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Title Page

Evidence for *De Novo* Synthesis of Lysophosphatidic Acid in the Spinal Cord through Phospholipase A_2 and Autotaxin in Nerve Injury-induced Neuropathic Pain

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Running Title Page

(a) Running title: *De novo* lysophosphatidic acid synthesis after nerve injury

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(d) List of abbreviations:

LPA: lysophosphatidic acid; ATX: autotaxin; LPC: lysophosphatidylcholine; *atx*$^{+/-}$: ATX gene heterozygous mutant mice; WT: wild-type mice; AACOCF3: arachidonyl trifluoromethyl ketone; BEL: bromoenol lactone; DMEM: Dulbecco's modified Eagle's medium; SC: dorsal horn of the lumbar spinal cord; DR: dorsal roots; DRG: dorsal root ganglions; SPN: spinal nerves; SCN: sciatic nerves; B103 (+): LPA_1 receptor-expressing B103 cells; CSF: cerebrospinal fluid; PLA: phospholipase A; $cPLA_2$: cytosolic phospholipase A_2; $iPLA_2$: calcium-independent phospholipase A_2; PC: phosphatidylcholine; B103 (-): LPA_1 receptor-lacking B103 cells; IC_{50}: half-maximal inhibitory concentration; NK1 receptor: neurokinin 1 receptor; NMDA receptor: N-methyl-D-aspartate receptor; SP: substance P; Glu: glutamate.

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Abstract

We previously reported that lysophosphatidic acid (LPA) initiates nerve injury-induced neuropathic pain and its underlying mechanisms. In addition, we recently demonstrated that intrathecal injection of LPA induces *de novo* LPA production through the action of autotaxin (ATX), which converts lysophosphatidylcholine (LPC) to LPA. Here, we examined nerve injury-induced *de novo* LPA production using a highly sensitive biological titration assay with B103 cells expressing LPA_1 receptors. Nerve injury caused high levels of LPA production in the ipsilateral sides of the spinal dorsal horn and dorsal roots, but not in the dorsal root ganglion, spinal nerve or sciatic nerve. Nerve injury-induced LPA production reached its maximum at 3 h after injury, followed by a rapid decline by 6 h. The LPA production was significantly attenuated in ATX heterozygous mutant mice, while the concentration and activity of ATX in cerebrospinal fluid were not affected by nerve injury. On the other hand, the activities of cytosolic phospholipase A_2 ($cPLA_2$) and calcium-independent phospholipase A_2 ($iPLA_2$) were enhanced, with peaks at 1 h after injury. Both *de novo* LPA production and neuropathic pain-like behaviors were substantially abolished by intrathecal injection of arachidonyl trifluoromethyl ketone (AACOCF3), a mixed inhibitor of cPLA2 and iPLA2, or bromoenol lactone (BEL), an iPLA2 inhibitor, at 1 h after injury. However, administration of these inhibitors at 6 h after injury had no significant effects on neuropathic pain. These findings provide evidence that PLA_2- and ATX-mediated *de novo* LPA production in the early phase is involved in nerve injury-induced neuropathic pain.

Introduction

Lysophosphatidic acid (LPA) is a bioactive lipid mediator that exerts a variety of biological activities including promotion of cell proliferation, prevention of apoptosis, and modulation of cell shape and cell migration (Aoki, 2004; Aoki et al., 2008). Consequently, LPA has been demonstrated to play important roles in numerous pathological and physiological situations, such as wound healing, lung fibrosis, cancer, reproduction and hair growth (Balazs et al., 2001; Mills and Moolenaar, 2003; Ye et al., 2005; Pasternack et al., 2008; Tager et al., 2008). Moreover, LPA has been detected in several biological fluids, including serum, saliva, seminal fluid, follicular fluid and even ascites from ovarian cancer patients (Xu et al., 1995; Tokumura et al., 1999; Aoki et al., 2002; Hama et al., 2002; Sugiura et al., 2002). Furthermore, LPA is a lipid metabolite that is produced after tissue injury (Eichholtz et al., 1993; Tigyi et al., 1995).

Previously, we reported that LPA_1 receptor signaling initiated nerve injury-induced neuropathic pain and its underlying machineries, including demyelination and altered expressions of pain-related molecules (Inoue et al., 2004; Ueda, 2006; Ueda, 2008). In addition, nerve injury-induced neuropathic pain could be caused by a single intrathecal injection of LPA, and blocked by LPA_1 receptor knockdown at the early, but not late, stage (Inoue et al., 2004). Moreover, deletion of the LPA_1 receptor gene did not change the basal nociceptive threshold (Inoue et al., 2004), thus providing evidence that nerve injury-induced neuropathic pain is initiated by *de novo* LPA synthesis via defined biosynthetic pathways (Aoki, 2004; Aoki et al., 2008). Very recently, we found that intrathecal administration of LPA caused feed-forward LPA production at the early phase (Ma et al., 2009b). Based on these findings, we speculate that nerve injury may induce the production of LPA at the early phase and

subsequently cause LPA_1 receptor activation to induce neuropathic pain. Moreover, we previously demonstrated that autotaxin (ATX), which converts lysophosphatidylcholine (LPC) to LPA (Aoki, 2004; Aoki et al., 2008), is involved in nerve injury-induced neuropathic pain, because neuropathic pain was significantly attenuated in ATX gene heterozygous mutant (*atx*$^{+/-}$) mice (Inoue et al., 2008a). In addition, LPC conversion to LPA mediated by ATX has been implicated in LPA-induced LPA production (Ma et al., 2009b). In addition, we found that intense stimulation of spinal cord slices with combined pain transmitters or capsaicin, which is thought to induce the release of pain transmitters, caused biosynthesis of LPC, which was subsequently converted to LPA by ATX (Inoue et al., 2008b). Taken together, these findings suggest that nerve injury-induced neuropathic pain occurs after LPC production, with subsequent LPA production via LPC conversion by ATX and activation of LPA_1 receptor signaling. The present study represents an initial neurochemical examination of the processes underlying nerve injury-induced *de novo* LPA production, and provides evidence of PLA_2- and ATX-mediated early biosynthesis of LPA after nerve injury.

Methods

Animals

Male C57BL/6J mice (Tagawa Experimental Animal Laboratory, Nagasaki, Japan), *atx*$^{+/-}$ mice (Tanaka et al., 2006) and their sibling wild-type (WT) mice from the same genetic background were used in this study. The mice weighed 20-24 g. They were kept in a room maintained at 21±2ºC and 55±5% relative humidity with a 12-h/12-h light/dark cycle and had free access to a standard laboratory diet and tap water. The procedures were approved by the Nagasaki University Animal Care Committee, and complied with the fundamental guidelines for the proper conduct of animal experiments and related activities in academic research institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology, Japan.

Drugs

LPC was purchased from Sigma (St. Louis, MO). Arachidonyl trifluoromethyl ketone (AACOCF3) and bromoenol lactone (BEL) were purchased from Cayman Chemicals (Ann Arbor, MI). For *in vitro* experiments, LPC was dissolved in Dulbecco's modified Eagle's medium (DMEM) containing 0.1% fatty acid-free bovine serum albumin (A-6003; Sigma-Aldrich, St. Louis, MO). For *in vivo* experiments, AACOCF3 and BEL were dissolved in artificial cerebrospinal fluid (125 mM NaCl, 3.8 mM KCl, 1.2 mM KH_2PO_4, 26 mM $NaHCO_3$, 10 mM glucose).

Partial sciatic nerve ligation

Partial ligation of the sciatic nerves was performed under anesthesia with pentobarbital (50 mg/kg, i.p.), according to modified methods (Rashid et al., 2003).

The common sciatic nerve of the right hind limb was exposed at the high thigh level through a small incision and the dorsal half of the nerve thickness was tightly ligated with a silk suture. A sham-operation was performed similarly except without touching the sciatic nerve.

Sample preparation from tissues

At different time points after sciatic nerve injury, mice were anesthetized with pentobarbital (50 mg/kg, i.p.) as previously reported (Ma et al., 2009b). The unilateral dorsal horn (laminae I-V) of the lumbar (L4-6) spinal cord (SC), L4-6 dorsal roots (DR), L4-6 dorsal root ganglions (DRG), L4-6 spinal nerves (SPN) and L4-6 sciatic nerves (SCN) on the ipsilateral or contralateral side were then removed to enable the extraction of LPA, as shown in Fig. 1A. The average wet weights of the isolated unilateral SC, DR, DRG, SPN and SCN in each mouse were 4, 2, 2, 2 and 2.5 mg, respectively. Following their isolation, the tissue samples were placed in 1.5-ml polypropylene tubes and homogenized by sonication in 300 μl of serum-free DMEM for approximately 30 s. To extract LPA from the homogenates using a solid-phase lipid extraction method, the samples were slowly loaded onto Oasis HLB cartridges (Millipore, Tokyo, Japan), which had been pre-conditioned with 3 ml of methanol followed by 3 ml of distilled water. The columns were then washed with 3 ml of distilled water and 1 ml of chloroform. Subsequently, LPA was eluted with 600 μl of methanol and dried with N_2 gas. The final samples were dissolved in 100 μl of DMEM and stored at -80ºC until analysis.

Biological titration method

B103 cells expressing LPA_1 receptors and enhanced green fluorescent protein (B103

(+) cells) were used for quantitative measurement of LPA, according to a modified method (Inoue et al., 2008b; Ma et al., 2009b) based on an earlier report (Ishii et al., 2000). The cells were maintained as monolayer cultures on tissue culture dishes in DMEM supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Carlsbad, CA), penicillin and streptomycin (final concentrations: 100 U/ml and 100 μg/ml, respectively). Cells were seeded at 2.5×10^4 cells/cm^2 onto 8-well glass slides coated with poly-L-lysine (Sigma; final concentration, 100 mg/l) and collagen (BD Bioscience, San Jose, CA; final concentration, 5 μg/cm^2). The cells were then cultured in DMEM containing 10% heat-inactivated fetal bovine serum at 37°C in a 5% CO_2 atmosphere for 10 h. Subsequently, the cells were cultured in serum-starved DMEM for 15 h.

In the biological assay, a standard LPA solution or a diluted tissue sample was applied to B103 (+) cells. After incubation at 37ºC for 20 min, the medium was replaced with 4% paraformaldehyde followed by incubation at 25ºC for 60 min. The glass slide was then cover-slipped with Fluoromount™ (DBS, Pleasanton, CA) and examined under a fluorescence microscope (Keyence, Osaka, Japan). The percentage of cells exhibiting a rounded morphology among at least 500 cells in each well was determined.

Collection of cerebrospinal fluid

Cerebrospinal fluid (CSF) was collected according to a previously described method (Inoue et al., 2008b). In this method, mice were anesthetized with pentobarbital (50 mg/kg, i.p.), and the L4-5 vertebral column was carefully exposed. Next, SP8 polyethylene tubing (ID: 0.20 mm; OD: 0.50 mm; Natsume, Tokyo, Japan) connected to a syringe was inserted into the subarachnoid space of the exposed L4-5 vertebral

column, and the CSF was suctioned using the syringe.

Western blotting

Western blotting analysis for ATX was performed as previously reported (Inoue et al., 2008b). Briefly, CSF was collected at 2 h after nerve injury or sham operation. The collected CSF (0.5 μl) was applied to an SDS-polyacrylamide gel (8%). An anti-ATX antibody described in a previous report (Tanaka et al., 2006) was used at a dilution of 1:100. A horseradish peroxidase-conjugated anti-rat antibody (Zymed Laboratories, Carlsbad, CA) was used as the secondary antibody at a dilution of 1:1000. Immunoreactive bands were detected using an enhanced chemiluminescent substrate (SuperSignal® West Pico Chemiluminescent Substrate; Pierce Biotechnology, Rockford, IL) for horseradish peroxidase. The intensities of the immunoreactive bands were analyzed by NIH Imaging for Macintosh.

Phospholipase A_2 activity assays

The activities of cytosolic phospholipase A_2 ($cPLA_2$) and calcium-independent phospholipase A_2 ($iPLA_2$) were detected using the following assays as described previously (Smani et al., 2003). Briefly, at different time points after sciatic nerve injury, mice were anesthetized with pentobarbital (50 mg/kg, i.p.), and the ipsilateral side of the spinal dorsal horn was removed. After sonication and centrifugation at 20000×*g* for 20 min at 4ºC, the supernatant was collected and kept on ice. The protein concentration of the supernatant was determined by the Lowry method, and the assays were performed on the same day using a $cPLA_2$ assay kit (Cayman Chemicals) to evaluate the $cPLA_2$ activity or a modified $cPLA_2$ assay kit (Cayman Chemicals) to evaluate the $iPLA_2$ activity, as described previously (Smani et al., 2003). In the $cPLA_2$

assay, the tissue samples were incubated with both BEL, an $iPLA_2$ inhibitor (Ackermann et al., 1995), and a substrate, arachidonoyl thio-PC, at 20ºC for 1 h in a assay buffer. The reactions were stopped by DTNB/EGTA for 5 min, and the absorbances were determined at 405 nm using a standard plate reader. To detect the activity of $iPLA_2$, but not $cPLA_2$, the samples were incubated with the substrate, arachidonoyl thio-PC, at 20ºC for 1 h in a modified Ca^{2+}-free buffer (4 mM EGTA, 160 mM HEPES pH 7.4, 300 mM NaCl, 8 mM Triton X-100, 60% glycerol, 2 mg/ml of BSA). The reactions were stopped by the addition of 5,5'-dithiobis(nitrobenzoic acid) for 5 min. The activity of PLA_2 was defined as the percentage of the control activity as follows: injured tissues (absorbance/mg of protein) / normal tissues (absorbance/mg of protein) × 100.

Intrathecal injection

The intrathecal injection was carried out as previously reported (Ma et al., 2009a) according to the modified method (Hylden and Wilcox, 1980). In this method, unanesthetized mouse was held by the pelvic girdle in one hand, and the syringe was held in another hand. The needle was inserted into the tissue between the dorsal aspects of lumbar region 5 and 6, at an angle of about 20° above the vertebral column, then it slipped into the groove between the spinous and transverse processes. Changing the angle of the syringe to 10°, the needle was carefully moved forward to the intervertebral space, and 5 μl of drug solution was injected. The whole operation was performed within one minute. After injection, no specific behavior or sign of distress was observed in injected-mouse.

Nociceptive tests

In thermal paw withdrawal tests, nociception was measured as the latency to paw withdrawal evoked by exposure to a thermal stimulus (Hargreaves et al., 1988; Rashid et al., 2003; Ma et al., 2009a). Unanesthetized animals were placed in Plexiglas cages on top of a glass sheet and allowed an adaptation period of 1 h. A thermal stimulator (IITC Inc., Woodland Hills, CA) was positioned under the glass sheet and the focus of the projection bulb was aimed precisely 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 paw pressure test was performed as described previously (Rashid et al., 2003; Ma et al., 2009a). Mice were placed in a Plexiglas chamber on a 6×6-mm wire mesh grid floor and allowed to acclimatize for 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.). The pressure required to induce a flexor response was defined as the pain threshold. All behavioral experiments were performed under double-blinded conditions.

Statistical analysis

Statistical analyses were carried put using Student's *t*-test and Tukey's multiple comparison *post hoc* analysis following one-way ANOVA. The criterion of significance was set at $p<0.05$. All results are expressed as means ± S.E.M.

Results

Nerve injury-induced *de novo* LPA production in the ipsilateral sides of the spinal dorsal horn and dorsal roots

To quantify LPA production after sciatic nerve injury, we developed a biological titration method using LPA_1 receptor-expressing B103 cells (B103 (+) cells), based on previously described methods (Ishii et al., 2000; Inoue et al., 2008b; Ma et al., 2009b). Using our method, we evaluated the percentages of cells showing a rounded morphology induced by the addition of LPA, and examined at least 500 cells in each well. The measurements were specific for LPA, since high levels of both LPC and S1P were reported to have no effects on these cells (Inoue et al., 2008b; Ma et al., 2009b). In addition, our most recent study demonstrated that LPA did not induce any morphological rounding of B103 cells lacking LPA_1 receptor expression (B103 (-) cells), even if LPA was present at a high concentration (Ma et al., 2009b). In the present study using B103 (+) cells, the calibration curve for the LPA-induced cell-rounding activity was linear as the LPA concentration increased from 0.15 to 5 pmol, after subtracting the basal cell-rounding activity. Experiments were carried out in 100-μl wells. The equation was defined as $y=5.454x+5.66$ ($R^2=0.991$; x: $\log_{10}$[LPA (pmol)]; y: percentage of rounded cells). In subsequent experiments, LPA-equivalents for the tissue extracts of the different regions were estimated using this equation based on the linear LPA concentration-dependent responses, as mentioned in our recent report (Ma et al., 2009b).

As shown in Fig. 1B, the basal level of LPA-equivalents in the SC of control mice was 0.79 pmol/mg tissue. However, the LPA levels increased in a time-dependent manner in the ipsilateral side of the SC after sciatic nerve injury. The maximal production (74.8 pmol/mg tissue) was obtained at 3 h after nerve injury, and had

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substantially disappeared by 6 h. Increases in the nerve injury-induced LPA level were also observed in the ipsilateral side of the DR with a similar time course (Fig. 1B). In contrast, no significant increases in the LPA level were observed in the ipsilateral side of the DRG, SPN or SCN at 3 h after injury (Fig. 1C). In addition, no LPA increases were observed in the contralateral side of the SC or DR at 3 h after nerve injury (Fig. 1C), suggesting that the newly produced LPA is unlikely to diffuse throughout the CSF.

ATX involvement in nerve injury-induced LPA production

The LPA levels in *atx*$^{+/-}$ mice at 3 h after nerve injury were evaluated. The LPA levels were significantly attenuated in the ipsilateral sides of the SC and DR in *atx*$^{+/-}$ mice, compared with WT mice (Fig. 2).

Lack of nerve injury-induced changes in the concentration and activity of ATX in CSF

To examine whether the concentration or enzyme activity of ATX in CSF was affected by nerve injury, we collected CSF from mice at 2 h after nerve injury or sham operation. In a Western blot analysis for ATX, there were no differences in the ATX amounts in CSF (0.5 μl) between the presence and absence of nerve injury (Fig. 3A). In addition, when the CSF (0.5 μl) was incubated with LPC (10 pmol) for 30 min, there were no significant changes in the LPA-equivalents between these preparations in the biological titration assay (Fig. 3B). These findings suggest that the concentration and enzyme activity of ATX are not affected by nerve injury.

Nerve injury-induced activation of spinal cPLA$_2$ and iPLA$_2$ at the early stage

We attempted to evaluate the signal transduction pathway leading to LPA production. Since the enzymes $cPLA_2$ and $iPLA_2$, which are expressed in the spinal cord (Karin Killermann et al., 2005), catalyze phosphatidylcholine (PC) conversion to LPC (Aoki, 2004; Aoki et al., 2008; Inoue et al., 2008b), the ipsilateral side of the SC in injured or control mice was collected and analyzed by $cPLA_2$ and $iPLA_2$ activity assays. As shown in Fig. 4A, the $cPLA_2$ activity was significantly increased at 1 h after nerve injury compared with the control group, and the increase was followed by remarkable declines at 3 and 6 h after injury. A significant increase in the $iPLA_2$ activity was also observed at 1 h after injury, but only slight decreases were observed at 3 and 6 h after injury (Fig. 4B).

Nerve injury-induced LPA production through PLA_2

To assess the roles of $cPLA_2$ and $iPLA_2$ in the nerve injury-induced LPA production, AACOCF3, a mixed inhibitor of $cPLA_2$ and $iPLA_2$ (Street et al., 1993; Ackermann et al., 1995), or BEL, an $iPLA_2$ inhibitor (Ackermann et al., 1995), was administered (10 nmol, i.t.) at 1 h after injury. At 3 h after injury, the ipsilateral sides of the SC and DR were removed to measure the LPA levels. The nerve injury-induced LPA productions in the SC and DR were significantly blocked by post-injury treatment with AACOCF3 (Fig. 5A). Similar results were found in the BEL treatment group (Fig. 5B).

Blockade of neuropathic pain by early treatment with PLA_2 inhibitors

To assess the pharmacological effects of AACOCF3 or BEL in behavioral tests, different doses of AACOCF3 or BEL were administered at 1 h after nerve injury, and nociceptive tests were performed on 3, 5 and 7 days after injury. Sciatic nerve injury

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caused robust mechanical allodynia at 3 days after nerve injury in the ipsilateral side, and the allodynia was blocked by intrathecal injection of AACOCF3 or BEL at 1 h after injury in a dose-dependent manner, while no effects on the nociceptive thresholds of the contralateral side were observed (Fig. 6A and B). Since the maximal effect of AACOCF3 or BEL was observed at 10 nmol, we adopted this dose in subsequent experiments. However, no abnormal behaviors were observed following AACOCF3 or BEL treatment at 10 or 30 nmol. As shown in Fig. 6C and D, sciatic nerve injury-induced thermal hyperalgesia and mechanical allodynia lasted for at least 7 days in the ipsilateral side, and these neuropathic pain behaviors were significantly attenuated by post-injury treatment with AACOCF3 or BEL at 10 nmol, while no differences were observed in the contralateral side.

To evaluate the critical time period for the antagonistic effects of AACOCF3 and BEL, they were each administered (10 nmol, i.t.) at 6 h post-injury. In accordance with the above time course, nociceptive tests were performed on 3, 5 and 7 days after injury. However, neither of the inhibitors had any effects on nerve injury-induced thermal and mechanical neuropathic pain (Fig. 7A and B).

Discussion

In the present study, we have demonstrated for the first time that sciatic nerve injury causes *de novo* synthesis of LPA *in vivo* by the action of endogenous ATX, using a previously described method (Inoue et al., 2008b; Ma et al., 2009b). This method, in which LPA_1 receptor-mediated cell-rounding activity was measured as a quantitative evaluation of the LPA levels in processed extracts from tissue samples, has been confirmed to be a highly sensitive and specific assay for the evaluation of low levels of LPA in extracts (Inoue et al., 2008b; Ma et al., 2009b), because LPA concentration was detectable from 0.15 pmol (equivalent: 1.5 nM) in this assay, showing the higher sensitivity compared with the enzymatic cycling method, another widely used method for LPA determination that is only suitable for concentrations over 100 nM LPA (Kishimoto et al., 2003). It should be noted that the LPA levels in the SC and DR of control mice were negligible (0.79 and 1.81 pmol/mg tissue, respectively). When the sciatic nerve was injured, the LPA levels were elevated by 95- and 48-fold (74.8 and 86.9 pmol/mg tissue, respectively) in these regions at 3 h on the ipsilateral side, while there was no significant elevation on the contralateral side. This injury-induced production of LPA is consistent with the previous finding that LPA was generated after hemorrhagic brain injury (Tigyi et al., 1995). However, there were no increases in the LPA levels in the ipsilateral DRG, SPN or SCN, suggesting that LPA production occurs in the spinal cord and migrates to the vicinity of the dorsal root along the nerve fiber (Fig. 8).

Sciatic nerve injury caused time-dependent increases in the LPA levels in the SC and DR that lasted until 3 h post-injury. These elevations may be attributed to the sum of the original *de novo* LPA production plus the produced LPA-induced feed-forward LPA production (Fig. 8), since we recently reported that a low level of LPA induced

feed-forward LPA synthesis through ATX and LPA_3 receptor in both *in vivo* and *in vitro* experiments (Ma et al., 2009b). Furthermore, the increased LPA levels were followed by significant decreases at 6 h after injury. These decreases may be caused by end-product inhibition of ATX, since ATX activity is inhibited by high levels of LPA (van Meeteren et al., 2005).

Two major pathways of LPA production have been proposed, namely intracellular LPA generation from phosphatidic acid by phospholipase A_1 or PLA_2 and extracellular generation from LPC by ATX (Aoki, 2004; Aoki et al., 2008). However, based on the observations that both stimulation-induced LPA synthesis and LPA-induced LPA production absolutely required the presence of ATX in *in vitro* studies using spinal cord slices (Inoue et al., 2008b; Ma et al., 2009b), the latter pathway seems to be more important for the *de novo* synthesis of LPA in the spinal cord after nerve injury. Indeed, a significant level of ATX is present in CSF (Sato et al., 2005; Inoue et al., 2008b). In the present study, $atx^{+/-}$ mice exhibited significant attenuation of nerve injury-induced LPA production at 3 h after injury, and this finding is consistent with our previous observation that there was a partial, but significant, attenuation of nerve injury-induced neuropathic pain in $atx^{+/-}$ mice (Inoue et al., 2008a). Therefore, it is evident that nerve injury-induced *de novo* LPA production largely depends on the action of ATX in CSF *in vivo*. On the other hand, in the present study, we found that the concentration and activity of ATX in CSF were not changed by nerve injury, indicating that LPC biosynthesis is the rate-limiting process for *de novo* biosynthesis of LPA following nerve injury. Taken together, these findings suggest that the rapid production of LPA after injury can be attributed to the more rapid LPC production and subsequent ATX-mediated conversion of LPC to LPA. Experiments using an ATX inhibitor may support this hypothesis. However,

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since the commercially available ATX inhibitor shows some affinity for the LPA_3 receptor, which is involved in LPA-induced LPA production (Ma et al., 2009b), we should await for a specific ATX inhibitor to be available.

In the present study, we attempted to clarify the signal transduction after nerve injury that leads to the *de novo* biosynthesis of LPC and LPA. Since the enzymes $cPLA_2$ and $iPLA_2$, which are expressed in the spinal cord (Karin Killermann et al., 2005), catalyze PC conversion to LPC (Aoki, 2004; Aoki et al., 2008; Inoue et al., 2008b), we propose that both $cPLA_2$ and $iPLA_2$ are involved in the nerve injury-induced production of LPC and LPA. In fact, we found that the activities of both $cPLA_2$ and $iPLA_2$ were remarkably increased at 1 h post-injury, which is consistent with a previous report that PLA_2 activity was rapidly and significantly elevated after spinal cord injury (Nai-Kui et al., 2006). There is a contradictory report that the activities of both $cPLA_2$ and $iPLA_2$ were not altered by carrageenan-induced inflammatory pain (Karin Killermann et al., 2005). Although the detailed mechanisms underlying this difference remain unclear, it may be attributable to the differences between neuropathic pain and inflammatory pain.

It should be noted that the $cPLA_2$ activation was transient, while the $iPLA_2$ activation was sustained for more than 6 h. Considering that the injury-induced LPA production observed in the present study and the LPA_1 receptor signaling-mediated initiation of neuropathic pain in a previous study (Ma et al., 2009a) were terminated within 6 h, $cPLA_2$ seems more likely to be related to the *de novo* LPA production. Indeed, AACOCF3, a mixed inhibitor of $cPLA_2$ (half-maximal inhibitory concentration (IC_{50}): 2-8 μM) and $iPLA_2$ (IC_{50}: 15 μM) (Street et al., 1993; Ackermann et al., 1995), substantially abolished the nerve injury-induced LPA production and neuropathic pain. However, BEL, a more potent and selective $iPLA_2$

inhibitor (IC_{50}: 60 nM) (Ackermann et al., 1995) also substantially abolished the injury-induced LPA production and neuropathic pain. Therefore, both $iPLA_2$ and $cPLA_2$ are considered to play roles in nerve injury-induced LPA production and neuropathic pain (Fig. 8). The difference between the short-term LPA production within 6 h and the sustained $iPLA_2$ activation for more than 6 h may be explained by end-product inhibition of ATX by LPA (van Meeteren et al., 2005). The roles of $cPLA_2$ and $iPLA_2$ proposed in the present study are also consistent with our recent finding that the activation of both $cPLA_2$ and $iPLA_2$ as the final step caused *de novo* biosynthesis of LPC from PC following intense stimulation of the primary afferent or simultaneous activation of neurokinin 1 (NK1) and N-methyl-D-aspartate (NMDA) receptors by substance P (SP) and glutamate (Glu), respectively (Inoue et al., 2008b) (Fig. 8). Moreover, given that $cPLA_2\alpha$ and $iPLA_2$ are the predominant PLA_2 messages in spinal cord (Karin Killermann et al., 2005), and *S*-isomers and *R*-isomers of BEL were reported to be more selective for $iPLA_2\beta$ and $iPLA_2\gamma$, respectively (Jenkins et al., 2002), we speculate that the α isoform of $cPLA_2$ may be mainly involved in the injury-induced LPA synthesis, but the detailed subtype of $iPLA_2$ involved in this study is still unclear. Using the specific inhibitor or antisense oligodeoxynucleotide for each PLA_2 to determine the subtype would be the next important issue.

On the other hand, it currently remains unknown which cell types are involved in nerve injury-induced LPA production, although there are some reports that LPA can be synthesized and secreted by primary neurons and Schwann cells *in vitro* (Fukushima et al., 2000; Weiner et al., 2001). It is particularly difficult to clarify whether the *de novo* LPA synthesis occurs in specific neurons or highly differentiated cell types. Alternatively, LPA synthesis may occur through neuron-glia interactions or

in an autocrine manner. In future research, an important aim will be the evaluation of LPA production in individual or cocultured specific cell types. Another issue to be investigated in the future is the clarification of which species of LPA molecules are involved in the injury-induced *de novo* synthesis, since there are several subspecies of LPA (Aoki, 2004; Aoki et al., 2008). The development of an advanced method utilizing mass spectrometry with highly efficient purification and condensation would be required for such studies.

The present study also provides information regarding the mechanisms underlying nerve injury-induced neuropathic pain. In a series of previous studies, we have demonstrated that LPA_1 receptor signaling initiates nerve injury-induced neuropathic pain and its underlying mechanisms (Inoue et al., 2004; Ueda, 2006; Ueda, 2008). Recently, a pharmacological study demonstrated that LPA_1 signaling can initiate neuropathic pain within a timeframe of 2-4 h (Ma et al., 2009a). Therefore, our present study provides the first demonstration that the *de novo* biosynthesis of LPA at 2-3 h after nerve injury is essential for the development of neuropathic pain. Targeted inhibition of PLA_2- and ATX-mediated LPA synthesis may be a potential strategy for the prevention of nerve injury-induced neuropathic pain.

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Footnotes

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Legends for figures

Fig. 1. Nerve injury-induced *de novo* LPA production in the ipsilateral sides of the spinal dorsal horn and dorsal roots. (A) Exact locations of the removed samples, including the dorsal horn of the lumbar spinal cord (SC), dorsal roots (DR), dorsal root ganglions (DRG), spinal nerves (SPN) and sciatic nerves (SCN). (B) Quantification of LPA production in the ipsilateral sides of the SC and DR at different time points after sciatic nerve injury. The capital letter "C" represents the control group (naive mice). (C) Evaluation of LPA production after nerve injury in different preparations from the ipsilateral sides of the SC, DR, DRG, SPN and SCN and the contralateral sides of the SC and DR at 3 h post-injury. The capital letter "I" represents the injured group. The LPA measurements were carried out in triplicate for each sample. All data represent means ± S.E.M. from three separate experiments. Cell-rounding morphology was evaluated in at least 500 enhanced green fluorescent protein-positive cells. *$p<0.05$, versus with the control group.

Fig. 2. ATX involvement in nerve injury-induced LPA production. Quantification of LPA production in the SC and DR of injured wild-type (WT) mice and ATX gene heterozygous mutant ($atx^{+/-}$) mice at 3 h post-injury. The LPA measurements were carried out in triplicate for each sample. All data represent means ± S.E.M. from three separate experiments. *$p<0.05$, versus with the WT group.

Fig. 3. Lack of nerve injury-induced changes in the concentration and activity of ATX in CSF. CSF was collected at 2 h after nerve injury or sham operation, and the concentration and enzyme activity of ATX in CSF (0.5 μl) was analyzed by Western blotting (*panel A*) and a biological titration method (*panel B*). All data represent

means ± S.E.M.

Fig. 4. Nerve injury-induced activation of spinal $cPLA_2$ and $iPLA_2$ at the early stage. The activities of $cPLA_2$ (*panel A*) and $iPLA_2$ (*panel B*) in the SC after nerve injury were determined by $cPLA_2$ and $iPLA_2$ activity assays. The capital letter "C" represents the control group (naive mice). All data represent means ± S.E.M. from three to five separate experiments. *p<0.05, versus the control group.

Fig. 5. Nerve injury-induced LPA production through PLA_2. AACOCF3 (*panel A*) or BEL (*panel B*) was administered (10 nmol, i.t.) at 1 h post-injury, and the ipsilateral sides of the SC and DR were removed at 3 h post-injury to evaluate the LPA levels. The LPA measurements were carried out in triplicate for each sample. All data represent means ± S.E.M. from three separate experiments. *p<0.05, versus the vehicle group.

Fig. 6. Blockade of neuropathic pain by early treatment with PLA_2 inhibitors. (A and B) Difference doses of AACOCF3 (*panel A*) or BEL (*panel B*) were administered intrathecally at 1 h post-injury, and the paw pressure test was performed at 3 days post-injury. (C and D) AACOCF3 or BEL was administered (10 nmol, i.t.) at 1 h post-injury. Thermal paw withdrawal tests (*panel C*) and paw pressure tests (*panel D*) were performed at days 3, 5 and 7 after injury. The results represent the thresholds of the latency (in s) or pressure (in g) to thermal or mechanical stimulation, respectively. PWL, paw withdrawal latency; PWT, paw withdrawal threshold. All data represent means ± S.E.M. from three or four mice. *p<0.05, versus the vehicle group or contralateral side.

Fig. 7. Delayed treatment with PLA_2 inhibitors has no effect on neuropathic pain. (A and B) AACOCF3 or BEL was administered (10 nmol, i.t.) at 6 h post-injury. Thermal paw withdrawal tests (*panel A*) and paw pressure tests (*panel B*) were performed at days 3, 5 and 7 after injury. All data represent means ± S.E.M. from three or four mice. *$p<0.05$, versus the contralateral side.

Fig. 8. Proposed hypothesis for the mechanisms underlying *de novo* production of LPA following sciatic nerve injury. Sciatic nerve injury causes intense activation of NK1 and NMDA receptors by substance P (SP) and glutamate (Glu), respectively, leading to subsequent activation of both $cPLA_2$ and $iPLA_2$, which catalyze a conversion of PC to LPC. ATX then converts LPC to LPA, which further induces an LPA production in ATX and LPA_3 receptor-mediated feed-forward system. The produced LPA in the spinal cord migrates to the vicinity of the dorsal root along the nerve fiber.

Fig.1

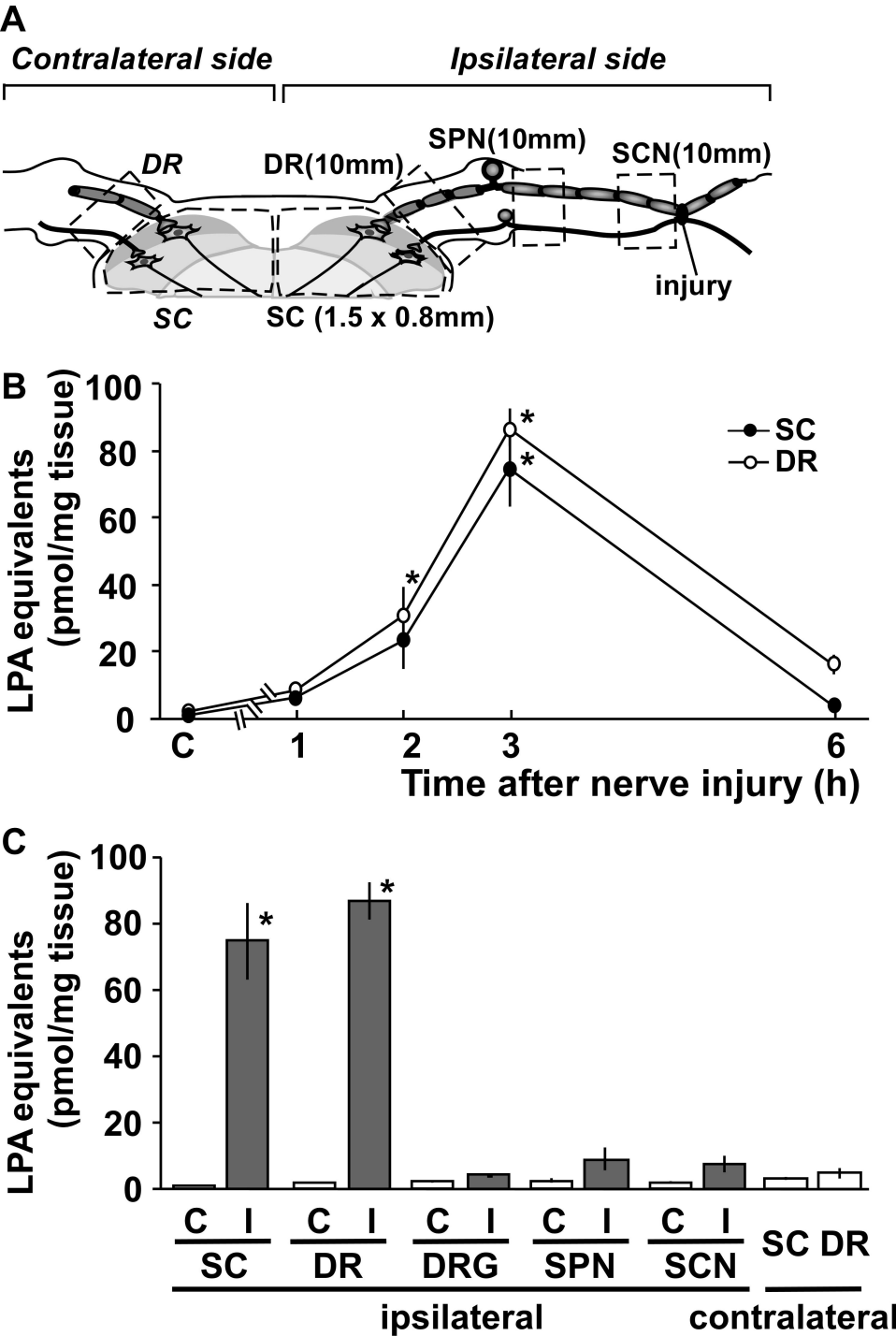

A
Contralateral side
Ipsilateral side
DR
DR(10mm)
SPN(10mm)
SCN(10mm)
injury
SC
SC (1.5 x 0.8mm)
B
LPA equivalents (pmol/mg tissue)
100
80
60
40
20
0
SC
DR
*
C
1
2
3
6
Time after nerve injury (h)
C
LPA equivalents (pmol/mg tissue)
100
80
60
40
20
0
*
C I
SC
DR
DRG
SPN
SCN
SC DR
ipsilateral
contralateral

Fig.2

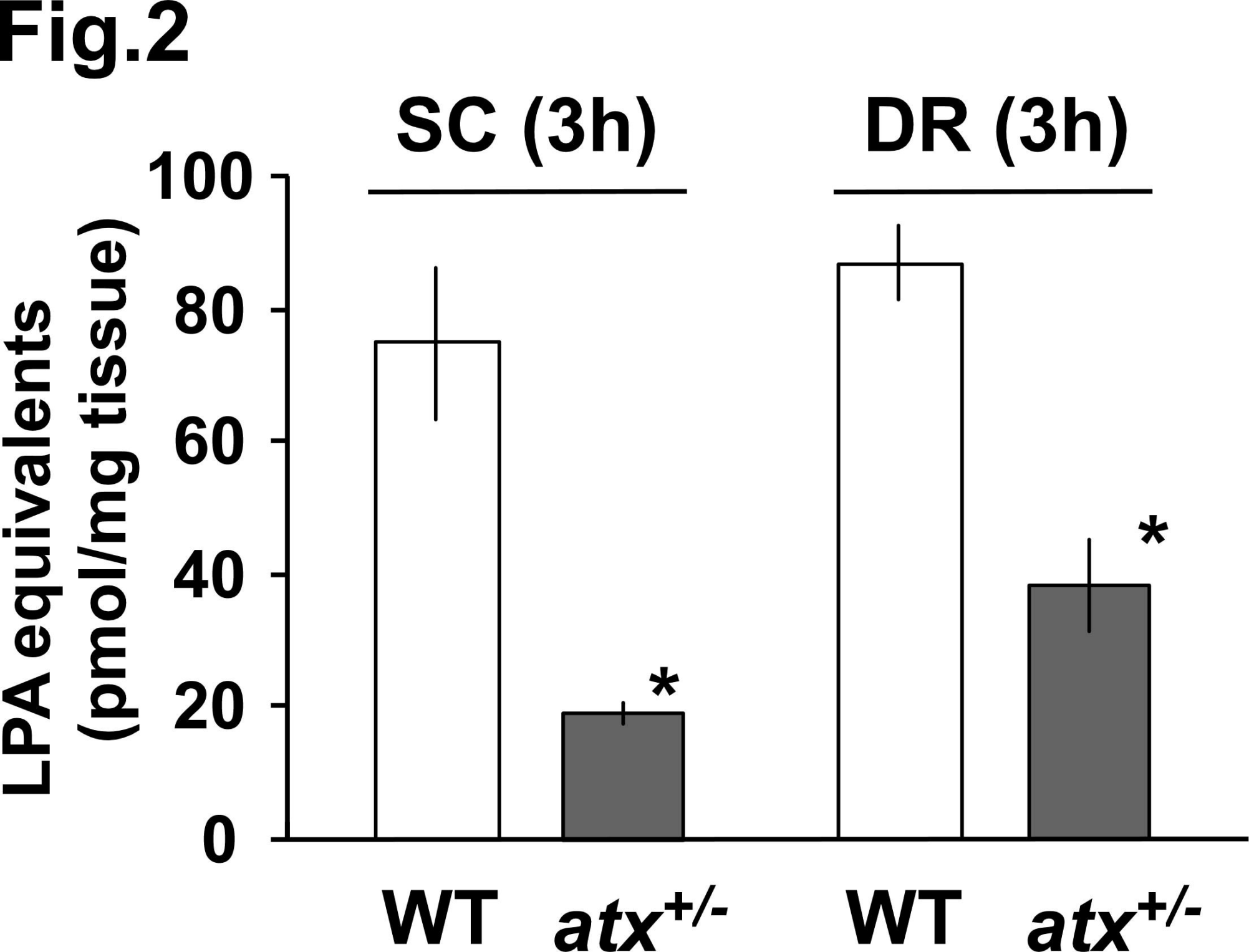

SC (3h)
DR (3h)
LPA equivalents
(pmol/mg tissue)
100
80
60
40
20
0
*
*
WT
atx+/-
WT
atx+/-

Fig.3

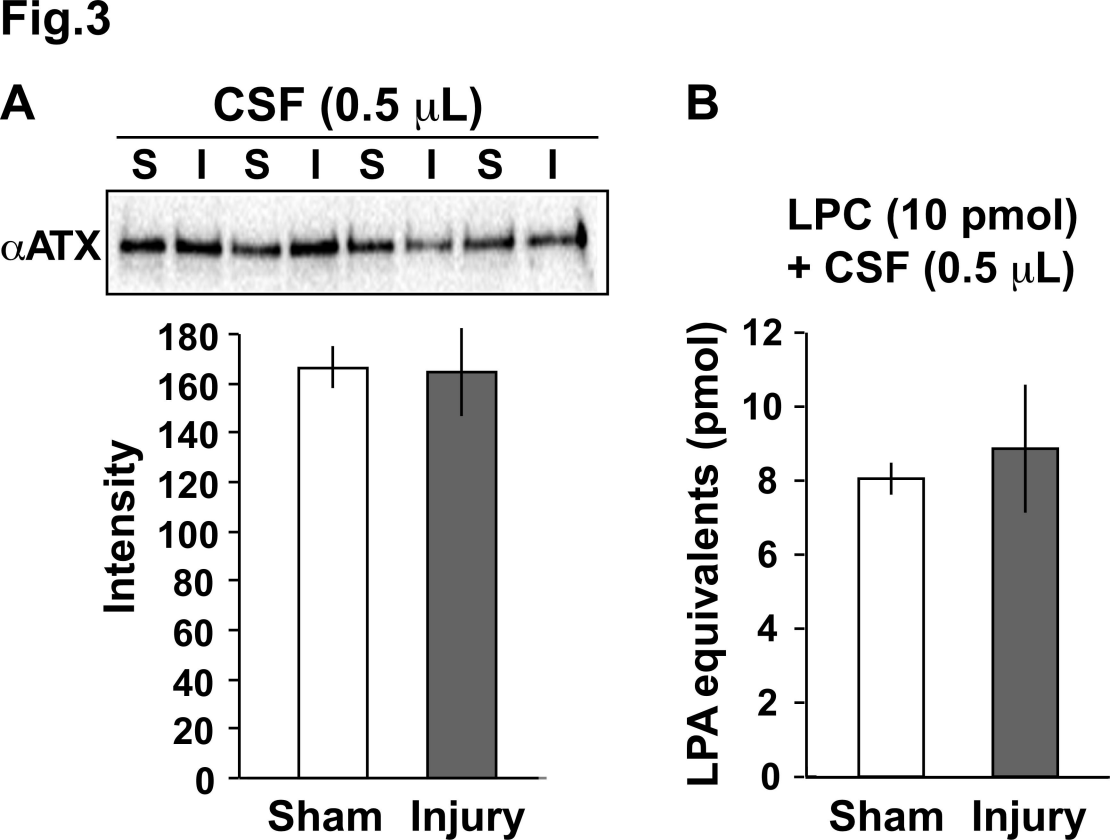

A
CSF (0.5 μL)
S I S I S I S I
αATX
Intensity
180
160
140
120
100
80
60
40
20
0
Sham
Injury
B
LPC (10 pmol)
+ CSF (0.5 μL)
LPA equivalents (pmol)
12
10
8
6
4
2
0
Sham
Injury

Fig.4

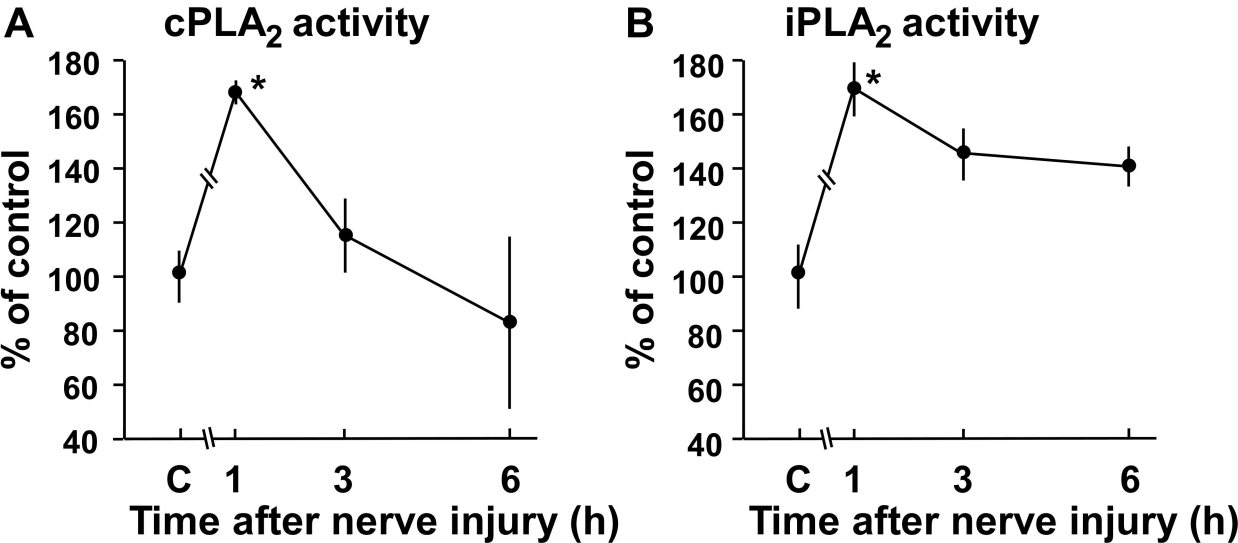
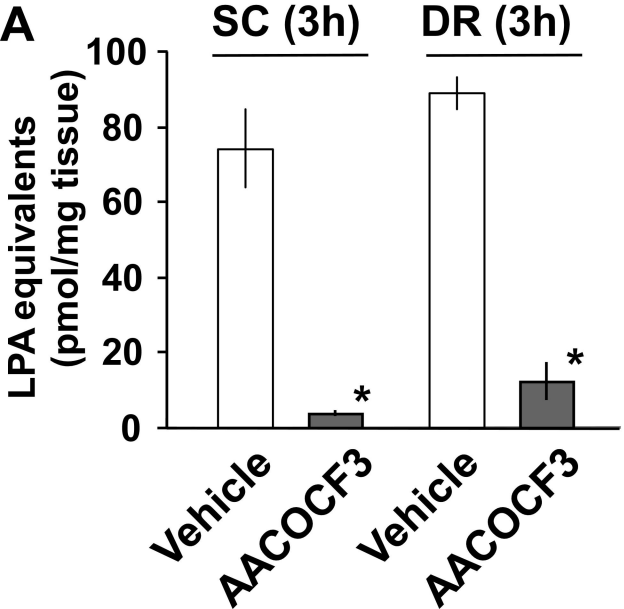

A
cPLA2 activity
180
160
140
120
100
80
60
40
% of control
*
C
1
3
6
Time after nerve injury (h)
B
iPLA2 activity
180
160
140
120
100
80
60
40
% of control
*
C
1
3
6
Time after nerve injury (h)

Fig.5


A
SC (3h)
DR (3h)
LPA equivalents (pmol/mg tissue)
100
80
60
40
20
0
*
*
Vehicle
AACOCF3
Vehicle
AACOCF3

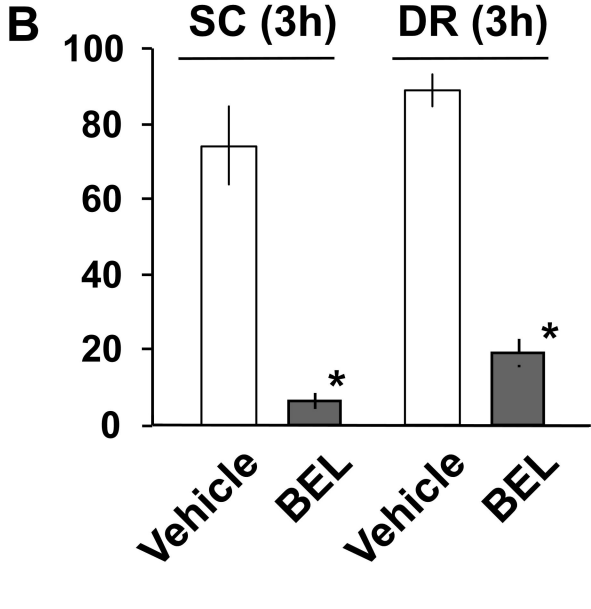

B
SC (3h)
DR (3h)
100
80
60
40
20
0
*
*
Vehicle
BEL
Vehicle
BEL

Fig.6

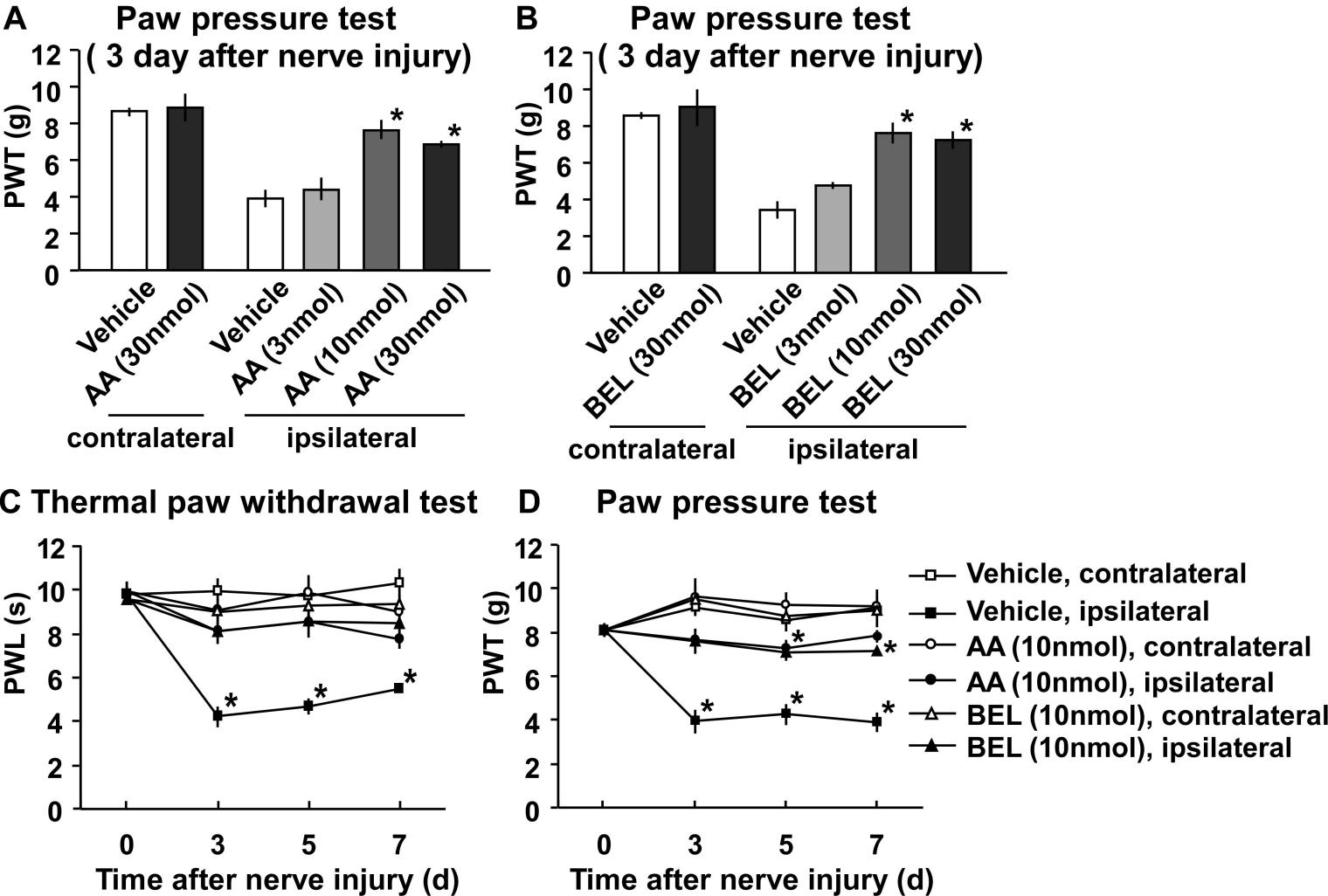

A
Paw pressure test
(3 day after nerve injury)
PWT (g)
12
10
8
6
4
2
0
Vehicle
AA (30nmol)
Vehicle
AA (3nmol)
AA (10nmol)
AA (30nmol)
contralateral
ipsilateral
B
Paw pressure test
(3 day after nerve injury)
PWT (g)
Vehicle
BEL (30nmol)
Vehicle
BEL (3nmol)
BEL (10nmol)
BEL (30nmol)
contralateral
ipsilateral
C Thermal paw withdrawal test
PWL (s)
0
3
5
7
Time after nerve injury (d)
D
Paw pressure test
PWT (g)
Time after nerve injury (d)
Vehicle, contralateral
Vehicle, ipsilateral
AA (10nmol), contralateral
AA (10nmol), ipsilateral
BEL (10nmol), contralateral
BEL (10nmol), ipsilateral

Fig.7

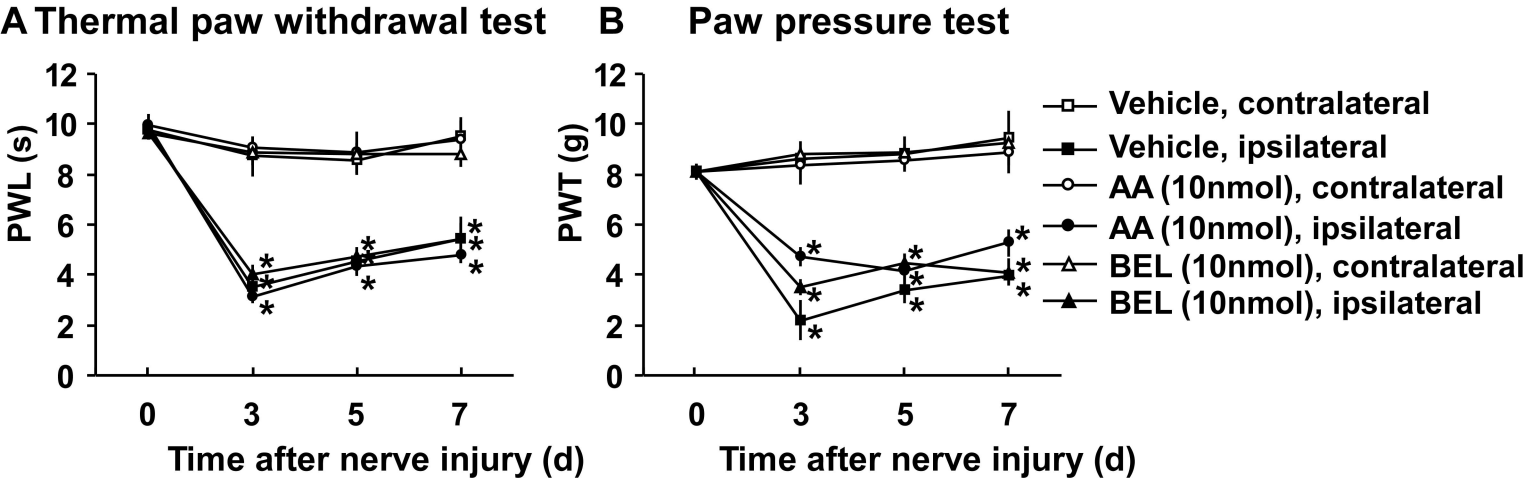
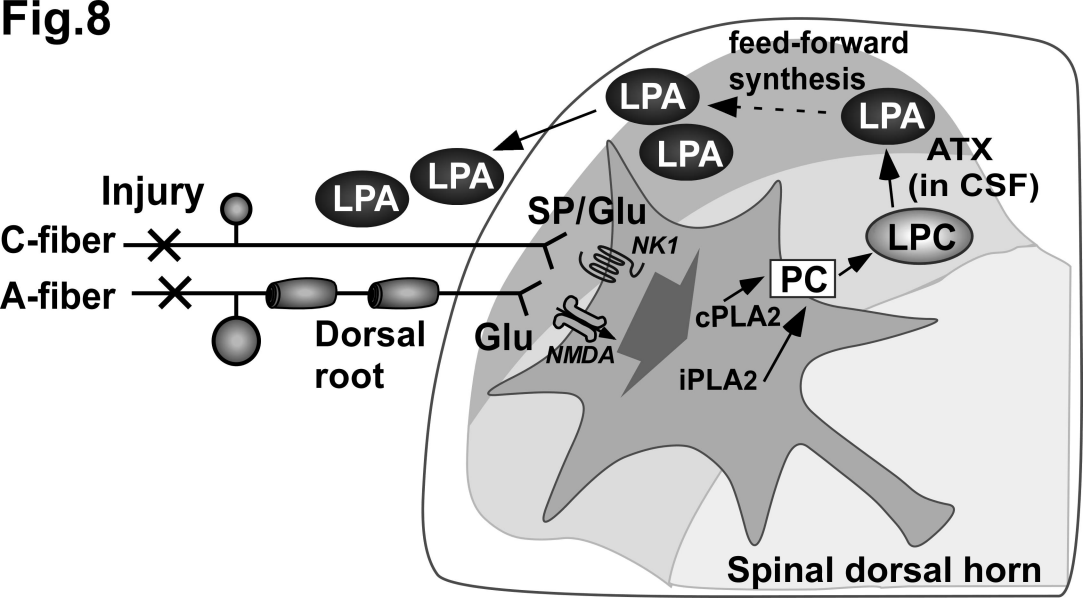

A Thermal paw withdrawal test
PWL (s)
12
10
8
6
4
2
0
0
3
5
7
Time after nerve injury (d)
B
Paw pressure test
PWT (g)
12
10
8
6
4
2
0
0
3
5
7
Time after nerve injury (d)
Vehicle, contralateral
Vehicle, ipsilateral
AA (10nmol), contralateral
AA (10nmol), ipsilateral
BEL (10nmol), contralateral
BEL (10nmol), ipsilateral

Fig.8


feed-forward
synthesis
LPA
LPA
LPA
LPA
LPA
ATX
(in CSF)
Injury
C-fiber
A-fiber
SP/Glu
NK1
LPC
PC
cPLA2
Glu
NMDA
iPLA2
Dorsal
root
Spinal dorsal horn