

Initiation of neuropathic pain requires lysophosphatidic acid receptor signaling

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Lysophosphatidic acid (LPA) is a bioactive lipid with activity in the nervous system mediated by G-protein-coupled receptors. Here, we examined the role of LPA signaling in the development of neuropathic pain by pharmacological and genetic approaches, including the use of mice lacking the LPA₁ receptor. Wild-type animals with nerve injury develop behavioral allodynia and hyperalgesia paralleled by demyelination in the dorsal root and increased expression of both the protein kinase C γ -isoform within the spinal cord dorsal horn and the $\alpha_2\delta_1$ calcium channel subunit in dorsal root ganglia. Intrathecal injection of LPA induced behavioral, morphological and biochemical changes similar to those observed after nerve ligation. In contrast, mice lacking a single LPA receptor (LPA₁, also known as EDG2) that activates the Rho–Rho kinase pathway do not develop signs of neuropathic pain after peripheral nerve injury. Inhibitors of Rho and Rho kinase also prevented these signs of neuropathic pain. These results imply that receptor-mediated LPA signaling is crucial in the initiation of neuropathic pain.

Human peripheral nerve injury can lead to a pain state referred to as neuropathic pain, with symptoms including continuous burning pain and abnormal sensory sensations such as allodynia (pain as a result of non-noxious stimuli) and hyperalgesia (an increased response to a normally painful stimuli)^{1,2}. The use of experimental animal models has identified many associated cellular events, ranging from segmental demyelination of peripheral nerves to central sensitization^{1,2}. Inflammatory mediators and products of tissue damage such as growth factors^{3,4} are candidates for initiators of neuropathic pain^{5–8}. Lipid metabolites are an additional class of possible initiators, although comparatively little is known about their role in the development of neuropathic pain.

LPA is one such lipid metabolite that is released after tissue injury^{9,10} and, as such, is an attractive candidate for a signaling molecule involved in the development of neuropathic pain. LPA is a small phospholipid that produces activation of cognate G-protein-coupled receptors^{11,12}. A major potential source of LPA is serum, in which it is produced by activated platelets and can reach micromolar concentrations¹¹. Damage to the nervous system that results in serum leakage at the site of an injury can result in substantial exposure to LPA for neural cells¹³.

LPA receptors have numerous activities in the nervous system^{14–16} and can activate several signaling pathways¹⁷ by means of multiple G-proteins^{11,12}. Direct stimulation of peripheral nociceptor endings by LPA through LPA₁ (also known as EDG2) receptors also implies that LPA has a role in nociceptive processes^{18,19}. Receptor-mediated LPA signaling through G $\alpha_{12/13}$ activates the small GTPase RhoA^{11,12,20,21}. In the active state, Rho translocates to the plasma membrane and relays extracellular signals to multiple downstream effectors including Rho kinase (ROCK), which can be inhibited by a pyridine derivative

compound, Y-27632. Inhibition of the Rho pathway can also be accomplished by selective adenosine diphosphate (ADP)-ribosylation of RhoA using *Clostridium botulinum* C3 exoenzyme (BoTXC3).

Previously, we found that intrathecal injection of BoTXC3 before peripheral nerve injury blocked the development of hyperalgesia in mice²², indicating that LPA receptors might pathophysiologically activate Rho in neuropathic pain states involving peripheral nerve injury. Using a standard animal model, we report here that activation of the LPA₁ receptor and its downstream Rho–ROCK pathway is required for the development of neuropathic pain.

RESULTS

Induction of allodynia and hyperalgesia by LPA

A single intrathecal injection of LPA in mice produced thermal hyperalgesia at 24 h in a dose-dependent manner in the tail-flick test (Fig. 1a). A single LPA injection also produced mechanical allodynia that persisted for at least 7–8 d before returning to baseline levels at day 13 (Fig. 1b). Further, LPA produced hyperalgesia in the thermal paw-withdrawal test (Supplementary Fig. 1 online). However, another LPA-like phospholipid, sphingosine 1-phosphate (S1P, intrathecal), which signals through the S1P receptors^{11,12}, did not produce any sign of mechanical allodynia (Fig. 1c).

Selective ADP-ribosylation of RhoA using BoTXC3 dose-dependently blocked the LPA-induced thermal hyperalgesia in the tail-flick test, whereas it had no effects on the latency of control, vehicle-treated mice (Fig. 1d). Similarly, BoTXC3 abolished mechanical allodynia (Fig. 1e) and thermal hyperalgesia in the thermal paw-withdrawal test (data not shown). All results indicate that these effects of LPA required activation of RhoA. Systemic pretreatments with Y-27632, a reversible

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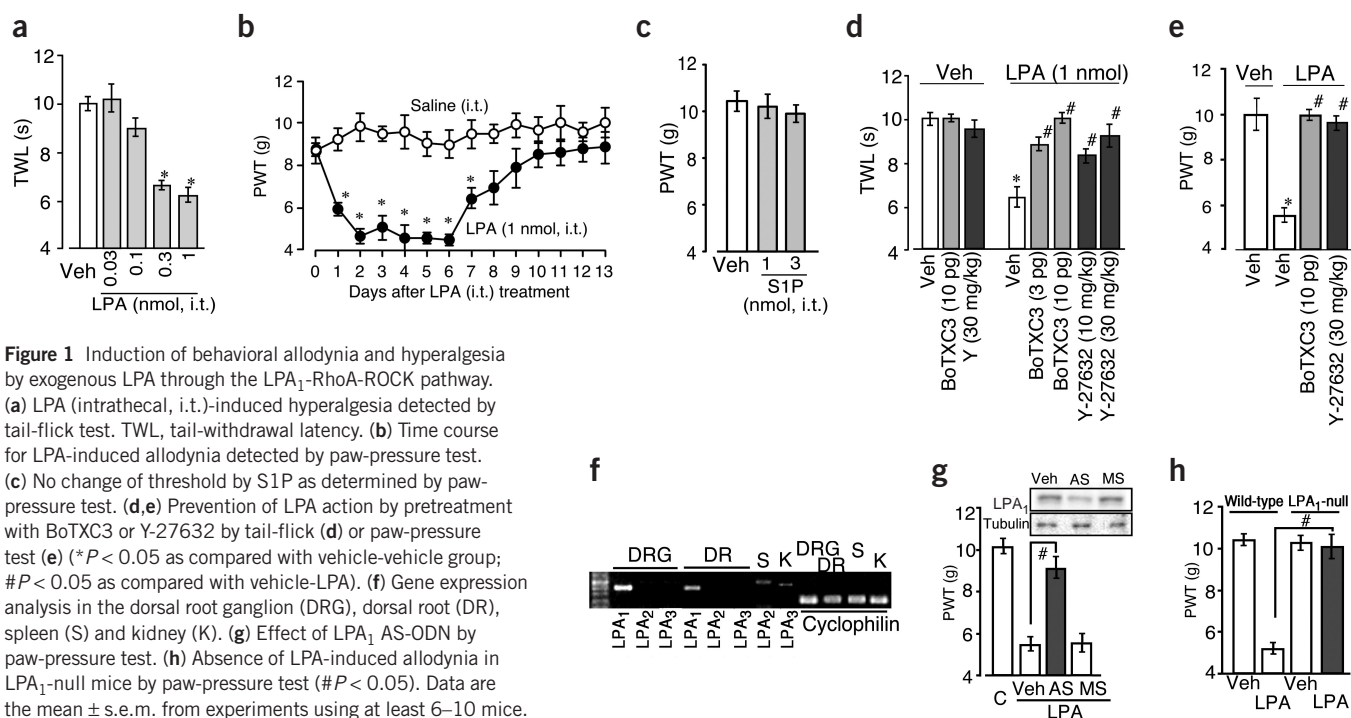


Figure 1 Induction of behavioral allodynia and hyperalgesia by exogenous LPA through the LPA₁-RhoA-ROCK pathway. (a) LPA (intrathecal, i.t.)-induced hyperalgesia detected by tail-flick test. TWL, tail-withdrawal latency. (b) Time course for LPA-induced allodynia detected by paw-pressure test. (c) No change of threshold by S1P as determined by paw-pressure test. (d,e) Prevention of LPA action by pretreatment with BoTXC3 or Y-27632 by tail-flick (d) or paw-pressure test (e) (**P* < 0.05 as compared with vehicle-vehicle group; #*P* < 0.05 as compared with vehicle-LPA). (f) Gene expression analysis in the dorsal root ganglion (DRG), dorsal root (DR), spleen (S) and kidney (K). (g) Effect of LPA₁ AS-ODN by paw-pressure test. (h) Absence of LPA-induced allodynia in LPA₁-null mice by paw-pressure test (#*P* < 0.05). Data are the mean ± s.e.m. from experiments using at least 6–10 mice.

inhibitor of ROCK (ref. 23), also completely abolished the LPA-induced hyperalgesia in the tail-flick test (Fig. 1d) and the thermal paw-withdrawal test (data not shown) and allodynia in the paw-pressure test (Fig. 1e). In addition, a single intrathecal pretreatment with Y-27632 also dose-dependently blocked the LPA-induced hyperalgesia in the tail-flick test (Supplementary Fig. 1 online). Intrathecal injection of Y-27632 (10 nmol) also prevented LPA-induced allodynia (Supplementary Fig. 1 online). Y-27632 had no effect on the withdrawal latency of control, vehicle-treated mice (Fig. 1d). These data indicate that the activation of RhoA and ROCK is essential for the development of hyperalgesia and allodynia induced by LPA treatment.

LPA can act through G-protein-coupled LPA receptors designated LPA₁, LPA₂ (EDG4) and LPA₃ (EDG7); a recently reported fourth receptor, LPA₄, shows different G-protein interactions and was not examined here. To confirm the type and location of LPA receptors that mediate intrathecal LPA-induced events, we first measured gene expression in neurons and Schwann cells of the dorsal root ganglion

(DRG), in the dorsal root. By RT-PCR, we confirmed that mainly LPA₁, but not LPA₂ or LPA₃, was expressed in both DRG and dorsal root (Fig. 1f), consistent with a previous study with cultured Schwann cells and sciatic nerve²⁴. We found that DRG cells, which mainly contain neuronal cells, treated with cytosine arabinoside expressed LPA₁ at a level similar to that in untreated DRG cells (Supplementary Fig. 2 online), indicating that LPA₁ mRNA obtained from the DRG is predominantly of neuronal origin. When mice were pretreated intrathecally with LPA₁ antisense oligodeoxynucleotide (AS-ODN) before LPA treatment, the development of LPA-induced mechanical allodynia was completely prevented, whereas the missense ODN (MS-ODN) had no effect (Fig. 1g). Similar blockade of LPA-induced mechanical allodynia was also observed in LPA₁-deficient mice. LPA₁ AS-ODN administered intrathecally significantly reduced the expression of LPA₁ in DRG (*P* < 0.05; Fig. 1g and Supplementary Fig. 2 online). On the other hand, LPA₁ expression was also observed in the spinal cord but not in the sympathetic ganglia (Supplementary Fig. 2 online). However, the

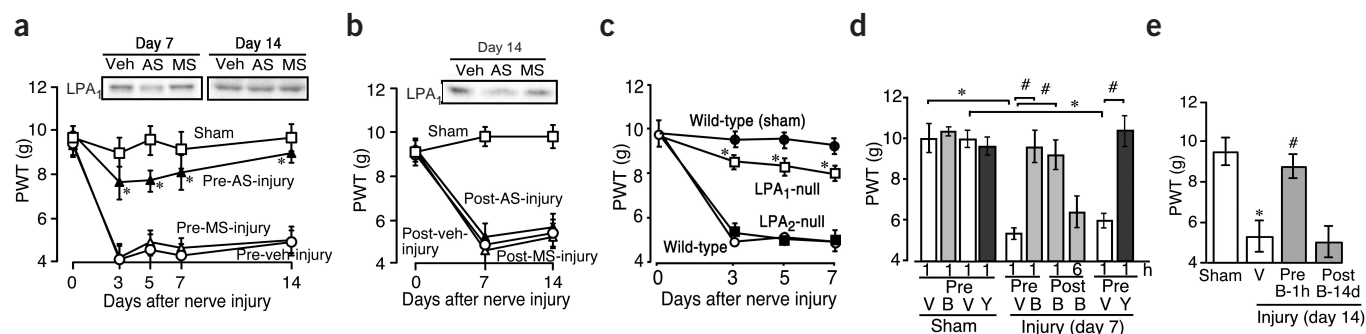
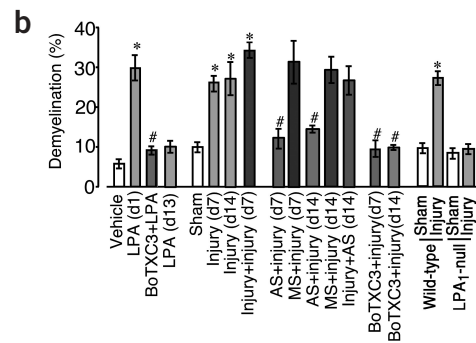
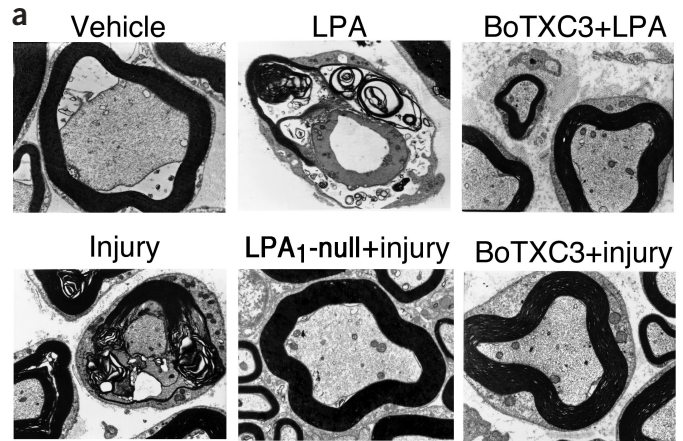


Figure 2 Attenuated neuropathic pain after nerve injury in LPA₁-deficient mice and BoTXC3-treated mice, as detected by paw-pressure test. (a) Effect of preinjury injection of LPA₁ AS-ODN in nerve-injured mice. (b) Effect of postinjury injection of AS-ODN. (c) Absence of injury-induced allodynia in LPA₁-deficient mice. (d) Prevention of allodynia by BoTXC3. V, vehicle; B, BoTXC3; Y, Y-27632; 1, 1 h; 6, 6 h. (e) Effects of BoTXC3 at day 14. BoTXC3 was delivered 1 h before (pre-B-1h) or at day 14 (post-B-14d). **P* < 0.05. #*P* < 0.05. Data in all figures were the mean ± s.e.m. from experiments using 6–10 mice.

Figure 3 Absence of injury-induced dorsal root demyelination in LPA₁-deficient or BoTXC3-treated mice. **(a)** LPA (top)- or injury (bottom)-induced dorsal root demyelination at 1 d after LPA injection or 7 d after injury. There was no demyelination after injury in LPA₁-deficient or BoTXC3-treated mice. **(b)** Percent of demyelination in LPA-treated and nerve-injured mice with various treatments (LPA at days 1, 13; LPA with BoTXC3; nerve injury days 7, 14 with preinjury AS-ODN treatment; nerve injury with BoTXC3; and LPA₁-deficient mice; *n* = 3. Injury + injury represents data from animal treated with simultaneous ligation to one-half and residual half of sciatic nerve to make full ligation. **P* < 0.05 as compared with vehicle- or sham-operated group. #*P* < 0.05 as compared with LPA-treated or injured group).



AS-ODN caused a slight, but not significant, decrease in expression, possibly caused in part by limited penetrability of intrathecal AS-ODN into the spinal cord²⁵. Thus, these findings indicate that primary afferent neurons may be important in mechanical allodynia mediated through the LPA₁ receptor, although the contribution of spinal mechanisms cannot be excluded.

Neuropathic pain through LPA₁ and Rho-ROCK

As LPA is a lipid metabolite that is released after tissue injury^{9,10}, LPA and its receptor (LPA₁) could plausibly underlie signaling mechanisms for the development of neuropathic pain. Partial sciatic nerve injury in mice caused robust allodynia and hyperalgesia starting from day 3 after nerve injury, which persisted throughout days 5, 7 and 14 (Fig. 2a and Supplementary Fig. 1 online), consistent with previous reports^{26,27}. The LPA₁ AS-ODN, when introduced into mice before the nerve injury operation, significantly inhibited the injury-induced allodynia and hyperalgesia, but the MS-ODN did not (*P* < 0.05; Fig. 2a and Supplementary Fig. 1 online). The effect of AS-ODN continued at day 14, even when it was discontinued from day 5 after injury (Fig. 2a and Supplementary Fig. 1 online). Significant reduction of LPA₁ expression in DRG, but not in spinal cord, by AS-ODN at day 7 and return of expression at day 14 owing to discontinuation was also confirmed by western blot analysis (*P* < 0.05; Fig. 2a and Supplementary Fig. 2 online). However, postinjury injection of AS-ODN did not prevent the allodynia or hyperalgesia induced by nerve injury, although

LPA₁ expression in DRG was decreased (Fig. 2b and Supplementary Figs. 1 and 2 online). We further observed that LPA₁-deficient mice did not develop allodynia and hyperalgesia at days 3, 5 and 7 after nerve injury (Fig. 2c and Supplementary Fig. 1 online). However, LPA₁-deficient mice developed robust allodynia and hyperalgesia (Fig. 2c and Supplementary Fig. 1 online).

Intrathecal injection of the Rho inhibitor BoTXC3 1 h before or 1 h after, but not 6 h after the nerve injury completely prevented the development of mechanical allodynia observed at 7 d after partial sciatic nerve ligation (Fig. 2d). Systemic pretreatments with the ROCK inhibitor Y-27632 also completely abolished the allodynia induced by nerve injury (Fig. 2d). In addition, intrathecal pretreatment with Y-27632 1 h before nerve injury also prevented the development of allodynia in the injured mice (Supplementary Fig. 1 online). BoTXC3 and Y-27632 similarly prevented the development of allodynia in a different strain of mouse (C57BL6/J mice; data not shown). BoTXC3 and Y-27632 pretreatments also prevented development of hyperalgesia in nerve-injured mice (Supplementary Fig. 1 online). However, these treatments had no effect on the withdrawal threshold or latency of control sham-operated mice (Fig. 2d and Supplementary Fig. 1 online). Moreover, whereas a single, preinjury intrathecal injection of

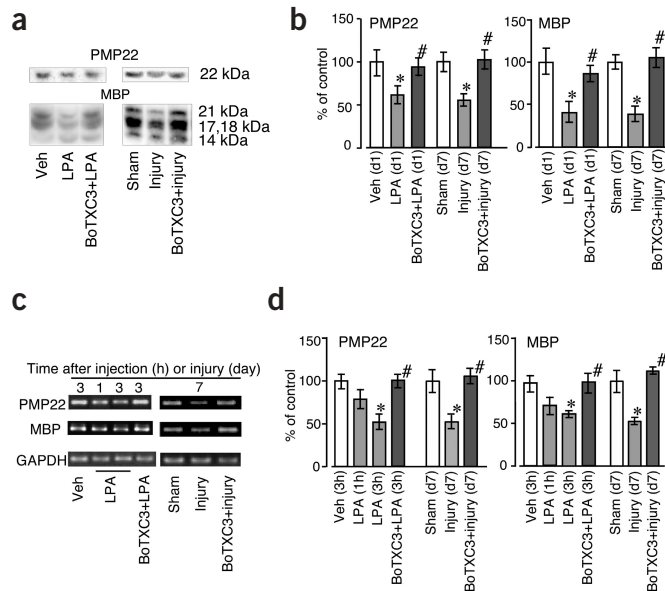


Figure 4 Blockade of LPA- or injury-induced reduction of MBP and PMP22 expression in the dorsal root by BoTXC3. **(a,b)** Protein expression of MBP and PMP22 in dorsal root after LPA treatment or nerve injury at 1 d after LPA injection or 7 d after nerve injury, and effects of BoTXC3 (10 pg) pretreatment, as detected by western blotting. **(c,d)** mRNA expression of MBP and PMP22 in dorsal root after LPA treatment or nerve injury at 1 h or 3 h after LPA injection or 7 d after nerve injury, as detected by RT-PCR. Quantification of western blot **(b)** and RT-PCR **(d)** data for PMP22 and MBP expression is shown at right. **P* < 0.05. #*P* < 0.05.



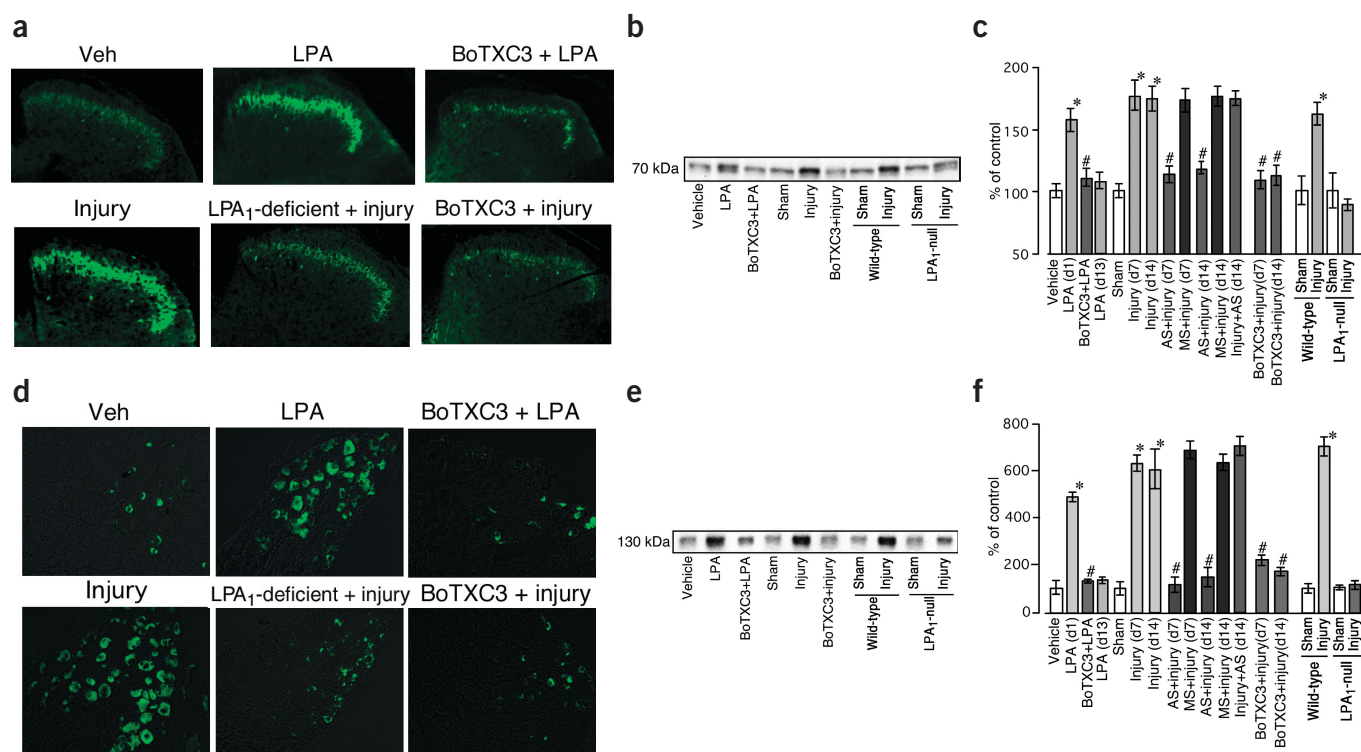


Figure 5 Absence of injury-induced upregulation of PKC γ and Ca α 2 δ 1 expression in LPA $_1$ -deficient mice or BoTXC3-treated mice. (a, d) Photomicrographs showing PKC γ immunostaining in the spinal dorsal horn (a) or Ca α 2 δ 1 immunostaining in DRG (d). All experiments were evaluated 1 d after LPA treatment or 7 d after injury with and without BoTXC3 (10 pg) pretreatment. (b, c, e, f) Western blot analyses of PKC γ in the spinal cord (b, c) or Ca α 2 δ 1 in DRG (e, f). * P < 0.05 as compared with vehicle-treated group or sham-operated group. # P < 0.05 as compared with LPA-treated group or nerve injured group.

BoTXC3 prevented the development of nerve injury-induced allodynia and hyperalgesia as observed at day 14 after injury, postinjury acute intrathecal injection of BoTXC3 at day 14 did not block the injury-induced allodynia or hyperalgesia (Fig. 2e and Supplementary Fig. 1 online). Together, these results indicate that activation of the LPA $_1$ receptor and its downstream Rho-ROCK pathway during the early phase after nerve injury is crucial for the development of allodynia and hyperalgesia.

Demyelination through LPA $_1$ and Rho

Aberrant myelination is associated with neuropathic pain, and receptor-mediated LPA signaling influences the morphology of Schwann cells²⁴. Thus, we examined the demyelination induced by LPA or nerve injury as well as the influences of targeted deletion of LPA $_1$ and inhibition of downstream RhoA mechanisms. Intrathecal injection of LPA (1 nmol) caused a demyelination of the dorsal root after 24 h, which was abolished by BoTXC3 (Fig. 3a, top). The demyelination was reversed, with myelination returning to a near-normal level, by day 13 after intrathecal LPA (Fig. 3b), by which time the behavioral hyperalgesia and allodynia had disappeared. Partial sciatic nerve injury in mice also caused demyelination in the distal dorsal root (Fig. 3a, b) and the proximal sciatic nerve (data not shown) at days 7 and 14 after injury. Although demyelination in the dorsal root was no different in sham-operated LPA $_1$ -deficient mice than in sham-operated wild-type mice, the injury-induced demyelination in the dorsal root was abolished in LPA $_1$ -deficient mice (Fig. 3a, b). Furthermore, the injury-induced dorsal root demyelination was also blocked by BoTXC3 treatment (Fig. 3a, b). A single, intrathecal preinjury injection of BoTXC3 prevented the demyelination at both day 7 and day 14

after injury (Fig. 3b). Moreover, preinjury injection with LPA $_1$ AS-ODN also prevented the injury-induced demyelination in dorsal root at days 7 and 14, even after discontinuation of AS-ODN injection from day 5 after injury (Fig. 3b). The simultaneous ligation of the residual half of sciatic nerves showed only a slight increase in demyelination (Fig. 3b), implying that only a fraction of damaged fibers is necessary to cause the maximal demyelination, possibly through LPA, an extracellular signal.

We also measured the expression of myelin-associated proteins such as myelin basic protein (MBP) and peripheral myelin protein 22 kDa (PMP22) in the dorsal roots after LPA injection or peripheral nerve injury. Both LPA treatment and injury caused a significant decrease in the expression of MBP and PMP22 protein or mRNA, indicating the presence of demyelination after such treatments (Fig. 4). Moreover, pretreatment with BoTXC3 normalized both LPA-induced and nerve injury-induced reduction of expression of myelin proteins and mRNAs (Fig. 4). Considering the expression of LPA $_1$ receptors within the spinal cord (Supplementary Fig. 2 online), we measured the expression of MBP, a common marker of myelinating cells in the peripheral and central nervous systems, in the spinal cord of intrathecal LPA-treated or nerve-injured mice by western blot analysis to determine whether there is any demyelination. We found that intrathecal LPA caused a statistically significant reduction in MBP expression in spinal cord, whereas no changes in MBP expression were observed in spinal cord after nerve injury (Supplementary Fig. 2 online (P < 0.05)). These results indicate that injury-induced release of LPA from the area of the affected sciatic nerve did not extend out within the spinal cord, and thus caused demyelination mainly in the primary afferent neurons.

Expression of protein kinase C γ and Ca $\alpha_2\delta_1$

Peripheral nerve injury causes several biochemical changes in spinal cord and DRG neurons. Among them, the increased expressions of the γ -isoform of protein kinase C (PKC γ) in the spinal dorsal horn²⁸ and the $\alpha_2\delta_1$ subunit of the voltage-gated calcium channel (Ca $\alpha_2\delta_1$) in the DRG (ref. 29,30) are important markers of neuropathic pain. Intrathecal injection of LPA caused an increase in the expression of PKC γ in the spinal dorsal horn in a BoTXC3-reversible manner (Fig. 5a, top). Upregulation of PKC γ was also observed after partial sciatic nerve injury and was abolished by targeted deletion of LPA $_1$ and pretreatment with BoTXC3 (Fig. 5a, bottom). Western blot analysis further supported these LPA $_1$ receptor- and Rho-mediated changes of PKC γ upregulation (Fig. 5b,c). Similarly, upregulation of Ca $\alpha_2\delta_1$, was observed after LPA treatment of nerve injury and was abolished by targeted deletion of LPA $_1$ and pretreatment with BoTXC3 (Fig. 5d). Western blot analysis also supported the observation of upregulation (Fig. 5d–f).

DISCUSSION

Nerve injury recapitulated the behavioral, morphological and biochemical changes that are often observed in neuropathic pain patients, such as mechanical allodynia, thermal hyperalgesia and demyelination, as well as upregulation of PKC γ and Ca $\alpha_2\delta_1$ expression, which have also been documented in experimental animals^{26–30}. Notably, these changes after nerve injury were abolished by pretreatment with AS-ODN for LPA $_1$ or by targeted deletion of LPA $_1$, and were mimicked by the intrathecal injection of LPA. The similarity between the underlying mechanisms of LPA-induced and nerve injury-induced changes is also observed in their sensitivity to pretreatment with the RhoA inhibitor BoTXC3 and the ROCK inhibitor Y-27632. Also, the fact that only preinjury, but not postinjury, injection of LPA $_1$ AS-ODN and BoTXC3 prevented the nerve injury-induced behavioral, morphological and biochemical changes implies that Rho- and ROCK-mediated events are involved in the initiation, rather than the maintenance, of neuropathic pain. It is also notable that the onset of mechanical allodynia became refractory to BoTXC3 as early as 6 h after nerve injury, further indicating that the injury-induced LPA release and activation of Rho is likely to occur at an early, upstream phase of the process.

Although the mechanisms underlying demyelination, and its relationship to allodynia or hyperalgesia induced by LPA or nerve injury, remain to be determined, it is evident that the LPA $_1$ receptor and Rho-mediated mechanisms are involved, as cellular and behavioral changes were sharply attenuated in LPA $_1$ -deficient mice and in mice pretreated with BoTXC3. There are reports that significant amounts of LPA $_1$ receptor exist on Schwann cells and that Rho GTPases regulate PMP22-dependent changes in altering the morphology of cultured Schwann cells^{31,32}, which may tie LPA signaling to other identified phenomena. For example, when the myelin sheath is damaged, action potentials generated in one axon can lead to membrane potential changes in adjacent axons that put them closer to threshold, perhaps even causing an ectopic action potential. It has been reported that Wallerian degeneration-induced release of cytokines is a crucial factor in the development of hyperalgesia in animal models of neuropathic pain⁴. Support for a role of myelin sheath breakdown in the development of neuropathic pain also comes from observations of individuals with Guillain-Barre syndrome³³ and Charcot-Marie-Tooth type I disease³⁴ and from mice deficient in the myelin protein periaxin³⁵, in which neuropathic pain develops as a result of aberrant myelination (splitting, detachment and loss of myelin). We speculate that LPA signaling might also participate in these peripheral myelin diseases.

Peripheral nerve injury in humans and animals causes several biochemical changes in the DRG and spinal cord that are also regarded as markers of neuropathic pain^{36–38}. Markers of neuropathic pain include increased expression of PKC in spinal dorsal horn and of calcium channels in DRG (refs. 30,31,38,39). The activation of PKC in the spinal cord dorsal horn, which triggers sustained activation of *N*-methyl-D-aspartate receptors, is believed to underlie the process of central sensitization^{38,39}. Among different isoforms of PKC, the γ -isoform is well studied with regard to neuropathic pain. Increased expression of PKC γ is well documented in animal models of peripheral neuropathy^{40,41}. Reduced hyperalgesia has also been observed after peripheral nerve injury in mice lacking PKC γ ²⁸. Increased expression of Ca $\alpha_2\delta_1$ in the DRG is also likely to be important in the development of allodynia induced by peripheral nerve injury^{29,30}. We observed the prevention of both LPA- and nerve injury-dependent increased expression of PKC γ and Ca $\alpha_2\delta_1$ in LPA $_1$ -deficient mice and in mice treated with BoTXC3, indicating that LPA $_1$ and Rho activation are among the cellular events involved in the initiation of neuropathic pain. Although we do not know exactly how LPA-induced increases in the expression of PKC γ and Ca $\alpha_2\delta_1$ initiated neuropathic pain, one possibility might be through increased excitability of spinal and DRG neurons followed by central sensitization. Finally, LPA-induced behavioral and functional changes were reversed earlier than injury-induced functional and behavioral changes, which are reported to continue longer²⁷. Our results imply that although LPA $_1$, Rho and ROCK signaling pathways are involved in the development of nerve injury-induced neuropathic pain, other factors are likely to be involved in the maintenance of such pain.

An overall model of the system and interactions is presented in **Supplementary Figure 3**. In the nerve injury-induced model, LPA at high concentrations is generated at the site of the wound by activated platelets, degenerating axons and/or damaged neurons. The LPA $_1$ receptors, also present on Schwann cells, mediate demyelination and upregulate the expression of Ca $\alpha_2\delta_1$ in DRGs. The demyelination and Ca $\alpha_2\delta_1$ upregulation are thought to underlie the sensitization of A δ / β nociceptive transmission, possibly through ephapsis, ectopic discharge and collateral sprouting leading to allodynia and hyperalgesia. PKC γ upregulation in lamina II of the dorsal spinal cord may now be involved in the process of central sensitization, which is thought to be a common cause of neuropathic allodynia and hyperalgesia^{28,40,41}. Altogether, these results indicate that LPA, signaling through the LPA $_1$ receptor and RhoA-ROCK activation, is crucial for the development of allodynia and hyperalgesia in the sciatic nerve injury model of neuropathic pain. Therefore, LPA, which can be easily released by activated platelets at the site of injury, might work as a signaling molecule to drive the induction of neuropathic pain. These findings also indicate that antagonists or inhibitors of these components of the LPA signaling pathway may have promise as analgesics for the treatment of initial phases of neuropathic pain.

METHODS

Animals. We used male standard ddY-strain and C57BL6/J mice weighing 20–25 g in most experiments. In some experiments LPA $_1$ -deficient, LPA $_2$ -deficient and sibling wild-type mice from the same genetic background^{42,43} were used. The baseline pain threshold with thermal and mechanical nociception test was the same in LPA $_1$ -deficient and wild-type mice. All procedures were approved by the Nagasaki University Animal Care Committee and complied with the recommendations of the International Association for the Study of Pain⁴⁴.

LPA injection and drug treatments. LPA (Avanti Polar-Lipids) was dissolved in 0.25% methanol in saline and injected intrathecally between lumbar regions 5 and 6. Most experiments were conducted 24 h after injection of LPA (1 nmol in

5 μ l). BoTXC3 was delivered intrathecally (3 or 10 μ g in 2 μ l). The ROCK inhibitor Y-27632 (Mitsubishi Pharma) was given intrathecally 1 h before LPA injection or nerve injury, or subcutaneously every 12 h starting from 1 h before LPA injection or nerve injury until the test day, when nociception tests were carried out 12 h after the final injection. AS-ODN (5' -AGCTGCCATGACAGTGTGT-3') and MS-ODN (5' -AGCAGCGTTGCTCACTGCAGT-3') for LPA₁ were synthesized. AS-ODN was injected intrathecally in a dose of 10 μ g/2 μ l on the first, third and fifth days, then nerve injury was done with subsequent injections of AS-ODN on days 1, 3 and 5 after nerve injury. The mice were used for assessing the hyperalgesia or the expression of LPA₁ on day 7 or 14 after injury (pre-AS-ODN treatments). AS-ODN or MS-ODN was injected on days 7, 9, 11 and 13 after injury, and experiments were performed on days 7 and 14 (post-AS-ODN treatments).

Injury of sciatic nerve and drug treatments. Partial injury to the sciatic nerve was carried out according to described methods²⁷. Briefly, the common sciatic nerve of the right hindlimb was exposed at the level of the high thigh through a small incision, and the dorsal one half of the nerve thickness was tightly ligated with a silk suture.

Quantification of demyelination. Semi-thin sections (1 μ m) were cut and stained with alkaline toluidine blue. The stained sections were observed a light microscopy and quantification was done according to a previous report⁴⁵, in which myelinated fibers with abnormally thick myelin sheaths were counted.

Immunohistochemistry, SDS-polyacrylamide gel electrophoresis and RT-PCR. Immunohistochemistry of PKC γ (1:500; Santa Cruz Biotechnology) and Ca $\alpha_2\delta_1$ (1:200; Sigma) was carried out as described²⁹. Western blot analyses were done according to described methods^{19,46}. The primary antibodies were used in the following dilutions: MBP (1:200; Ultraclone), PMP22 (1:500; Upstate), Ca α_2 (1:200; Sigma), PKC γ (1:200 Santa Cruz), β -tubulin (1:500 Santa Cruz) and LPA₁ (1:500; provided by J. Chun, The Scripps Research Institute, CA). RT-PCR analyses were carried out according to described methods^{19,46}. PCR amplification was done using *Taq* DNA polymerase (Takara) and primers specific for *Pmp22* (5'-GCAGCCTTCCTTCGTGTAACC-3' and 5'-ATGTCTATGAGGGTTCGGCGC-3'), *Mbp* (5'-GCAGCCTTCCTTCGTGTAACC-3' and 5'-ATGTCTATGAGGGTTCGGCGC-3'), *Edg2* (5'-ATCTTTGGC-TATGTTCCGA-3' and 5'-TTGCTGTGAACnCCAGCCA-3'), *Edg4* (5'-TGGCCTACTCTTCTCATGTTCCA-3' and 5'-GTGTCCAGCACACCACA AATGCC-3'), *Ppia* (cyclophilin) (5'-ATGGTCAACCCACCGTGT-3' and 5'-TATGGCGTGTGAAGTACCA-3') or *Gapd* (GAPDH) (5'-GTGAAGGTGCGTGTGAACGGATTT-3' and 5'-CACAGTCTTCTGGGTGGCAGTAT-3'). Data are presented graphically as a ratio of PMP22 (or MBP) to GAPDH mRNA. (Normalization was done using GAPDH mRNA levels as controls in the same reaction.)

Evaluation of nociceptive responses. Mechanical and thermal paw-withdrawal and tail-flick tests were performed as described⁴⁶. In the paw-pressure test, a mechanical stimulus was delivered to the mouse's paw using an automated Transducer filament with a tip diameter of 0.8–0.9 mm (Model 1601, IITC Inc.). It has the advantage over conventional von Frey filaments in that it measures the paw-withdrawal threshold at once and the result is shown digitally on a display screen. With this apparatus, a control response of 10 g was earlier adjusted for naive mice. This control response in naive mice is higher than the usual control response with von Frey filaments owing to the greater diameter of the tip of the transducer (0.8–0.9 mm, as compared with 0.1–0.2 mm in von Frey filaments) and consequent greater area of stimulation on the paw. Thresholds and latencies were determined as the average of three measurements per paw over a 10-min test period. Observers scoring the responses were blinded to the pretreatment given.

Statistical analysis. Statistical evaluations were done using the Scheffe's test for multiple comparisons, after one-way or repeated measured analysis of variance. Data were expressed as mean \pm s.e.m. Significance was established at $P < 0.05$.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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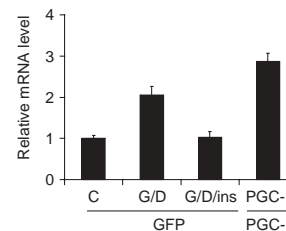
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ERRATUM: GC-1 promotes insulin resistance in liver through PPAR- α -dependent induction of TRB-3

Seung-Hoi Koo, Hiroaki Satoh, Stephan Herzig, Chih-Hao Lee, Susan Hedrick, Rohit Kulkarni, Ronald M Evans, Jerrold Olefsky & Marc Montminy
Nature Medicine 10, 530-534 (2004)

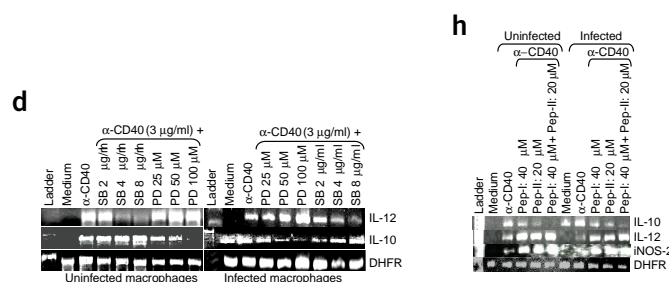
In the version of this article initially published online, the legend descriptions of Figure 3a and e were incorrect. They should read "(a) Western blot analysis of PGC-1, TRB-3 and Hsp-90 from fasted livers of diabetic mice injected with either GFP or PGC-1 RNAi adenovirus," and "(e) Western blot analysis of PGC-1, TRB-3 and Hsp-90 in livers of 6 h fasted PPAR- α -deficient mice injected with either GFP or PGC-1 RNAi adenovirus," respectively. In addition, Figure 3b contained an incorrect label ("PGC-1 RNAi"); the corrected version of the panel is shown to the right.



ERRATUM: Reciprocal CD40 signals through p38MAPK and ERK-1/2 induce counteracting immune responses

Ram Kumar Mathur, Amit Awasthi, Pallavi Wadhone, Boppana Ramanamurthy & Bhaskar Saha
Nat. Med. 10, 540-544 (2004)

In the version of this article originally published online, labels at the right side of Figures 2 were cut off. In Figure 2d, the labels should read (top to bottom) "IL-12" "IL-10", and "DHFR"; in Figure 2h, the labels should read (top to bottom) "IL-10", "IL-12", "iNOS-2" and "DHFR". The complete version of both panels are shown to the right.



In addition, the units for values reported in Figure 3g, lanes 4–6, were incorrectly listed as mmol (millimolar). The correct units should be μ mol (micromolar).

ERRATUM: Initiation of neuropathic pain requires lysophosphatidic acid receptor signaling

Makoto Inoue, Md Harunor Rashid, Ryousuke Fujita, James J A Contos, Jerold Chun & Hiroshi Ueda
Nat. Med. 10, 712-718 (2004)

In the version of this article initially published online, on p. 715, at the beginning of the second column, the antisense oligonucleotide was identified incorrectly. The first complete sentence of the column should begin, "Moreover, preinjury injection with LPA1 AS-ODN..."

In addition, the bottom curve in Figure 2c was labeled incorrectly. The label should read "Wild-type". The corrected figure panel is printed to the right.

