INTRODUCTION

Neuropathic pain is produced by nerve lesions or neurological conditions such as multiple sclerosis, diabetes, and cancer and affects an estimated 10% of the general population. Treatment options for individuals affected by neuropathic pain are limited and ineffective, often leading to a worsened condition and disability. Initiation and propagation of pain signaling occurs through afferent nerve fibers that relay peripheral signals through dorsal root ganglia (DRG) to signal...
via the central nervous system (CNS) spinal cord dorsal horn and brain (reviewed in 2-4). Neuropathic pain involves central sensitization, a process that results in allodynia (painful response to normally innocuous stimuli) and hyperalgesia (increased pain sensation to noxious stimuli).5

One identified modulator of neuropathic pain is the bioactive lipid lysophosphatidic acid (LPA). LPA normally signals through six known G protein-coupled receptors, \(LPA_1-6\), which are involved in myriad biological and pathological processes affecting most of the physiological systems in the body, including the nervous system.6-13 \(LPA_1\) is expressed in the peripheral nervous system (PNS) and CNS. Schwann cells represent one of the \(LPA_1\) expressing cell types that may be involved in the induction of neuropathic pain. LPA signaling through this receptor influences Schwann cell morphology, migration, and survival.14,15 In vivo, sciatic nerves of \(Lpar1\) deficient mice show abnormalities including an increased number of apoptotic Schwann cells, reduced myelin thickness, and a proportionately lower number of small nerve fiber interacting Schwann cells.14,16 Neurons can also be affected through \(LPA_1\)-mediated changes to cell morphology, motility, growth cone collapse, calcium signaling, and proliferation.16-23 Mice deficient for this receptor display alterations in cortical development and neurogenesis as well as behavioral abnormalities.22-24

A role for LPA in pain sensation was first identified through intrathecal (i.t.) injection of LPA, where mice that received a single i.t. injection of LPA developed thermal hyperalgesia and mechanical allodynia.25 LPA-induced neuropathic pain was accompanied by other sequelae including demyelination in the dorsal root and increased expression of pain associated markers including, protein kinase Cγ (PKCγ) in the spinal cord dorsal horn, and voltage-gated calcium channel Ca\(\text{a}_2\delta1\) in the DRG.25 Interestingly, i.t. injection of LPA also induced de novo production of LPA in the dorsal horn and dorsal root, implicating a feed-forward role in pain generation.26,27 De novo LPA production was also observed in the dorsal horn and dorsal root following PSNL.28-30 Wildtype (Wt) mice subject to PSNL displayed pain behaviors similar to those of mice that received i.t. LPA, and showed similar demyelination as well as upregulation of PKCγ and Ca\(\text{a}_2\delta1\).25

LPA’s effects in PSNL were shown to be receptor-dependent through the use of constitutive null receptor mutants. \(Lpar1\) null mutant mice were protected from both PSNL and i.t. LPA injection induced mechanical allodynia and did not show accompanying increases of PKCγ and Ca\(\text{a}_2\delta1\).25 \(Lpar5\) null mutant mice were also protected from PSNL-induced neuropathic pain, albeit through CNS mechanisms distinct from those of \(Lpar1\) null mutants.31

While \(Lpar1\) null mutant mice are protected from PSNL-induced neuropathic pain, the cell types responsible for mediating this protection remain unclear. To address this issue, we generated a \(Lpar1\) conditional null mutant mouse and targeted deletion of \(Lpar1\) in all neural lineages, peripheral and CNS neurons, Schwann cells, and microglia/myeloid cells to identify the cell types responsible for mediating \(Lpar1\)'s protective effect in the PSNL neuropathic pain model.

## 2 MATERIALS AND METHODS

### 2.1 Mice

All procedures performed on animals were IACUC approved and performed in accordance with the regulations of The Scripps Research Institute (TSRI) Department of Animal Resources and the Sanford Burnham Prebys Medical Discovery Institute animal care and use committee. Mice used in this study were nestin-cre (Jackson Laboratory Stock Number 003 771), \(P0\)-cre (Jackson Laboratory Stock Number 017 927), synapsin-cre (Jackson Laboratory Stock Number 003 966), and \(CD11b\)-cre (obtained from Don Cleveland) transgenic lines.

### 2.2 Synthesis of the \(Lpar1\) conditional gene targeting vector

Creation of the \(Lpar1\) conditional gene targeting vector was accomplished by PCR amplification of mouse \(Lpar1\) genomic fragments using a bacterial artificial chromosome (BAC RP23-149020 Children’s Hospital Oakland Research Institute (CHORI)) containing the \(Lpar1\) genomic locus as a template. PCR amplification was performed using \(Pfx50\) DNA polymerase (Invitrogen) and amplified genomic fragments were assembled into pBluescript II. During the process of assembly, a loxp site was inserted into a HindIII site 5’ of \(Lpar1\) exon 3 and a neomycin cassette under the control of the phosphoglycerate kinase promoter (PGK-neo) flanked by loxp sites was inserted directionally (all loxp sites in the same orientation) into an Xbal site 3’ of \(Lpar1\) exon 3 (Figure 1A). The construct was engineered so that 3.4 and 6.7 kb of \(Lpar1\) genomic DNA flanked the PGK-neo insertion site. To aid in cloning, BamHI and AattII restriction enzyme sites were added to the distal 5’ and 3’ ends of the \(Lpar1\) genomic segment chosen for targeting vector design. An EcoRI restriction enzyme site was included in the loxp flanked PGK-neo cassette to identify ES cell clones containing an allele that recombined homologously with the targeting vector.

### 2.3 Production of \(Lpar1^{lox/lox}\) and \(Lpar1^{lox/lox}\)-cell type-specific null mutant mice

To create the \(Lpar1^{lox/lox}\) mice, \(1 \times 10^7\) R1 ES cells were mixed with 50 µg of linearized \(Lpar1\) targeting vector in a 0.4 cm electroporation cuvette and the cells were pulsed with
**FIGURE 1**  Conditional gene targeting of the Lpar1 gene locus and identification of ES cells positive for homologous recombination. A, Schematic of the Lpar1 genomic locus, the region used for gene targeting, and the Lpar1 targeting vector. In the targeting vector, loxP sites flank Lpar1 exon 3 and the neomycin cassette used for ES cell drug resistance selection screening, an introduced EcoRI site allows for identification of homologous recombination events with the indicated external probe. Asterisks represent artificial restriction enzyme sites used in the construction of the targeting vector. B, Southern blot of EcoRI digested ES cell DNA hybridized with the radiolabeled probe shown in (A) identified an ES cell clone positive (+) for homologous recombination, as indicated by the presence of a 4.2 kb band. An ES cell clone with an incorrect recombination event (−) is shown for comparison and shows only the Wt 7.9 kb Lpar1 band. C, Four identified ES cell clones (9, 28, 37, and 65) were grown and homologous recombination was reconfirmed by Southern blotting. These clones were chosen for loxP site retention screening, clones 37 and 65 were used for used for blastocyst injections.

Male mice were chosen because cre is expressed in the germ-line of nestin-cre male mice. Offspring were then screened by PCR for the presence of the 5′ loxP site with the primers listed above, for the presence or absence of the PGK-neo cassette with primers A1 Exon 3 Forward 5′-gtaatgtgcttgcttgctgc-3′ and Neo Reverse 5′-tggaattgcttgctgctg-3′, and Neo Reverse 5′-tggaattgcttgctgctg-3′, and for retention of the loxP site 3′ to Lpar1 exon 3 with primers 3′ loxP Forward 5′-tggaattgcttgctgctg-3′ and 3′ loxP Reverse 5′-gtgagctgtgctgctgctg-3′. Mice that retained the loxP sites 5′ and 3′ to Lpar1 exon 3 but deleted the PGK-neo cassette were selected for propagation and crossing with nestin-cre, P0-cre, synapsin-cre, and CD11b-cre transgenic mice.32-35

PCR genotyping of the Lpar1 conditional mutant mice was done with the following primers: 5′ loxP Forward 5′-gtgagctgtgctgctgctg-3′, 3′ loxP Reverse 5′-tggaattgcttgctgctg-3′, and A1 Exon 3 Forward 5′-gtaatgtgcttgcttgctgc-3′. PCR amplification of genomic DNA with these primers identified Wt, Lpar1\(^{\text{flx}}\), and Lpar1 deleted products of 316, 354, and 242 bp, respectively. Synapsin-cre, CD11b, P0-cre, and nestin-cre transgenes were identified by PCR amplification.
of genomic DNA with a common reverse PCR primer, (Cre Reverse 5′-CAG CAT TGC TGT CAC TTG GTC-3′), and forward primers specific for synapsin (SynCreForward 5′-CCCAAGAAGAGGAAAGGTG-3′), CD11b (CD11b Forward 5′-ACACCTCAGCTCTCAGTAG-3′), P0 (MPZ Forward (P0 Cre) 5′-ATT GGT CAC TGG CTC AAG AC-3′), and nestin (Nestin Prom: 5′-ACT CCC TTC TCT AGT GCT CCA-3′) yielding products of 350 bp, 1 kb, 525 bp, and 550 bp, respectively.

2.4 | Southern blotting and DNA hybridization

ES cell clones were screened for homologous recombination by digesting 10 µg of ES cell DNA with EcoRI, running the DNA on a 0.8% TAE agarose gel, and transferring the digested DNA to Nytran SuPerCharge membrane (GE Healthcare Life Sciences) in 20 × SSPE. Transferred DNA was UV crosslinked to the membrane and hybridized with a 32P-labeled (Prime-It II Random Primer Labeling Kit, Agilent) Lpar1 probe with sequence external to the 5′ end of the targeting vector. The 800 bp probe was produced by PCR from a BAC containing Lpar1 with the following primers: A1 Ext Forward 5′-actgaggtcacttactcagag-3′ and A1 Ext Reverse 5′-gtctatggctgtggaattcaag-3′. Probe hybridization was carried out overnight at 42°C in a 0.05 M pH 7.4 phosphate buffer containing 50% formamide, 5 × SSPE, 1 × Denhart’s, 1% SDS, containing 1% denatured 10 mg/mL salmon sperm DNA following a 1 hour pre-hybridization. Blots were washed and visualized using a phosphorimager. The presence of a 4.2 kb recombined band and a 7.9 kb Wt band was indicative of ES cells with homologous recombination events.

2.5 | Partial sciatic nerve ligation and behavioral testing

The partial sciatic nerve ligation (PSNL) procedure was performed as described.31 Adult Lpar1\textsuperscript{flox/flox} and Lpar1\textsuperscript{flox/cre} transgenic mice in a C57BL/6J background were anesthetized via nosecone delivery of isoflurane and the right limb sciatic nerve exposed and tightly ligated with 10-0 fine sutures. The wound and skin were closed and stitched, and the animals allowed to recover. For behavioral testing, animals were acclimated in cages with wire mesh bottoms for 1 hour prior to testing in an environmentally controlled testing room. Paw withdrawal threshold (gram (g)) against increasing mechanical stimuli (0-50 g in 20 seconds) were measured before and following PSNL surgery with tests conducted four separate times with at least a 1 minute interval between tests. The average response was normalized to presurgery controls ± SEM.

2.6 | Immunohistochemistry

DRG were isolated from the lumbar region of Lpar1\textsuperscript{flox/flox} and Lpar1\textsuperscript{flox/cre} conditional null-mutant mice. Tissues were embedded in OCT compound and 5 µM sections were cut and immunolabeled with antibodies to mouse LPA1 (PA1 10401, Thermo Fisher Scientific) and MBP (ab134018, Abcam). Secondary antibodies were used against the listed primary antibodies and 60x images were acquired on a Zeiss Axio Imager D2 microscope.

2.7 | Reverse transcription PCR

DRG were isolated from the lumbar region of Lpar1\textsuperscript{flox/flox} and Lpar1\textsuperscript{flox/cre} conditional null-mutant mice. DRG were placed in 1 mL of TRIzol Reagent (Thermo Fisher Scientific) and total RNA was isolated according to the manufacturer’s directions. cDNA was synthesized from total RNA using a Bio-Rad iScript cDNA synthesis kit and β-actin and Lpar1-specific oligonucleotide primer pairs were used to amplify target gene transcripts. Primers used to amplify a 350 bp product from β actin cDNA were M β Actin Forward 5′-gacaccatgatgagccttctg-3′ and M β Actin Reverse 5′-CCCAAGAAGAAGAGGAAGGTG-3′, Probe hybridization was carried out overnight at 42°C in a 0.05 M pH 7.4 phosphate buffer containing 50% formamide, 5 × SSPE, 1 × Denhart’s, 1% SDS, containing 1% denatured 10 mg/mL salmon sperm DNA following a 1 hour pre-hybridization. Blots were washed and visualized using a phosphorimager. The presence of a 4.2 kb recombined band and a 7.9 kb Wt band was indicative of ES cells with homologous recombination events.

3 | RESULTS

3.1 | Generation of Lpar1 conditional null mutant mice

We selected a portion of the Lpar1 genomic locus for conditional gene targeting in embryonic stem (ES) cells to create a mutant mouse (Lpar1\textsuperscript{flox/flox}) where Lpar1 exon 3 is selectively deleted in the presence of the cre recombinase (Figure 1A). The targeting vector contained a loxP site that was introduced into a restriction enzyme site 5′ of exon 3, and a neomycin drug selection cassette flanked by loxP sites in a restriction enzyme site 3′ of exon 3. Following electroporation of the linearized Lpar1 targeting construct, drug selection, and screening of DNA isolated from selected ES cell clones by Southern blotting and hybridization, several clones with a homologously recombined Lpar1 allele were identified (Figure 1B,C). PCR with primers flanking the 5′ loxP site was used to select ES cell clones for blastocyst injection. Mouse positive for germline transmission of the recombined allele were then crossed with nestin-cre transgenic mice to produce Lpar1\textsuperscript{flox/flox-cre}}
mice. Because nestin is expressed in the testis, male Lpar1\textsuperscript{flox}\textsuperscript{flox}\textsuperscript{-nestin-cre} mice were bred to C57BL/6J female mice to produce offspring with germline cre-mediated loxP site recombination. Selective deletion of the floxed neomycin cassette and retention of the 5′ loxP site in offspring were identified by PCR (Figure 2A,B). Heterozygous Lpar1\textsuperscript{flox/+} mice with the correct recombination events were then crossed together to produce Wt, Lpar1\textsuperscript{flox/+}, and Lpar1\textsuperscript{flox/flox} mice (Figure 2C). A high level of embryonic lethality was observed for Lpar1 constitutive null mutant mice in a C57BL/6J background, whereas Lpar1\textsuperscript{flox/flox} mice in this background strain were healthy and indistinguishable from Wt littermates. Wt, Lpar1\textsuperscript{flox/+}, and Lpar1\textsuperscript{flox/flox} mice were differentiated by PCR (Figure 2C) and are behaviorally the same.

### 3.2 Cre-mediated Lpar1 targeted deletion

To delete Lpar1 in all neural cell types, neurons, Schwann cells, and myeloid lineage cells, Lpar1\textsuperscript{flox/flox} mice were crossed to nestin, synapsin, P0, and CD11b-cre transgenic mice, respectively.\textsuperscript{32-35} To confirm that Lpar1 was deleted in the presence of cre, genomic DNA was isolated from DRG of Lpar1\textsuperscript{flox/flox} and Lpar1\textsuperscript{flox/flox}\textsuperscript{-nestin-cre} mice and PCR was used to verify genomic recombination of the Lpar1 genomic locus to produce a null allele (Figure 3A). DRG contain both neural and nonneural cells, with conditional deletion limited to neural cells, thus producing a recombined (neural) and unrecombined (nonneural) signal in conditional mutants. As expected, PCR products indicative of both an unrecombined and recombinated Lpar1\textsuperscript{flox/flox} allele can be amplified from genomic DNA isolated from Lpar1\textsuperscript{flox/flox}\textsuperscript{-nestin-cre} DRG, while only an unrecombined product can be produced from the DRG of control Lpar1\textsuperscript{flox/flox} mice (Figure 3A). In agreement with genomic deletion of Lpar1, RT-PCR showed Lpar1 mRNA transcripts are absent in Lpar1\textsuperscript{flox/flox}\textsuperscript{-nestin-cre} DRG (Figure 3B). Following Schwann cell-specific P0-cre crossing, PCR analyses of sciatic nerve showed deletion of Lpar1 (Figure 3C) compared to Wt. Neuronal deletion was confirmed in the cerebral cortex (Ctx) of Lpar1\textsuperscript{flox/flox}\textsuperscript{-synapsin-cre} mice (Figure 3D). Immunofluorescent labeling of peripheral myelinated axons for myelin basic protein (MBP, red) and satellite glia expressing LPA1 (green) in Wt DRG (Figure 3E) was not observed in Lpar1\textsuperscript{flox/flox}\textsuperscript{-nestin-cre} mice (Figure 3F). These data demonstrate conditional deletion of Lpar1 in the presence of targeted cre recombinase expression.

### 3.3 Lpar1 expressing neural cell types contribute to PSNL-induced neuropathic pain phenotypes

To determine which Lpar1 expressing neural cell types mediate PSNL-induced neuropathic pain protection, paw...
withdrawal threshold responses following cre recombination for \textit{Lpar1\textsuperscript{flox/flox-nestin-cre}} transgenic (F/F NC Tg) DRG shows genomic deletion of \textit{Lpar1} exon 3 DNA from the tail of a Wt mouse is shown for comparison. B, qPCR products of cDNA prepared from \textit{Lpar1\textsuperscript{flox/flox}} (F/F) and \textit{Lpar1\textsuperscript{flox/flox-nestin-cre}} transgenic (F/F NC Tg) DRG shows that \textit{Lpar1} transcripts are lost in neural tissues. C, PCR of genomic DNA isolated from the tail of a Wt mouse (Wt) and the sciatic nerve of \textit{Lpar1\textsuperscript{flox/flox}} (F/F) and \textit{Lpar1\textsuperscript{flox/flox-P0-cre}} (F/F P0) mice. D, PCR amplification of genomic DNA isolated from the sciatic nerve (SN) and cortex (Ctx) of \textit{Lpar1\textsuperscript{flox/flox}} and \textit{Lpar1\textsuperscript{flox/flox-synapsin-cre}} mice. The PCR primers used for amplification of Wt (316 bp), \textit{Lpar1} floxed alleles (354 bp), and \textit{Lpar1} deleted products (242 bp) are identical to those used in Figure 2B. The 100, 200, 300, and 400 bp bands of the 1kb plus DNA ladder are indicated for reference. E and F, Immunofluorescent labeling of peripheral myelinated axons identify Wt LPA\textsubscript{1} immunolabeling in \textit{Lpar1\textsuperscript{flox/flox}} mice (E) and its absence (F) in \textit{Lpar1\textsuperscript{flox/flox-nestin-cre}} transgenic mice. LPA\textsubscript{1} labeling is in green and MBP (myelin) in red for individual samples. Scale bar = 100 µM.

**FIGURE 3** Functional deletion of \textit{Lpar1} is cre-dependent. A, PCR products of DNA isolated from \textit{Lpar1\textsuperscript{flox/flox}} (F/F) and \textit{Lpar1\textsuperscript{flox/flox-nestin-cre}} transgenic (F/F NC Tg) DRG shows genomic deletion of \textit{Lpar1} exon 3 DNA from the tail of a Wt mouse is shown for comparison. B, qPCR products of cDNA prepared from \textit{Lpar1\textsuperscript{flox/flox}} (F/F) and \textit{Lpar1\textsuperscript{flox/flox-nestin-cre}} transgenic (F/F NC Tg) DRG shows that \textit{Lpar1} transcripts are lost in neural tissues. C, PCR of genomic DNA isolated from the tail of a Wt mouse (Wt) and the sciatic nerve of \textit{Lpar1\textsuperscript{flox/flox}} (F/F) and \textit{Lpar1\textsuperscript{flox/flox-P0-cre}} (F/F P0) mice. D, PCR amplification of genomic DNA isolated from the sciatic nerve (SN) and cortex (Ctx) of \textit{Lpar1\textsuperscript{flox/flox}} and \textit{Lpar1\textsuperscript{flox/flox-synapsin-cre}} mice. The PCR primers used for amplification of Wt (316 bp), \textit{Lpar1} floxed alleles (354 bp), and \textit{Lpar1} deleted products (242 bp) are identical to those used in Figure 2B. The 100, 200, 300, and 400 bp bands of the 1kb plus DNA ladder are indicated for reference. E and F, Immunofluorescent labeling of peripheral myelinated axons identify Wt LPA\textsubscript{1} immunolabeling in \textit{Lpar1\textsuperscript{flox/flox}} mice (E) and its absence (F) in \textit{Lpar1\textsuperscript{flox/flox-nestin-cre}} transgenic mice. LPA\textsubscript{1} labeling is in green and MBP (myelin) in red for individual samples. Scale bar = 100 µM.

withdrawal threshold responses following cre recombination for \textit{Lpar1\textsuperscript{flox/flox-nestin}}. \textit{Lpar1\textsuperscript{flox/flox-synapsin}}, \textit{Lpar1\textsuperscript{flox/flox-P0}}, and \textit{Lpar1\textsuperscript{flox/flox-CD11b-cre}} were assessed. A rescued pain phenotype was observed for all genotypes compared to controls (Figure 4A-D). \textit{Lpar1\textsuperscript{flox/flox-nestin-cre}} conditional mutant mice challenged with PSNL had similar paw withdrawal threshold responses compared to previously defined \textit{Lpar1} constitutive null mutant mice\textsuperscript{25} (Figure 4A). By contrast, \textit{Lpar1\textsuperscript{flox/flox-P0-cre}} mice initially responded like control mice at early time points (Figure 4B; days 3 and 6), but then showed sustained protection at later time points (Figure 4E; day 9 through day 21). \textit{Lpar1\textsuperscript{flox/flox-synapsin-cre}} mice were initially refractory to PSNL-induced neuropathic pain (Figure 4C) but lost protection over time (Figure 4E; day 12 through day 21). Pain rescue was observed in \textit{Lpar1\textsuperscript{flox/flox-CD11b-cre}} mice compared to \textit{Lpar1\textsuperscript{flox/flox}} controls, with statistically significant protection observed at day 9 post-PSNL (Figure 4D). It is notable that the combined protection of \textit{P0} and \textit{synapsin-cre} recombination approximated the protection produced by \textit{nestin-cre} recombination (Figure 4A), implicating an additive rescue effect produced by both Schwann cells and neuronal LPA\textsubscript{1} activation in PSNL-initiated pain.

### 4 DISCUSSION

\textit{Lpar1} conditional null mutant mice were generated and shown to undergo cre-mediated recombination, enabling identification of \textit{Lpar1}-expressing neurons and Schwann cells as functionally important for the PSNL phenotype. In the absence of cre, \textit{Lpar1\textsuperscript{flox/flox}} mice developed a pain phenotype comparable to Wt control mice,\textsuperscript{25} demonstrating that this new floxed mutant gene functions normally in mice subject to PSNL. \textit{Lpar1\textsuperscript{flox/flox-nestin-cre}} mice with a
pan-neural lineage deletion of \( Lpar1 \) were protected from PSNL-induced neuropathic pain which supports neural LPA\(_1\) signaling as important despite \( Lpar1 \)'s ubiquitous tissue expression. By comparison, \( P0 \) and synapsin-cre recombination produced only partial rescue with complementary temporal phases of protection that appeared additive to account for the degree of rescue by \( nestin \)-cre recombination. Rescue from neuropathic pain was only observed at day 9 in \( Lpar1^{\text{flx/flx}}-CD11b\)-cre mice. It was recently reported that microglial depletion protects mice from PSNL-induced thermal hyperalgesia, therefore, we cannot exclude the possibility that CD11b expressing myeloid lineage cells also play a role in PSNL-induced neuropathic pain.36

The actions of LPA\(_1\) in Schwann cells affecting PSNL phenotypes have not, to our knowledge, been previously

\[ \text{FIGURE 4} \] Deletion of \( Lpar1 \) in neuronal lineages protects against PSNL induced neuropathic pain. A, Targeted \( nestin \)-cre-mediated deletion of \( Lpar1 \) in all neural lineages protects against neuropathic pain in the PSNL mouse model. B, Schwann cell-specific deletion of \( Lpar1 \) through a \( P0 \)-cre transgene protects mice from PSNL at later, but not earlier, time points. C, Specific deletion of \( Lpar1 \) in neurons protects mice from PSNL induced neuropathic pain only at early time points. D, Deletion of \( Lpar1 \) in CD11b expressing cell types provides protection from neuropathic pain at day 9 post-PSNL. E, Schwann cell-specific deletion of \( Lpar1 \) occurs at later time points and is long-lasting. The plotted data are the average paw withdrawal threshold time observed for \( Lpar1 \) conditional null mutants normalized to \( Lpar1^{\text{flx/flx}} \) control animal responses ± SEM. For (A, B, and C), \( N = 10 \text{~} Lpar1^{\text{flx/flx}}, N = 10 \text{~} Lpar1^{\text{flx/flx}}-nestin-cre, N = 9 \text{~} Lpar1^{\text{flx/flx}}-P0 \text{ cre, and } N = 8 \text{~} Lpar1^{\text{flx/flx}}-synapsin-cre \text{ animals. For (D), } N = 4 \text{~} Lpar1^{\text{flx/flx}} \text{, and } N = 4 \text{~} Lpar1^{\text{flx/flx}}-CD11b\text{-cre. For (E), } N = 2 \text{ for all genotypes used. Statistical analysis was performed using a two-way ANOVA followed by a Sidak’s multiple comparisons test and differences were considered significant when } P \leq 0.05 \text{ (} * = P \leq 0.05, ** \leq 0.01, *** \leq 0.001, **** \leq 0.0001 \text{) } \]
reported, and the observed phenotype was unexpected with regard to the clear and differential time-dependence of the effect. Explanations for these temporal changes in pain protection may be due to differences in de novo synthesis of LPA and the varied activation states documented for LPA₁ that may occur in neurons and Schwann cells. Such LPA signaling effects could be altered by receptor removal to produce the time-course differences observed for PSNL-initiated pain rescue. Long-lasting protection from neuropathic pain at later time points may also reflect changes in nerve myelination that may interfere with the transmission of pain stimuli as previously suggested.²⁴,²⁵ Nerve fibers in Lpar₁<sup>fl<sub>ox</sub>/fl<sub>ox</sub>-P0-cre</sup> mice may be abnormally myelinated, and nerve injury-induced demyelination may alter normal pain signal transmission. However, we note that the nerve fibers that respond to noxious stimuli are lightly myelinated Aδ fibers and unmyelinated C-fibers,²³ requiring a more complex scenario that might involve central pain consolidation through myelinated fibers.

Effects of Lpar₁ deletion from neurons in Lpar₁<sup>fl<sub>ox</sub>/fl<sub>ox</sub>-synapsin-cre</sup> mice showed early protection in PSNL, contrasting with later protection of Schwann cell receptor deletion, while supporting the involvement of neurons in LPA₁-mediated PSNL-induced pain. Synapsin-cre deletion is effective in CNS neurons but can be less effective in peripheral (DRG) neurons,³³,⁴² implicating central neuronal mechanisms. A possible explanation for rescue at early timepoints could involve a lack of de novo LPA synthesis from Lpar₁ deficient neurons. LPA can be released by neurons following nerve transection and neurons can synthesize LPA de novo through an LPA receptor dependent feed-forward mechanism (as evidenced by LPA₃).¹⁵,²⁶,⁴³ De novo LPA synthesis from other cell types following PSNL may result in LPA accumulation to drive neuropathic pain at later time points, particularly through activation of Schwann cell receptors in the neuron-specific mutants. Alternatively, PSNL may cause damage and vascular leakage that exposes peripheral nerves to LPA by activating cognate receptors to produce aberrant pain signaling.⁹,⁴⁴-⁴⁶

Other LPA receptor subtypes can contribute in distinct ways to neuropathic pain based on analyses of different LPA receptor-null mutants.¹³,¹⁶,²⁵,³¹,³⁹,⁴⁷,⁴⁸ Lpar₅ null mutant mice are also protected from PSNL-induced neuropathic pain and show decreased sensitivity to acute pain stimuli and faster recovery responses when challenged in an inflammatory pain model.³¹,⁴⁹ Additionally, deletion of Lpar₃ in mice prevents i.t. LPA-induced novo production of LPA in the dorsal horn and dorsal root and also prevents LPA-induced allodynia and hyperalgesia,²⁶ suggesting an LPA₃ mediated feed-forward mechanism for LPA in neuropathic pain initiation.²⁷ Prevention of LPA de novo synthesis and neuropathic pain in the i.t. LPA and PSNL neuropathic pain models using minocycline combined with Lpar₁ expression in microglia indicate that this feed-forward mechanism is likely mediated by microglia.⁵⁰,⁵¹ In the present study, we observed a rescue effect of Lpar₁ loss from microglia only at day 9 post-PSNL, suggesting that maintained LPA₂ could sustain PSNL-initiated pain, and the possible involvement of Lpar₁ expressing CD11b expressing myeloid cell-types. Cx3cr<sub>1<sup>C<sub>re<sub>E</sub>⁰</sub></sup> transgenic mice that express the cre recombinase fused to a mutant estrogen ligand-binding domain, would be useful in delineating the contribution of microglia vs other CD11b expressing cell types in PSNL,⁵² which could be pursued in the future.

The generated Lpar₁ conditional mutant mice will be useful in identifying other cell types involved in LPA₁ signaling in neuropathic pain models, as recently described for astrocytes,³⁶ as well as many other conditions and disease models.⁶-¹²,³⁹,⁴⁷,⁵³,⁵⁴ The tractability of LPA₁ as a member of the lysophospholipid receptor family supports its potential as a druggable GPCR⁸,¹⁰,³⁷,³⁸ and the development of novel therapies that target LPA₁.

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CONFLICT OF INTEREST
The authors declare no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS
R. Rivera, M. Lin, and J. Chun designed research; R. Rivera, M. Lin, and E. Bornhop performed research; R. Rivera, M. Lin, and J. Chun designed research; R. Rivera, M. Lin, and J. Chun wrote the paper.

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