

Chronic Pain Induces Anxiety with Concomitant Changes in Opioidergic Function in the Amygdala

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Clinically, it has been reported that chronic pain induces depression, anxiety, and reduced quality of life. The endogenous opioid system has been implicated in nociception, anxiety, and stress. The present study was undertaken to investigate whether chronic pain could induce anxiogenic effects and changes in the opioidergic function in the amygdala in mice. We found that either injection of complete Freund's adjuvant (CFA) or neuropathic pain induced by sciatic nerve ligation produced a significant anxiogenic effect at 4 weeks after the injection or surgery. Under these conditions, the selective μ -opioid receptor agonist [D-Ala², N-MePhe⁴, Gly⁵-o]-enkephalin (DAMGO)- and the selective δ -opioid receptor agonist (+)-4-[(α R)- α -(2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl]-3-methoxybenzyl]-N,N-diethylbenzamide (SNC80)-stimulated [³⁵S]GTP γ S binding in membranes of the amygdala was significantly suppressed by CFA injection or nerve ligation. CFA injection was associated with a significant increase in the κ -opioid receptor agonist 2-(3,4-dichlorophenyl)-N-methyl-N-[(1S)-1-phenyl-2-(1-pyrrolidinyl)ethyl]acetamide hydrochloride (ICI199,441)-stimulated [³⁵S]GTP γ S binding in membranes of the amygdala. The intracerebroventricular administration and microinjection of a selective μ -opioid receptor antagonist, a selective δ -opioid receptor antagonist, and the endogenous κ -opioid receptor ligand dynorphin A caused a significant anxiogenic effect in mice. We also found that thermal hyperalgesia induced by sciatic nerve ligation was reversed at 8 weeks after surgery. In the light–dark test, the time spent in the lit compartment was not changed at 8 weeks after surgery. Collectively, the present data constitute the first evidence that chronic pain has an anxiogenic effect in mice. This phenomenon may be associated with changes in opioidergic function in the amygdala. *Neuropsychopharmacology* (2006) **31**, 739–750. doi:10.1038/sj.npp.1300858; published online 10 August 2005

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INTRODUCTION

Clinically, it has been reported that chronic pain induces depression, anxiety, and reduced quality of life (Gallagher *et al*, 1995; Von Korff and Simon, 1996). There have been many reports on the relation between pain and emotion. Many of these reports deal with the effects of enhanced emotional regulation on pain, and the relation between emotional distress and the seeking of treatment by individuals who are experiencing pain. It has been estimated that over 50% of patients who suffer from chronic pain also express clinically diagnosable symptoms of depression (Dworkin and Gitlin, 1991). Based on reports of the comorbidity between chronic pain and depressive illness in human patients, it is possible that these disease states are linked.

Opioid receptors belong to the large superfamily of G-protein-coupled receptors. Three opioid receptor types, μ -, δ -, and κ -opioid receptors, have been successfully cloned (Evans *et al*, 1992; Kieffer *et al*, 1992; Chen *et al*, 1993; Fukuda *et al*, 1993; Meng *et al*, 1993; Wang *et al*, 1993; Yasuda *et al*, 1993). Opioid analgesics and endogenous opioid peptides have a wide range of physiological and behavioral effects on pain perception, motivation, locomotion, regulation of intestinal motility, immunomodulation, thermo-regulation, neuroendocrine secretion, and rewarding effects (Pasternak, 1988; Mansour *et al*, 1988; Cahill *et al*, 2001). It is well known that endogenous opioid peptides are released in the central nervous system in response to stressful stimuli, and activation of μ -opioid receptor plays an important role in stressful conditions (Kiritsoy-Roy *et al*, 1986; degli Uberti *et al*, 1995). δ -Opioid receptors have also been associated with anxiety and depression, since δ -agonists reduced the immobility of rats in a forced swimming test (Broom *et al*, 2002). Furthermore, κ agonists produce a dysphoria similar to that noted in depression and chronic stress (Pfeiffer *et al*, 1986).

The present study was then undertaken to investigate whether chronic pain could induce anxiogenic effects and

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alter opiodergic functions in the amygdala, which is a key structure in the regulation of anxiety and the expression of emotional responses to stress in mice.

EXPERIMENTAL PROCEDURES

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 Ministry of Education, Culture, Sports, Science and Technology of Japan. All efforts were made to minimize the number of animals used and their suffering.

Animals

Male C57BL/6J mice (CLEA Japan, Inc., Tokyo, Japan), weighing 18–23 g, and SD rats, weighting 250–280 g, were used in the present study. Animals were kept in a room with an ambient temperature of $23 \pm 1^\circ\text{C}$ and a 12-h light–dark cycle (lights on 0800 to 2000). Food and water were available *ad libitum*. All animals were housed individually. All behavioral studies were performed during the light period.

Nerve Injury Pain Model

The mice were anesthetized with sodium pentobarbital (70 mg/kg, i.p.). We produced a neuropathic pain model by tying a tight ligature with 8–0 silk suture around approximately 1/3–1/2 the diameter of the sciatic nerve located in the right hind paw side (ipsilateral side) as described previously (Narita *et al*, 2000). In sham-operated mice, the nerve was exposed without ligation.

Chronic Inflammatory Pain Model

A persistent inflammatory pain model was produced by unilateral intraplantar injection of complete Freund's adjuvant (CFA; *Mycobacterium tuberculosis*; Sigma, St Louis, MO, USA) in a volume of 50 μl into the plantar surface of the right hind paw (ipsilateral side) of mice under anesthesia with sodium pentobarbital (70 mg/kg, i.p.) (Ohsawa *et al*, 2000). Control mice were given saline into the plantar surface of the right hind paw.

Measurement of Thermal and Tactile Thresholds

To assess the sensitivity to thermal stimulation, the right plantar surface of mice was tested individually using a well-focused, radiant heat light source (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 naive mice. The paw-withdrawal latency was determined as the average of three measurements per paw. 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 measurements. Before the behavioral responses to

the thermal stimulus were tested, mice were habituated for at least 30 min in a clear 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 data represent the average value for the paw-withdrawal latency of the right hind paw. To ascertain the acute effect of a selective benzodiazepine receptor agonist etizolam (1 mg/kg, s.c.) on the increased sensitivity to the thermal stimulation, each paw was measured just before and 30 min after etizolam (1 mg/kg, s.c.) injection at 7 days after nerve ligation. In the chronic experiment, mice were treated repeatedly with etizolam once a day for 3 weeks (from day 7 to day 28) after nerve ligation and the paw withdrawal latency was measured one day after last injection.

To quantify the sensitivity to a tactile stimulus, paw withdrawal in response to a tactile stimulus was measured using a bending force (0.02 g) applied by von Frey filaments (North Coast Medical, Inc., Morgan Hill, CA, USA) (Yajima *et al*, 2005). A von Frey filament was applied to the plantar surface of each hind paw for 3 s, and this was repeated three times with an inter-trial interval of at least 5 s. Each of the hind paws was tested individually. Paw withdrawal in response to a tactile stimulus was 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 or licking; 3, an intense withdrawal response away from the stimulus with brisk flinching and/or licking. The 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 more than 3 min between the measurements. Before the behavioral responses to tactile stimuli were tested, mice were habituated for at least 30 min on an elevated nylon mesh floor. Under these conditions, paw withdrawal in response to a tactile stimulus was tested.

Light–Dark Test

To investigate changes in anxiogenic-like effects, mice tested using the light–dark paradigm (Bilkei-Gorzo *et al*, 1998; Schramm *et al*, 2001). We used a box consisting of a small (mouse: $18 \times 13 \times 18 \text{ cm}^3$; rat: $40 \times 25 \times 40 \text{ cm}^3$) dimly lit compartment with black walls and a black floor, connected by a tunnel (mouse: 5 cm long; rat: 15 cm long) to a large (mouse: $18 \times 18 \times 18 \text{ cm}^3$; rat: $40 \times 40 \times 40 \text{ cm}^3$) intensely lit (500 lux) compartment with a white walls and a white floor. Each animal was placed in the dark compartment at the beginning of the observation session. Compartment entry and exit were defined as all four paws into and out of an arm, respectively. The time spent in the lit compartment was recorded for 10 min.

Elevated Plus-Maze Test

We used the elevated plus-maze test as another measurement of anxiety. The elevated plus-maze test has been used extensively to identify novel anxiolytic agents and to investigate the physiological and neurochemical basis of anxiety (Dawson and Tricklebank, 1995). The elevated

plus-maze consisted of two opposing open and two closed arms ($30 \times 6 \text{ cm}^2$ each) joined by a common central platform ($9 \times 9 \text{ cm}^2$). The maze was elevated 40 cm above the floor. The closed arms were enclosed by 15 cm high walls, and the open arms were surrounded by 0.3-cm ledges. Illumination of the open and closed arms and the central platform was approximately equal (100 lux). Arm entry and exit were defined as all four paws into and out of an arm, respectively. The results were calculated as mean ratios of the time spent in the open arms to the total time spent in both the open and closed arms. Entries to the open and closed arms were also scored. In order to ascertain the anxiolytic effect of etizolam, a single injection of etizolam (1 mg/kg, s.c.) was performed 30 min before the light–dark or the elevated-plus maze procedures. In the chronic experiment, mice were used for this procedure following repeated treatment with etizolam once a day for 3 weeks day 7 to day 28 after nerve ligation.

Guanosine-5'-*o*-(3-[^{35}S]thio) Triphosphate ([^{35}S] GTP γ S) Binding Assay

The saline- or CFA-injected mice and sham-operated or sciatic nerve-ligated mice were decapitated at 4 weeks after injection or surgery. For membrane preparation, the brain was quickly removed and dissected including the basolateral amygdala, central nucleus, and medial nucleus of the amygdala (about $2 \times 2 \times 2 \text{ mm}^3$ cubic weighting 35 mg per mouse) according to the brain atlas of Paxinos G and Franklin KBJ (*The Mouse Brain*, 2nd edn. Academic Press: CA, USA). The tissue was homogenized using a Potter-Elevehjem tissue grinder with a Teflon pestle in 20 vol (w/v) of ice-cold Tris-Mg $^{2+}$ buffer containing 50 mM Tris-HCl, pH 7.4, 5 mM MgCl $_2$, and 1 mM EGTA for the [^{35}S]GTP γ S binding assay. The homogenate was centrifuged at 4°C for 10 min at 48 000g. The pellet was resuspended in ice-cold Tris buffer or [^{35}S]GTP γ S binding assay buffer containing 50 mM Tris-HCl, pH 7.4, 5 mM MgCl $_2$, and 1 mM EGTA and 100 mM NaCl and centrifuged at 4°C for 10 min at 48 000g. The resultant pellet was resuspended on ice-cold [^{35}S]GTP γ S binding assay buffer and stored at –70°C until use.

The membrane homogenate (3–8 μg protein per assay) was incubated at 25°C for 2 h in 1 ml of assay buffer with various concentrations of agonist, 30 μM GDP, and 50 pM [^{35}S]GTP γ S (specific activity, 1000 Ci/mmol; Amersham Biosciences). The reaction was terminated by filtration using a Brandle cell harvester and Whatman GF/B glass filters presoaked in 50 mM Tris-HCl, pH 7.4, and 5 mM MgCl $_2$ at 4°C for 2 h. Filters were then washed three times with 5 mM ice-cold Tris-HCl buffer, pH 7.4, transferred to scintillation-counting vials containing 0.5 ml of Soluene-350 and 4 ml of Hionic Fluor, 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 GTP γ S. Comparable results were obtained from at least three independent sets and in each set experiments were performed in triplicate.

Intracerebroventricular Injection

Intracerebroventricular administration was performed as described previously (Haley and McCormick, 1957). A few

days before intracerebroventricular injection, the mice were lightly anesthetized with diethyl ether and a 2 mm double-needle tip: 27G \times 2 mm and base: 22G \times 10 mm (Natsume Seisakusho Co. Ltd, Tokyo, Japan) attached to a 25- μl Hamilton microsyringe was inserted into the unilateral injection site; a hole was made in the skull for the injection. The solution was injected in a volume of 4 μl per mouse.

Surgery and Microinjection

After 3 days of habituation to the main animal colony, all rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). The anesthetized animals were placed in a stereotaxic apparatus. The skull was exposed and a small hole was made using a dental drill. A guide cannula (AG-9, Eicom Co., Kyoto, Japan) was implanted into the basolateral amygdala (from bregma: posterior –2.8 mm, lateral +5.0 mm, ventral –7.9 mm) according to the atlas of Paxinos and Watson (1998). The guide cannula was fixed to the skull with cranioplastic cement. At 7 days after surgery, the animals were injected with a selective μ -opioid receptor antagonist CTOP, a δ -opioid receptor antagonist NTI or dynorphin A into the basolateral amygdala 10 min before testing. In the microinjection study, we used the injection cannula (AMI-9.5, Eicom Co.) extended beyond the guide cannula by 0.5 mm. A stainless steel injection cannula was inserted into that guide cannula for each animal. The injection cannula was connected through polyethylene tubing to a 10 μl Hamilton syringe that was preloaded with CTOP, NTI, or dynorphin A (0.1, 1, or 10 pmol/0.3 μl) or vehicle. The receptor antagonist or vehicle was delivered by motorized syringe pump in a volume of 0.3 μl over 60 s.

Drugs

SNC80 ((+)-4-[(α R)- α -(2S, 5R)-4-Allyl-2,5-dimethyl-1-piperazinyl]-3-methoxybenzyl]-N,N-diethylbenzamide (Tocris Cookson Ltd, Ballwin, MO, USA)) and ICI 199 441 (Tocris Cookson Ltd, Ballwin, MO, USA) were dissolved in assay buffer containing 20% ethanol. DAMGO ([D-Ala 2 , N-Me-Phe 4 , Gly 5 -ol] enkephalin) (Sigma Co., St Louis, MO, USA) was dissolved in assay buffer. D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH $_2$ (CTOP; Sigma Chemical Co., MO, USA) and dynorphin A (Peptide Institute Inc., Osaka, Japan) were dissolved in saline for *in vivo* experiments. Naltrindole hydrochloride (NTI; Tocris Cookson Inc., MO, USA) was dissolved in saline containing 1% dimethyl sulfoxide. Etizolam (Wako Pure Chemical Industries Ltd, Osaka, Japan) was dissolved in distilled water containing 0.5% carboxymethyl cellulose sodium salt.

Statistical Analysis

All data are presented as the mean \pm SEM. The statistical significance of differences between groups was assessed with repeated measures analysis of variance (ANOVA) and one-way ANOVA followed by the Bonferroni–Dunn multiple comparison test or Student's *t*-test.

RESULTS

Unilateral intraplantar injection of a CFA solution into the mouse hind paw caused a significant decrease in the latency of paw withdrawal after the thermal stimulus only on the ipsilateral side in mice (vs saline group, $F_{1,8}=177.133$, $p<0.001$, Figure 1a). CFA-injected mice also exhibited a significant increase in paw withdrawal in response to the tactile stimulus only on the ipsilateral hind paw in mice (vs saline group, $F_{1,8}=154.223$, $p<0.001$, Figure 1b).

Partial nerve ligation of the sciatic nerve caused a significant decrease in the latency of paw withdrawal after the thermal stimulus only on the ipsilateral side of mice (vs sham group, $F_{1,8}=272.452$, $p<0.001$, Figure 1a). The sciatic

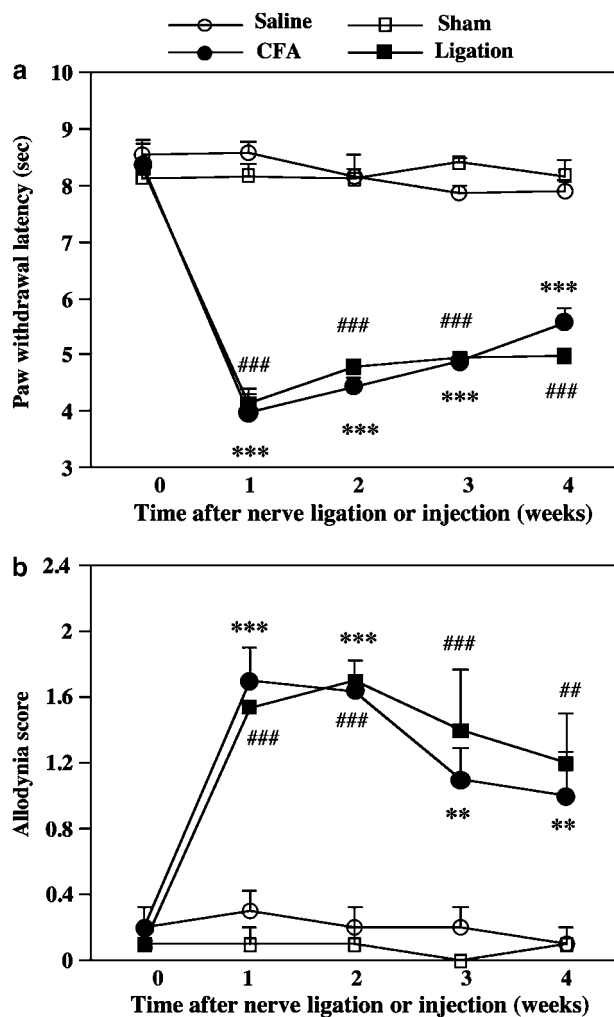


Figure 1 Changes in the latency of paw withdrawal after thermal and mechanical stimulation induced by the injection of complete Freund's adjuvant (CFA) or sciatic nerve ligation in mice. The data represent the results of (a) paw-withdrawal latencies after the thermal stimulus and (b) paw withdrawal in response to a tactile stimulus on the ipsilateral side at 1, 2, 3, and 4 weeks after injection or surgery. There was no difference in the basal response before injection or surgery between saline- and CFA-injected or sham-operated and sciatic nerve-ligated mice. The tactile stimulus was applied using filaments with a bending force of 0.02g. Each point represents the mean \pm SEM of 6–8 mice. *** $p<0.01$ and **** $p<0.001$: saline group vs CFA group. ## $p<0.01$ and ### $p<0.001$: sham group vs ligation group.

nerve-ligated mice also exhibited a significant increase in the paw-withdrawal response to the tactile stimulus only on the ipsilateral hind paw in mice (vs sham group, $F_{1,8}=50.43$, $p<0.001$, Figure 1b). Paw-withdrawal latencies and responses on the contralateral side of mice were not changed by injection or surgery (data not shown).

Under these conditions, we investigated whether a chronic pain-like state could induce an anxiogenic effect in mice. In the light–dark test, the time spent in the lit side was similar in both CFA-injected and sciatic nerve-ligated groups at 1, 2, or 3 weeks after injection or surgery. However, CFA-injected and sciatic nerve-ligated mice exhibited a significant decrease in the time spent in the lit side at 4 weeks after CFA injection or surgery (*** $p<0.001$ vs saline 4 weeks group, Figure 2a) (** $p<0.01$ vs sham 4 weeks group, Figure 2b). In the elevated plus-maze test, the percentage of time spent in the open arms was significantly reduced in CFA-treated mice (** $p<0.01$ vs saline 4 weeks group, Figure 3a) and sciatic nerve-ligated mice ($^{\#}p<0.05$ vs

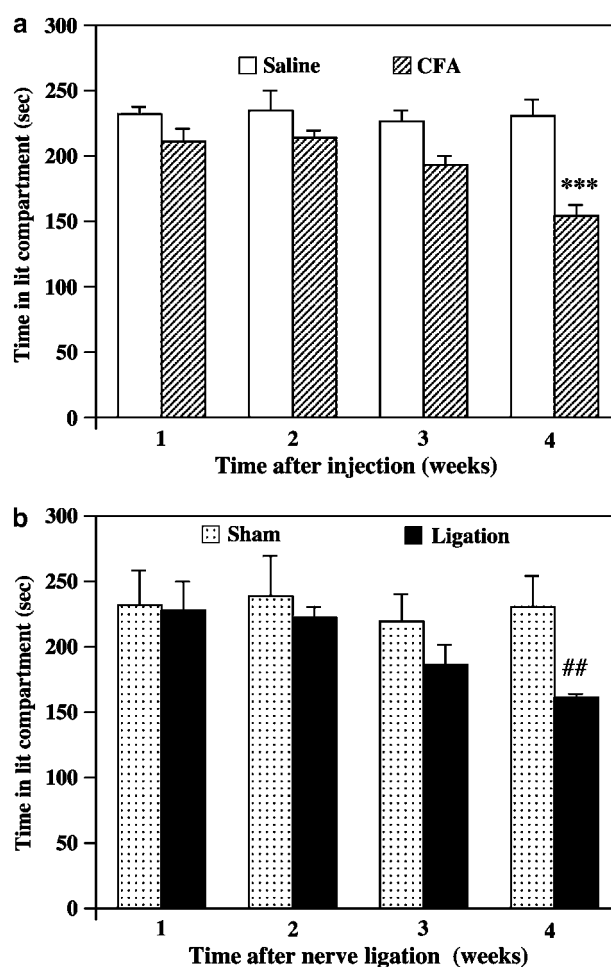


Figure 2 Changes in anxiety behavior in the light–dark test under an inflammatory pain-like state or neuropathic pain-like state in mice. (a) An inflammatory pain-like state caused a significant decrease in the time spent in the lit compartment at 4 weeks after injection. (b) A neuropathic pain-like state induced a significant decrease in the time spent in the lit compartment at 4 weeks after surgery. Each column represents the mean \pm SEM of 6–8 mice. *** $p<0.001$: saline 4 weeks group vs CFA 4 weeks group. ## $p<0.01$: sham 4 weeks group vs ligation 4 weeks group.

sham 4 weeks group, Figure 3d) at 4 weeks after injection or surgery. However, the number of entries into the open arms was not significantly reduced in CFA-injected mice (Figure 3b) or sciatic nerve-ligated mice (Figure 3e). The number of entries into closed arms, which was taken as an index of general activity, was not affected by CFA injection (Figure 3c) or sciatic nerve ligation (Figure 3f).

We next investigated whether a chronic pain-like state could affect G-protein activation through the stimulation of μ -, δ -, and κ -opioid receptors in membranes of the amygdala. The ability of the selective μ -opioid receptor agonist DAMGO, the selective δ -opioid receptor agonist SNC80, and the κ -opioid receptor agonist ICI 199,441 to stimulate G-proteins in membranes of the amygdala

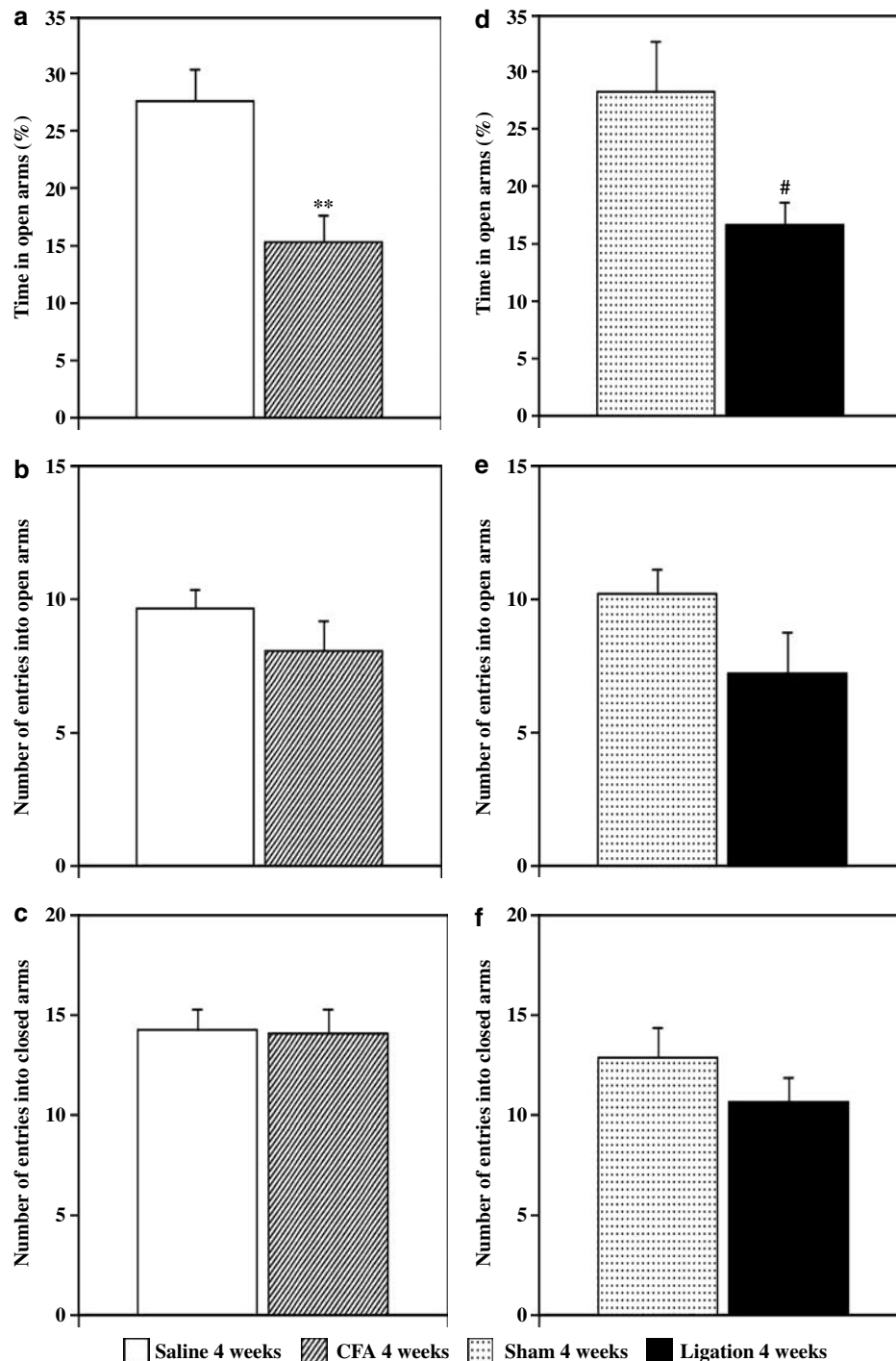


Figure 3 Inflammatory pain- or neuropathic pain-induced anxiogenic effect in the elevated plus-maze test in mice. The percentage of time spent in the open arms was significantly decreased by CFA injection (a) or sciatic nerve ligation (d) at 4 weeks after injection or surgery, respectively. The number of entries into the open arms was similar in both groups (b and e). There was no change in the number of entries into the closed arms in both groups (c and f). Each column represents the mean \pm SEM of 6–8 mice. ** $p < 0.01$: saline 4 weeks group vs CFA 4 weeks group. # $p < 0.05$: sham 4 weeks group vs ligation 4 weeks group.

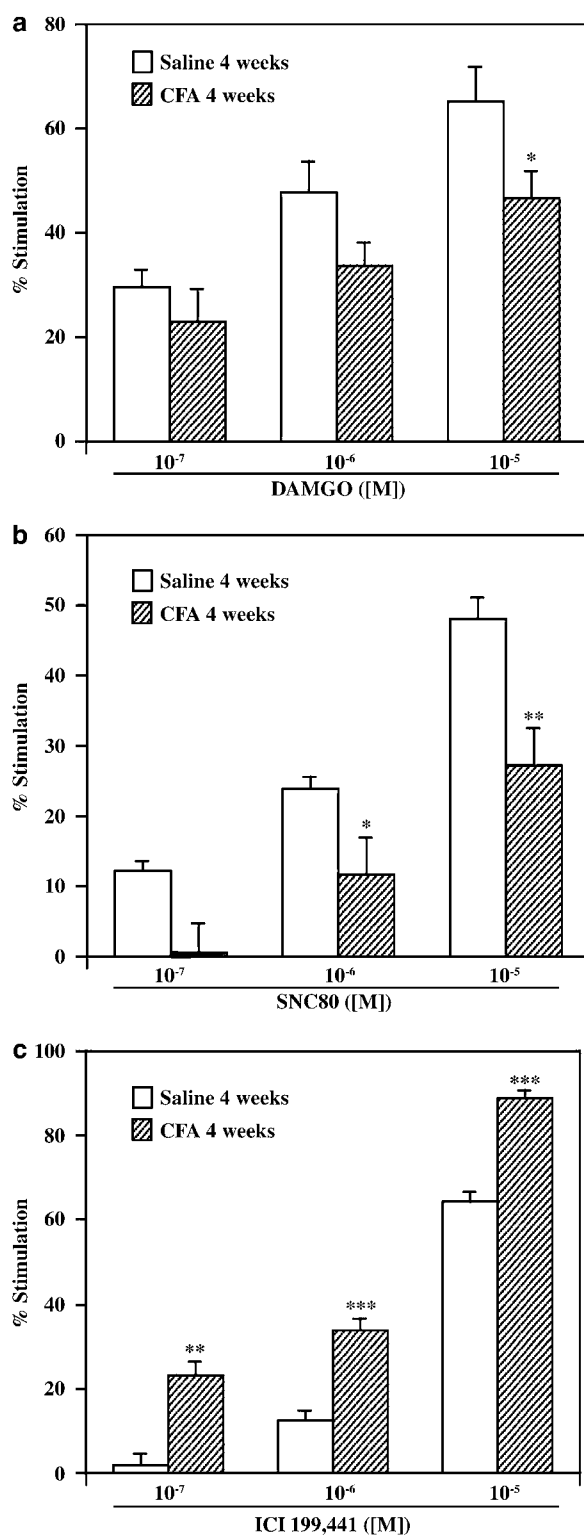


Figure 4 Effects of the selective μ -opioid receptor agonist DAMGO (a), the selective δ -opioid receptor agonist SNC80 (b) and κ -opioid receptor agonist ICI199,441 (c) on $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding to membranes of the amygdala obtained from saline- or CFA-injected mice at 4 weeks after injection. Membranes were incubated with $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ (50 pM) and GDP (30 μM) with or without each agonist. The data are shown as the percentage of the basal $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding measured in the presence of GDP and the absence of each agonist. Each column represents the mean with SEM * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$: saline 4 weeks group vs CFA 4 weeks group.

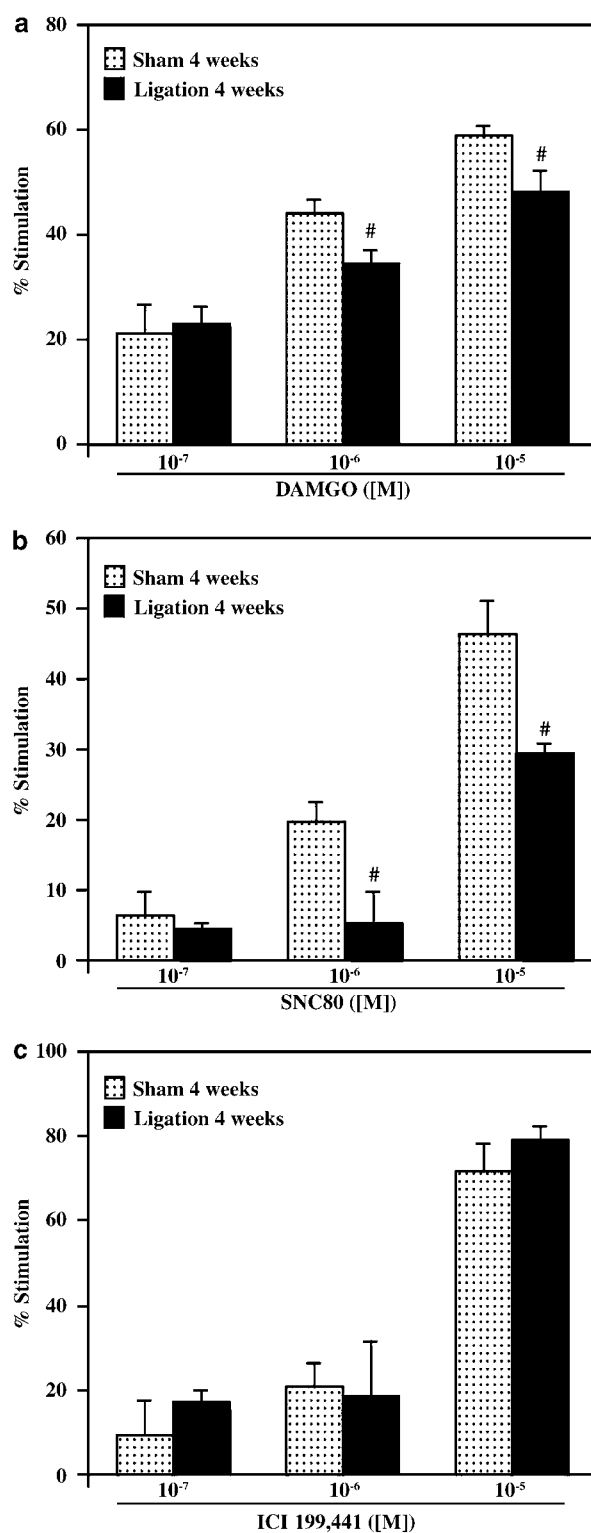


Figure 5 Effects of the selective μ -opioid receptor agonist DAMGO (a), the selective δ -opioid receptor agonist SNC80 (b) and the κ -opioid receptor agonist ICI199,441 (c) on $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding to membranes of the amygdala obtained from sham-operated or sciatic nerve-ligated mice at 4 weeks after surgery. Membranes were incubated with $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ (50 pM) and GDP (30 μM) with or without each agonist. The data are shown as the percentage of the basal $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding measured in the presence of GDP and the absence of each agonist. Each column represents the mean with SEM # $p < 0.05$: sham 4 weeks group vs ligation 4 weeks group.

obtained from saline- or CFA-injected mice, sham-operated or sciatic nerve-ligated mice was examined by the binding of [35 S]GTP γ S. The stimulatory effect of DAMGO on [35 S]GTP γ S binding to the amygdala membrane was significantly decreased by CFA injection ($*p < 0.05$ vs saline 4 weeks group, Figure 4a) or sciatic nerve-ligation ($*p < 0.05$ vs sham 4 weeks group, Figure 5a) as compared to saline injection or sham operation. The stimulatory effect of SNC80 on [35 S]GTP γ S binding to membranes of the amygdala was significantly decreased by CFA injection ($*p < 0.05$, $**p < 0.01$ vs saline 4 weeks group, Figure 4b) or sciatic nerve-ligation ($*p < 0.05$ vs sham 4 weeks group, Figure 5b) compared to saline injection or sham operation, respectively. The stimulatory effect of ICI 199,441 on [35 S]GTP γ S binding to membranes of the amygdala was significantly increased by CFA injection compared to saline injection ($**p < 0.01$, $***p < 0.001$ vs saline 4 weeks group, Figure 4c), while sciatic nerve ligation had no effect on ICI 199,441-stimulated [35 S]GTP γ S binding to membranes of the amygdala (Figure 5c). At 1 week after sciatic nerve ligation, there were no significant changes in G-protein activation through μ - and δ -opioid receptors in membranes of the amygdala (Figure 6).

To elucidate the interactions between opiodergic functions and anxiety, we next investigated whether intracerebroventricular injection of μ -, δ -opioid receptor antagonist, or dynorphin A could induce an anxiogenic effect using the light–dark test. The time spent in the lit compartment was significantly decreased at 30 min after intracerebroventricular injection of the selective μ -opioid receptor antagonist CTOP ($^{##}p < 0.01$ vs saline treatment group, Figure 7a) or selective δ -opioid receptor antagonist naltrindole ($***p < 0.001$ vs vehicle treatment group, Figure 7b). The intracerebroventricular injection of dynorphin A also produced a significant decrease in the time spent in the lit compartment at 30 min after injection ($*p < 0.05$ vs saline treatment group, Figure 7c).

To further ascertain the direct involvement of opiodergic function in the amygdala in the expression of anxiety, we examined whether microinjection of μ - or δ -opioid receptor antagonist, or dynorphin A into the amygdala could cause an anxiogenic effect. Using the rat light–dark test, the time

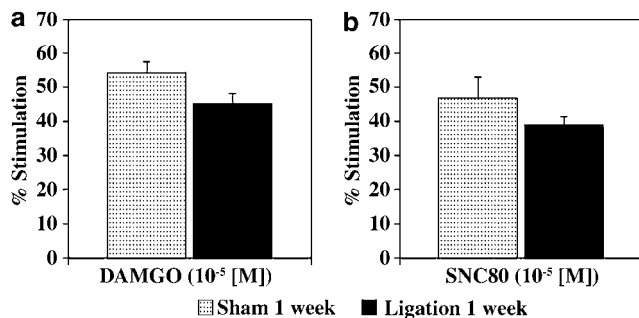


Figure 6 Effect of the selective μ -opioid receptor agonist DAMGO (a) and the δ -opioid receptor agonist SNC80 (b) on [35 S]GTP γ S binding to membranes of the amygdala obtained from sham-operated or sciatic nerve-ligated mice at 1 week after surgery. Membranes were incubated with [35 S]GTP γ S (50 pM) and GDP (30 μ M) with or without each agonist. The data are shown as the percentage of that in the presence of GDP and the absence of each agonist. Each column represents the mean with SEM.

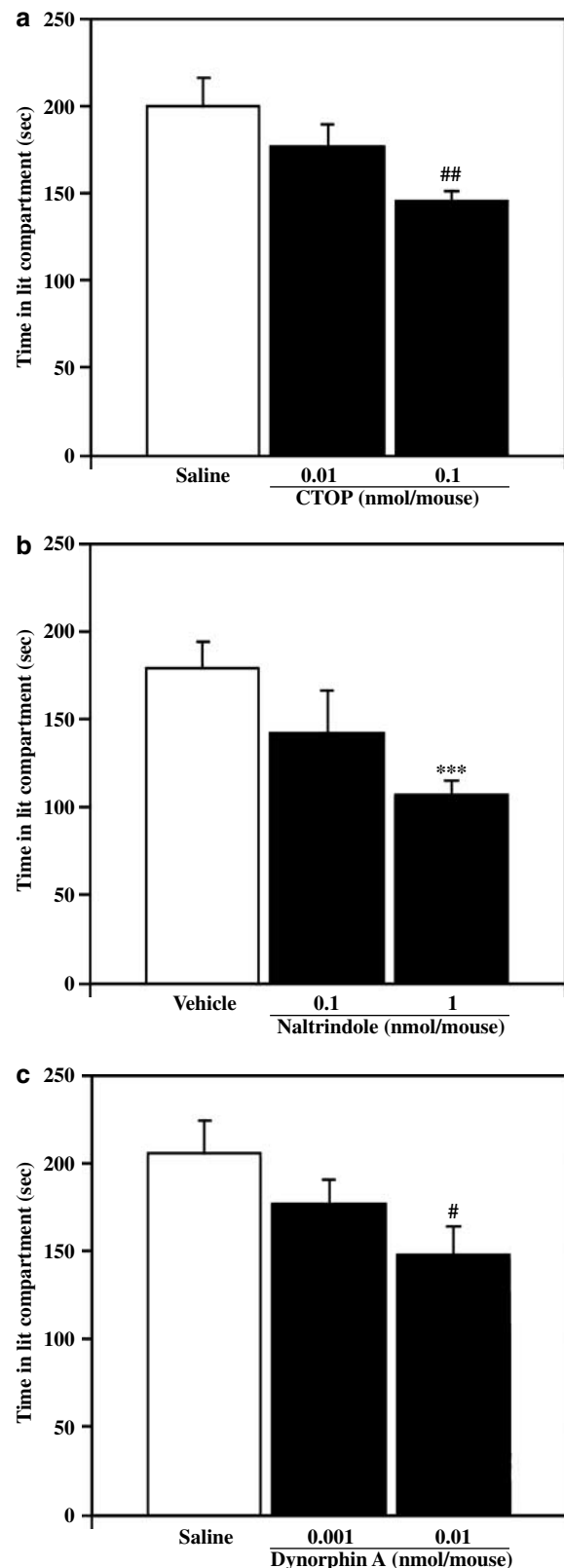
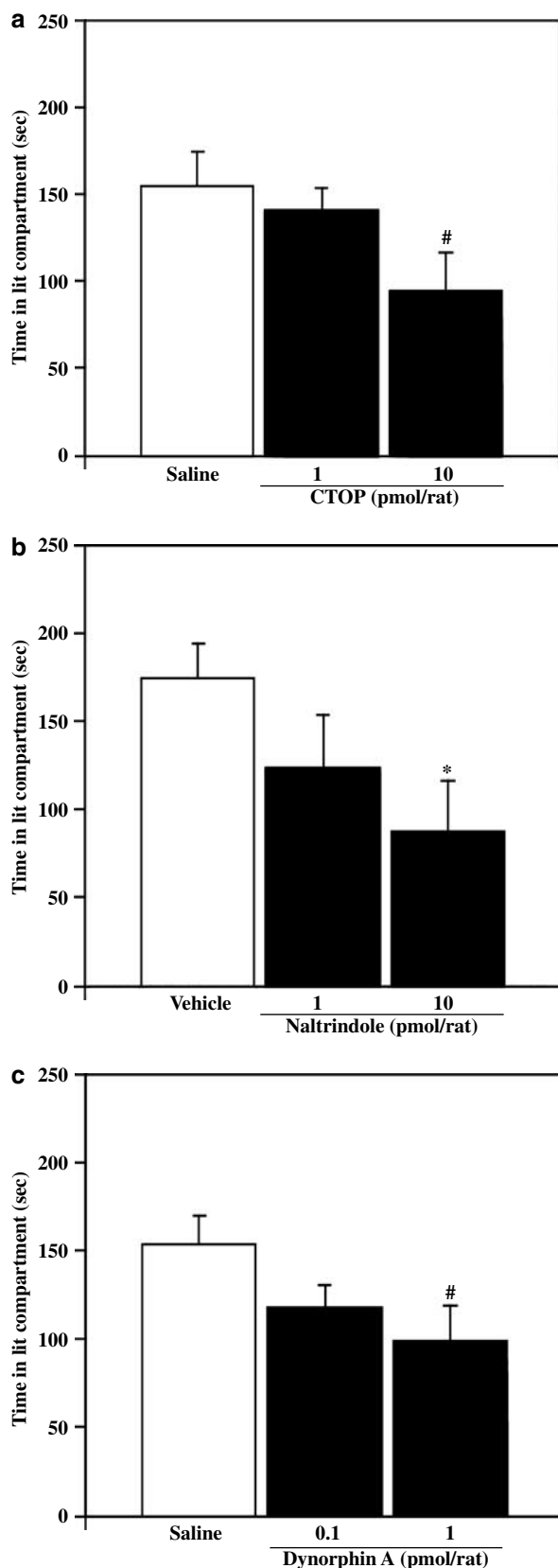


Figure 7 Anxiogenic effects of the selective μ -opioid receptor antagonist CTOP (a), the selective δ -opioid receptor antagonist naltrindole (b) and dynorphin A (c) in mice. The time spent in the lit compartment was significantly decreased at 30 min after intracerebroventricular injection using the light–dark test. Each column represents the mean \pm SEM of 5–11 mice. $***p < 0.001$: vs vehicle treatment group. $*p < 0.05$ and $^{##}p < 0.01$: vs saline treatment group.

spent in the lit compartment was significantly decreased at 10 min after the microinjection of either CTOP ($^{\#}p < 0.05$ vs saline treatment group, Figure 8a) or naltrindole into the



amygdala ($^*p < 0.05$ vs vehicle treatment group, Figure 8b). The microinjection of dynorphin A into the rat amygdala also produced a significant decrease in the time spent in the lit compartment at 10 min after injection ($^{\#}p < 0.05$ vs saline treatment group, Figure 8c).

At 8 weeks after surgery, thermal hyperalgesia induced by sciatic nerve ligation was reversed (Figure 9a). In the light–dark test, the decrease in the time spent in the lit compartment was also reversed at 8 weeks after surgery (Figure 9b). Eight weeks after sciatic nerve ligation, there were no significant changes in the G-protein activation through μ - and δ -opioid receptors in membranes of the amygdala (Figure 10).

Finally, we investigated whether the anxiolytic agent could affect the pain perception. To ascertain the effect of s.c. injection of etizolam on paw withdrawal latencies at the ipsilateral and the contralateral sides in sham-operated or nerve-ligated mice, each paw was measured just before and 30 min after etizolam injection. At the beginning of this experiment, we confirmed that acute treatment with etizolam (1 mg/kg, s.c.) produced a significant increase in a percent of the open arm entries and the percent of time spent in the open arms of the maze in the plus-maze test (Figure 11a–c) and time spent in the lit zone in the light–dark test ($^*p < 0.05$ vs vehicle treatment group, Figure 11d), indicating the possible expression of an anxiolytic-like effect induced by etizolam at 1 mg/kg through the stimulation of GABA_A receptor. Under these conditions, the decreased thermal threshold on the ipsilateral side in nerve-ligated mice was not affected by a single s.c. injection of etizolam treatment at 1 mg/kg (Figure 11e). To further investigate the effect of chronic etizolam treatment on the sensitivity to the thermal stimulation, mice were treated repeatedly with etizolam (1 mg/kg, s.c.) once a day for 3 weeks (from day 7 to day 28) after nerve ligation. As a

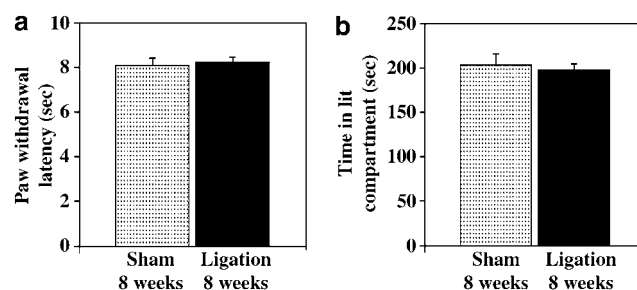


Figure 9 Recovery from the sciatic nerve ligation-induced-anxiogenic effect at 8 weeks after surgery. (a) The latency of paw withdrawal after exposure to a thermal stimulus was not changed at 8 weeks after surgery. (b) The time spent in the lit compartment was no change at 8 weeks after the surgery using the light–dark test. Each column represents the mean \pm SEM of eight mice.

Figure 8 Role of the rat amygdala in the expression of anxiogenic effects induced by the selective μ -opioid receptor antagonist CTOP (a), the selective δ -opioid receptor antagonist naltrindole (b) and dynorphin A (c). The time spent in the lit compartment was a significant decrease at 10 min after each microinjection using the light–dark test. Each column represents the mean \pm SEM of 5–11 rats. $^*p < 0.05$; vs vehicle treatment group. $^{\#}p < 0.05$; vs saline treatment group.

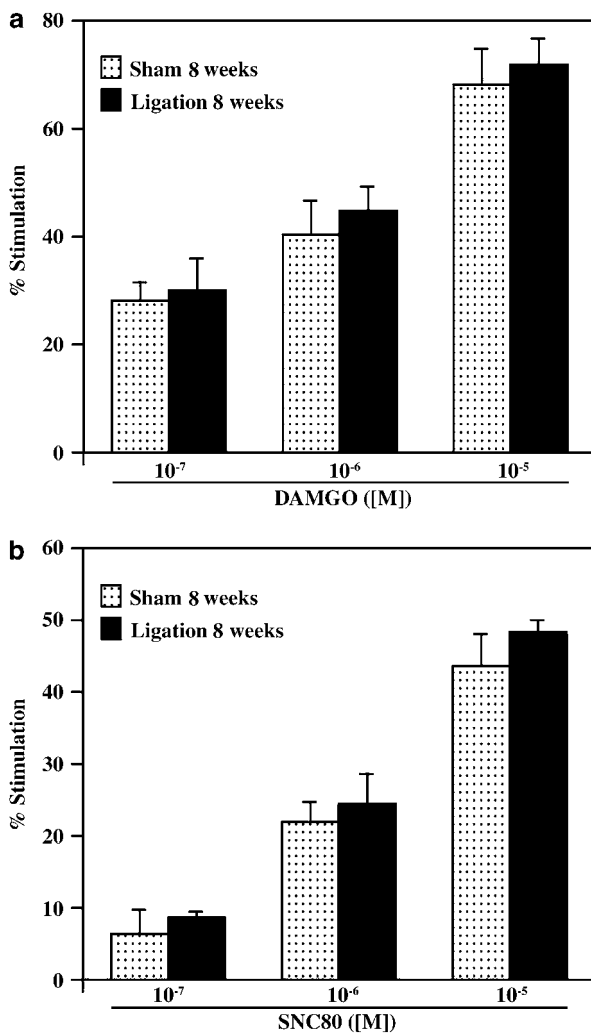


Figure 10 Effects of the selective μ -opioid receptor agonist DAMGO (a) and the δ -opioid receptor agonist SNC80 (b) on $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding to membranes of the amygdala obtained from sham-operated or sciatic nerve-ligated mice at 8 weeks after surgery. Membranes were incubated with $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ (50 pM) and GDP (30 μM) with or without each agonist. The data are shown as the percentage of the results in the presence of GDP and the absence of each agonist. Each column represents the mean with SEM.

result, there were no changes in the decreased thermal threshold between on the ipsilateral sides in vehicle- and etizolam-treated mice after nerve ligation (Figure 11j), whereas the increased anxiety-like behavior after nerve ligation was abolished by chronic treatment with etizolam (Figure 11f–i).

DISCUSSION

The relationship between depression and pain is well known. Some functional changes in the emotionality-related brain regions must occur in association with chronic pain. However, little is known about the molecular mechanism that underlies pain-induced anxiety. The present study was undertaken to investigate whether chronic pain could induce an anxiogenic effect in mice. Thermal hyperalgesia and tactile allodynia induced by either CFA injection or

sciatic nerve ligation lasted for at least 4 weeks after injection or surgery. Under these conditions, we found that either an inflammatory or neuropathic pain-like state led to an anxiogenic effect at 4 weeks after CFA injection or surgery, respectively, in mice. Furthermore, thermal hyperalgesia induced by sciatic nerve ligation was reversed at 8 weeks after surgery. In the light–dark test, the time spent in the lit compartment was not changed at 8 weeks after surgery. These findings suggest that pain and anxiety are closely connected.

Many studies have shown that the amygdala plays a key role in fear and anxiety (Davis *et al*, 1994; Moller *et al*, 1997). The distributions of μ -, δ -, and κ -opioid receptor mRNAs overlap in many brain regions including the amygdala (Mansour *et al*, 1995). The amygdala also has high levels of endogenous opioid peptides (Mansour *et al*, 1995). Therefore, we next investigated whether chronic pain could change opioidergic function in the amygdala. In the present study, the stimulatory effect of the selective μ -opioid receptor agonist DAMGO or the selective δ -opioid receptor agonist SNC80 on $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding to the amygdala membranes was significantly decreased by either CFA injection or sciatic nerve ligation as compared to saline injection or sham operation. In contrast, κ -opioid receptor agonist ICI 199,441-induced G-protein activation in the amygdala was significantly increased by CFA injection, but not sciatic nerve ligation. This phenomenon is consistent with our previous findings that inflammatory pain, but not nerve injury, causes a marked activation of endogenous κ -opioidergic system (Ozaki *et al*, 2002; Narita *et al*, 2005). At 1 week after the sciatic nerve ligation, thermal hyperalgesia and allodynia, but not an anxiogenic effect, were observed with nerve ligation. Under these conditions, there were no significant changes in G-protein activation through μ - and δ -opioid receptors in membranes of the amygdala. These findings raise the possibility that chronic pain-induced changes in opioidergic function in the amygdala may lead to the anxiogenic effect.

In the present study, we also found that intracerebroventricular administration and microinjection of either μ -opioid receptor antagonists, a δ -opioid receptor antagonist, or dynorphin A into the amygdala caused a significant anxiogenic effect in mice. Recently, it has been reported that δ -opioid receptor knockout mice exhibit more anxiety behavior (Filliol *et al*, 2000). In addition, intracerebroventricular injection of dynorphin A increases forced swimming-induced immobility in mice (Katoh *et al*, 1990). These data support the idea that the dysfunction of μ -, δ -opioid receptors (and the possible enhancement of κ -opioid receptor function) in the amygdala may, at least in part, play a role in the mood altering effect of chronic pain.

As previously reported in healthy human subjects, sustained pain induces a reduction of μ -opioid receptor availability in the amygdala as measured by positron emission tomography (Zubieta *et al*, 2001). Based on previous work in experimental animals, it was hypothesized that a painful condition could be associated with an increased release of endogenous opioids in the amygdala (Manning, 1998). These data support the hypothesis that sustained pain induces a regionally selective release of endogenous opioids interacting with μ -opioid receptors in

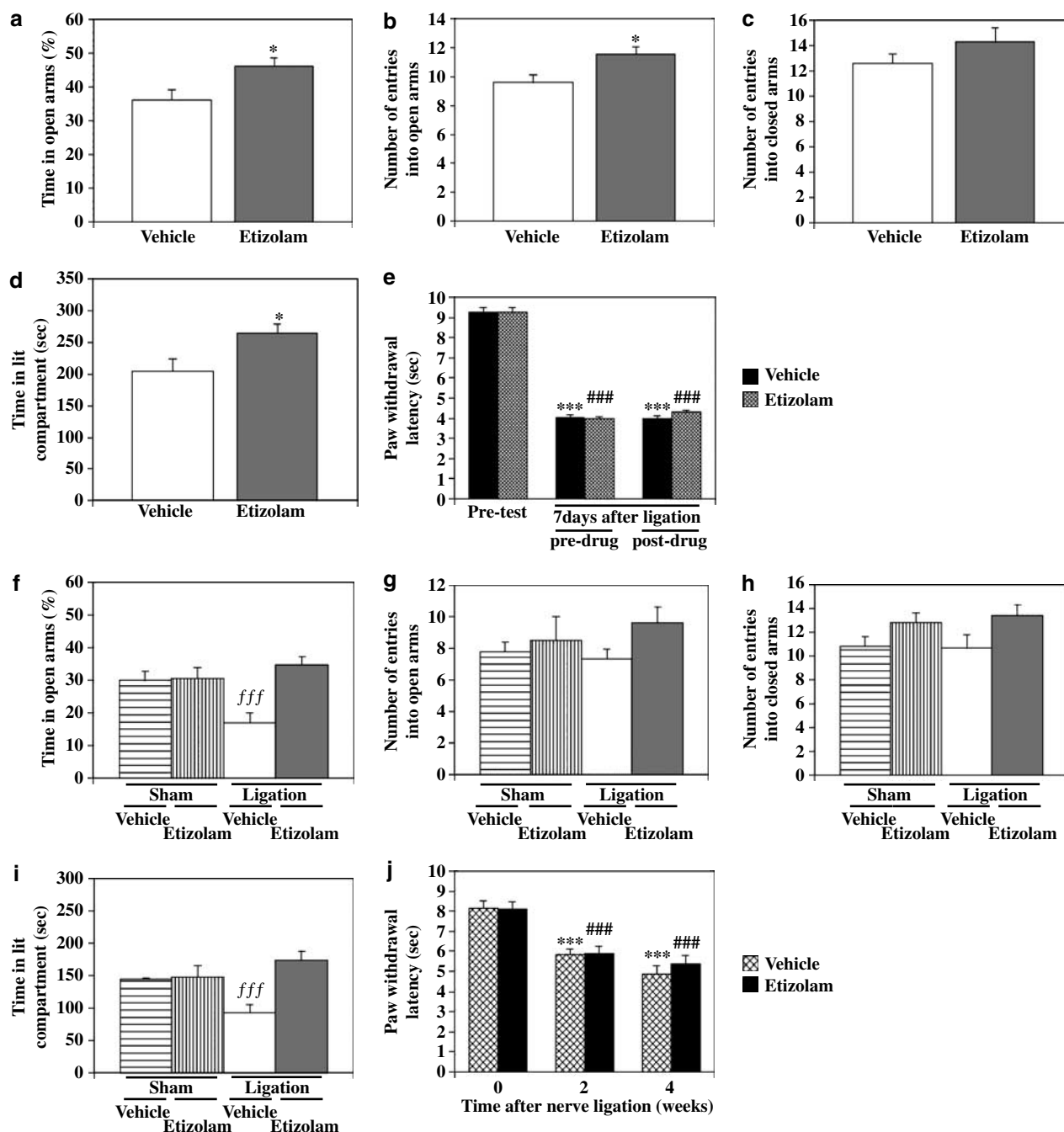


Figure 11 Effects of the selective benzodiazepine receptor agonist etizolam on anxiogenic (a–d and f–i) and hyperalgesic (e and j) effects induced by sciatic nerve ligation. Acute treatment with etizolam (1 mg/kg, s.c.) produced a significant increase in the percentage of the time spent in open arms (a) and the number of entries into open arms (b) in the elevated plus-maze test (* $p < 0.05$: vehicle vs etizolam). (c) There was no difference in the number of entries into closed arms between the vehicle- and etizolam-treated mice. (d) Acute treatment with etizolam (1 mg/kg, s.c.) caused a significant increase in time spent in the lit compartment using the light–dark test (* $p < 0.05$: vehicle vs etizolam). (e) At 7 days after nerve ligation, the thermal threshold was significantly decreased (***, ### $p < 0.001$: pretest vs prevehicle and pre-etizolam, respectively). After confirming the predrug threshold at 7 days after nerve ligation, mice were treated with a single injection of vehicle or etizolam (1 mg/kg, s.c.) and the thermal threshold was measured 30 min after the injection (postdrug). The decreased thermal threshold on the ipsilateral side in nerve-ligated mice was not affected by a single injection of etizolam (1 mg/kg, s.c., no significance; predrug vs postdrug). (f–i) Neuropathic pain induced the anxiogenic effect in the elevated plus-maze (f–h) and the light–dark (i) tests. Groups of mice were operated with or without sciatic nerve ligation and each group was divided into two groups: chronic vehicle treatment and chronic etizolam treatment. Mice were repeatedly injected with vehicle or etizolam (1 mg/kg, s.c.) once a day for 3 weeks (from day 7 to day 28) after nerve ligation. The assay was performed one day after last injection. The decreased percentage of time spent in the open arms was completely recovered by repeatedly s.c. treatment with etizolam (f) (fff $p < 0.001$: vs sham-vehicle). There was no change in the number of entries into the open (g) and closed (h) arms in each group. The decreased time spent in the lit compartment was significantly recovered by repeatedly s.c. treatment with etizolam (i) (fff $p < 0.001$: vs sham-vehicle). (j) At 2 and 4 weeks after nerve ligation, the thermal threshold was significantly decreased (***, ### $p < 0.001$: vehicle 0 week vs vehicle 2 and 4 weeks, ### $p < 0.001$: etizolam 0 week vs etizolam 2 and 4 weeks, respectively). Under these conditions, there was no difference in the decreased thermal threshold on the ipsilateral sides between vehicle- and etizolam-treated mice following nerve ligation. Each column represents the mean \pm SEM of 6–8 mice.

the amygdala, resulting in the internalization and recycling of μ -opioid receptors.

We previously reported that morphine failed to induce rewarding effects under a neuropathic pain-like state in the rat and mouse (Ozaki *et al*, 2002, 2003). Furthermore, this pain-like state is associated with a reduction in μ -opioid receptor function in the ventral tegmental area (Ozaki *et al*, 2002, 2003). We also found that an inflammatory pain-like state could cause a sustained activation of the κ -opioidergic system in the nucleus accumbens, leading to suppression of the morphine-induced rewarding effect in rats (Suzuki *et al*, 1999; Narita *et al*, 2005). Taken together, these findings suggest that a state of pain may cause physiological changes in opioid transmission at supraspinal levels. Furthermore, there may be differences in the mechanisms between the neuropathic and inflammatory pain-like states. Although further study is needed, this phenomenon could explain why the κ -opioid receptor function in the amygdala was changed by inflammation, but not by nerve injury.

Another key finding was that acute and chronic treatments with a selective benzodiazepine receptor agonist etizolam (1 mg/kg, s.c.), which clearly showed a significant anxiolytic effect in normal mice and a complete suppression of the expression of anxiety under a neuropathic pain-like state, failed to improve the increased pain-sensitivity after nerve injury. In our preliminary study, we found that chronic psychological stress produced a significant decrease in thermal thresholds, whereas subchronic treatment with a selective benzodiazepine receptor inverse agonist FG7142 failed to alter the increased pain-sensitivity under a chronic pain-like state (data not shown). Thus, we propose that, although chronic pain and negative emotions including anxiety and depression are concomitant constructs because of similar biological foundations and the negative emotions can be increased by chronic pain, chronic pain is not always suppressed by the treatment with anxiolytic drugs. In contrast, we cannot exclude the possibility that chronic pain-induced negative emotions cause some pain and/or lead to an increase of pain-sensitivity (Figure 12).

In conclusion, the present data constitute the first evidence that chronic pain has an anxiogenic effect in mice, which may be associated with changes in opioidergic function in the amygdala.

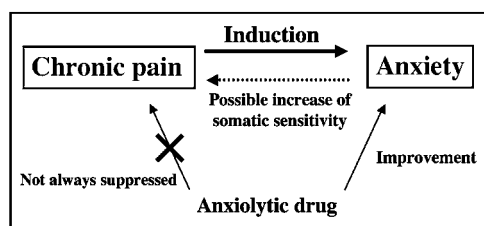


Figure 12 Conceptual frameworks for the presumed interaction between chronic pain and anxiety. Negative emotions such as anxiety can result from the experience of chronic pain. When the anxiolytic drug is treated under a chronic pain-like state, the anxiety can be improved by the drug. However, it is likely that chronic pain is not always suppressed by the anxiolytic drug. In contrast, anxiety could increase somatic sensitivity.

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