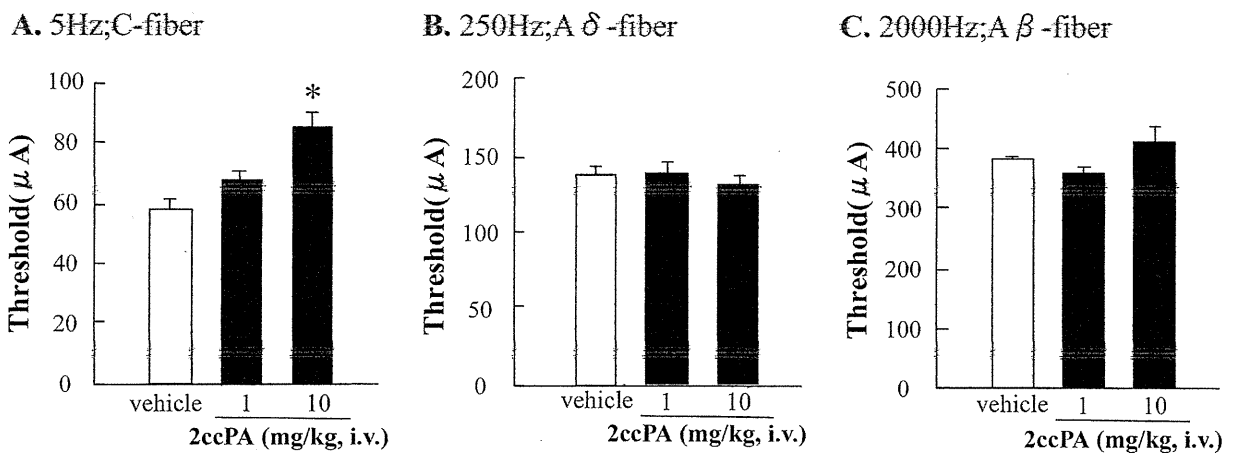

Figure 4 Effects of cPA on the Electrical Stimulation-Induced Paw Withdrawal (EPW) test. The threshold represents the minimum intensity (μA) required to elicit a paw withdrawal response to electrical stimulation with 5 Hz (C-fiber) (A), 250 Hz (Aδ-fiber) (B), and 2000 Hz (Aβ-fiber) (C). The EPW test was performed 10-15 min after 2ccPA (i.v.) injection. All data represent the mean ± S.E. from 3-6 individual mice per group. *P < 0.05; significantly different from the control response by Tukey's multiple comparison.

4.4. Analgesic effects of 2ccPA on mouse formalin-evoked licking and biting behavior

In the formalin-induced nociception tests, ICR (CD1) mice were given an s.c. injection of formalin solution to the hind paw. As shown in Figure 5A, the time course of the total time spent in licking and biting comprised 2 phases. Morphine or i.v. injection of 2ccPA at 30 min prior to formalin injection markedly reduced phase II licking and biting. Quantitative analysis revealed that 2ccPA exerted dose-dependent inhibition of phase II responses, with significant inhibition observed at a 10-mg/kg i.v. dose (Figure 5B). As a reference, significant analgesia was also achieved with 3 mg/kg morphine.

4.5. Pre-injury administration of 2ccPA prevents neuropathic pain development

LPA is produced by ATX in the early phase after nerve injury [13,14]; therefore, we administered 2ccPA (10 nmol, i.t. or 100 nmol, i.t.) at 30 min prior to inducing nerve injury. 2ccPA prevented thermal hyperalgesia and mechanical allodynia in a dose-dependent manner at 5 and 7 days after nerve injury (Figure 6). However, 2ccPA (i.t.) injection in naïve C57BL/6 mice had no significant effect on nociceptive latency at 90 min or 1 or 7 days after injection (additional file 1).

4.6. Repeated administration of 2ccPA induces analgesia against established neuropathic pain in mice

We examined the effects of 2ccPA on established neuropathic pain in C57BL/6 mice. In the thermal withdrawal test, mice with sciatic nerve injury exhibited a decreased threshold at day 7. Under these conditions, a single 2ccPA dose (100 nmol, i.t.) significantly increased nociceptive latency at 30 min on the day 7 after injury (considered as day 1), as shown in Figure 7Ba. When 2ccPA (i.t.) was administered daily to the injured mice, the basal latency (before the i.t. injection) time-dependently increased from day 1 to 7, though no significant change was observed on the 7th day (Figures 7Ba-d). A significant increase was observed at 90 min after the 2ccPA injection on the day 4 and at all time points until 120 min on the 7th day (Figure 7Bc). The reason for more pronounced 2ccPA analgesia on the 7th day may be attributed to the fact that there is some, but not significant increase in the basal latency on the 7th day. A single 2ccPA injection (10 mg/kg i.v.) had no effect on thermal hyperalgesia (Figure 7Ca). There was significant analgesia by 2ccPA (i.v.) on the seventh day following daily injections (i.v.), with no change in basal latency throughout the 7 days (Figures 7Ca-d).

In the paw-pressure test, repeated post-injection (i.t. or i.v.) of 2ccPA yielded similar effects on day 8 (Figure 8). Following repeated i.t. injection, there was an increasing trend in basal latency on day8, while there was no change with i.v. injection. Significant analgesia was also observed at several time points after the 2ccPA i.t. injection but not after the i.v. injection. When the area under the curve (AUC) was evaluated, there was significant analgesia with both i.t. and i.v. injections on day 8 (Figure 8D).

4.7. Repeated 2ccPA administration induces analgesia against established neuropathic pain in rats

Similar studies to examine the analgesic effects of 2ccPA against established chronic pain were performed using a different chronic-pain model in rats. In the chronic

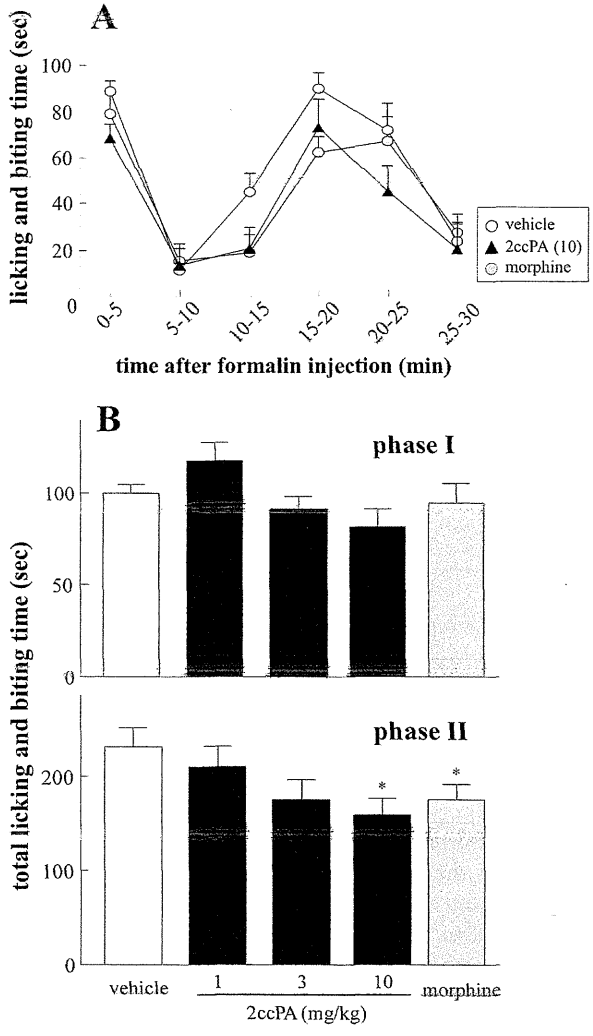

Figure 5 Effects of i.v. 2ccPA on licking and biting time. A, Time variations of licking and biting time of mice s.c. injected with 30 μL of 2% formalin in the mouse hind paw immediately after i.v. injection with 10 mg/kg (closed triangle) 2ccPA or vehicle (open circle). Morphine (3 mg/kg i.p.) was applied 30 min prior to formalin injection as a control. Each data point represents the average licking and biting time for 8-13 mice. B, Graphical summary of the total phase I and II licking and biting times of mice at 1 mg/kg, 3 mg/kg, or 10 mg/kg 2ccPA. All data represent the mean ± S.E. *P < 0.05; significantly different from the control response by Student's t-test.

constrictive sciatic nerve injury (CCI) model, the same experimental schedule was performed, as described above (Figure 9). In the thermal withdrawal test, there was significant analgesia at 2 h after i.v. injection of 2ccPA (10 mg/kg) on the seventh day following repeated injections, with no significant change in the basal latency at time 0 min (Figure 9B). There was weak but not significant analgesia at 2 h with 3 mg/kg (i.v.) on the seventh day (Figure 9C), while significant analgesia was observed on the fourth day with 10 mg/kg (Figure 9D). The 2ccPA-induced analgesia was slightly weaker, but comparable to the analgesic effects of gabapentin (90 mg/kg i.v.). Similar results were observed with the paw-pressure test (Figures 9E-9G). In this case, weak but significant analgesia against mechanical allodynia was observed only with 10 mg/kg at 4 h on the seventh day. However, the analgesic effect was much lower than that with gabapentin.

5. Discussion

We designed and chemically synthesized the metabolically stabilized derivatives of cPA, to avoid cPA hydrolysis in animals [10]. In 2ccPA, the phosphate oxygen of cPA is replaced with a methylene group at *sn*-2 (Figure 1). This study showed that the effective dose of 2ccPA was almost 10-fold less than that of natural cPA, consistent with other studies [6,7,10]. Differences in chemical stability and/or structural traits might account for the difference in the effective dose of natural cPA and 2ccPA required to achieve analgesia. The specificity of these compounds has been extensively reported [7,10].

Our initial examination demonstrated that intragastral administration of 1 mg/kg of 2ccPA resulted in a remarkable 40% reduction of the somato-cardiac sympathetic C-reflex (additional file 2), suggesting practical stability against gastric digestion as well as rapid gastric absorption of 2ccPA. We examined another carba-cPA, 3ccPA, in which the phosphate oxygen of cPA is replaced with a methylene group at *sn*-3, and found that effective doses of 3ccPA 16:1 were similar to those of 2ccPA for suppression of somato-cardiac sympathetic reflexes (additional file 3). In this report, we demonstrated that cPA and 2ccPA suppressed the supraspinal sympathetic and spinal kinetic reflexes, specifically the C-fiber, but not the A-fiber reflex, in anesthetized animals. This result was consistent with the experiments with mice, in which 2ccPA increased the nociceptive threshold only for Neurometer™ electrical stimulus with 5 Hz, which is supposed to stimulate C-fibers, but not with 250 or 2000 Hz, which are supposed to stimulate Aδ and Aβ-fibers [16]. These results suggest that both cPA and 2ccPA suppress nociceptive responses by primary afferent C-fibers. Both compounds are reported to possess selective and potent ATX inhibitory activities [10]. Endogenous LPA in the peripheral tissues or plasma exerts tonic stimulation of C-fibers, as evidenced by previous findings that LPA injected into the hind paw of mice caused nociceptive flexor responses, partially via substance P release from the nociceptor endings of C-fibers [11,28]. As there was no significant analgesia with i.t.-injected 2ccPA (additional file 1), the inhibitory responses of these compounds are unlikely to be mediated by inhibition of LPA synthesis in naïve

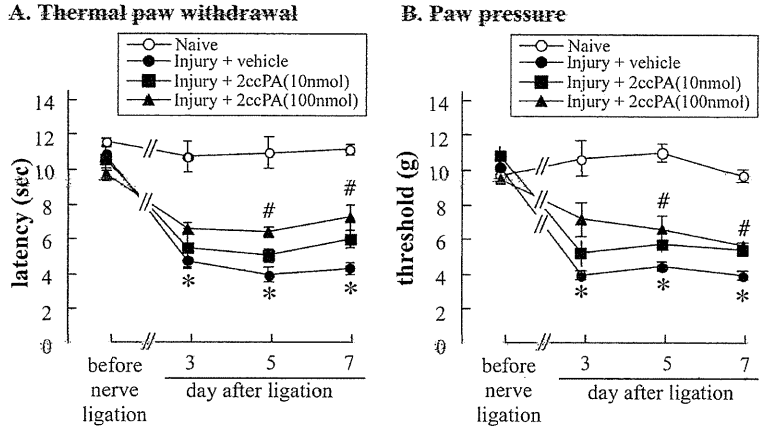

Figure 6 Pre-injury administration of 2ccPA (i.t.) prevents development of neuropathic pain. Neuropathic pain was induced by partial sciatic nerve injury in mice. 2ccPA (10 or 100 nmol/5 μL i.t.) was injected at 30 min prior to nerve injury. The threshold was measured on days 3, 5, and 7 after nerve injury, using the thermal withdrawal (A) and paw-pressure (B) tests. All data represent the mean ± S.E. from 3-6 individual mice per group. *P < 0.05; significantly different from the control response by Tukey's multiple comparison tests.

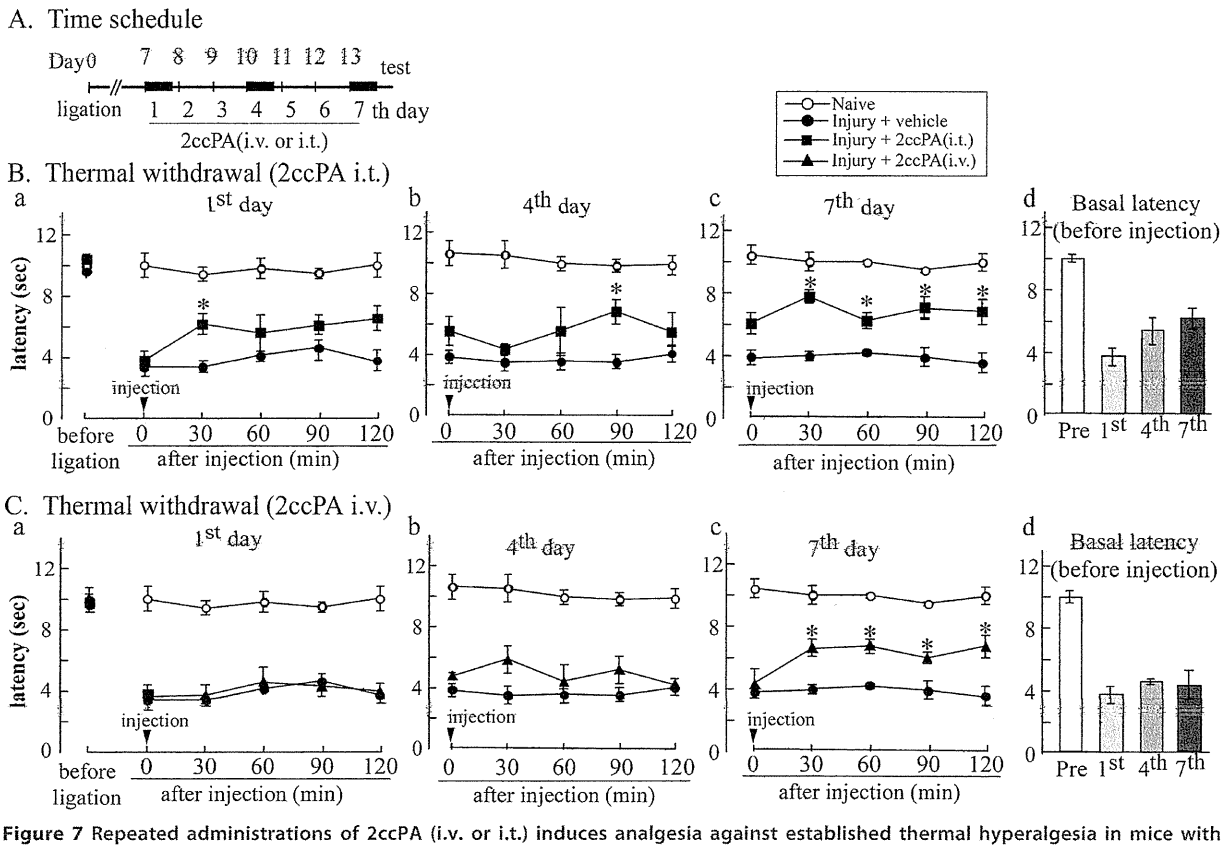

Figure 7 Repeated administrations of 2ccPA (i.v. or i.t.) induces analgesia against established thermal hyperalgesia in mice with neuropathic pain. A, Neuropathic pain in mice was induced by partial sciatic nerve injury. Vehicle or 2ccPA (i.v. or i.t.) was injected daily for 7 days, starting 7 days after injury. B, Vehicle or 2ccPA (100 nmol/5 μL 1-3 days and 10 nmol/5 μL 4-7 days, respectively, i.t.) was daily injected for 7 days, starting 7 days after injury. The withdrawal latency was measured at 30, 60, 90, and 120 min after the injection of 2ccPA (i.t.) on the first (a), fourth (b), or seventh day (c). The basal latency was measured before injection of 2ccPA (i.t.) (d). (C) Vehicle or 2ccPA (10 mg/kg, i.v.) was daily injected for 7 days, starting 7 days after injury. The withdrawal latency was measured at 30, 60, 90, and 120 min after the injection of 2ccPA (i.v.) on the first (a), fourth (b), or seventh day (c). The basal latency was measured before injection of 2ccPA (i.t.) (d). All data represent the mean ± S.E. from 3-6 individual mice per group. *P < 0.05 indicates significant difference from the vehicle response by Tukey's multiple comparison test.

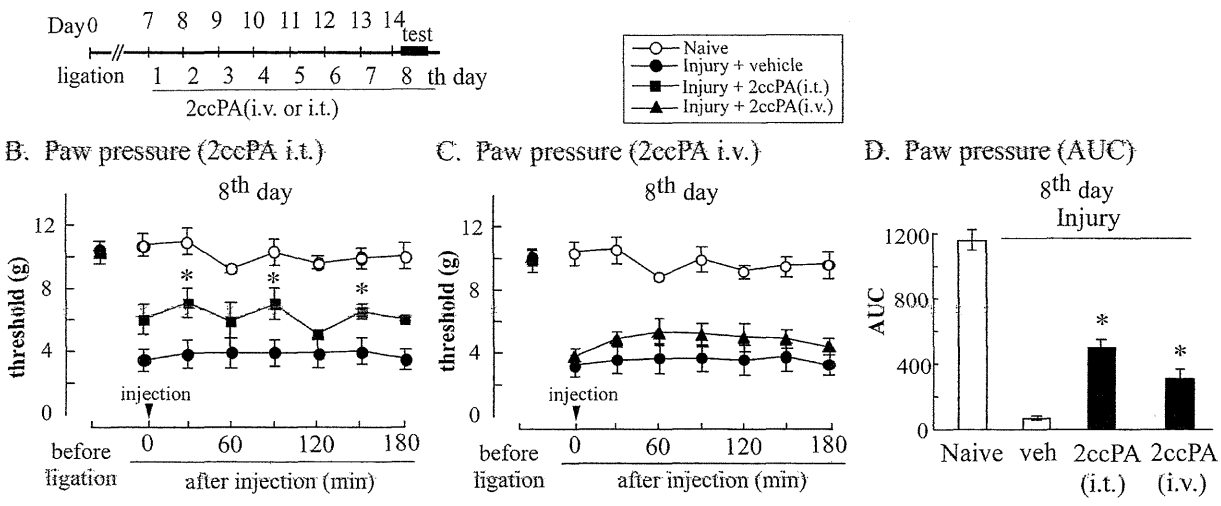

Figure 8 Repeated administration of 2ccPA (i.v. or i.t.) induces analgesia against established mechanical allodynia in neuropathic pain mice. Vehicle or 2ccPA (10 mg/kg i.v.) was daily injected for 8 days, starting 7 days after injury (A). The threshold in the paw pressure test was measured at 30, 60, 90, 120, 150, and 180 minutes after 2ccPA application (i.t. or i.v.) on the eighth day (B and C). Quantitative analyses with 2ccPA (i.t. or i.v.) were performed with the area under the curve (AUC) (D). AUC data represent increased thresholds by subtracting the thresholds before injection in vehicle-treated mice. All data represent the mean ± S.E. from 3-6 individual mice per group. *P < 0.05 indicates significant difference from the vehicle response by Tukey's multiple comparison test.

mice. However, we cannot exclude the possibility that cPA and/or 2ccPA may have inverse agonist actions on C-fiber nociceptor endings, since they have some weak actions on LPA receptors [7,10].

We also found that 2ccPA exerted anti-nociceptive effects in the formalin test. Formalin-induced characteristic behaviors in phase I are the result of direct C-fiber-evoked excitation, whereas the behaviors in phase II are evoked by repetitive C-fiber stimulation [29,30]. 2ccPA reduced both phase responses, but the inhibition of phase II responses was significant. We speculate that repetitive C-fiber stimulation may cause the ATX-catalyzed production of LPA in the periphery and stimulate C-fibers in an autocrine manner.

It should be noted that 2ccPA attenuated neuropathic pain possibly via the central nervous system. Our initial study revealed that i.t. injection of 2ccPA prevented nerve injury-induced neuropathic pain in mice. This finding is consistent with a series of studies by Ueda and colleagues, in which nerve injury induces LPA production by ATX in the spinal cord and causes neuropathic pain through the LPA_1 receptor [12,13,31]. The most striking evidence is that repeated administration of 2ccPA through i.t. and i.v. routes produced significant analgesia against established neuropathic pain in mice and rats. The i.t. injection of 2ccPA on day 7 after injury produced weak analgesia against thermal hyperalgesia. More pronounced analgesia was observed when it was given daily by the seventh i.t. injection on day 13 after injury. As there is some, but not significant, recovery of the basal threshold before the seventh injection of 2ccPA, LPA production may occur in the late phase to maintain the neuropathic pain status, as well as at the early phase to trigger the initiating mechanisms [12]. Similar analgesic effects were observed with i.v. injection by the seventh injection on day 13 after injury, though there was no tendency to recover the basal threshold. The difference of basal latency following repeated injections between i.t. and i.v. routes may be related to the fact that there are some residual increases at as late as 120 min in the case with i.t., but not i.v. injections at the 1st and 4th day. Although the lack of elevation in the basal threshold cannot be explained at this time, it seems to occur after repeated i.v. treatments: i.v.-injected 2ccPA-induced analgesia was equivalent to that yielded by the first i.t. injection. We previously reported that cPA and carba derivatives penetrate into the central nervous system through the blood-brain barrier [32]. In the present study, the effective dosage of 2ccPA for mice were about five times higher than that for rats, both for i.v. and i.p. injection, possibly because these two animals exhibit different sensitivities against administered drugs depending on their chemical species [33].

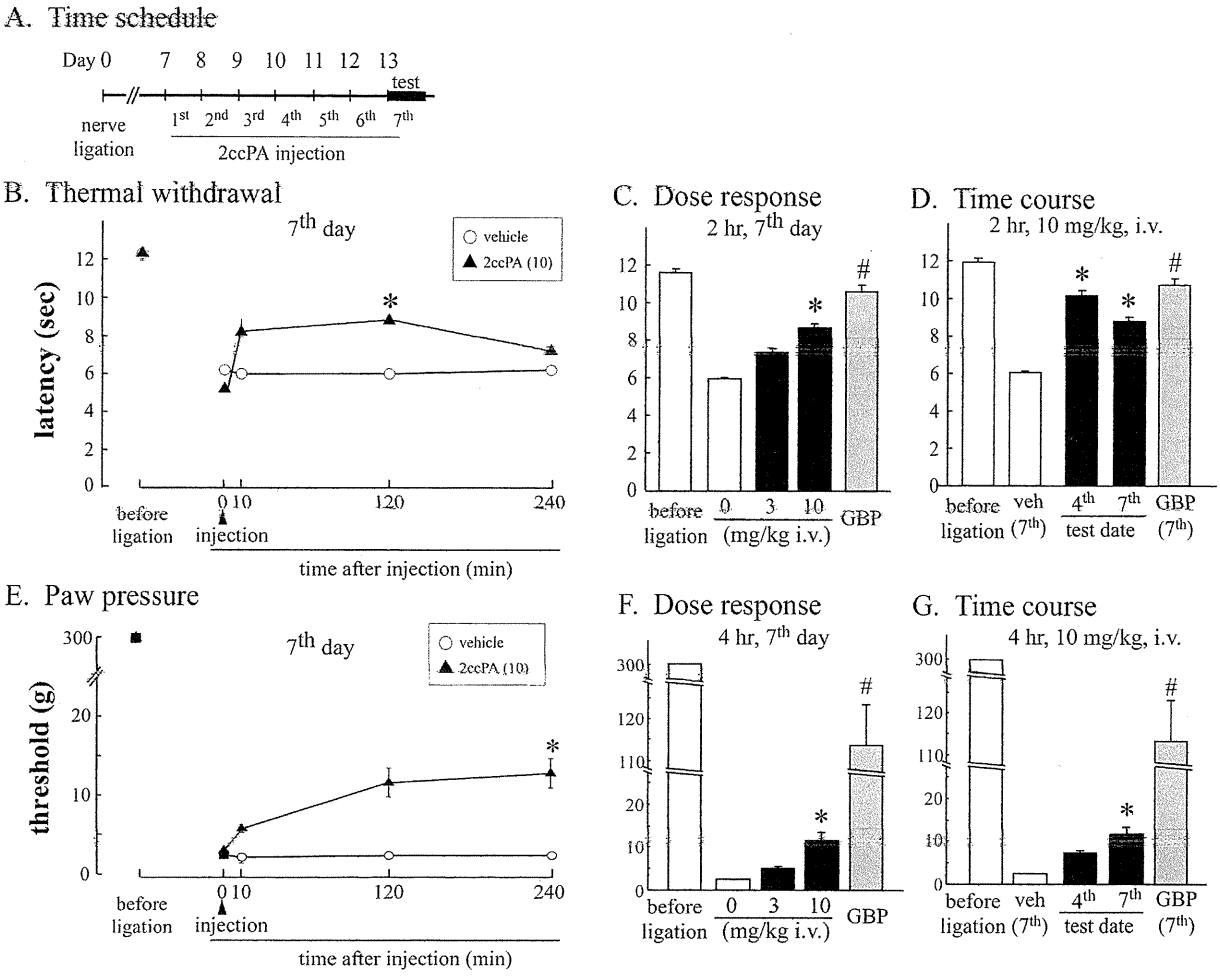

Figure 9 Effects of i.v. 2ccPA on thermal paw-withdrawal and mechanical allodynia of nerve-ligated rats. A, The rat sciatic nerve was ligated 7 days prior to i.v. injection of 2ccPA, applied once daily for 7 consecutive days. B, A hind paw was irradiated with infrared light to evoke thermal withdrawal, and the time variation of the withdrawal latency was measured prior to and at 10 min, 2 h, or 4 h after the application of 2ccPA on the seventh day. C, Dose response; 2-h injection of 2ccPA at 3 or 10 mg/kg applied once daily for 7 consecutive days. D, Time course; 2-h injection of 2ccPA (10 mg/kg) applied once daily for 4 or 7 consecutive days. Gabapentin (90 mg/kg i.v.) was applied as a control. *P < 0.05 and #P < 0.05 indicates significant difference from the vehicle response by Dunnett's test and by Student's t-test or Welch's test, respectively. E, The responses of rats to tactile stimulation was tested with 5 von Frey filaments, and threshold filament sizes to evoke paw withdrawal were measured prior to and at 10 min, 2 h, or 4 h after application of 2ccPA on the seventh day. F, Dose response; 4-h injection of 2ccPA at 1, 3 or 10 mg/kg applied once daily for 7 consecutive days. G, Time course; 4-h injection of 2ccPA (10 mg/kg) applied once daily for 4 or 7 consecutive days. Gabapentin (90 mg/kg i.v.) was applied as a control. *P < 0.05 and #P < 0.05 indicates significant difference from the vehicle response by Steel's test and by Wilcoxon test, respectively.

Recently, we demonstrated LPA-induced LPA production; i.t. injection of LPA or the addition of LPA to spinal cord slices markedly increased the LPA level in a time-dependent manner with the peak occurring at 3 h [14,34]. This finding indicates the presence of feed-forward amplification of LPA production in initiating neuropathic pain. As LPA production declines, however, there may be end-product inhibition. Although it is a fascinating mechanism that cPA is a natural ATX inhibitor [10] produced by ATX, it remains to be seen whether the amounts of cPA produced are sufficient to exert this effect.

6. Conclusion

Our results indicate that cPA and 2ccPA are potent inhibitors of nociceptive transmission by C-primary-afferents and reverse inflammatory and neuropathic pain. These chemicals may be good candidates for use in clinical pain management.

Additional material

Additional file 1: No effect of 2ccPA (i.t.) injection in naïve mice. The thresholds were measured at 90 min (A) and on days 1 and 7 (B) after 2ccPA injection, using the thermal withdrawal test. All data represent the mean ± S.E. from 3-6 individual mice per group.

Additional file 2: Time variation of relative C-reflex level after oral administration of 2ccPA (16:1). Relative C-reflex levels were measured after oral administration of 2ccPA (16:1) at 1 mg/kg and plotted over time until 120 min. Each data point represents the average four independent measurements, and vertical bar represents S.E. **P < 0.01 and *P < 0.05; significantly different from time 0 by one-way ANOVA, Dunnett's multiple comparison test.

Additional file 3: Effects of i.v. 3ccPA on the somato-carcdiac C-reflex. Relative C-reflex levels of the somato-cardiac response were measured after i.v. injection of 3ccPA (18:1) at 100 μg/kg (n = 3). Vertical bar represents S.E. **P < 0.01; significantly different from the vehicle by Student's t-test.

List of Abbreviations Used

aCSF: artificial cerebrospinal fluid; ATX: autotoxin; ccPA: carba-cyclic phosphatidic acid; cPA: cyclic phosphatidic acid; i.p.: intraperitoneal; i.v.: intravenous; LPA: lysophosphatidic acid; PBS: phosphate-buffered saline; s.c.: subcutaneously

Acknowledgements

This work was supported in part by the Princess Takamatsu Cancer Research Fund to KM and a Grant-in-Aid for Scientific Research (KAKENHI, No 22790203) from the Ministry for Education, Culture, Sports, Science and Technology of Japan to MG.

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Authors' contributions

YK and JN participated in the experimental designing, collection and analyses of data, and drafted the manuscript in equal contribution. MG performed the statistical analyses and drafted the manuscript. HH participated in the designing of the study, carried out surgical manipulation, data collection, and drafted the manuscript. HM conceived of the study, and participated in its design. TO participated in EPW assay. HU and KM conceived of the study, participated in its design and coordination. All authors read and approved the final manuscript.

Conflicts of interests

The authors declare that they have no competing interests.

Received: 14 January 2011 Accepted: 14 May 2011
Published: 14 May 2011

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doi:10.1186/1744-8069-7-33
Cite this article as: Kakiuchi *et al.*: Antinociceptive effect of cyclic phosphatidic acid and its derivative on animal models of acute and chronic pain. *Molecular Pain* 2011 **7**:33.

Nishiyori et al. Molecular Pain 2011, 7:69
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MOLECULAR PAIN

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Permanent relief from intermittent cold stress-induced fibromyalgia-like abnormal pain by repeated intrathecal administration of antidepressants

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Abstract

Background: Fibromyalgia (FM) is characterized by chronic widespread pain, which is often refractory to conventional painkillers. Numerous clinical studies have demonstrated that antidepressants are effective in treating FM pain. We previously established a mouse model of FM-like pain, induced by intermittent cold stress (ICS).

Results: In this study, we find that ICS exposure causes a transient increase in plasma corticosterone concentration, but not in anxiety or depression-like behaviors. A single intrathecal injection of an antidepressant, such as milnacipran, amitriptyline, mianserin or paroxetine, had an acute analgesic effect on ICS-induced thermal hyperalgesia at post-stress day 1 in a dose-dependent manner. In addition, repeated daily antidepressant treatments during post-stress days 1-5 gradually reversed the reduction in thermal pain threshold, and this recovery was maintained for at least 7 days after the final treatment. In addition, relief from mechanical allodynia, induced by ICS exposure, was also observed at day 9 after the cessation of antidepressant treatment. In contrast, the intravenous administration of these antidepressants at conventional doses failed to provide relief.

Conclusions: These results suggest that the repetitive intrathecal administration of antidepressants permanently cures ICS-induced FM pain in mice.

Keywords: fibromyalgia, cold stress, vicious circle, antidepressant, allodynia, hyperalgesia

2. Background

Fibromyalgia (FM) is characterized by generalized tenderness and chronic widespread pain that affects 2-4% of the population in industrialized nations and primarily affects females [1]. Although its etiology and pathogenesis are largely unknown, emerging evidence indicates that pain amplification within the central nervous system (CNS) plays a critical role in the pathology of FM pain [2]. Recent studies, including functional imaging, have revealed that this central amplification process depends, in part, on deficits in endogenous descending pain inhibitory pathways [3,4] and abnormal pain processing [5]. In addition, FM pain is often refractory to treatment using conventional painkillers, such as non-steroidal anti-inflammatory drugs and opioids [6]. However, numerous studies have demonstrated the effectiveness of antidepressants and antiepileptics, such as gabapentin and pregabalin, in the treatment of FM pain [7,8].

There are several animal models of FM pain, induced by either intramuscular injection of acidic saline [9], vagotomy [10], sound stress [11] or depletion of biogenic amines [12]. However, in order to better understand the molecular basis of the underlying pain mechanisms, it is necessary to establish an animal model which accurately reflects the pathological and pharmacotherapeutic features of the disease.

Recently, we established a mouse model of FM using intermittent cold stress (ICS), which produces long-lasting thermal hyperalgesia and mechanical allodynia, predominantly in females [13]. We found that gabapentin,

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particularly when injected intracerebroventricularly, had potent anti-hyperalgesic and anti-allodynic effects in this model [13]. In addition, systemically and intracerebroventricularly-administered morphine was found to have no analgesic effect in ICS-exposed mice, due to a failure to activate descending pain inhibitory pathways [14]. These findings indicate that our ICS model might accurately reflect the pathological and pharmacotherapeutic features of FM pain. In this study, we examine whether various antidepressants can ameliorate the abnormal pain sensations in this model.

3. Materials and methods

3.1. Animals

Male C57BL/6J mice weighing 18-22 g were used. They were kept in a room with an ambient temperature of 21 ± 2°C, with free access to a standard laboratory diet and tap water. All procedures were approved by the Nagasaki University Animal Care Committee and complied with the recommendations of the International Association for the Study of Pain [15].

3.2. Drug treatments

Antidepressants were obtained from Sigma (St. Louis, MO, USA). Milnacipran, paroxetine, and amitriptyline were dissolved in artificial cerebrospinal fluid (aCSF; 125 mM NaCl, 3.8 mM KCl, 2.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 1.2 mM KH_2PO_4, 26 mM $NaHCO_3$, 10 mM glucose, pH 7.4). Mianserin was dissolved in physiological saline. For vehicle treatments, aCSF or saline was injected. Intrathecal (i.t.) injections were administered according to Hylden and Wilcox [16] using a 30-gauge needle. The site of injection was chosen to be between spinal L5 and L6–near where the spinal cord ends and the cauda equina begins. This allowed us to maximize inter-vertebral accessibility and to minimize the possibility of spinal damage. After sufficient training, the experimenters were able to perform the technique without causing injury to the animals.

3.3. Experimental model of fibromyalgia

ICS exposure and constant cold stress (CCS) were performed as previously reported [13]. Briefly, for the ICS model, mice were placed on stainless mesh plate in a cold room at 4°C overnight (from 4:30 pm to 10:00 am), followed by ICS with environmental temperatures alternating between 24 and 4°C every 30 min, from 10:00 am to 4:30 pm. These procedures were repeated twice. On day 3, the mice were adapted to 24°C for 1 h before behavior testing. We designated day 3 following the onset of stress exposure as day 1 post-stress exposure (P1). For the CCS model, mice were placed in the cold room from 4:30 pm on day 1 to 10:00 am on day 3, followed by adaptation at 24°C for 1 h. Mice in the control group were kept at 24°C for all 3 days (from 4:30 pm on day 1 to 10:00 am on day 3). During the stress period, two mice were kept in each cage (12 × 15 × 10.5 cm), with free access to food and agar as alternate drink water in place of fluid. Although the body weight of mice was decreased during and after the ICS stress, it attained to the control mice level as early as 4 day after the stress (Figure 1).

3.4. Measurement of plasma corticosterone

Plasma corticosterone levels were measured as described previously [17]. Briefly, plasma was separated by centrifugation at 3 000 *g* for 15 min at 4°C and collected into ice-chilled tubes containing 0.1% EDTA and stored at -80°C until use. Blood samples were collected at 9:00 pm in order to exclude the effect of circadian rhythms on circulating plasma corticosterone. The plasma corticosterone level was estimated fluorometrically, according to the method of Zenker and Bernstein [18].

3.5. Assessment of stress-related behaviors

Spontaneous locomotor activity was measured in the open filed (22 × 33 cm) for 3 min, using SCANET apparatus (Melquest, Japan). In the elevated plus-maze test used to estimate anxiety, the time spent in the open arm was recorded during a 6-min period. To assess depression-like behaviors, the tail-suspension test was performed [19,20]. Mice were suspended 30 cm above the floor using adhesive tape, and the total duration of immobility during a 6-min period was measured.

3.6. Nociception tests

In the thermal paw withdrawal test, the nociception threshold was assessed using the latency of paw

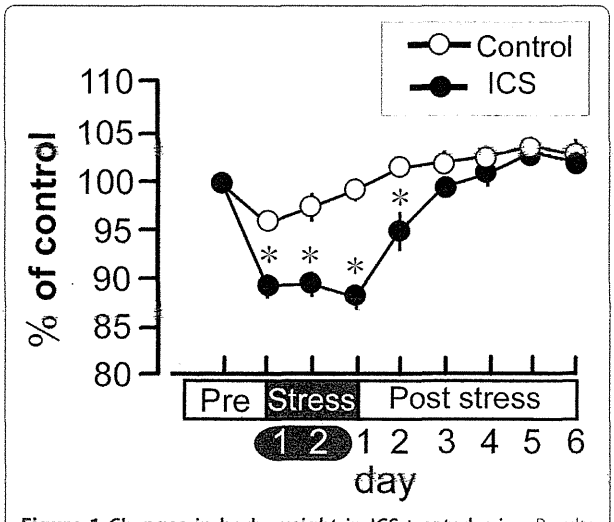

Figure 1 Changes in body weight in ICS treated mice. Results represent the percentage of body weight of mice, compared to the value at 1 day before ICS stress (Pre). *$p < 0.05$, *vs.* control group. Data are the means ± S.E.M; 6 mice per group.

withdrawal upon a thermal stimulus [21,22]. Unanesthetized animals were placed in plexiglass cage on top of a glass sheet and acclimated for 1 h. A thermal stimulator (IITC Inc., Woodland Hills, CA, USA) was positioned under the glass sheet and the focus of the projection bulb was aimed exactly at the middle of the plantar surface of the animal. A mirror attached to the stimulator permitted visualization of the plantar surface. A cut-off time of 20 s was set to prevent tissue damage.

The mechanical paw pressure test was performed as described previously [22]. Briefly, mice were placed in a plexiglass chamber on a 6 × 6 mm wire mesh grid floor and allowed to acclimate for a period of 1 h. A mechanical stimulus was then delivered to the middle of the plantar surface of the right-hind paw using a Transducer Indicator (Model 1601; IITC Inc., Woodland Hills, CA, USA). The pressure needed to induce a flexor response was defined as the pain threshold. A cut-off pressure of 20 g was set to avoid tissue damage. In these experiments using thermal and mechanical tests, the thresholds were determined from three repeated challenges at 10 min intervals, and the averages were used for statistical analysis. For the time-course experiments, we measured the paw-withdrawal latencies (PWL) at 30, 60, and 180 min after intrathecal injection of antidepressant. In the area under the curve (AUC) analysis of antidepressant-induced analgesia, we calculated the AUC generated by plotting analgesic threshold (after deducting the control threshold from each threshold point) against time, from 30 to 180 min after antidepressant treatment, using a trapezoidal method.

3.7. Statistical analysis

In Figure 2 and Table 1, data were analyzed using Student's *t*-test. In Figures 1, 3, 4, 5 and 6, Tables 2 and 3, the differences between multiple groups were analyzed using a one-way ANOVA with the Tukey-Kramer multiple comparison post-hoc analysis. Significance was set at $p < 0.05$. All results are expressed as means ± S.E.M.

4. Results

4.1. Effects of ICS stress exposure on plasma corticosterone levels and anxiety and depression-like behaviors

We previously designed an improved mouse model for dysautonomia, also referred to as the specific alternation of rhythm in temperature (SART) model [23], and found that ICS, but not CCS, caused long-lasting abnormal pain sensations [13]. In the present study, we used plasma corticosterone levels as a biomarker for stress. As shown in Figure 2A, we found that ICS exposure caused a transient increase in plasma corticosterone levels at P1. In contrast, CCS exposure had no effect on plasma corticosterone levels between P1 and P12 (Figure 2A). ICS had no effect on spontaneous locomotor activity at P1 (Figure 2B). Furthermore there was no significant change in the duration of time spent in the open arm in the elevated plus-maze test or in the total duration of immobility in the tail-suspension test at P1

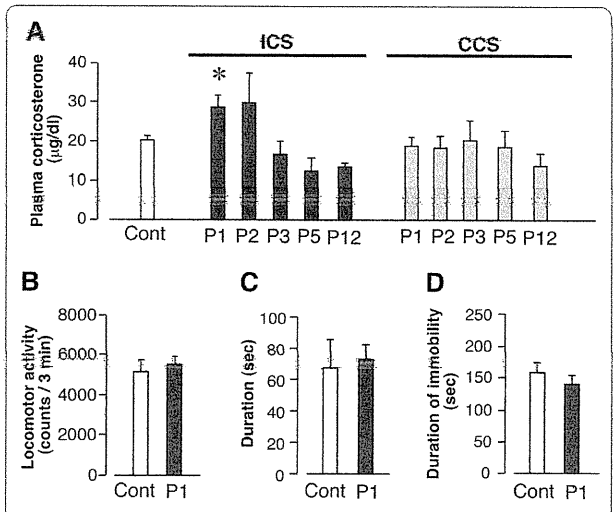

Figure 2 Effects of stress exposure on plasma corticosterone levels, spontaneous locomotor activity, and anxiety and depression-like behaviors. (A) Time-course of plasma corticosterone levels after ICS or CCS exposure. (B-D) Lack of changes in spontaneous locomotor activity in the SCANET apparatus (B), duration of time spent in the open arm of the plus-maze test (C), and total duration of immobility in the tail suspension test (D) at P1. *$p < 0.05$, *vs.* control group. Data are expressed as the means ± S.E.M; 3-5 mice per group.

Table 1 Dose-dependent acute analgesic effects of antidepressants on ICS-induced thermal hyperalgesia

Drug	Dose (μg)	n	AUC
Milnacipran	0.03	4	50.6 ± 21.5
	0.1	6	281.9 ± 71.3*
	0.3	4	166.2 ± 31.3*
Amitriptyline	5	3	51.1 ± 85.2
	15	7	252.6 ± 42.2*
	30	3	235.5 ± 64.4*
Mianserin	10	3	144.7 ± 171.5
	20	4	527.8 ± 103.2*
Paroxetine	2	3	40.7 ± 62.4
	5	7	211.2 ± 38.6*
	10	3	251.3 ± 797*

Thermal pain threshold was assessed at P1, using thermal paw withdrawal tests. Acute anti-hyperalgesic effects of milnacipran, amitriptyline, mianserin, and paroxetine were evaluated by AUC, as described in Materials and Methods. *$p < 0.05$, *vs.* vehicle-treated control group.

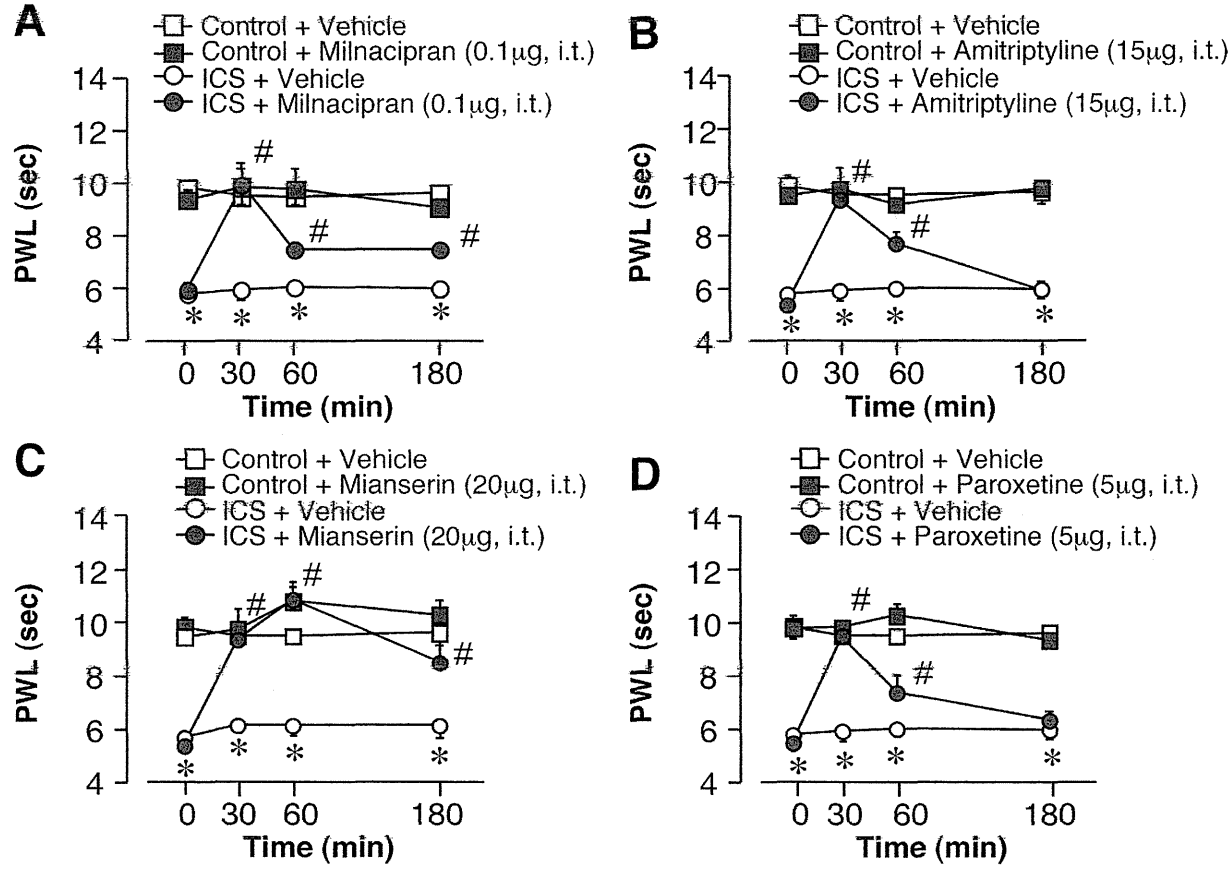

Figure 3 Antidepressant-induced acute analgesic effects in ICS treated mice. Thermal pain threshold was assessed at P1 after control or ICS treatment, using the thermal paw withdrawal test. Results represent the time course of thermal paw withdrawal latencies (PWL, in seconds) after a single intrathecal injection of antidepressants. (A-D) Each data point in [control + vehicle] and [ICS + vehicle] groups is common. *$p < 0.05$, *vs.* vehicle-treated control group; #$p < 0.05$, *vs.* vehicle-treated and ICS-exposed groups. Data are expressed as the means ± S.E.M.; 4-8 mice per group.

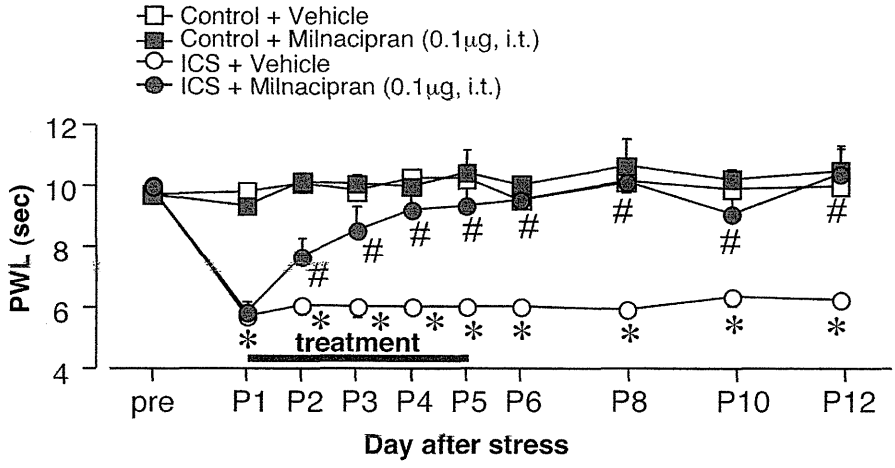

Figure 4 Permanent relief from ICS-induced thermal hyperalgesia by repeated intrathecal administration of milnacipran. Intrathecal injections of milnacipran (0.1 μg) were given once daily at 11:30 a.m. from P1-P5 after assessment of nociceptive thresholds at 11:00 a.m. Results represent the basal threshold as the latency to paw-withdrawal from thermal stimuli (PWL, in seconds), just before the daily injection of vehicle or milnacipran. *$p < 0.05$, *vs.* vehicle-treated control group; #$p < 0.05$, *vs.* vehicle-treated and ICS-exposed groups. Data are expressed as the means ± S.E.M.;4-8 mice per group.

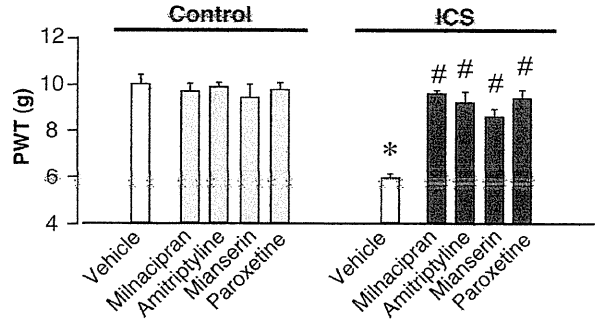

Figure 5 Complete relief from ICS-induced mechanical allodynia. Basal mechanical paw-withdrawal threshold (PWT, in grams) was assessed at P14, using paw pressure tests. Intrathecal injection of milnacipran (0.1 μg), amitriptyline (15 μg), mianserin (20 μg), or paroxetine (5 μg), was given once daily from P1-P5, as described in Figure 4. *$p < 0.05$, *vs.* vehicle-treated control group; #$p < 0.05$, *vs.* vehicle-treated and ICS-exposed groups. Data are expressed as the means ± S.E.M.; 3-6 mice per group.

(Figures 2C, D). In addition, there were no gross behavioral changes in mice as early as 1 h after the transfer from 4°C to 24°C room.

4.2. Antidepressant-induced acute analgesic effects on thermal hyperalgesia in ICS-exposed mice

Previous reports demonstrated that thermal hyperalgesia is elicited at P1 after ICS exposure and lasts for at least 12 days [13,14]. As shown in Figure 3, the nociceptive thermal threshold was significantly reduced and stable throughout experiments for 180 min. A single intrathecal injection of milnacipran (0.1 μg) had no effect on the nociceptive threshold in control mice (Figure 3A), but produced significant anti-hyperalgesic effects that persisted for at least 180 min post-injection at P1 (Figure 3A). This effect of milnacipran was dose-dependent in the range of 0.03-0.1 μg, but declined at 0.3 μg (Table 1). Statistical significance was observed at 0.1 and 0.3 μg. Similar results were observed with other antidepressants, such as amitriptyline (5-30 μg), mianserin (10 and 20 μg), and paroxetine (2-10 μg), as shown in Figures 3B-D and Table 1. However, with 20 μg of mianserin, a significant analgesic effect was observed at 60 min in the control mice, and anti-hyperalgesic effects were observed until 180 min (Figure 3C). Both amitriptyline and paroxetine showed significant anti-hyperalgesic effects, but no significance was observed at 180 min (Figures 3B, D).

4.3. Permanent relief of abnormal pain by repeated central administration

As the anti-hyperalgesic effect of milnacipran remained 180 min after intrathecal administration at day P1 after ICS stress (threshold: ~ 7.46 ± 0.2 s), we measured the nociceptive threshold at 11:00 a.m. on day P2. As seen in Figure 4, a significant anti-hyperalgesic effect still remained (threshold: ~ 7.67 ± 0.6 s). The second administration of milnacipran was performed at 11:30 a.m. The basal nociceptive threshold at 11:00 a.m. on day P3 further increased to 8.56 ± 0.8 s. The increase in basal threshold was maintained by daily administration of milnacipran. Complete recovery to the normal pain threshold was observed on P6, the day following the last administration, and lasted until P12. Similar complete reversals of hyperalgesia on P5 and P12 were observed after 5-day administrations of amitriptyline (15 μg), mianserin (20 μg), and paroxetine (5 μg), as seen in Table 2. Complete recovery was also observed with ICS-induced mechanical allodynia, even on P14, following a 5-day administration of the antidepressants (Figure 5).

4.4. Lack of beneficial effects by repeated systemic administration

When milnacipran was given by intravenous (i.v.) injection (10 mg/kg), there was a significant analgesic effect in the thermal nociception test at 30 min in control mice. However, there was no significant suppression in the ICS mouse model using this dose of antidepressant up to 180 min on P1 (Figure 6A). The absence of an ameliorative effect on ICS-induced hyperalgesia was also observed with amitriptyline (3 mg/kg, i.v.), mianserin (10 mg/kg, i.v.), and paroxetine (1 mg/kg, i.v.), despite producing significant acute analgesia at 30 min in control mice (Figures 6B-D). In addition, the repeated systemic administration of milnacipran for 5 days did not affect the basal threshold throughout the experiment (Figure 6E). Repeated administrations of amitriptyline, mianserin or paroxetine also did not provide relief from ICS-induced hyperalgesia (Table 3).

5. Discussion

Patients with FM exhibit widespread pain, with diverse symptoms, such as fatigue, depression, and sleep disturbance. Although the pathogenesis of FM is not clearly understood, certain biological stressors, such as autonomic nervous system disorder and psychological distress seem to be closely related to the development of FM [24]. An important role for such stressors is supported by studies using animal models in which rats or mice are subjected to stressors, such as chemical, sound, or surgery stress, which induce long-lasting abnormal pain [9-11,25]. Recently, we reported that ICS produces long-lasting thermal hyperalgesia and mechanical allodynia in mice [13,14]. The ICS-induced pain is bilateral and female-predominant (after gonadectomy) [13], which are also features found in FM patients [26].

In this study, mice subjected to ICS exhibited a transient increase in plasma corticosterone levels on P1. In

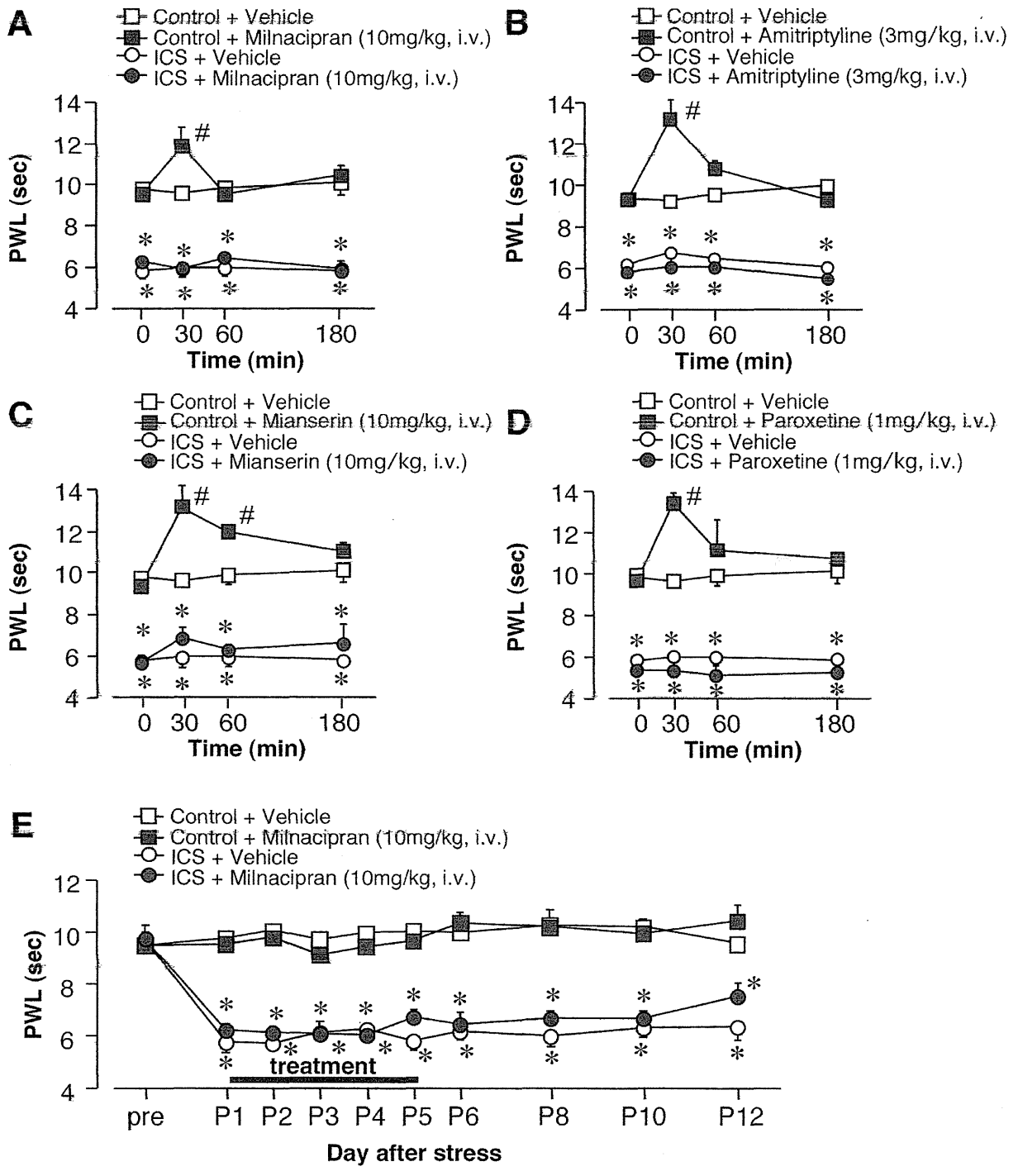

Figure 6 Lack of anti-hyperalgesic effects by systemic administration of antidepressants. Thermal pain threshold was assessed at P1 after control or ICS-treatment, using thermal paw withdrawal tests. (A-D) Results represent the time-course of thermal paw-withdrawal latencies (PWL, in seconds) after a single i.v. injection of antidepressants. (A-D) Each data point in [control + vehicle] and [ICS + vehicle] groups is common. (E) Milnacipran was given i.v. once daily for 5 days, as described in Figure 4. Results represent the basal threshold as the latency to paw-withdrawal from thermal stimuli (PWL, in seconds), just before the daily injection of vehicle or milnacipran. *$p < 0.05$, *vs.* vehicle-treated control group; #$p < 0.05$, *vs.* vehicle-treated and ICS-exposed groups. Data are expressed as the means ± S.E.M.; 3-6 mice per group.

Table 2 Permanent relief from ICS-induced thermal hyperalgesia by repeated intrathecal (i.t.) administration of amitriptyline, mianserin or paroxetine

	n	Pre (sec)	P1 (sec)	P5 (sec)	P12 (sec)
Control-vehicle (i.t.)	8	9.84 ± 0.33	9.81 ± 0.45	10.24 ± 0.23	9.99 ± 0.22
ICS-vehicle (i.t.)	8	10.22 ± 0.28	5.76 ± 0.15*	6.04 ± 0.16*	6.28 ± 0.21*
ICS- amitriptyline (15 μg, i.t.)	4	10.48 ± 0.23	5.35 ± 0.36	8.44 ± 0.16#	9.35 ± 0.66#
ICS-mianserin (20 μg, i.t.)	4	9.68 ± 0.30	5.35 ± 0.36	8.13 ± 0.25#	9.95 ± 0.66#
ICS- paroxetine (5 μg, i.t.)	4	9.82 ± 0.44	5.47 ± 0.13	9.08 ± 0.46#	9.52 ± 0.21#

Antidepressants were administered between P1-P5, as described in Figure 3. Thermal pain threshold was assessed using the thermal paw withdrawal test. *$p < 0.05$, *vs.* vehicle-treated control group; #$p < 0.05$, *vs.* vehicle-treated and ICS-exposed groups.

contrast, there was no significant change in corticosterone levels in mice subjected to CCS. Considering that the abnormal pain in CCS mice was only transient, and not long-lasting [13,14], the rise in corticosterone levels in ICS mice likely played a role in the appearance of abnormal pain. A recent report suggests that the stress-induced increase in corticosterone concentration may be related to abnormal pain behavior in an FM-like animal model, possibly through a mechanism involving epinephrine release [27].

In our ICS model, the mice did not show significant changes in the tail-suspension test, a behavioral test designed to assess depression-like behavior [28]. This is in contrast to a study using less frequent temperature alternation (the SART model), in which mice exhibited hyperalgesia for only a week [29], and there was a transient reduction of immobility duration in forced swimming test, followed by gradual recovery in 5-6 days [30]. As the forced swimming causes a facilitation of immobility in an antidepressant-reversible manner [31], it is not clear whether the transient reduction of immobility duration reflects depression. From this point of view, the tail suspension test seems to be a better method for evaluation of depression-related despair behavior. Gabapentin and pregabalin are widely used to treat FM patients in the clinic [32,33]. These medicines alleviate abnormal pain and the accompanying fatigue and insomnia, without affecting depressive symptoms [33,34]. Therefore, the presence of depression-like behavior is unlikely to be necessary in animal models of FM. Consequently, the ICS model may be more clinically relevant than the SART model for evaluating long-term pain.

Various antidepressants have been used for FM in the clinic [35,36]. Recently milnacipran and duloxetine, serotonin/norepinephrine reuptake inhibitors, and serotonin-specific reuptake inhibitors have been approved for treating FM pain by the United States Food and Drug Administration. As the antinociceptive activities of these compounds are largely independent of their effects on mood, making them potentially efficacious for patients with or without depressive [37], it appears to reflect the

Table 3 Lack of anti-hyperalgesic effects by repeated systemic administration of antidepressants

	n	Pre (sec)	P1 (sec)	P5 (sec)	P12 (sec)
Control-vehicle (i.v.)	8	9.51 ± 0.22	9.80 ± 0.18	10.00 ± 0.29	9.57 ± 0.30
ICS-vehicle (i.v.)	8	9.67 ± 0.23	5.78 ± 0.36	6.02 ± 0.12	6.36 ± 0.21
ICS- amitriptyline (3 mg/kg, i.v.)	4	9.55 ± 0.42	5.85 ± 0.37	5.79 ± 0.36	6.72 ± 0.35
ICS-mianserin (10 mg/kg, i.v.)	4	9.77 ± 0.34	5.14 ± 0.56	5.98 ± 0.39	6.66 ± 0.41
ICS- paroxetine (1 mg/kg, i.v.)	4	9.34 ± 0.36	5.29 ± 0.34	6.89 ± 0.30	6.96 ± 0.65
ICS- milnacipran (10 mg/kg, i.v.)	4	9.75 ± 0.47	6.25 ± 0.25	6.71 ± 0.21	7.54 ± 0.53

Amitriptyline, mianserin, paroxetine or milnacipran were given intravenously (i.v.) and assessment of basal nociceptive thresholds was performed as described in Figure 5. Thermal pain threshold was assessed using the thermal paw withdrawal test. *$p < 0.05$, *vs.* vehicle-treated control group.

importance of central descending monoaminergic pathways in pain regulation [38,39]. Recent studies revealed that polymorphisms in the 5-HT receptor, transporter, and metabolic enzyme can contribute to the etiology of FM [40-42]. The fMRI study also demonstrates that brain regions involved in descending pain inhibitory pathways appear to have decreased activity in FM patients [43]. Although serotonergic and/or noradrenergic pathways are well documented as descending pain inhibitory pathways [39], there is no report that the abnormality of such descending monoaminergic systems is observed in FM patients. However, it would be challenging to examine the effects of representative antidepressants on ICS-induced abnormal pain by introducing the drugs into the intrathecal space, very close to target regions.

Our study shows that the repeated intrathecal administration of different antidepressants gradually suppressed ICS-induced pain. The gradual reversal of abnormal pain may be related to the down-regulation of β-adrenoceptors or abnormal monoaminergic metabolism [44-46]. Alternative mechanisms may include the altered expression of multiple receptors and ion channels, such as the NMDA receptor, opioid receptors, and sodium channels [47-49]. It should be noted that the reversal of abnormal pain continued after the cessation of drug treatment, for each of the antidepressants tested. Although further investigation is required to clarify the molecular mechanisms of antidepressant action and to provide a permanent cure for ICS-induced abnormal pain, it is interesting to speculate that the chronic pain may be due to a vicious cycle of pain elicited by reduced inhibitory input from monoaminergic pathways. Thus, the rescue of pain-inhibitory mechanisms by repeated antidepressant treatment should halt chronic pain. Similar observations were made in our previous study using central administration of gabapentin [13,14]. In that study, using the ICS model, a single intracerebroventricular administration of gabapentin produced a 4-day period of anti-hyperalgesia. As the injection had no effect on peripheral nerve injury-induced neuropathic pain [13,14], and the gabapentin was unlikely to have remained in the brain for 4 days, it is interesting to speculate that the observed effect is due to the inhibition of the pain cycle, possibly through enhancement of inhibitory transmission. However, the present study demonstrates that systemic administration of various antidepressants had no significant beneficial effect on ICS-induced hyperalgesia, though they had a significant acute analgesic effect in control mice. As the clinically beneficial effects of oral antidepressants to FM patients were evident when they are treated for more than several weeks [50], the lack of effects of intravenous antidepressants in the present study may be attributed to the shortage of treatments (5 days). In this meaning it is surprising that only 5 days repetitive intrathecal treatments abolishes abnormal pain even after the cession of treatments. Furthermore, although the mechanisms underlying the lack of antihyperalgesic effect remain elusive, it may be worthwhile to investigate possible involvements of interference of spinal effects by peripheral pain facilitating serotonergic actions or by descending pain facilitating monoaminergic systems [39]. Thus, we expect that repetitive intrathecal administration of antidepressants are likely to be more effective at treating FM-like pain in mouse models.

Finally, this study demonstrates that the ICS model has similarities to clinical features of FM in terms of the sensitivity to analgesics or adjuvant analgesics. In our previous findings, we observed that the effective dose of gabapentin was 3 mg/kg for ICS-induced pain, but was over 30 mg/kg for nerve injury-induced neuropathic pain in mice [13,14], consistent with the fact that the clinically-effective dose of gabapentin for FM patients is lower than that for neuropathic pain [51]. In addition, we observed that ICS-induced thermal hyperalgesia was resistant to morphine treatment [13,14], consistent with the clinical evidence [52]. Considering that other experimental animal models of FM-like pain exhibit morphine analgesia (albeit with low potency) [53-56], the ICS model may be pharmacologically distinct from the others.

6. Conclusion

This study demonstrates that repeated intrathecal antidepressant treatment provides a complete cure of ICS-induced FM-like abnormal pain. Based on the pharmacological similarity of ICS-induced pain to clinical FM, the ICS model appears to be suitable for investigating the pathogenesis of FM and for evaluating therapeutic strategies for this debilitating illness.

List of abbreviations used

aCSF: artificial cerebrospinal fluid; AUC: area under the curve: CCS: constant cold stress; FM: fibromyalgia; ICS: intermittent cold stress; PWL: paw withdrawal latency; PWT: paw withdrawal threshold.

Acknowledgements

The authors thank Drs. L Ma and W Xie for technical assistance. This study was supported in part by MEXT KAKENHI (17109015 to Hiroshi Ueda) and Health Labor Sciences Research Grants from the Ministry of Health, Labor and Welfare of Japan (to Hiroshi Ueda): *Research on Allergic disease and Immunology* and *Third Term Comprehensive Control Research for Cancer (398-49)*.

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Authors' contributions

MN participated in the experimental designing, collection and analyses of data, and drafted the manuscript in equal contribution. HU and JN

performed the statistical analyses and carried out surgical manipulation, data collection, and drafted the manuscript. KA and TM performed stress exposing and participated nociceptive behavior assay. SK measured plasma corticosterone levels. HU conceived of the study, participated in its design and coordination. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Received: 11 August 2011 Accepted: 21 September 2011
Published: 21 September 2011

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doi:10.1186/1744-8069-7-69
Cite this article as: Nishiyori *et al.*: Permanent relief from intermittent cold stress-induced fibromyalgia-like abnormal pain by repeated intrathecal administration of antidepressants. *Molecular Pain* 2011 7:69.

特集「臨床を裏づける神経障害性疼痛の本態」によせて

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Introduction
Essentials of neuropathic pain which support clinical data
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痛みはあくまでも主観的な感覚であり，病状を視覚的に明らかに示すことはできない．しかも，それが実験動物であり，ヒトでも客観的な評価を行うことが難しいとされる慢性疼痛である場合には，その研究成績の正当性を証明することは困難を極めるといわれている．しかし，慢性痛に限らず創薬を試みるには，この実験動物における基礎研究の段階を素通りしてなし得ることはほとんどない．そんな中で最も現実的に臨床と基礎とを関連づけることができるアプローチは，薬理学的評価である．これまでの先人の優れた研究成果から慢性疼痛，特に神経障害性疼痛の優れた病態モデルが開発され，その病態生理，組織化学，生化学的特徴が報告され，臨床・基礎研究により，病態の鍵を握る重要な因子が明らかにされてきた．本特集では，神経障害性疼痛の病態形成から治療戦略まで，薬理学的視点から最新の話題を解説することを目的としている．

第一稿では，永井・植田により，神経障害性疼痛（術後痛など）のモルヒネによる先制鎮痛機構が述べられている．ここでは，筆者らが一連の研究において明らかにしてきた，神経障害性疼痛の初発原因分子としてのリゾホスファチジン酸（LPA）の生合成と関連づけた研究成果を明らかにしている．第二稿で，大久保・川畑は，硫化水素（H_2S）の疼痛情報伝達の役割を解説している．H_2Sは，知覚神経終末に発現する$Ca_V3.2$T型カルシウムチャネルを活性化し，体性痛や内臓痛を促進することが知られているが，本稿では，末梢神経障害や化学療法薬による神経障害性疼痛時におけるH_2S合成と$Ca_V3.2$T型カルシウムチャネル発現変動との関連が紹介されている．第三稿では，田辺によって，ガバペンチンによる鎮痛効果の上位中枢における新たな作用機構が解説されている．神経障害性疼痛治療としてのガバペンチン/プレガバリンの作用点としては，これまで，末梢知覚神経におけるカルシウムチャネルの$Ca_V\alpha_2\delta$に対する抑制効果として解説されてきたが，本稿では下行性ノルアドレナリン疼痛抑制系やそのGABA神経による制御との関連が紹介されている．第四稿で，柴田・小泉は，臨床的に治療効果が確認されている生薬ブシ末について，神経障害性疼痛抑制効果のメカニズムを，脊髄のアストロサイトの脱活性化との関連で詳細に解説している．第五稿で，宮野・上園・仲田は，化学療法薬であるパクリタキセルにより誘発される末梢神経障害のメカニズムとして，C線維からのサブスタンスP遊離の関与を解説している．このメカニズムは，これまで報告されてきたA線維に対する機構と対比して興味深いものである．第六稿

では，木口・岸岡が，末梢神経障害性疼痛における神経炎症の役割を解説している．本稿では，サイトカイン・ケモカインを中心に，炎症性メディエーターが末梢神経や中枢神経において複雑なネットワークを形成することで慢性神経炎症を誘導することを述べている．従来，神経障害性疼痛は炎症と対比して捉えられてきたことを合わせて考えると，治療薬理学的ツールの活用という観点から本稿は大変興味深い．

近年，治療薬が承認されるようになり，神経障害性疼痛はもはや「時限付き」の難治性疾患になりつつある．本稿で見られるように，臨床で用いられている治療薬が新たな分子機構とともに世界中で認知されることが，今後の慢性疼痛治療克服の確かな道のりであれと祈るばかりである．

※　　※　　※

神経障害性疼痛に対するモルヒネ先制鎮痛

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要　旨
神経障害時に生じる強力な痛み刺激が異常痛の慢性化に至る神経可塑的変化を誘発するならば，あらかじめその痛みを抑制することで慢性痛の記憶を予防できると考えられる．実際，先制鎮痛という治療法において，この概念が術後痛の予防として臨床で証明されている．一方，筆者らは，神経障害性疼痛の初発因子として，脂質メディエーターであるリゾホスファチジン酸（LPA）を同定しており，近年ではこのLPAは強い痛み刺激によって脊髄で産生されることを明らかにしている．本稿では，モルヒネを用いた先制鎮痛のメカニズムについて，痛み刺激によって産生されるLPA合成の観点から解説する．

（ペインクリニック 32：1457-1463，2011）
キーワード：モルヒネ，先制鎮痛，リゾホスファチジン酸

はじめに

神経障害によって引き起こされる痛みの多くは，原因から解除されても持続する長期性であり，記憶機構と深く関連することが知られている．長期化した記憶性の痛みは，痛み自体が誘発する新たな分子機構によって，末梢知覚神経，脊髄および上位脳の神経機能の可塑性を増幅する，いわゆる「痛みの悪循環」によって正常時の痛みと大きく異なった複雑な痛みの伝達がなされている．したがって，原因となる発症初期の段階での疼痛管理は，神経障害性疼痛の予防・抑制にとって非常に重要である．先制鎮痛は，術中などのあらかじめ予想される侵害刺激に対して予防的に鎮痛薬を処置し，神経障害発症初期のメカニズムを抑制することで術後痛などに効果を上げている．しかしながら，先制鎮痛の詳細なメカニズムは依然として不明のままである．筆者らは，神経障害性疼痛の誘発分子として，リゾホスファチジン酸（lysophosphatidic acid：LPA）を発見しているが，最近，この先制鎮痛がLPA産生の抑制と連関することを明らかにできたので，本稿ではこの研究成果を紹介する．

1．神経障害性疼痛に対する先制鎮痛効果

神経障害性疼痛の形成および維持機構の特徴の一つとして，反復痛み刺激によって脊髄後角ニューロンの感受性が亢進する中枢性感作が挙げられる[1]．そこで，この初期に痛み刺激によって引き起こされる神経活動の一連のカスケードを抑制することが，その後，長期的に続く過敏応答を防ぐことができると考えられる．

〈Special Article〉 Essentials of neuropathic pain which support clinical data
Pre-emptive morphine treatment for neuropathic pain
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