Usefulness of Antidepressants for Improving the Neuropathic Pain-Like State and Pain-Induced Anxiety through Actions at Different Brain Sites

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Clinically, it is well known that chronic pain induces depression, anxiety, and a reduced quality of life. There have been many reports on the relationship between pain and emotion. We previously reported that chronic pain induced anxiety with changes in opioidergic function in the central nervous system. In this study, we evaluated the anxiolytic-like effects of several types of antidepressants under a chronic neuropathic pain-like state and searched for the brain site of action where antidepressants show anxiolytic or antinociceptive effects. Sciatic nerve-ligated mice exhibited thermal hyperalgesia and tactile allodynia from days 7 to 28 after nerve ligation. At 4 weeks after ligation, these mice showed a significant anxiety-related behavior in the light–dark test and the elevated plus–maze test. Under these conditions, repeated administration of antidepressants, including the tricyclic antidepressant (TCA) imipramine, the serotonin noradrenaline reuptake inhibitor (SNRI) milnacipran, and the selective serotonin reuptake inhibitor (SSRI) paroxetine, significantly prevented the anxiety-related behaviors induced by chronic neuropathic pain. These antidepressants also produced a significant reduction in thermal hyperalgesia and tactile allodynia. Moreover, the microinjection of paroxetine into the basolateral amygdala or cingulate cortex reduced anxiety-related behavior, and microinjection into the primary somatosensory cortex significantly attenuated thermal hyperalgesia. These findings suggest that serotonergic antidepressants are effective for treating anxiety associated with chronic neuropathic pain and may be useful for treating neuropathic pain with emotional dysfunction such as anxiety. Furthermore, SSRIs show anxiolytic and antinociceptive effects by acting on different brain regions.
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INTRODUCTION

Clinically, it has been recognized that patients with chronic pain often suffer affective disorders such as anxiety and depression (Gallagher *et al*, 1995; Von Korff and Simon, 1996). Many of these reports deal with the effect of enhanced emotional regulation on pain and the relation between emotional distress and the seeking of treatment by individuals experiencing pain (Ettinger and Argoff, 2007;

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Vogt, 2005). Antidepressant drugs are efficacious and have been widely used in the management of chronic pain conditions (Max *et al*, 1987; Richeimer *et al*, 1997). Firm evidence has shown that tricyclic antidepressants (TCAs) should be considered the gold standard treatment for neuropathic pain (Coluzzi and Mattia, 2005; Sindrup *et al*, 2005). It has also been reported that the analgesic effect of antidepressants is independent of their antidepressive action, and occurs at lower doses and with a faster onset of action in a clinical situation (Pilowsky *et al*, 1982; Onghena and Van Hondenhobe, 1992; Richeimer *et al*, 1997). This effect can be explained by several pharmacological mechanisms. Some TCAs block sodium channels, which may contribute to their antihyperalgesic efficacy (Dick *et al*, 2006). They also apparently block calcium ion channels (Choi *et al*, 1992; Chaplan *et al*, 1994). However, it has also been considered that antidepressants modulate pain perception by blocking the reuptake of monoaminergic neurotransmitters in noradrenergic and serotonergic systems, which originate from the brain stem and project

to the spinal cord dorsal horn (Valverde *et al*, 1994). Moreover, the antihistaminergic action of TCAs may have a general analgesic effect (Rumore and Schlichting, 1986). Recent findings have shown that antidepressants also interact with the opioidergic system (Gray *et al*, 1998) and that they act as NMDA-receptor antagonists (Reynolds and Miller, 1988). Antidepressants, especially selective serotonin reuptake inhibitors (SSRIs) and TCAs, are also indicated for anxiety disorders, including generalized anxiety disorder, panic disorder, obsessive–compulsive disorder, and post-traumatic stress disorder. Serotonin noradrenaline reuptake inhibitors are also used to treat anxiety disorders including generalized anxiety disorder, obsessive–compulsive disorder, and attention-deficit hyperactivity disorder. However, very few studies have investigated the relationship between the analgesic effect of antidepressants and changes in emotionality under a chronic pain-like state.

Large distributed brain networks have been found to be active during painful stimulation (Brooks and Tracey, 2005) and have been identified by neuroimaging methods. The cortical and subcortical brain regions were found to be commonly activated by nociceptive stimulation of the anterior cingulate cortex (aCG), insula, frontal cortices, primary somatosensory cortex (S1), secondary somatosensory cortex (S2), and amygdala, often referred to as the 'pain matrix' (Brooks and Tracey, 2005). Among these areas, S1 and S2 are primarily thought to play a role in discriminating the location and intensity of painful stimuli, and the aCG is involved in the affective (cognitive–evaluative) component of pain (Brooks and Tracey, 2005). With regard to emotional dysfunction such as anxiety and depression, the amygdala plays a major role in its acquisition and maintenance, as reported in several studies using an animal model (Nelson and Trainor, 2007).

The present study was undertaken to investigate whether antidepressants are effective for the treatment of anxiety concomitant with neuropathic pain. We also evaluated the possible anxiolytic or antinociceptive effects of antidepressants when they were restrictively administered in each brain site.

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 the 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

In the present study, we used male C57BL/6J mice (CLEA Japan Inc., Tokyo, Japan), weighing 18–23 g, and male Sprague–Dawley rats (Tokyo Laboratory Animals Science, Tokyo, Japan), weighing 250–300 g. The animals were kept in a room with an ambient temperature of $23 \pm 1^\circ C$ and a 12-h light–dark cycle (lights on 08:00–20:00). Food and water were available *ad libitum*. All behavioral studies were performed during the light period.

Nerve Injury Pain Model

The mice were anesthetized with isoflurane (3%). The rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) in diethyl ether. We produced a neuropathic pain model by tying a tight ligature with an 8–0 (with mice) or 7–0 (with rats) silk suture around approximately 1/3–1/2 the diameter of the sciatic nerve located on the right hind-paw side (ipsilateral side), as described previously (mice, Narita *et al*, 2005; rats, Seltzer *et al*, 1990). This model may mimic important characteristics of chronic neuropathic pain in patients following peripheral nerve injury (Cui *et al*, 1997). We refer to these sciatic nerve partially ligated animals as 'sciatic nerve-ligated' animals in this study. In sham-operated animals, the nerve was exposed without ligation.

Measurement of Thermal and Tactile Thresholds

To assess the sensitivity to thermal stimulation, the right plantar surface of animals was tested 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 animals. The paw-withdrawal latency was determined as the average of three measurements per paw. Only quick hind-paw movements (with or without the 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, the animals were habituated for at least 30 min in a clear acrylic cylinder (mice, 15 cm high and 8 cm in diameter; rats, 30 cm high and 12 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 the antidepressant on the increased sensitivity to thermal stimulation, each paw was measured just before and 30 min after injection, using imipramine (10 mg/kg, s.c.), milnacipran (10 mg/kg, s.c.), or paroxetine (4 mg/kg, s.c.), at 7 days after nerve ligation.

In the chronic experiment, the mice were treated repeatedly with either imipramine, the selective serotonin reuptake inhibitor paroxetine, or the selective SNRI milnacipran, once a day for 3 weeks (from days 7 to 28) after nerve ligation, and the paw-withdrawal latency was measured one day after the last injection.

To quantify the sensitivity to a tactile stimulus, paw withdrawal in response to a tactile stimulus was measured using a bending force (0.16 g) applied by von Frey filaments (North Coast Medical Inc., Morgan Hill, CA, USA) (Narita *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 scoring as follows: 0, no response; 1, a slow or slight response to the stimulus; 2, a quick withdrawal response away from the

stimulus without flinching and/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 measurements. Before the behavioral responses to tactile stimuli were tested, the 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. All animals received just one treatment. All behavioral experiences were carefully measured in a non-blinded fashion.

Light–Dark Test

To investigate changes in anxiogenic-like effects, the mice were tested using the light–dark paradigm (Bilkei-Gorzo et al, 1998; Schramm et al, 2001). We used a box consisting of a small ($18 \times 13 \times 18\,cm^3$) dimly lit compartment with dark black walls and a black floor, connected by a tunnel (5 cm long) to a large ($18 \times 18 \times 18\,cm^3$) intensely lit (500 lx) compartment with 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 a compartment, respectively. The time spent in the lit compartment was recorded for 10 min.

Elevated Plus-Maze Test

We also used the elevated plus-maze test to measure anxiety. This 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 (mice, $30 \times 6 \times 0.3\,cm^2$ each; rats, $60 \times 10 \times 4\,cm^2$ each) and closed arms (mice, $30 \times 6 \times 15\,cm^2$ each; rats, $60 \times 10 \times 40\,cm^2$ each) joined by a common central platform (mice, $9 \times 9\,cm^2$; rats, $11 \times 11\,cm^2$). The maze was elevated 40 cm (mice) or 50 cm (rats) above the floor. The open and closed arms and the central platform were subjected to approximately equal illumination. Arm entry and exit were defined as all four paws into and out of an arm, respectively. The results were calculated as the mean ratios of the time spent in the open arms to the total time spent in both the open and closed arms. Entries into the open and closed arms were also scored.

To ascertain the anxiolytic effect of acute treatment with imipramine, a single injection of imipramine (3, 10 mg/kg, s.c.) was administered 30 min before the light–dark or elevated-plus maze procedures.

In the chronic experiment, mice were used for this procedure following repeated treatment with imipramine, milnacipran, or paroxetine once a day for 3 weeks (days 7 to 27) after nerve ligation, and this procedure was performed on the day after the last injection. The same mice were subjected to all behavioral measurements. On day 28, the mice were subjected to the elevated plus-maze test and the light–dark test, and paw-withdrawal latencies and responses were measured on day 29.

Cannulation and Microinjection Using the Rat Neuropathic Pain Model

To find the brain site at which antidepressants act, we used the microinjection method with the rat neuropathic pain
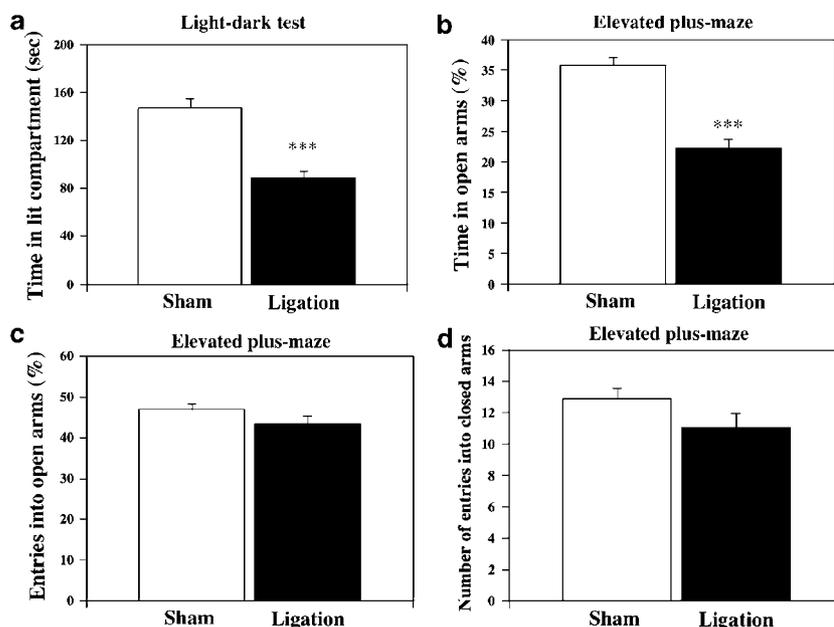

Figure 1 Chronic pain-induced anxiogenic effect. (a) Sciatic nerve ligation induced a significant decrease in the time spent in the lit compartment of the light–dark box 4 weeks after surgery. (b) The percentage of time spent in the open arms in the elevated plus–maze was significantly decreased by sciatic nerve ligation 4 weeks after surgery. (c) There was no difference in the percentage of entries into the open arms between sham-operated and nerve-ligated mice. (d) The number of entries into the closed arms, which indicates general activity-related behavior, was similar in both groups. Each column represents the mean ± SEM of 18 mice. ***$p < 0.001$ vs sham-operated mice.

model. All rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and placed in a stereotaxic apparatus. The skull was exposed and a small hole was made with a dental drill. A contralateral guide cannula (AG-4 or AG-9; Eicom, Kyoto, Japan) was implanted in the cingulate cortex (CG) (from Bregma: AP +1.7, ML +0.4, V −2.0), basolateral amygdala (BLA) (Bregma: AP −2.8, ML +5.0, V −7.9), or S1 (Bregma: AP −0.8, ML +1.9, V −1.8) according to the atlas of Paxinos and Watson (1998). The guide cannula was fixed to the skull with cranioplastic cement. After surgery, the rats were housed individually and allowed to recover for at least 3 days before sciatic nerve ligation. When not used for injection, the guide cannulas were occluded with dummy cannulas (AD-4 or AD-9; Eicom, Kyoto, Japan).

More than 3 days after cannulation surgery, the rats were anesthetized and the sciatic nerve was ligated. At day 4 after sciatic nerve ligation, we started microinjection with paroxetine into the CG, BLA, or S1 after the measurement of thermal thresholds. Microinjection was performed once a day from days 4 to 6 after nerve ligation. In the microinjection method, we used an injection cannula (AMI 4.5 or 9.5; Eicom) that extended 0.5 mm beyond the guide cannula. A stainless-steel injection cannula was inserted into the contralateral guide cannula for each animal. The injection cannula was connected through polyethylene tubing to a 10 μl Hamilton syringe that had been preloaded with paroxetine (5 nmol/0.3 μl) or vehicle.
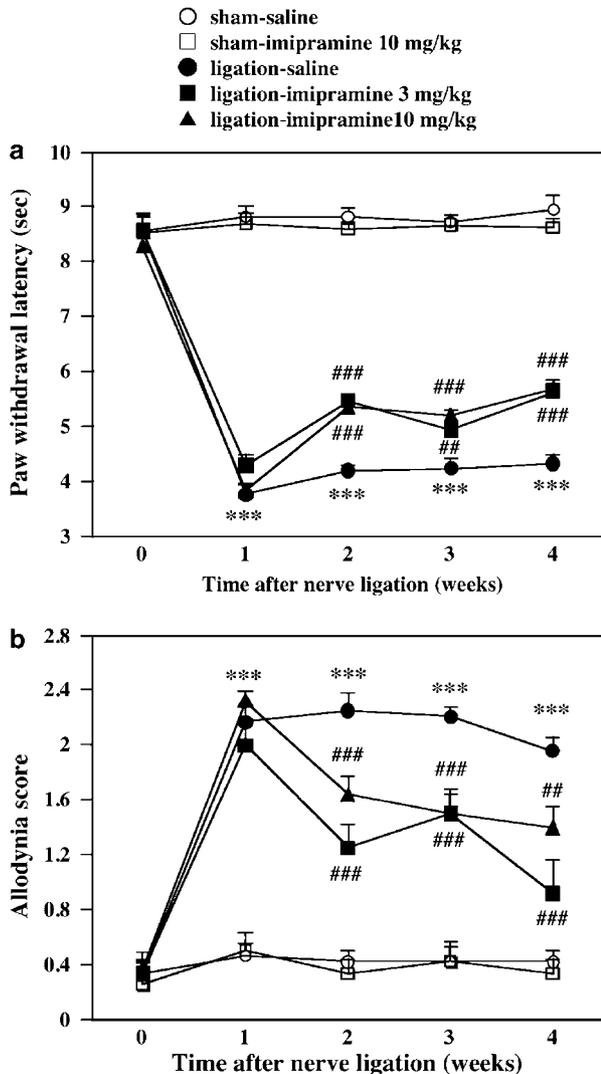

Figure 2 Antinociceptive effects of repeated s.c. imipramine on thermal hyperalgesia (a) and tactile allodynia (b) in sham-operated or nerve-ligated mice. A tactile stimulus was applied using filaments with a bending force of 0.16 g. Groups of mice were repeatedly treated subcutaneously with imipramine (3 or 10 mg/kg) or saline once a day from days 7 to 28 after nerve ligation. Imipramine-treated mice showed (a) a significant increase in the decreased thermal threshold and (b) a significant decrease in sensitivity to tactile stimuli on the ipsilateral side. Each point represents the mean ± SEM of 6–12 mice. ***$p < 0.001$ vs sham-operated and saline-treated mice. ###$p < 0.001$ and ##$p < 0.01$ vs the nerve-ligated and saline-treated groups.
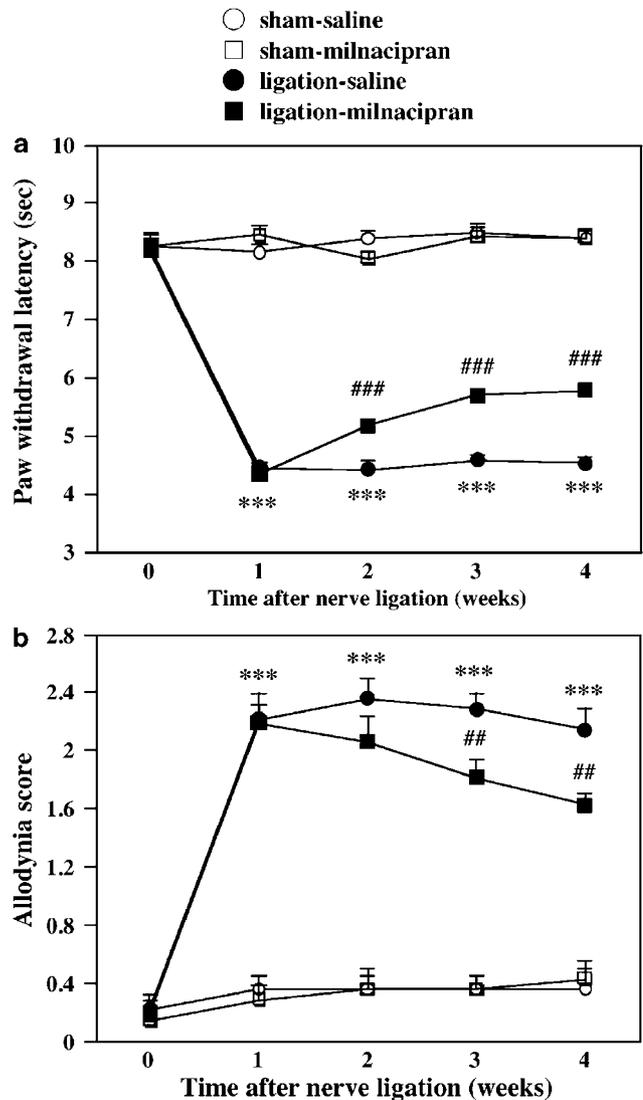

Figure 3 Antinociceptive effects of repeated s.c. milnacipran on thermal hyperalgesia (a) and tactile allodynia (b) in sham-operated or nerve-ligated mice. A tactile stimulus was applied using filaments with a bending force of 0.16 g. The groups of mice were repeatedly treated subcutaneously with milnacipran (10 mg/kg) or saline once a day from days 7 to 28 after nerve ligation. Milnacipran-treated mice showed (a) a significant increase in the decreased thermal threshold and (b) a significant decrease in sensitivity to tactile stimuli on the ipsilateral side. Each point represents the mean ± SEM of seven or eight mice. ***$p < 0.001$ vs sham-operated and saline-treated mice. ###$p < 0.001$ and ##$p < 0.01$ vs nerve-ligated and saline-treated group.

Paroxetine or the vehicle was delivered by a motorized syringe pump in a volume of 0.3 μl over 60 s. At 7 days after nerve ligation, the thermal thresholds, and anxiety-related behaviors of the rats were tested using the elevated plus-maze apparatus.

Drugs

Imipramine hydrochloride (10,11-dihydro-N,N-dimethyl-5H-dibenz[b,f] azepine-5-propanamine hydrochloride; Sigma-Aldrich, St Louis, MO, USA), milnacipran hydrochloride [(1R,2S)-rel-2-[aminomethyl]-N,N-diethyl-1-phenylcyclopropane-carboxamide hydrochloride; Sigma-Aldrich], and paroxetine maleate [(3S,4R)-3-[(1,3-benzodioxol-5-yloxy)methyl]-4-(4-fluorophenyl)-piperidine maleate; Tocris Cookson Inc., MO, USA] were dissolved in saline.

Statistical Analysis

All data are presented as the mean ± SEM of the animals in each groups. The statistical significance of differences between groups was assessed with two-way ANOVA or one-way ANOVA followed by the Bonferroni/Dunn multiple-comparison test or with Student's t-test.

RESULTS

Sciatic Nerve Injury-Induced Anxiety-Related Behavior

There was no difference in the latencies of paw withdrawal between sham-operated and sciatic nerve-ligated mice before surgery. The withdrawal latencies of the ipsilateral paw in response to the thermal and tactile stimuli were significantly decreased at 1 week after sciatic nerve ligation, and the changes in paw withdrawal latencies and responses lasted for at least 4 weeks after ligation (cf. sham-saline group and ligation-saline group in Figures 2–4). All of the ligated animals showed distinct neuropathic pain-like behavior. Paw-withdrawal latencies and responses on the contralateral side of mice were not changed by surgery (data not shown).

Under these conditions, we confirmed whether a chronic pain-like state could induce anxiogenic-like behavior in mice. In the light–dark test, the time spent on the lit side at 1 week after surgery was not changed by sciatic nerve ligation compared with the results after a sham operation (data not shown). However, sciatic nerve-ligated mice showed a significant decrease in the time spent on the lit side at 4 weeks after surgery ($p < 0.001$ vs sham 4-week group, Figure 1a). In the elevated plus-maze test, the percentage of time spent in the open arms was significantly reduced in sciatic nerve-ligated mice ($p < 0.001$ vs sham 4-week group, Figure 1b) at 4 weeks after surgery. On the other hand, the number of entries into the closed arms, as a general activity-related parameter, was similar in both groups (Figure 1d). There were no significant differences in the percentages of entries into the open arms between sham-operated and nerve-ligated mice (Figure 1c). These results suggest that chronic pain induces anxiety-like behavior in mice.
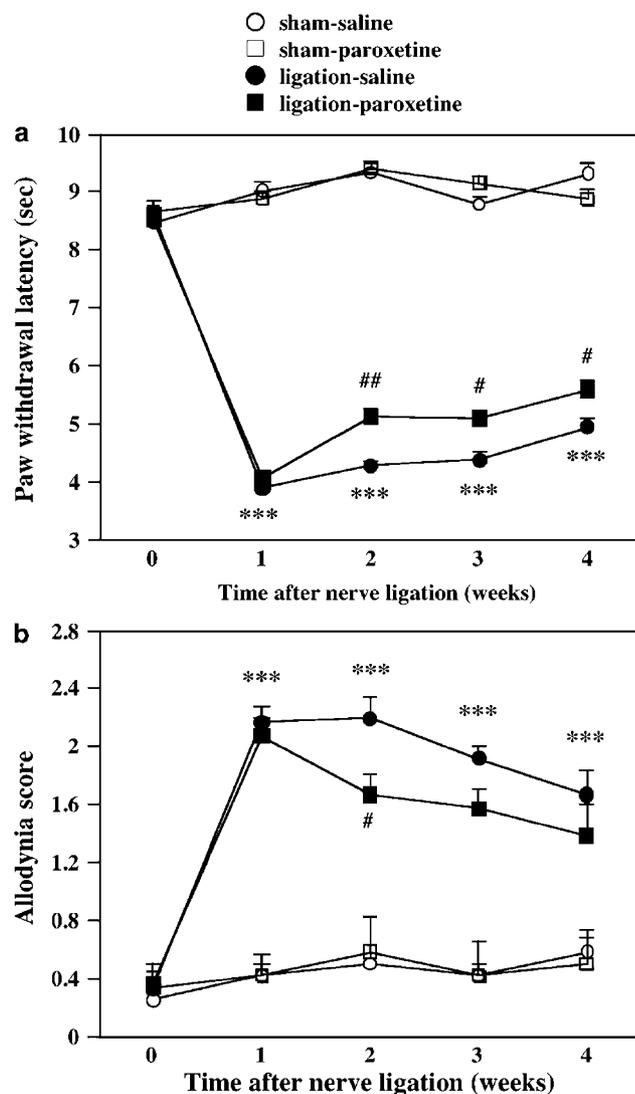

Figure 4 Antinociceptive effects of repeated s.c. paroxetine on thermal hyperalgesia (a) and tactile allodynia (b) in sham-operated or nerve-ligated mice. A tactile stimulus was applied using filaments with a bending force of 0.16 g. Groups of mice were repeatedly treated subcutaneously with paroxetine (4 mg/kg) or saline once a day from days 7 to 28 after nerve ligation. Paroxetine-treated mice showed (a) a significant increase in the decreased thermal threshold and (b) a significant decrease in sensitivity to tactile stimuli on the ipsilateral side. Each point represents the mean ± SEM of 6–7 mice. ***$p < 0.001$ vs the sham-operated and saline-treated groups. #$p < 0.05$ and ##$p < 0.01$ vs the nerve-ligated and saline-treated groups.

Antinociceptive Effects of Antidepressants for a Neuropathic Pain-Like State

Using this neuropathic pain-like state model, we investigated whether antidepressants could produce antiallodynic or antihyperalgesic effects. To ascertain the effect of s.c. injections of several antidepressants on paw-withdrawal latencies on the ipsilateral and contralateral sides in sham-operated or nerve-ligated mice, we measured each paw just before and 30 min after imipramine (10 mg/kg, s.c.), milnacipran (10 mg/kg, s.c.), and paroxetine (4 mg/kg, s.c.) injection. As a result, the decreased thermal threshold on the ipsilateral side in nerve-ligated mice was not affected by a single s.c. injection of these antidepressants at these doses

(data not shown), indicating that acute treatment with these antidepressants at this dose showed no antinociceptive effect. Next, to investigate the effect of chronic imipramine treatment on the sensitivity to thermal stimulation, the mice were treated repeatedly with imipramine (3, 10 mg/kg, s.c.) once a day for 3 weeks (from days 7 to 28) after nerve ligation. As a result, the decreased thermal threshold on the ipsilateral side was significantly suppressed in imipramine-treated mice after nerve ligation ($p < 0.01$ at 3 weeks after nerve ligation, and $p < 0.001$ at any other time vs the saline-treatment group, Figure 2a). Moreover, imipramine-treated mice showed a significant decrease in the sensitivity to tactile stimuli ($p < 0.001$ vs saline-treatment group, Figure 2b).

We also measured paw-withdrawal latencies and responses to thermal, and tactile stimuli following chronic treatment with either milnacipran (10 mg/kg, s.c.) or paroxetine (4 mg/kg, s.c.). The thermal hyperalgesia observed on the ipsilateral side in nerve-ligated mice was significantly suppressed by the repeated s.c. injection of milnacipran ($p < 0.001$ vs saline-treatment group, Figure 3a) and paroxetine ($p < 0.01$ at 2 weeks and $p < 0.05$ at 3 and 4 weeks after nerve ligation vs the saline-treatment group, Figure 4a) once a day from days 7 to 28 after nerve ligation. Furthermore, repeated s.c. injection of milnacipran ($p < 0.01$

at 3 and 4 weeks after nerve ligation vs the saline-treatment group, Figure 3b) or paroxetine ($p < 0.05$ at 2 weeks after nerve ligation vs the saline-treatment group, Figure 4b) significantly suppressed the increase in paw withdrawal in response to an innocuous stimulus induced by nerve ligation in mice.

Anxiolytic Effect of Antidepressants for Anxiety Induced by a Chronic Pain-Like State

Using this chronic pain-like state model that was associated with anxiety-related behaviors, we investigated whether antidepressants could exert an anxiolytic effect. To ascertain the effect of acute treatment with imipramine, a single s.c. injection (10 mg/kg s.c.) was administered 30 min before the light–dark procedure. Acute treatment with imipramine (10 mg/kg, s.c.) produced no changes in the time spent in the lit zone in the light–dark test, indicating that such treatment had no anxiolytic-like effect.

In contrast, chronic treatment with imipramine (10 mg/kg, s.c., once a day from days 7 to 27 after nerve ligation) was associated with a significant increase in the decreased time spent on the lit side in the light–dark test ($p < 0.01$ vs the saline-treatment group, Figure 5a). Furthermore, chronic imipramine-treated mice showed a significant
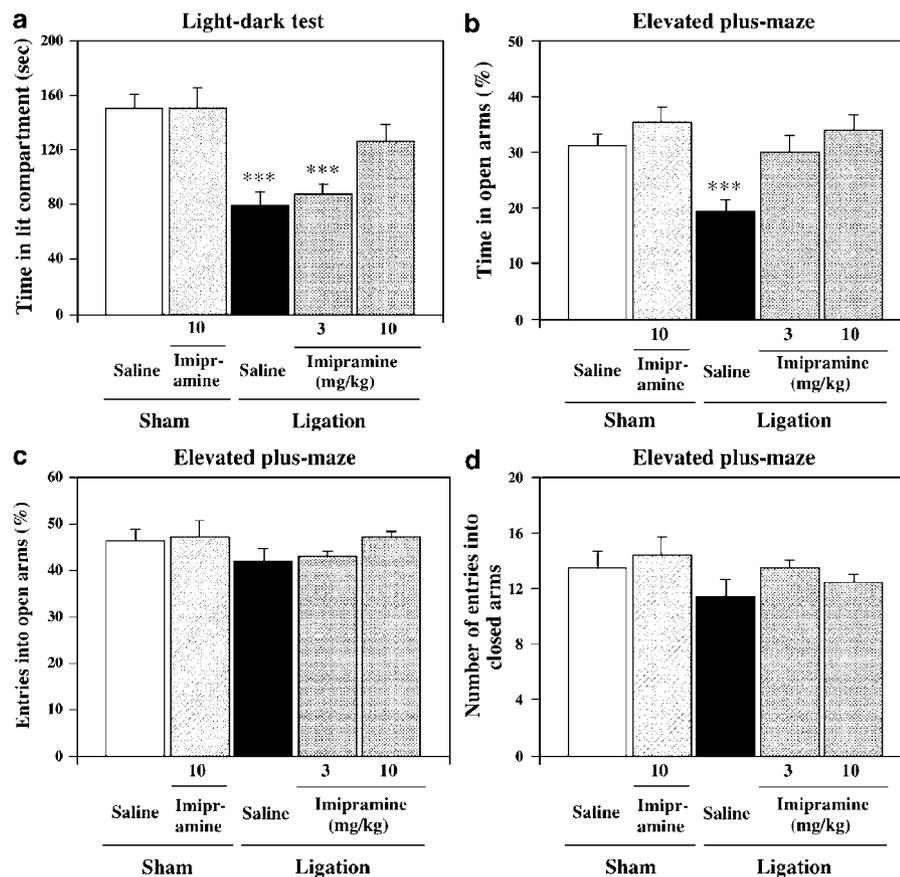
Figure 5 Anxiolytic effects of repeated s.c. imipramine on the light–dark test (a) and elevated plus–maze test (b, c, and d) in sham-operated or nerve-ligated mice. Anxiety-related behavior was measured at least 24 h after the last treatment. (a) The decreased time spent in the lit compartment was significantly increased by repeated s.c. treatment with imipramine 10 mg/kg. (b) The decreased percentage of time spent in the open arms was significantly increased by repeated s.c. treatment with imipramine 3 or 10 mg/kg. (c) The percentage of entries into the open arms was similar among the groups. (d) There was no significant difference among the groups in the number of entries into the closed arms, which is a parameter of general activity-related behavior. Each column represents the mean ± SEM of 6–12 mice. ***$p < 0.001$ vs sham-operated and saline-treated group.

improvement in the reduced percentage of time spent in the open arms in sciatic nerve-ligated mice ($p < 0.001$ at imipramine 10 mg/kg group and $p < 0.01$ at imipramine 3 mg/kg group *vs* the saline-treatment group, Figure 5b).

Next, we investigated the effect of chronic treatment with either milnacipran (10 mg/kg, s.c.) or paroxetine (4 mg/kg, s.c.) on anxiety-related behavior induced by sciatic nerve ligation. As a result, the anxiety-related behavior induced by sciatic nerve ligation was significantly suppressed by the repeated s.c. injection of milnacipran from days 7 to 27 after nerve ligation ($p < 0.01$ *vs* the saline-treatment group, Figure 6b). As with milnacipran, mice that had been treated with paroxetine showed a significant increase in the reduced time spent in the lit compartment using the light–dark test ($p < 0.05$ *vs* the vehicle treatment group, Figure 7a).

Anxiolytic or Antinociceptive Effects of Intrabrain Microinjection of Paroxetine

As in mice, rats also showed thermal hyperalgesia that lasted at least from days 4 to 7 after sciatic nerve ligation (see sham-saline group and ligation-saline group in Figures 8b, 9b and 10b). At day 4, the rats showed no anxiety-related behaviors in the elevated plus-maze test (data not shown). At 7 days after nerve ligation, however, the percentage of time spent in the open arms was significantly

reduced in sciatic nerve-ligated rats ($p < 0.05$ *vs* the sham-saline group, Figures 8c, 9c and 10c) in the elevated plus-maze test. These results suggest that chronic pain induces anxiety-like behavior in rats as well as mice.

TCAs including imipramine generally display a wide variety of pharmacological activities, including 5-HT and noradrenaline (NA) uptake inhibition and antagonist activity at several serotonergic, adrenergic, histaminergic, and cholinergic receptors (Vaishnavi *et al*, 2004). The SNRI milnacipran, which shows an equipotent inhibitory action on 5-HT and NA reuptake, is characterized by an absence of affinity for the receptors, such as histamine H$_1$ and muscarinic receptors, and α1-adrenoceptors (Gervasoni *et al*, 2002). Even though milnacipran displays an inhibitory action on 5-HT and NA receptor, this inhibitory action of milnacipran is likely to be much weaker than that of imipramine based on lower binding affinity (Mongeau *et al*, 1998). On the other hand, the SSRI paroxetine showed the greatest selectivity with high affinity for serotonin transporters (de Abajo *et al*, 2006) and also weakly inhibits the reuptake of NA and has affinity for cholinergic receptors. The inhibition of serotonin reuptake is an important and common mechanism among these three antidepressants. Chronic pain-induced anxiety is presumed to be elicited at the supraspinal level. Therefore, to investigate which brain areas contribute to the anxiolytic or antinociceptive effect
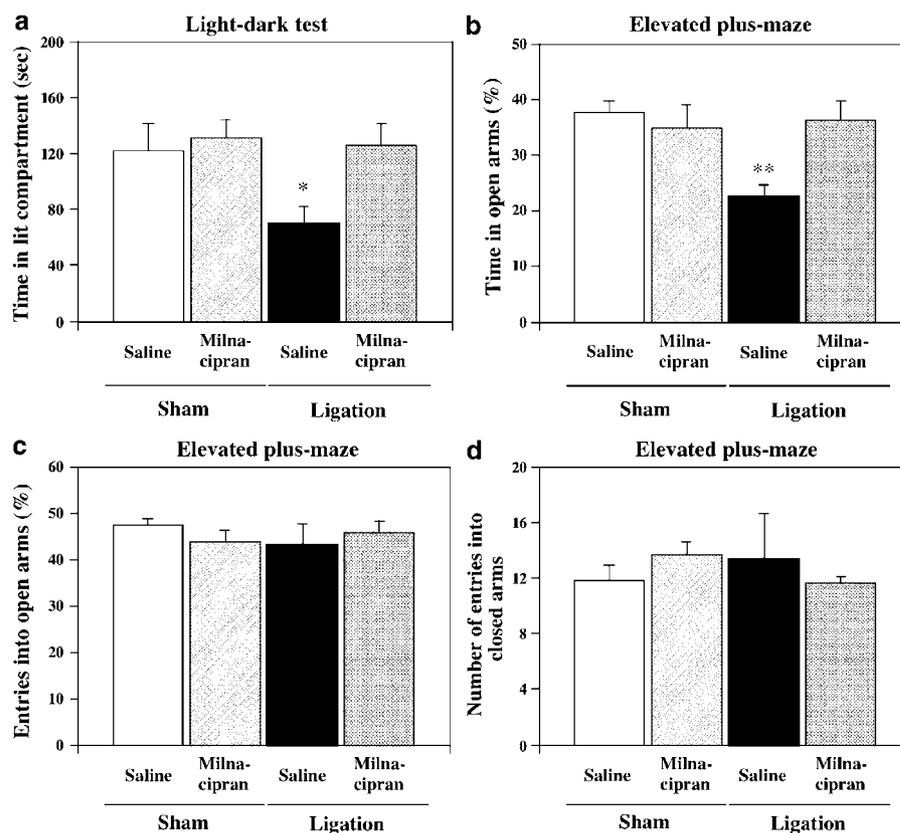

Figure 6 The anxiety-related behavior induced by sciatic nerve ligation was significantly suppressed by repeated s.c. injection of milnacipran from days 7 to 27 after nerve ligation. Anxiety-related behavior was measured at least 24 h after the last treatment. (a) Mice that chronically received milnacipran showed a relative decrease in anxiety-related behavior in the light–dark test. The subcutaneous administration of milnacipran at 10 mg/kg affected the time spent in the lit compartment. (b) The decreased percentage of time spent in the open arms was completely recovered by repeated s.c. treatment with milnacipran in the elevated plus–maze test. (c) The percentage of entries into the open arms was similar in all groups. (d) There was no difference in the number of entries into the closed arms among the groups. Each column represents the mean ± SEM of 7–8 mice. **$p < 0.01$, *$p < 0.05$ *vs* the sham-operated and saline-treated groups.
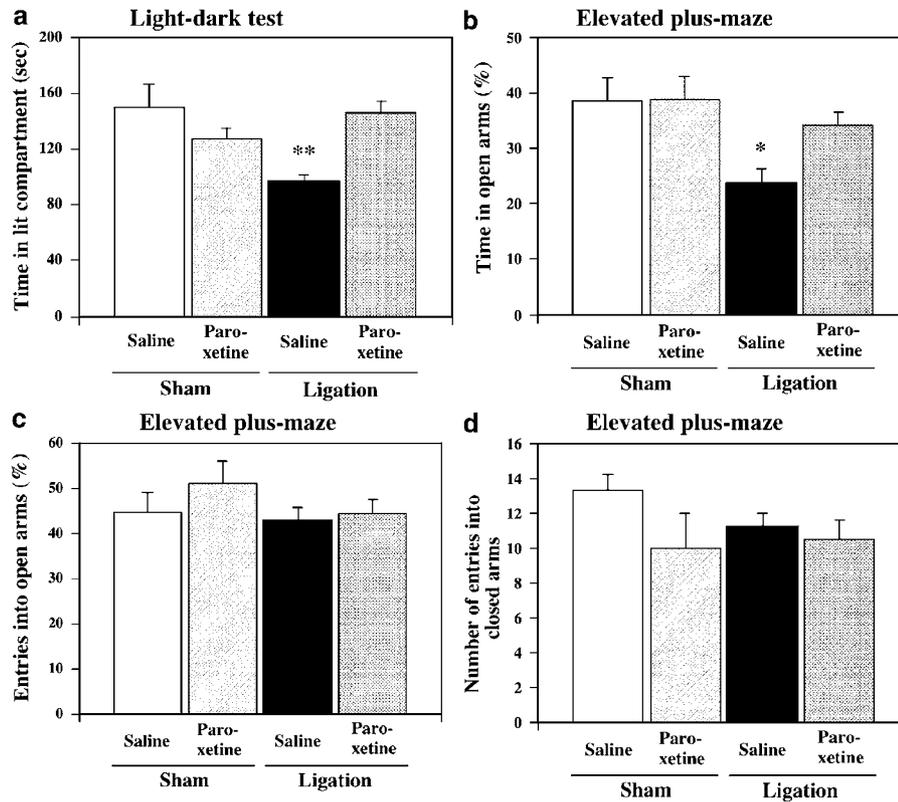
Figure 7 Expression of anxiety-related behavior in mice that had been chronically treated with paroxetine. The mice were injected with paroxetine at 4 mg/kg once a day from days 7 to 27 after surgery. Anxiety-related behavior was measured at least 24 h after the last treatment. (a) The decreased time spent in the lit compartment was significantly increased by repeated s.c. treatment with paroxetine in the light–dark box paradigm. Chronic treatment with paroxetine significantly affected the time spent in the lit compartment. (b) The decreased percentage of time spent in the open arms was significantly increased by repeated s.c. treatment with paroxetine in the elevated plus–maze test. (c) The percentage of entries into the open arms was similar in all groups. (d) There was no difference in the number of entries into the closed arms among these groups. Each column represents the mean ± SEM of 6–7 mice. $**p < 0.01$, $*p < 0.05$ vs the sham-operated and saline-treated groups.

induced by the SSRI paroxetine, we microinjected paroxetine into the CG, BLA, or S1. Figures 8a, 9a and 10a show the placement of microinjection cannulas within the rat CG, BLA, or S1. Guide cannulas were localized above the CG, BLA, or S1. Only data from rats in which guide cannulas had been accurately inserted in the CG, BLA, or S1 were used for subsequent statistical analysis. The diffusion of the microinjection with a volume of 0.3 μl was strictly observed inside the CG, BLA, or S1 area under confirmation with a stereoscopic microscope. None of the rats were excluded due to misdistribution in this study.

Using the rat elevated plus-maze test, we found that the decreased percentage of time spent in the open arms in sciatic nerve-ligated rats was significantly increased after the microinjection of paroxetine into the CG ($p < 0.01$ vs the nerve-ligated and saline-treatment groups, Figure 8c). With regard to the antinociceptive effect, the microinjection of paroxetine into the rat CG failed to exert an antinociceptive effect (Figure 8b). Furthermore, while the microinjection of paroxetine into the rat BLA also produced a significant increase in the reduced percentage of time spent in open arms in sciatic nerve-ligated rats ($p < 0.01$ vs the nerve-ligated and saline-treatment groups, Figure 9c), there was no change in thermal thresholds after an intra-BLA injection (Figure 9b). On the other hand, the microinjection of paroxetine into the S1 produced a significant increase in the reduced thermal threshold on the ipsilateral side after

nerve ligation ($p < 0.001$ vs the nerve-ligated and saline-treatment groups, Figure 10b). The percentage of time spent in the open arms was not significantly different between the ligation-saline group and ligation-paroxetine group, indicating there was no significant anxiolytic effect (Figure 10c).

DISCUSSION

Antidepressants have been used for more than 30 years in the clinical management of neuropathic pain (McQuay, 2002) and have been shown to provide analgesia for a variety of neuropathic pain syndromes regardless of the presence of depression (Richeimer et al, 1997; Mico et al, 2006). Many studies have demonstrated the efficacy of antidepressants for the management of diabetic neuropathy (Kvinesdal et al, 1984; Sindrup et al, 1989; Max et al, 1992), postherpetic neuralgia (Watson et al, 1982), poststroke pain (Leijon and Boivie, 1989), post-thoracotomy pain (Keller et al, 1994), and almost all types of neuropathic pain (Saarto and Wiffen, 2005; Sindrup et al, 2005). In this study, we demonstrated that chronic treatment with antidepressants produced significant antiallodynic and antihyperalgesic effects under a nerve-ligated neuropathic pain-like state.

The doses of antidepressants chosen in the present study were based on several earlier reports in which drug was administered intraperitoneally in mice. It has been reported
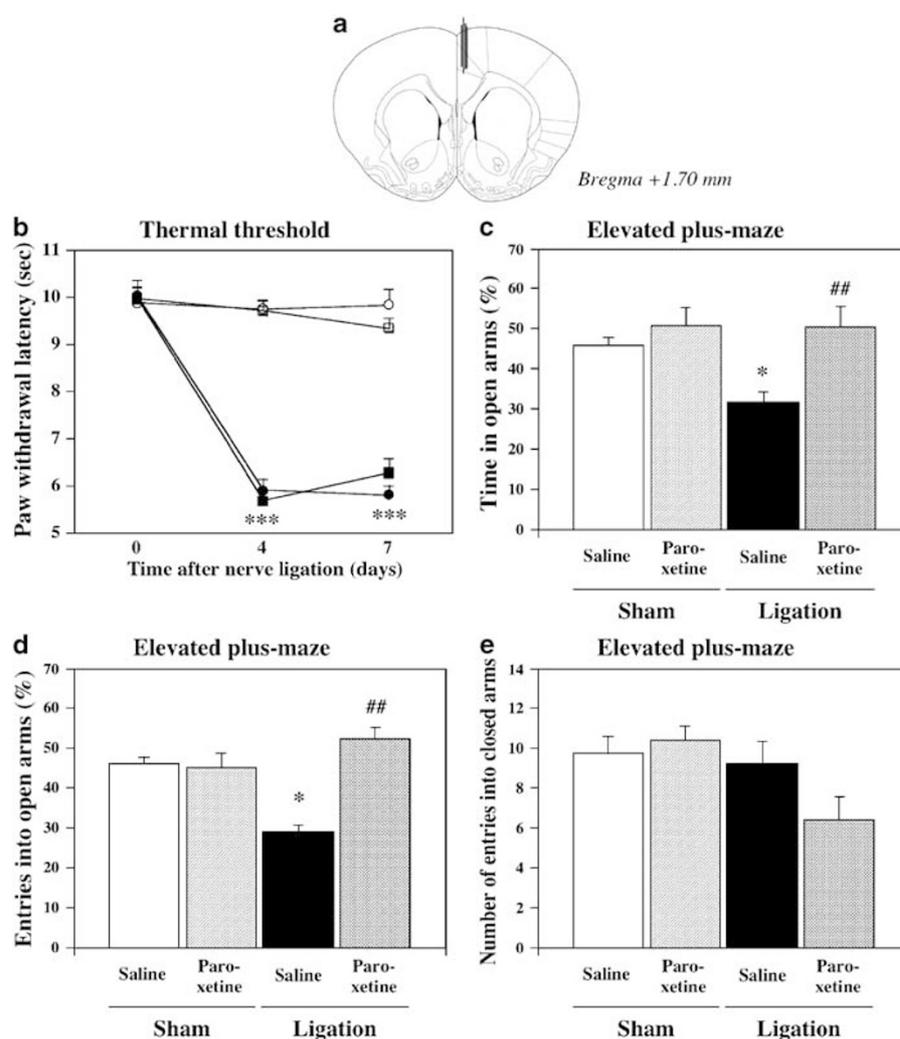
Figure 8 Antinociceptive (b) and anxiolytic (c, d, and e) effects of paroxetine microinjection into the cingulate cortex on thermal hyperalgesia (b) or the elevated plus-maze test (c, d, and e) in sham-operated or nerve-ligated rats. (a) Localization of microinjection sites in the rat cingulate cortex. Lines show traces of the region in the rat brain in which a cannula was inserted. Thermal hyperalgesia and anxiety-related behavior was measured for 12 h after the last treatment. Groups of rats were repeatedly treated with paroxetine (5 nmol/0.3 μl) or saline once a day from days 4 to 6 after nerve ligation. (b) Paroxetine-treated rats showed no significant increase in the decreased thermal threshold on the ipsilateral side. Each point represents the mean ± SEM of five rats. ***$p < 0.001$ vs sham-operated and saline-treated rats. Open circle: sham-operated and saline-treated groups; open square: sham-operated and paroxetine-treated groups; closed circle: nerve-ligated, and saline-treated groups; closed square: nerve-ligated and paroxetine-treated groups. (c) The decreased percentage of time spent in the open arms in the elevated plus–maze procedure in the nerve-ligated and saline-treated group was completely recovered by repeated microinjection of paroxetine into the cingulate cortex. (d) The decreased percentage of entries into the open arms was significantly increased in the nerve-ligated and paroxetine-treated group. (e) There was no significant difference among the groups in the number of entries into the closed arms, which is a parameter of general activity-related behavior. *$p < 0.05$ vs the sham-operated and saline-treated groups, ##$p < 0.01$ vs the nerve-ligated and saline-treated groups. Each column represents the mean ± SEM of 4–5 rats.

that imipramine produces a significant acute analgesic effect at 10–40 mg/kg in the formalin test (Sabetkasai et al, 1999; Zarrindast et al, 2000a, 2003), 10–15 mg/kg in the hot-plate test (Ghelardini et al, 2000; Sahebgharani and Zarrindast, 2001), and 20–30 mg/kg against a neuropathic pain-like state (Zarrindast et al, 2000b). It also induces an antidepressive effect at 10–32 mg/kg in the forced swimming test (Clenet et al, 2001; David et al, 2003; Rojas-Corrales et al, 2003). In contrast, some reports have stated that acute administration does not produce an anxiolytic effect (Cole and Rodgers, 1995). On the other hand, milnacipran has an anxiolytic effect at 8–32 mg/kg in the four-plate test (Hascoet et al, 2000; Bourin et al, 2005; Masse et al,

2005), whereas a few reports have shown an antinociceptive or analgesic effect (Aoki et al, 2006). Paroxetine has a significant antinociceptive effect at 5–20 mg/kg in the hot plate test (Duman et al, 2006) and at 5–30 mg/kg in the writhing test (Ormazabal et al, 2001; Kesim et al, 2005), and an anxiolytic effect at 4–16 mg/kg (Hascoet et al, 2000; Masse et al, 2005). In the present study, imipramine at 3 and 10 mg/kg, milnacipran at 10 mg/kg, and paroxetine at 4 mg/kg were chosen for chronic administration under a neuropathic pain-like state. As a result, we found that these doses were insufficient to produce an acute analgesic effect or an acute anxiolytic effect (unpublished observation). Furthermore, anxiety-related behavior was measured at
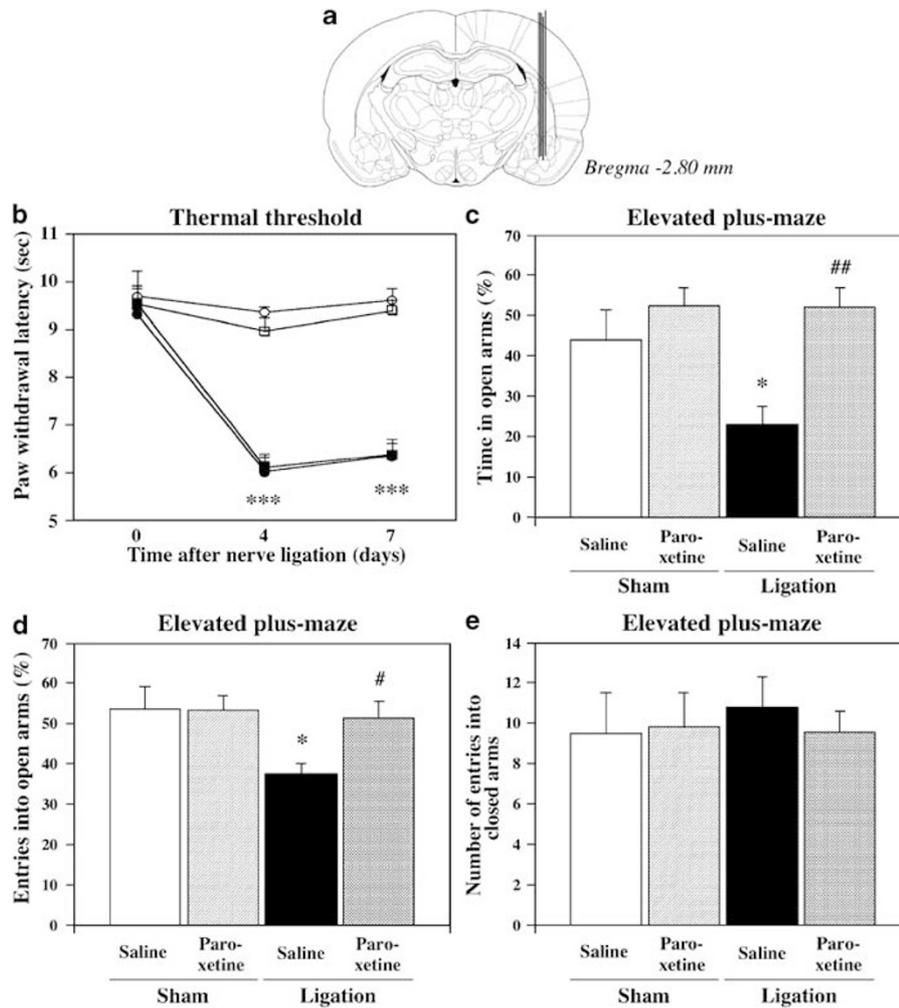
Figure 9 Antinociceptive (b) and anxiolytic (c, d, and e) effects of paroxetine microinjection into the basolateral amygdala on thermal hyperalgesia (b) or the elevated plus-maze test (c, d, and e) in sham-operated and nerve-ligated rats. (a) Localization of microinjection sites in the rat basolateral amygdala. Lines show traces of the region in the rat brain in which a cannula was inserted. Groups of rats were repeatedly treated with paroxetine (5 nmol/0.3 μl) or saline once a day from days 4 to 6 after nerve ligation. (b) Paroxetine-treated rats showed no significant increase in the decreased thermal threshold on the ipsilateral side. Each point represents the mean ± SEM of five rats. ***$p < 0.001$ *vs* sham-operated and saline-treated rats. Symbols are explained in the legend for Figure 8. (c) The decreased percentage of time spent in the open arms was completely recovered by repeated microinjection of paroxetine into the basolateral amygdala. (d) The decreased percentage of entries into the open arms was significantly increased in the nerve-ligated and paroxetine-treated groups. (e) There was no significant difference in the number of entries into the closed arms, which is a parameter of general activity-related behavior among the groups. *$p < 0.05$ *vs* the sham-operated and saline-treated groups, ##$p < 0.01$, #$p < 0.05$ *vs* the nerve-ligated and saline-treated groups. Each column represents the mean ± SEM of 4–5 rats.

least 24 h after the last treatment. In this way, we mimicked a clinical situation through the use of non-overdose, chronic administration. Consequently, anxiolytic and anti-nociceptive effects were present at 4 weeks after nerve ligation.

Previous functional imaging and stimulation studies in humans have shown that the contralateral side of cortical areas is activated by painful stimuli (Hsieh *et al*, 1995; Derbyshire *et al*, 1997). It has been recognized that nociceptive primary afferent axons terminate almost exclusively in the dorsal horn, and therefore this is the site of the first synapse in ascending pathways that convey to the contralateral side (the side opposite injury) of brain sensory information that underlies the conscious perception of pain. These areas include the somatosensory cortices, which are thought to be concerned with the sensory qualities of pain and other cortical areas including the cingulate cortex (CG) and insula and the prefrontal cortex, which are more closely aligned with affective-motivational aspects of pain. Neuron projecting from the medial prefrontal cortex to the basolateral amygdala (BLA) were detected by the injection of retrograde tracer into the medial frontal cortex (Hur and Zaborszky, 2005). It has been recognized that the BLA is mostly related to the modification of emotionality. Therefore, we injected paroxetine into the related cortices contralaterally, which resulted in anxiolytic or antinociceptive effects.

Cortical areas such as the amygdala and aCG are involved in modulation of the experience of pain (Mantyh, 2006). Among these areas, the CG is considered to be involved in pain perception and the emotional response to pain (Vogt, 2005). With regard to the amygdala, we reported previously
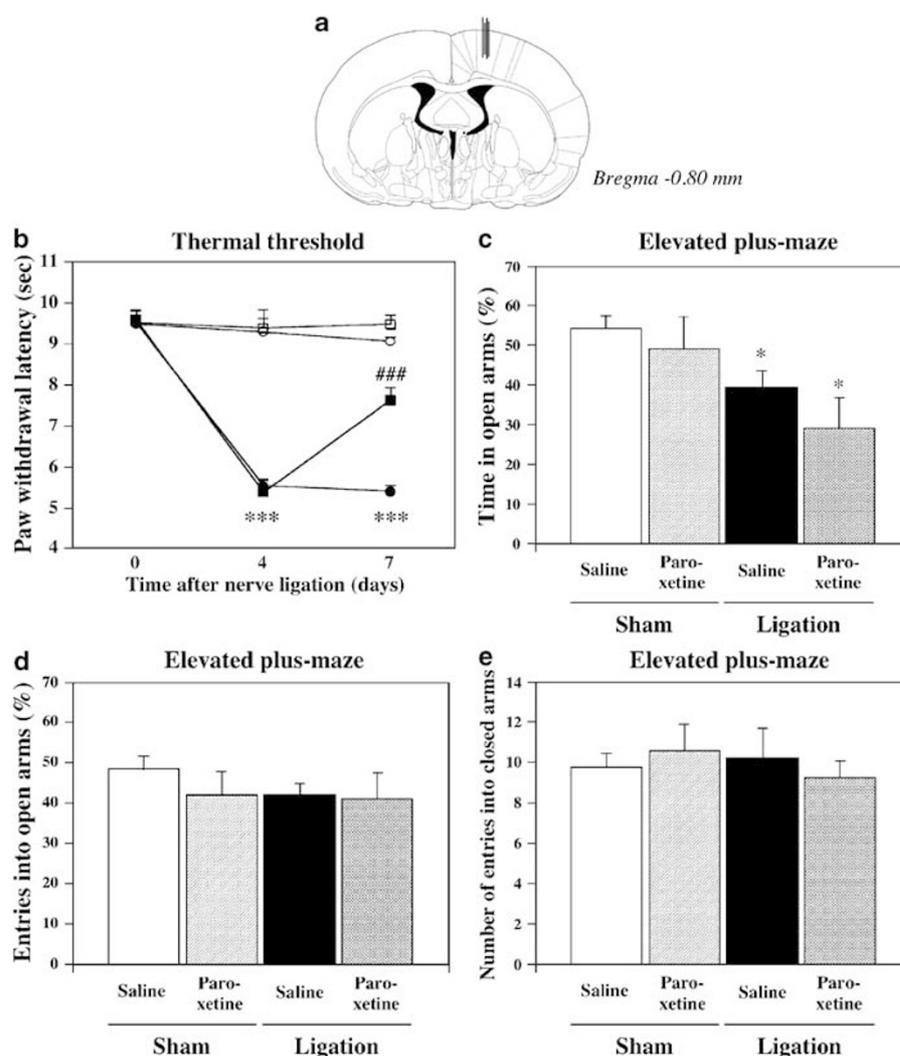
Figure 10 Antinociceptive (b) and anxiolytic (c, d, and e) effects of paroxetine microinjection into the S1 on thermal hyperalgesia (b) or the elevated plus-maze test (c, d, and e) in sham-operated and nerve-ligated rats. (a) Localization of microinjection sites in the rat S1. Lines show traces of the region in the rat brain in which a cannula was inserted. Groups of rats were repeatedly treated with paroxetine (5 nmol/0.3 μl) or saline once a day from days 4 to 6 after nerve ligation. (b) Paroxetine-treated rats showed dramatic increases in the decreased thermal threshold on the ipsilateral side. Each point represents the mean ± SEM of 5–6 rats. ***$p < 0.001$ *vs* sham-operated and saline-treated rats. ###$p < 0.001$ *vs* nerve-ligated and saline-treated rats. Symbols are explained in the legend for Figure 8. (c) The decreased percentage of time spent in the open arms did not change with the repeated microinjection of paroxetine. *$p < 0.05$ *vs* the sham-operated and saline-treated groups. (d) The percentage of entries into the open arms was similar among the groups. (e) There was no significant difference in the number of entries into the closed arms, which is a parameter of general activity-related behavior, among the groups. Each column represents the mean ± SEM of 4–6 rats.

that chronic pain-induced emotional dysfunction may be associated with changes in opioidergic function in the amygdala (Narita *et al*, 2006). Moreover, we observed changes in serotonin receptor function in the amygdala under a chronic pain-like state (unpublished observation). These findings suggest that the mechanism of action of SSRIs may be associated with the amygdala. In this study, microinjection of the SSRI paroxetine into either the CG or BLA had a dramatic anxiolytic effect in an animal model that showed chronic pain-induced anxiety, indicating that the change in serotonergic neurotransmission in the CG may be associated with the affective processing of emotional dysfunction during sustained pain. On the other hand, S1 is an important site for pain perception (Kakigi *et al*, 2004). In this study, microinjection of the SSRI paroxetine into the

contralateral S1 resulted in a dramatic antihyperalgesic effect in nerve-ligated rats, whereas the thermal threshold of sham-operated animals was unchanged. These results indicate that the inhibition of serotonin transporter in the S1 may play a role in the antihyperalgesic and antiallodynic mechanisms of serotonergic antidepressants. These observations raise the possibility that serotonergic antidepressants directly produce antihyperalgesic and antiallodynic effects, at least in part, via the supraspinal, but not the spinal level.

A possible anxiolytic mechanism of antidepressants is that antidepressant-mediated antihyperalgesia and antiallodynia may simply reduce pain and prevent the development of anxiety-related behavior induced by chronic pain. With regard to antidepressant-mediated antinociception and

changes in emotionality under neuropathic pain, only a few previous studies have been reported. To the best of our knowledge, only one report showed that the SNRI duloxetine attenuated escape avoidance behavior over a dose range that had no effect on reflex mechanical nociceptive responses using the chronic constriction injury model of neuropathic pain (Pedersen and Blackburn-Munro, 2006). In this study, repeated administration of the SNRI milnacipran attenuated the thermal hyperalgesia, allodynia, and anxiety-related behaviors following nerve injury. It is, however, likely that milnacipran is not a potent 5-HT and NA reuptake inhibitor based on *in vitro* binding affinity (Mongeau *et al*, 1998). Although milnacipran is generally considered to produce its pharmacological action via the dual blockade of 5-HT and NA reuptake, it has been reported that long-term milnacipran treatment enhances the efficacy of the 5-HT and reduces that of the NA transmission (Weiss *et al*, 2007). Taken together, these findings indicate that long-term milnacipran treatment may cause the attenuation of pain and its-related anxiety induced by nerve injury through the complex mechanism including the change in central catecholamine transmission in mice.

In the present study, the attenuation of hyperalgesia or allodynia by systemic administration of antidepressants was relatively modest, while anxiety-related behavior was almost fully suppressed under a neuropathic pain-like state. Additionally, we found that the microinjection of SSRI into the CG and the BLA prevented only anxiety-related behaviors, whereas the microinjection of SSRI into the S1 only restored the decreased thermal threshold after peripheral nerve injury. Therefore, we propose here that antidepressants may act on central restricted brain areas such as the CG and BLA to recover the emotional dysfunction induced by peripheral nerve injury, but they cannot always restore the decreased pain threshold. Although additional research is needed on the mechanisms that underlie the effect of antidepressants on chronic pain and its related emotional dysfunction, the present hypothesis can be supported by the notion that peripheral noxious stimuli eventually may activate many brain regions to recognize 'pain', which could be out of control even after the activation of emotionality-related brain areas can be controlled by post-treatment with antidepressants.

In summary, the present results showed that repeated s.c. administration of serotonergic antidepressants significantly prevented the thermal hyperalgesia and almost restored anxiety-related behaviors after sciatic nerve injury. Further-more, the microinjection of the SSRI paroxetine into the CG and the BLA prevented anxiety-related behaviors associated with sustained neuropathic pain, while the thermal thresh-old remains unchanged. On the other hand, rats that received microinjections in S1 showed a dramatic increase in the decreased thermal threshold after sciatic nerve ligation, but did not show an anxiolytic effect. These findings suggest that serotonergic antidepressants may be useful for treating neuropathic pain with emotional dysfunction. Furthermore, our findings may constitute the first evidence to show that SSRIs have an anxiolytic effect in the CG and BLA, and show an antinociceptive effect, at least in part, through the S1.

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