioactivity in the samples was determined with a liquid scintillation analyzer. Nonspecific binding was measured in the presence of 10 μM unlabeled GTPγS. In the present study, sample preparation was performed 10 days after partial sciatic nerve ligation.

Mouse *in vivo* microdialysis study was conducted as previously described [4]. Three days after sciatic nerve surgery, mice were implanted with microdialysis probe (D-I-6-01; 1 mm membrane length; Eicom) into the NAcc. (from bregma: anterior, +1.5 mm; lateral, +0.9 mm; ventral, −4.9 mm) according to the brain atlas [9]. At 24 h after implantation, the probe was perfused continuously at a flow rate of 2 μl/min. Outflow fractions were taken every 5 min. After 15 baseline fractions were collected, mice were given morphine (10 mg/kg, s.c.) or saline. Dialysis fractions were then analyzed using HPLC with ECD (HTEC-

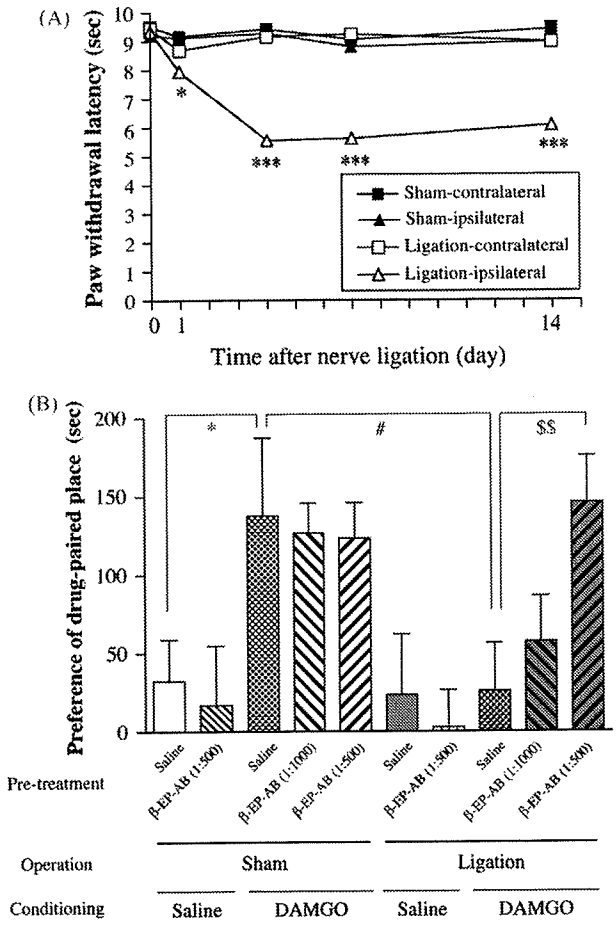

Fig. 1. (A) Effect of sciatic nerve ligation on withdrawal responses to thermal stimulation. There was no difference in the basal response between sham-operated and sciatic nerve-ligated rats before surgery (day 0). Thermal hyperalgesia was only observed on the ipsilateral side of sciatic nerve-ligated rats. The data are presented as the mean ± S.E.M. $^{*}p<0.05$ and $^{***}p<0.001$ vs. sham group. (B) Effect of microinjection of a specific antibody to β-endorphin into the VTA on the suppression of a place preference induced by DAMGO under a neuropathic pain-like state. Ordinate: mean differences (seconds) between time spent in the DAMGO- and saline-paired sides of the test box. Each column represents the mean conditioning score with S.E.M. $^{*}p<0.05$ vs. saline/sham/saline group. $^{\#}p<0.05$ vs. saline/sham/DAMGO group. $^{\$\$}p<0.01$ vs. saline/ligation/DAMGO group.

500; Eicom). Dopamine was identified according to the retention times of a dopamine standard, and amounts of dopamine were quantified by calculating with peak areas. The baseline microdialysis data were calculated as concentrations in the dialysates. Other microdialysis data are expressed as percentages of the corresponding baseline level.

The data are presented as the mean ± S.E.M. The statistical significance of differences between the groups was assessed by one- or two-way analysis of variance (ANOVA) followed by the Bonferroni/Dunnett test or Student's *t*-test.

Sciatic nerve-ligated rats exhibited a significant and persistent decrease in the latency of paw-withdrawal on the ipsilateral side ($p<0.001$: sham-ipsilateral vs. ligation-ipsilateral) (Fig. 1A). Under these conditions, we investigated whether sciatic nerve ligation could affect the place conditioning induced by the selective μ-opioid receptor agonist DAMGO in rats. In the present study, the place preference induced by the microinjection of DAMGO into the VTA was significantly suppressed in sciatic nerve-ligated rats ($p<0.05$ vs. sham-DAMGO groups). However, microinjection of β-endorphin antibody into the VTA just before and after three days of sciatic nerve ligation significantly reversed the suppression of DAMGO-induced place preference in rats with sciatic nerve ligation ($p<0.01$ vs. saline-ligation-DAMGO groups) (Fig. 1B).

To investigate a possible change in tyrosine hydroxylase activity in the VTA of sciatic nerve-ligated rats, immunohistochemical studies were performed. As shown in Fig. 2, p-TH (Ser31) immunoreactivity was prominently observed in the VTA of sham-operated rats (Fig. 2A(i) and 2A(ii): high magnification). Sciatic nerve ligation dramatically diminished the p-TH immunoreactivity in this region (Fig. 2B). Consistent with many previous studies which have characterized afferent projections to the NAcc., FG-containing cells were apparently detected in the VTA of sham-operated rats after microinjection of the retrograde tracer FG into the shell region of the unilateral NAcc. (Fig. 2A(iii)). Further immunostaining showed that a population of retrogradely labeled neurons in the VTA of sham-operated rats was also immunoreactive for p-TH (Ser31) (Fig. 2A(iv)).

The genotype of offspring from β-endorphin knockout (−/−) mice was confirmed by PCR analysis using DNA extracted from the ear. As shown in Fig. 3A (lanes 3 and 4), β-endorphin knockout (−/−) mice yielded a targeted deficient allele single amplification product. In contrast, β-endorphin (+/+) (wild-type) mice showed a single amplification product (Fig. 3A, lanes 1 and 2). Using these genotype mice, we performed a conditioned place preference test. In wild-type mice, the morphine-conditioned place preference was significantly attenuated following sciatic nerve ligation. ($p<0.001$ vs. sham-wild-type-morphine; Fig. 3B). In contrast, there were no significant changes in the morphine-induced place preference between sham- and nerve-ligated β-endorphin knockout (−/−) mice (Fig. 3B).

The ability of DAMGO to activate μ-opioid receptor linked to its G-proteins in the lower midbrain including the VTA obtained from sham-operated or sciatic nerve-ligated wild-type and β-endorphin knockout (−/−) mice was examined by monitoring the binding of [^{35}S]GTPγS. DAMGO (10^{-8}–10^{-5} M) produced a concentration-dependent increase in the binding of [^{35}S]GTPγS to lower midbrain membranes in sham-operated mice. Conversely, the level of [^{35}S]GTPγS binding to this area stimulated by DAMGO in nerve-ligated wild-type mice was significantly lower than that observed in sham-operated wild-type mice (10^{-5} M; $p<0.01$ vs. sham-operated wild-type group) (Fig. 3C(i)). In contrast, there were no significant changes in the DAMGO-induced increase in the binding of [^{35}S]GTPγS to lower midbrain membranes between nerve-ligated β-endorphin knockout (−/−) mice and sham-operated β-endorphin knockout (−/−) mice (Fig. 3C(ii)). The ability of DA to activate G-proteins in the limbic forebrain including the NAcc. of sham-operated and sciatic nerve-ligated mice was examined by monitoring the binding of [^{35}S]GTPγS to limbic forebrain membranes. DA (10^{-8} to 10^{-5} M) produced a concentration-dependent increase

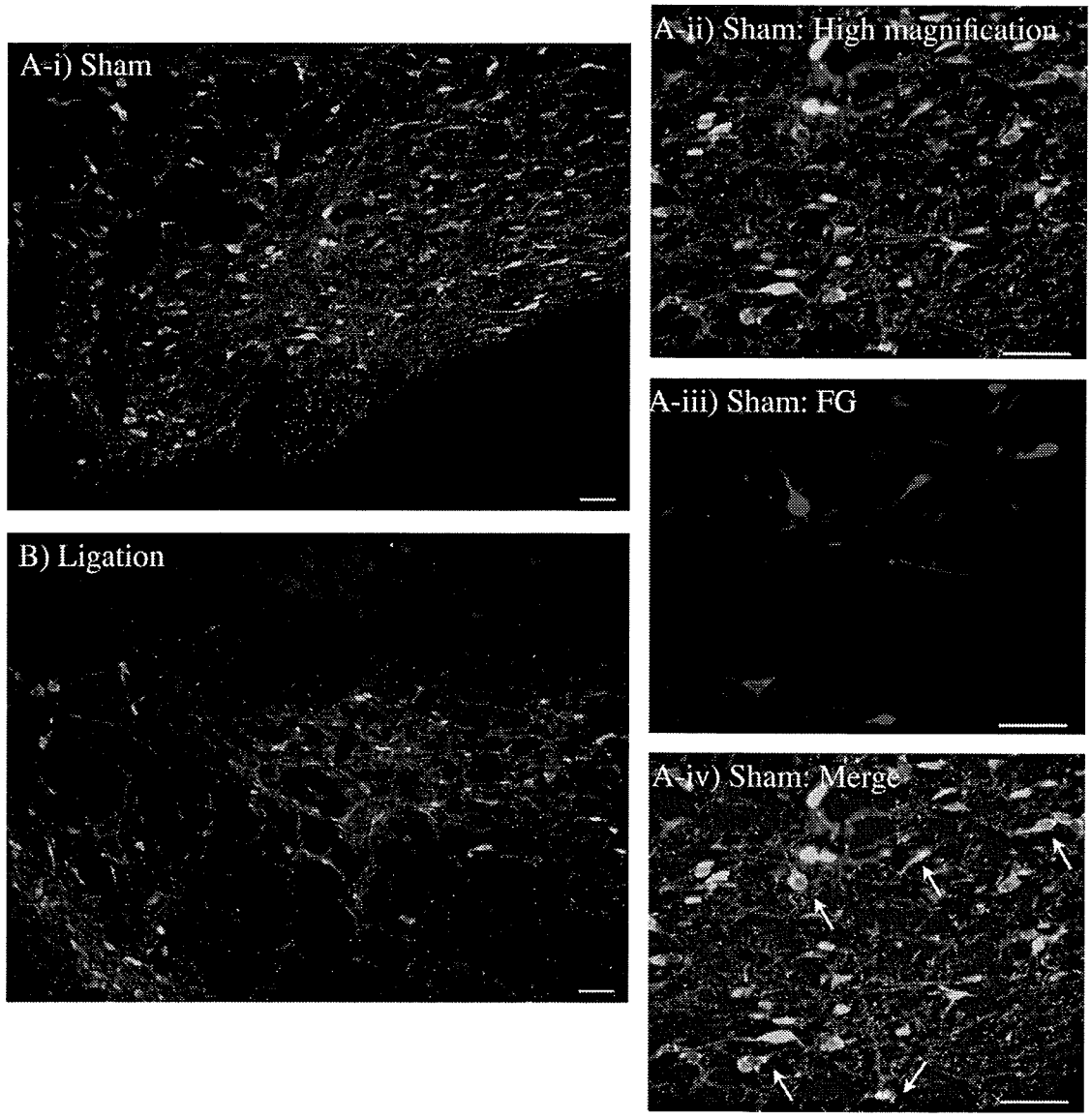

Fig. 2. Immunofluorescent staining for phosphorylated-tyrosine hydroxylase (Ser31) (p-TH)-like immunoreactivity (IR) in the VTA of sham-operated rats or nerve-ligated rats (A(i)). The images show p-TH-like IR in the VTA of nerve-ligated rats (B) or sham-operated rats (A(i)). (A(iv)) Apparent co-localization of p-TH (green: A(ii)) with FG-positive cells retrogradely labeled from the NAcc (blue: A(iii)) in the VTA of sham-operated rats. The sample was prepared 10 days after nerve ligation in rats. Scale bars = 50 μm.

in [^{35}S]GTPγS binding to limbic forebrain membranes from both sham-operated and sciatic nerve-ligated wild-type mice to the same degree (Fig. 3D(i)). Similarly, no differences in the increase in [^{35}S]GTPγS binding stimulated by DA were noted between sham-operated and sciatic nerve-ligated β-endorphin knockout (−/−) mice (Fig. 3D(ii)).

Fig. 4A shows the location of microdialysis probes within the mouse NAcc. Probe-inserted regions were localized in the NAcc. Only data from mice in which probes had been accurately inserted in the NAcc. were used for subsequent statistical analysis. The effect of the s.c. administration of morphine on the dialysate dopamine level in the mouse NAcc. is shown in Fig. 4B. The dopamine levels were markedly increased by morphine (10 mg/kg, s.c.) compared with that induced by saline treatment in sham-operated wild-type mice. Under these conditions, the increased level of dialysate dopamine in the NAcc. stimulated by morphine was significantly decreased in sciatic nerve-ligated wild-type mice ($F(1,234)=2.824$; $p<0.0001$, sham-operated wild-type mice treated with morphine vs. ligated wild-type mice treated with morphine). However, the increase in the level of dialysate dopamine stimulated by morphine was not affected in sciatic nerve-ligated β-endorphin knockout (−/−) mice.

In the present study, we confirmed that sciatic nerve ligation suppressed a place preference induced by microinjection of the selective μ-opioid receptor agonist DAMGO into the VTA of rats. Furthermore, we demonstrated that nerve injury reduced both the increase in the level of extracellular dopamine by s.c. morphine in the NAcc. in wild-type mice and the increase in [^{35}S]GTPγS binding to membranes of VTA obtained from wild-type mice induced by DAMGO. Moreover, p-TH (Ser31)

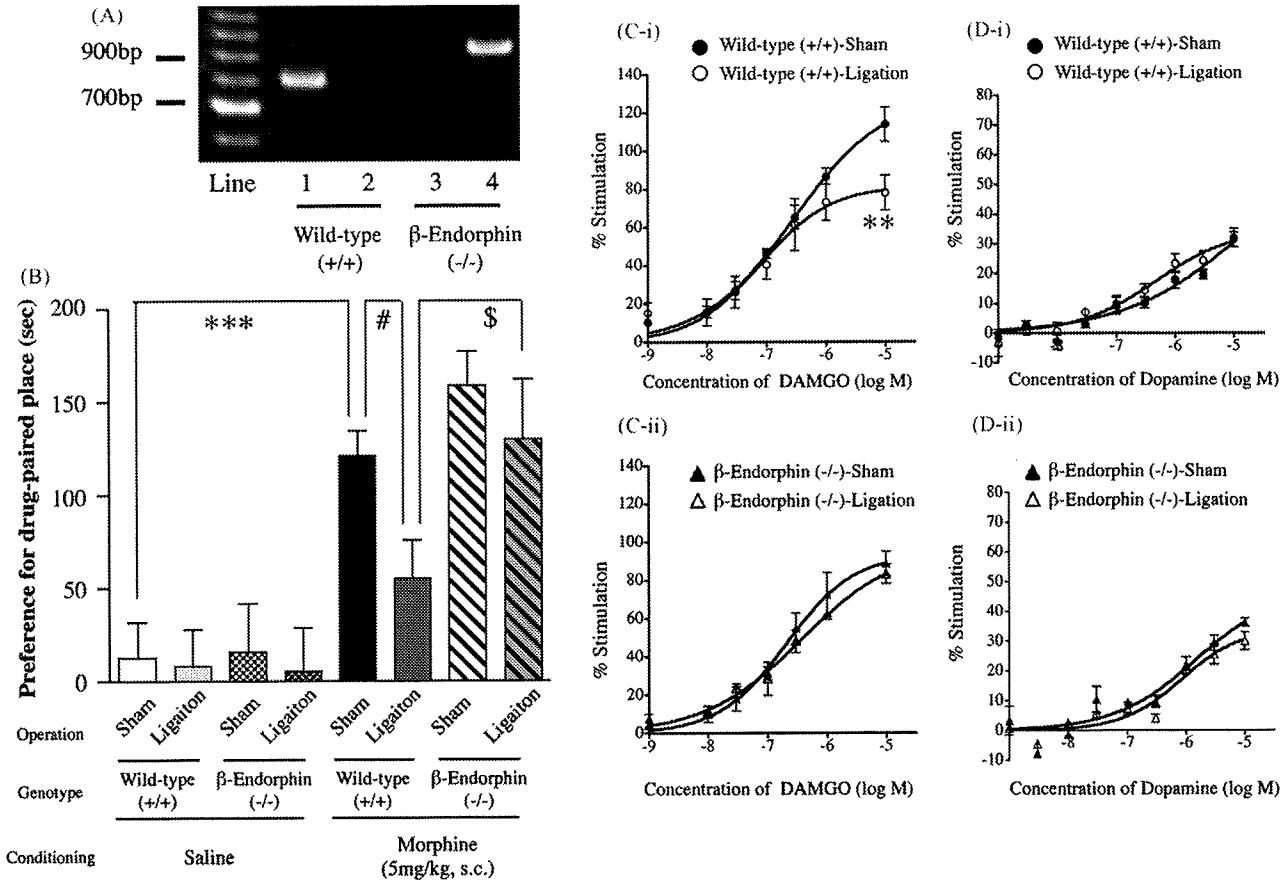

Fig. 3. (A) Representative PCR for *proopiomelanocortin* (*Pomc*) DNA extracted from the ear of either wild-type (+/+) or β-endorphin knockout (−/−) mice. β-Endorphin knockout (−/−) yielded a targeted deficient allele single amplification product (lanes 3 and 4), whereas wild-type mice showed a wild-type allele single amplification products (lanes 1 and 2). (B) Disappearance of the suppression of the morphine (5 mg/kg, s.c.)-induced place preference by sciatic nerve ligation due to the absence of the β-endorphin gene. The data represent the mean with S.E.M.: $^{***}p < 0.001$ vs. sham/wild-type/saline group; $^{\#}p < 0.05$ vs. sham/wild-type/morphine group; $^{\$}p < 0.05$ vs. ligation/wild-type/morphine group. (C and D) Concentration-response curve of DAMGO (C) or dopamine (D) on the binding of [^{35}S]GTPγS to membranes of the lower midbrain or limbic forebrain from wild-type (+/+) and β-endorphin knockout (−/−) mice after sciatic nerve ligation or sham-operation. Each value represents the mean ± S.E.M. $^{**}p < 0.01$ vs. sham-wild-type group.

immunoreactivity in the rat VTA was dramatically diminished by sciatic nerve ligation, and some of p-TH-positive neurons were directly projected to the NAcc. These findings provide further evidence that a neuropathic pain-like state causes a significant reduction in the activity of a μ-opioid receptor-mediated mesolimbic dopaminergic pathway in rodents.

Under these conditions, an intra-VTA injection of a specific antibody to β-endorphin reversed the suppression of the DAMGO-induced place preference by sciatic nerve ligation in rats. To further investigate the role of β-endorphin in the suppression of opioid reward under a neuropathic pain-like state, we next performed several experiments using β-endorphin gene knockout mice. These β-endorphin knockout mice exhibit no change in the expression of other peptide products (e.g. ACTH and MSH) from the POMC gene [13]. Using these transgenic mice, we demonstrated here that the suppression of a morphine-induced place preference by sciatic nerve ligation was abolished in β-endorphin gene knockout mice, indicating that endogenous β-endorphin is required for recovery from the suppression of opioid reward under a neuropathic pain-like state in rodents. The present result was mostly consistent with the recent finding by Petraschka et al. [11] that β-endorphin knockout mice did not show μ-opioid recepter phosphorylation-dependent tolerance to morphine antinociception and reduced place preference induced by morphine.

A key finding in the present study is that the increased [^{35}S]GTPγS binding by DAMGO to membranes in the VTA area obtained from wild-type mice was dramatically decreased by sciatic nerve ligation and this effect was abolished in mice that lacked the β-endorphin gene following sciatic nerve ligation. These results provide molecular evidence that endogenous β-endorphin corresponds to the down-regulation of μ-opioid receptor function to activate G-proteins in the VTA following nerve injury. Taken together, we propose here that β-endorphin released by chronic nociceptive stimuli may continuously activate μ-opioid receptors in the VTA and in turn lead to the down-regulation of μ-opioid receptor function.

Another key finding of the present study is that the increased extracellular dopamine in the NAcc. produced by s.c. treatment with morphine was diminished by sciatic nerve ligation in wild-type mice, and this effect was also eliminated in β-endorphin knockout mice with sciatic nerve ligation. This result

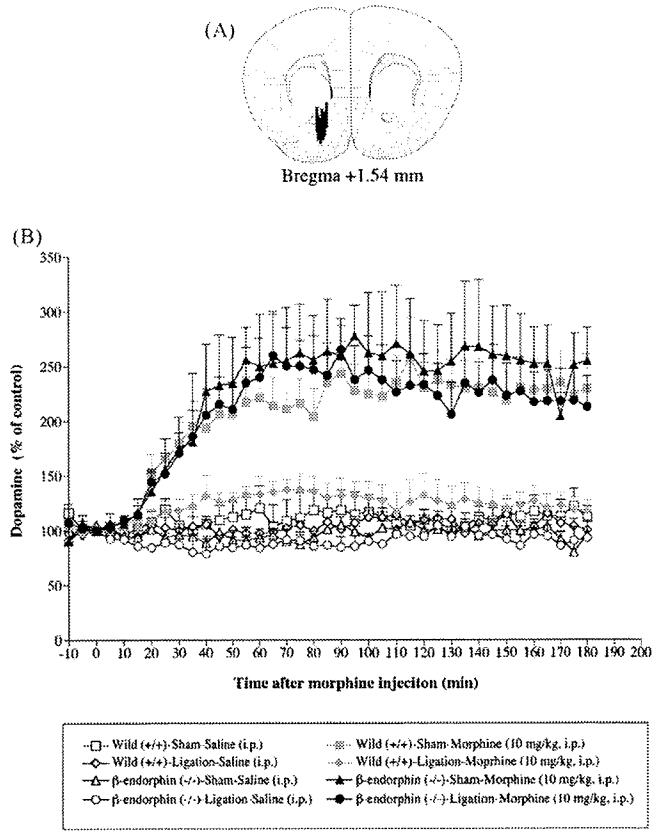

Fig. 4. Change in the increased dialysate dopamine level induced by morphine in β-endorphin knockout (−/−) mice. Localization of microdialysis probe sites in the mouse NAcc (A). Effects of treatment with morphine on the dialysate dopamine level in the NAcc. in sham- or nerve-ligated wild-type and these β-endorphin knockout (−/−) mice (B). Morphine (10 mg/kg, s.c.) or saline was injected at time 0. The data are expressed as percentages of the corresponding baseline levels with S.E.M. $F(1,234) = 2.824$, $p < 0.0001$; sham-operated wild-type mice treated with morphine vs. sham-operated β-endorphin knockout (−/−) mice treated with morphine.

strongly supports the idea that endogenous β-endorphin is responsible for the suppression of opioid reward linked to a mesolimbic dopaminergic system under a neuropathic pain-like state.

In conclusion, we demonstrated here that suppression of a μ-opioid-induced place preference by sciatic nerve ligation was abolished by microinjection of the β-endorphin antibody into the VTA and by deletion of the β-endorphin gene. Furthermore, the deletion of β-endorphin also eliminated the reductions in both μ-opioid receptor function in the VTA by nerve ligation and in the activity of mesolimbic dopaminergic transmission projecting from the VTA to the NAcc. regulated by μ-opioid receptors in the VTA. These findings provide further evidence that β-endorphin released in the VTA is a key player in regulating the dysfunction of μ-opioid receptor function to negative modulate opioid reward under a neuropathic pain-like state.

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ORIGINAL INVESTIGATION

Changes in the rewarding effects induced by tramadol and its active metabolite M1 after sciatic nerve injury in mice

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Abstract
Introduction The present study was designed to investigate the rewarding effects induced by tramadol and its active metabolite *O*-desmethyltramadol (M1) under a neuropathic pain-like state.
Results In opioid receptor binding and G protein activation, we confirmed that M1, but not tramadol, showed μ-opioid receptor (MOR) agonistic activity. Furthermore, we found that the subcutaneous (s.c.) injection of tramadol and M1 each produced a significant place preference in mice, and these effects were significantly suppressed by pretreatment with the MOR antagonist β-funaltrexamine. The dopamine level in the mouse nucleus accumbens was significantly increased by s.c. injection of either tramadol or M1. Mice with sciatic nerve ligation exhibited a marked decrease in the latency of paw withdrawal in response to a thermal stimulus only on the ipsilateral side. Under these neuropathic pain-like conditions, the rewarding effect induced by s.c. injection of either tramadol or M1 was dramatically inhibited after sciatic nerve ligation. Furthermore, the M1-induced G protein activation in the lower midbrain area was suppressed after sciatic nerve ligation.
Discussion Our present data support the notion that the rewarding effect induced by tramadol is mediated mainly through metabolism to its active metabolite M1 via MOR. Furthermore, the suppression of the M1-induced G protein activation in the lower midbrain area caused by sciatic nerve ligation may be responsible for inhibiting the rewarding effects induced by s.c. injection of tramadol and M1 under a neuropathic pain-like state.

Keywords Tramadol · Active metabolite M1 · Rewarding effect · Neuropathic pain · Opioid receptor binding · G protein activation · Nucleus accumbens · Ventral tegmental area · Dopamine · μ-Opioid receptor

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Introduction

Most of the opioids used clinically have been classified as μ-opioid receptor (MOR) agonists. Recent studies have shown that MOR agonists and their metabolites have interesting pharmacological differences (Ide et al. 2006; Lemberg et al. 2006; Peckham and Traynor 2006). Tramadol is used as an analgesic agent for the treatment of moderate to severe pain. It has been available in Germany since 1977 and in the United States since 1995. Tramadol, which has been found to be an opioid agonist with selectivity for the MOR, is a synthetic 4-phenyl-piperidine analog of codeine. Its affinity for the MOR is approximately tenfold less than that of codeine and 6,000-fold less than that of morphine (Raffa et al. 1992). Tramadol has an analgesic effect through the activation of MOR (Radbruch et al. 1996; Gibson 1996). In addition to its actions on brain opioid receptors, tramadol also inhibits the reuptake of serotonin and noradrenaline (Driessen and

Reimann 1992; Bamigbade et al. 1997; Sagata et al. 2002). Tramadol is a central analgesic that is in the second step of the WHO three steps ladder scale unlike conventional opioids. Tramadol is rapidly metabolized in the liver. The principal metabolic pathway, *O*-desmethylation and *N*-desmethylation, involve cytochrome P-450 isoenzymes 2D6, 2B6, and 3A4, respectively (Lintz et al. 1998). The primary metabolites *O*-desmethyltramadol (M1) and *N*-desmethyltramadol (M2) may be further metabolized to three additional secondary metabolites. The overall activity of tramadol originates from the specific action of its antinociceptive active metabolite M1, which shows high affinity for the MOR (Raffa et al. 1992). It has been reported that the metabolite M2 displayed only weak affinity and had no effect on GTPγS binding (Gillen et al. 2000).

Neuropathic pain is well-characterized by spontaneous burning pain, hyperalgesia (increased pain in response to painful stimuli), and allodynia (pain evoked by normally innocuous stimuli) and is very difficult to manage in the pain clinic. Because general analgesics such as acetaminophen and nonsteroidal antiinflammatory drugs often fail to improve neuropathic pain, it is widely accepted that complicated mechanisms may underlie this pain syndrome. Many studies have focused on the long-term changes in functions of the spinal dorsal horn neurons (Nichols et al. 1995; Mayer et al. 1999; Narita et al. 2000). However, the mechanisms that underlie neuropathic pain are not completely understood.

A growing body of clinical evidence suggests that when opioid analgesics including morphine are used to control pain in patients, psychological dependence is not a major concern. We previously reported that morphine failed to induce rewarding effects in rats that had been injected with formalin or carrageenan into the hind paw and that had been tying ligature of the sciatic nerve (Suzuki et al. 1996, 1999, 2001; Ozaki et al. 2002, 2003, 2004; Narita et al. 2005a, 2007). Furthermore, it has been documented that chronic pain attenuates the development of tolerance to the antinociceptive effect of morphine in rodents (Vaccarino et al. 1993; Imai et al. 2006). These findings suggest the possibility that pain could lead to physiological changes at supraspinal levels associated with the suppression of opioid dependence. It has been reported that tramadol has a potential for physical and psychological dependence (Yanagita 1978; Liu et al. 1999; Sprague et al. 2002; Tzschentke et al. 2002), but may possess considerably less abuse potential than morphine (Budd 1994). Recent clinical evidence has suggested that tramadol abuse has been suggested in humans (Cicero et al. 1999; Yates et al. 2001; Zacny 2005; Epstein et al. 2006). Although tramadol has been used clinically for many years, the effect of tramadol on the reward system is poorly understood.

The aim of the present study was to clarify the mechanisms of the rewarding effects of tramadol and its active metabolite M1. Furthermore, this study was designed to investigate whether a state of neuropathic pain induced by sciatic nerve ligation could alter the rewarding effects induced by tramadol and M1.

Materials and methods

The present study was conducted in accordance with Guiding Principles for the Care and Use of Laboratory Animals Hoshi University, as adopted by the Committee on Animal Research of Hoshi University. Every effort was made to minimize the numbers and any suffering of animals used in the following experiments.

Animals

Male ICR mice weighing 20–25 g (Tokyo Animal Laboratories, Tokyo, Japan) were used. Animals were kept in a room with an ambient temperature of 23±1°C and a 12-h light–dark cycle (lights on 0800–2000 h) and were allowed to adapt to this environment for 1 week before the experiments. Food and water were available ad libitum.

Drugs

The drugs used in the present study were tramadol (Mikasa Pharmaceutical, Tokyo, Japan), *O*-desmethyltramadol (M1; synthesized by Dr. Higashiyama), β-funaltrexamine hydrochloride (β-FNA). All drugs were dissolved in 0.9% physiological saline (Otsuka Pharmaceutical, Tokyo, Japan) for in vivo experiments or assay buffer for in vitro experiments.

Neuropathic pain model

The mice were anesthetized with 3% isoflurane. We produced a partial sciatic nerve injury by tying a tight ligature with 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 (Seltzer et al. 1990; Malmberg and Basbaum 1998). In sham-operated mice, the nerve was exposed without ligation.

Place conditioning

Place conditioning studies were conducted using an apparatus consisting of a shuttle box (15×30×15 cm, $w \times l \times h$), which was made of an acrylic resin board and divided into two equal-sized compartments (Suzuki 1996; Oe et al. 2004). One compartment was white with a textured floor and the other compartment was black with a smooth floor to create equally inviting compartments. The place conditioning

schedule consisted of three phases (preconditioning test, conditioning, and postconditioning test). The preconditioning test was performed as follows: the partition separating the two compartments was raised to 7 cm above the floor, a neutral platform was inserted along the seam separating the compartments, and mice that had not been treated with either drugs or saline were then placed on the platform. The time spent in each compartment during a 900-s session was then recorded automatically using an infrared beam sensor (KN-80; Natsume Seisakusyo, Tokyo, Japan). Groups of mice were allocated to their respective conditioning compartments based on their preconditioning test data. Conditioning sessions (three for tramadol or M1/three for saline) were started the next day after the preconditioning test and conducted once daily for 6 days. Immediately after subcutaneous (s.c.) injection of tramadol or M1, these animals were placed in the compartment opposite to that in which they had spent the most time in the preconditioning test for 1 h. On alternate days, these animals received saline and were placed in the other compartment for 1 h. On the day after the final conditioning session, a postconditioning test that was identical to the preconditioning test was performed.

Assessment of thermal hyperalgesia

To assess the sensitivity to thermal stimulation, each of the hind paws of mice was 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 8–10 s in naïve mice. Only quick hind paw movement (with or without licking of the 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 latency of paw withdrawal after the thermal stimulus was determined as the average of two measurements per paw. The paw withdrawal threshold to the thermal stimulus was measured before surgery and the day after measurement of the thermal threshold (days 1, 3, 5, 7, 9, 11, and 14).

Assessment of tactile stimulus

To quantify the sensitivity to a tactile stimulus, paw withdrawal in response to a tactile stimulus was measured using von Frey filaments (North Coast Medical, Morgan Hill, CA, USA) with two different bending forces (0.02 and 0.16 g). Each von Frey filament was applied to the plantar surface of the hind paws for 3 s, and this was repeated three times. Each of the hind paws of the mice was tested individually. Paw withdrawal in response to a tactile stimulus was evaluated by 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 or licking; 3, an intense withdrawal response away from the stimulus with brisk flinching and/or licking. Paw withdrawal in 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 3 min between the measurements. Before the behavioral responses to a tactile stimulus were tested, mice were habituated for at least 1 h on an elevated nylon mesh floor. Under these conditions, paw withdrawal in response to a tactile stimulus was tested. The paw withdrawal threshold to the tactile stimulus was measured before surgery and the day after measurement of the thermal threshold (days 2, 4, 6, 8, 10, 12, and 14).

Receptor binding assay

For membrane preparation, the mouse whole brain without cerebellum was quickly removed after decapitation and rapidly transferred to a tube filled with an ice-cold buffer. The homogenate was centrifuged at 4°C for 10 min at 1,000×*g* and the supernatant was centrifuged at 4°C for 20 min at 48,000×*g*. The pellet was homogenated and resuspended at 4°C for 20 min at 48,000×*g*. The resulting pellet was resuspended and retained as membrane fraction. The MOR binding assay was performed in duplicate with [tylosil-3,5-(3)*H*(*N*)]-[D-Ala(2),*N*-MePhe(4),Gly-ol(5)]enkephalin ([^{3}H] DAMGO) (specific activity, 59.0 Ci/mmol; Amersham Biosciences, Arlington Heights, IL, USA) at 2 nM in a final volume of 1.0 ml that contained 50 mM Tris–HCl buffer, pH 7.4, and 0.1 ml of the homogenated membrane fraction. The amount of membrane proteins used in each assay was in the range of 90–140 μg, as determined by the method of Narita et al. (2001a). The test tubes were incubated for 1 h at 25°C. Specific binding was defined as the difference in bindings observed in the absence and presence of 1 μM unlabeled DAMGO. Incubation was terminated by collecting membranes on Whatman GF/B filters using a Brandel cell harvester. The filters were then washed three times with 5 ml Tris–HCl buffer, pH 7.4, at 4°C and transferred to scintillation vials. Then, 4 ml of clear-sol 2 (Nacalaitesque, Kyoto, Japan) was added to the vials. After a 12-h equilibration period, radioactivity in the samples was determined in a liquid scintillation analyzer.

Guanosine-5′-*o*-(3-thio) triphosphate ([^{35}S]GTPγS) binding assay

For membrane preparation, the section of the mouse midbrain including the ventral tegmental area (VTA), as described

previously (Ozaki et al. 2004), was quickly removed after decapitation and rapidly transferred to a tube filled with an ice-cold buffer. The membrane homogenate (3–8 μg protein/assay) was prepared as described by us (Narita et al. 2001a) and incubated at 25°C for 2 h in 1 ml of assay buffer with various concentrations of the each agonist, 30 μM guanosine-5′-diphosphate (GDP) and 50 pM [^{35}S]GTPγS (specific activity, 1,000 Ci/mmol; Amersham, Arlington Heights, IL, USA). The reaction was terminated by the filtration using Whatman GF/B glass filters (Brandel, Gaithersburg, MD, USA) presoaked in 50 μM Tris–HCl, pH 7.4, and 5 μM $MgCl_2$ at 4°C for 2 h. The filters were washed three times with 5 ml of an ice-cold Tris–HCl buffer, pH 7.4, and then transferred to scintillation-counting vials. Then, 4 ml of clear-sol 2 (Nacalaitesque, Kyoto, Japan) was added to the vials and equilibrated for 12 h, and the radioactivity in the samples was determined with a liquid scintillation analyzer. Nonspecific binding was measured in the presence of 10 μM unlabeled GTPγS.

Mouse in vivo microdialysis study and quantification of dopamine

Stereotaxic surgery was performed under sodium pentobarbital (70 mg/kg, i.p.) anesthesia. Mice were placed in a stereotaxic apparatus, and the skull was exposed. A small hole was then made using a dental drill. A microdialysis probe (D-I-6-01; 1 mm membrane length; Eicom) was implanted into the nucleus accumbens (NAcc; from bregma: anterior, +1.5 mm; lateral, −0.9 mm; ventral, −4.9 mm) according to the atlas of Paxinos and Franklin (2001). The microdialysis probe was fixed to the skull with cranioplastic cement. At 24 h after implantation, mice were placed in the experimental cages (30 cm wide×30 cm long×30 cm high). The probe was perfused continuously at a flow rate of 2 μl/min with a CSF containing 0.9 mM $MgCl_2$, 147.0 mM NaCl, 4.0 mM KCl, and 1.2 mM $CaCl_2$. Outflow fractions were taken every 5 min. After 15 baseline fractions were collected, mice were given tramadol (70 mg/kg, s.c.), M1 (30 mg/kg, s.c.), or saline. For this experiment, dialysis samples were collected for 180 min after tramadol, M1, or saline treatment. Dialysis fractions were then analyzed using HPLC with ECD (HTEC-500; Eicom). Dopamine was separated by a column with a mobile phase containing 0.1 M NaH_2PO_4, 0.1 M Na_2HPO_4, 2.0 mM sodium 1-decane sulfonate, 0.1 mM EDTA (2Na), and 1% methanol. The mobile phase was delivered at flow rate of 550 μl/min. Dopamine was identified according to the retention times of a dopamine standard, and amounts of dopamine were calculated as concentrations in the dialysates. Other microdialysis data are expressed as percentages of the corresponding baseline level.

Statistical analysis

The data for place preference were shown as the mean±SEM of preference for drug-paired place. The data for hyperalgesic responses were shown as the mean±SEM of paw withdrawal latency or allodynia score. The statistical significance of differences between the groups was assessed with Student's *t* test. The data were expressed as the percentage of basal [^{35}S]GTPγS binding measured in the presence of GDP and absence of agonist and shown as the mean±SEM of the percentage of stimulation. Receptor binding curves were fitted using the GraphPad Prism 4.0 program. The baseline microdialysis data were calculated as concentrations in the dialysates. Other microdialysis data were expressed as percentages of the corresponding baseline level. The statistical significance of differences between the groups was assessed with one-way and two-way ANOVA followed by Bonferroni/Dunn multiple comparison test or Student's *t* test.

Results

Role of the MOR in the expression of tramadol- or M1-induced pharmacological effects

We determined the competitive displacement binding of the MOR ligand [^{3}H]DAMGO with graded concentrations (10^{-11} to 10^{-6} M) of unlabeled tramadol, M1, or morphine in membranes of the mouse brain (Fig. 1). [^{3}H]DAMGO binding was displaced by M1 in a concentration-dependent manner. In contrast, the binding of [^{3}H]DAMGO was not affected by tramadol, whereas [^{3}H]DAMGO was displaced by increasing concentrations of morphine as a positive control. As shown in Fig. 1, the IC_{50} values were determined by the displacement of [^{3}H] DAMGO (Fig. 1). The affinity of M1 for MOR binding was 15.9 times lower than that of morphine.

Role of MOR in the expression of tramadol- or M1-induced rewarding effects

The rewarding effects of both tramadol and M1 were assessed by the conditioned place preference (CPP) in mice. Tramadol (30–70 mg/kg, s.c.) and M1 (10–30 mg/kg, s.c.) each produced a dose-dependent place preference (Fig. 2). These effects were significantly suppressed by pretreatment with β-FNA (40 mg/kg, s.c.) ($p<0.05$, $p<0.01$ vs. saline–saline group; $p<0.05$ vs. saline–tramadol or saline–M1 group; Fig. 2a or b). The G protein activation induced by tramadol (10^{-8} to 10^{-5} M) or M1 (10^{-8} to 10^{-5} M) in the mouse lower midbrain including the ventral tegmental area