Supporting Information

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SI Materials and Methods

Animals and Human Tissue. Male C57BL/6J Bommince mice (A/S Bomholtgaard) (b.wt. 25–30 g) were kept under standard conditions on a 12 h day/night cycle with free access to food and water. Human DRGs were harvested from children with obstetric brachial plexus lesions who underwent reconstructive nerve surgery. All material was immersion-fixed in formalin (see below). Median age at operation was 4 months. All DRGs had avulsed central roots but were in continuity with the periphery, an injury type that in animal experiments have been shown to have very little impact on nerve cell survival (1).

Surgeries. Surgical procedures were performed under general anesthesia (1.7–2.0% isoflurane). Spared nerve injury (SNI) was made with a 10.0 silk tight ligation of the two branches of the left sciatic nerve, the common peroneal and the tibial nerves followed by transection and removal of a 2–4 mm nerve portion. The third branch, the sural nerve, remained intact and any contact or stretch to this nerve was carefully avoided (2). The animals were allowed to survive for 14 or 21 days after surgery ($n = 12$ for each time point plus 10 normal controls).

For analysis of the role of inflammation, animals received an intraplantar injection of 20 μL of carrageenan (1%, Sigma) into the plantar surface of the left hindpaw without anesthesia, and were allowed to survive for 15 min, 1 h or 3 days after injection ($n = 4$ for each time point plus 4 normal controls).

For dorsal rhizotomy, animals were anesthetized with isoflurane as above, and the left L4 to L6 dorsal roots were transected. After surgery, animals were allowed to survive for 14 days ($n = 5$ plus 2 normal controls).

In the studies on intraaxonal transport, the animals were anesthetized with isoflurane as above, and the left sciatic nerve was carefully dissected free. A watch-maker forceps was used to compress the nerve for 30 sec. Eight hours later the mice were processed for immunohistochemistry ($n = 3$ plus 2 controls).

Drug Administration. The phospholipase C (PLC) inhibitor U73122 was purchased from Tocris. Selection of drug dose (30 mg/kg, dissolved in 0.5% DMSO) was based on the study in ref. 3. Drug was administered i.p. in a volume of 200 μL using a 27-gauge hypodermic needle. Tests were carried out from 15 min up to 72 h after drug injection.

Behavioral Tests. Mechanical allodynia was examined 2–21 days after SNI. Animals ($n = 12$) were placed in transparent plastic domes on a metal mesh floor with a hole size 2 × 3 mm. After 30 min habituation, the threshold for paw withdrawal (both ipsi- and contralateral side) was measured by graded-strength von Frey monofilaments (0.008, 0.04, 0.07, 0.16, 0.4, 0.6, 1.0, 1.4, and 2.0g) (2, 4, 5). For mechanical hyperalgesia, the animals were placed on an elevated grid, and a pin prick test was performed using a safety pin. The lateral part of the plantar surface of the paw was briefly stimulated with an intensity that was sufficient to indent but not penetrate the skin (pin-prick test). The duration of paw withdrawal was recorded, with an arbitrary minimal of 0.5 second (for the brief normal response) and a maximal cut-off of 5 sec (6). For the cold allodynia test, a drop of acetone solution was carefully dropped onto the lateral plantar surface of the paw without touching the skin, using a blunt needle connected to a syringe. The duration of the withdrawal response was recorded with a arbitrary minimal value of 0.5 seconds and a maximum of 10 seconds (7).

Immunohistochemistry. Animals were deeply anesthetized with sodium pentobarbital (Mebumal; 50 mg/kg, i.p.), and transcardially perfused with 20 mL of warm saline (0.9%; 37 °C), followed by 20 mL of a warm mixture of paraformaldehyde (4%; 37 °C) with 0.4% picric acid in 0.16 M phosphate buffer (pH 7.2) (8, 9) and then by 50 mL of the same, but ice-cold fixative. The L5 DRGs and the L4-L5 segments of spinal cords were dissected out and postfixed in the same fixative for 90 min at 4 °C and subsequently stored in 10% sucrose in phosphate-buffer saline (PBS; pH 7.4) containing 0.01% sodium azide (Sigma) and 0.02% Bacitracin (Sigma) at 4 °C for 2 days. Tissues, including human, were embedded with OCT compound (Tissue Tek; Miles), frozen and cut in a cryostat (Microm, Heidelberg, Germany) at 12 (for DRGs) or 20 (for spinal cord) μm thickness and mounted onto alum-gelatin-coated slides. The sections were dried at room temperature (RT) for 30 min, and then rinsed in PBS for 15 min and incubated for 24 h at 4 °C in a humid chamber with guinea-pig anti-PLCβ3 antiserum (10) (1: 4,000). The antibody was diluted in PBS containing 0.2% (wt/vol) BSA, 0.03% Triton X-100 (Sigma). Immunoreactivity was visualized using a commercial kit (TSA Plus, NEN Life Science Products). Briefly, the slides were rinsed in TNT buffer (0.1 M Tris·HCl, pH 7.5; 0.15 M NaCl; 0.05% Tween 20) for 15 min at RT, blocked with TNB buffer (0.1 M Tris·HCl; pH 7.5; 0.15 M NaCl; 0.5% Du Pont Blocking Reagent) for 30 min at RT followed by 30 min incubation with horseradish peroxidase-labeled secondary antibody (1: 200) (Dako) diluted in TNB buffer (1: 200). After a simple wash (15 min) in TNT buffers all sections were exposed to biotinyl tyramide-fluorescein (1:100) diluted in amplification diluent for ≈15 min, and finally washed in TNT buffer for 30 min at RT. For studying the proportion of PLCβ3-positive NPs sections were counterstained for 15 min in 0.001% (wt/vol) propidium iodide (PI) (Sigma) in PBS. Double-staining experiments were carried out on a group of TSA processed sections using a polyclonal rabbit anti-calcitonin gene-related peptide (CGRP, 1: 4,000) antiserum (11), a polyclonal rabbit anti-galanin antiserum (1:400) (12) or a polyclonal rabbit anti-GFP-antiserum (1:4,000) (Molecular Probes), followed by staining with lissamine rhodamine B sulfonyl chloride (LRSC)-conjugated donkey anti-rabbit (1: 100; Jackson ImmunoResearch). Moreover, another group of TSA processed sections was incubated with the isolectin B4 from Griffonia Simplicifolia I (GSA I) (IB4; 2.5 μg/ml; Vector Laboratories) followed by incubation with a goat anti-GSA I antiserum (1: 4000, Vector Laboratories) and a Rhodamine Red X-conjugated donkey anti-goat antibody (1: 200; Jackson Laboratories). Finally, all slides were coverslipped with glycerol/PBS (9:1) containing 0.1% *para*-phenylenediamine (13, 14). Absorption experiments were carried out with a GSR fusion protein carrying PLCβ3 C terminus (10).

Image Analysis. The sections were analyzed in a Bio-Rad Radiance Plus confocal scanning microscope installed on a Nikon Eclipse E 600 fluorescence microscope equipped with 4× (0.2 N.A.) 10× (0.5 N.A.), 20× (0.75 N.A.), and 60× oil-immersion (1.40 N.A.) objectives. The fluorescein labeling was excited using the 488 nm line of the argon ion laser and detected after passing a HQ 530/60 (Bio-Rad) emission filter. For the detection of rhodamine, the 543 nm line of the green HeNe laser was used in combination with the HQ 570 (Bio-Rad) emission filter. All of the slides were scanned in a series of 1 μm thick optical section.

Afterward, the images were analyzed separately and then merged to detect possible colocalization.

Quantitative Evaluations. Percentage of immunoreactive (IR) neuron profiles (NPs) were counted on the 12 μm thick sections and every fifth DRG section was selected (Nike Microphot-FX microscope, 20× objective). The total number of PLCβ3-IR NPs was divided by the total number of PI-stained NPs (as above), and the percentage of positive NPs was calculated. Five to 6 sections of each DRG from 4 to 6 animals in each lesion group and normal control group were included in the analysis, and 1,500–3,000 NPs were counted in each DRG. The percentage of PLCβ3-positive NPs colocalized with IB4, CGRP, galanin or GalR2-EGFP were counted on fluorescein isothiocyanate- and LRSC- stained sections. The relative fluorescence levels (intensity) of PLCβ3-LI in DRGs and spinal dorsal horn (lamina I-II) were measured using a Sarastro 1000 (Molecular Dynamics) confocal laser-scanning system. Images were recorded with 10x/0.45 or 20x/0.75 air objective and stored in a computer for subsequent analysis in Image Space Software (Molecular Dynamics). The size of PLCβ3-IR NPs with a visible nucleus were also measured (6 animals in each group) using the Sarastro 1000 confocal laser-scanning system with Image Space Software (as above). The profiles of DRG neurons were divided into small, medium-sized and large ones according to previous studies (15, 16). Small NPs had a somal area of 80–600 μm^2; medium-sized NPs 600–1,400 μm^2; and large NPs >1,400 μm^2.

Statistics. The percentage of PLCβ3-IR NPs and the intensity of PLCβ3-LI in DRG neurons and spinal dorsal horn and the comparison between ipsilateral and contralateral sides of DRG neurons in each treated group were evaluated by paired student *t* test. Kruskal-Wallis ANOVA test (one-way ANOVA on ranks) was used for the comparison of data among groups. $P < 0.05$ was chosen as the significant level.

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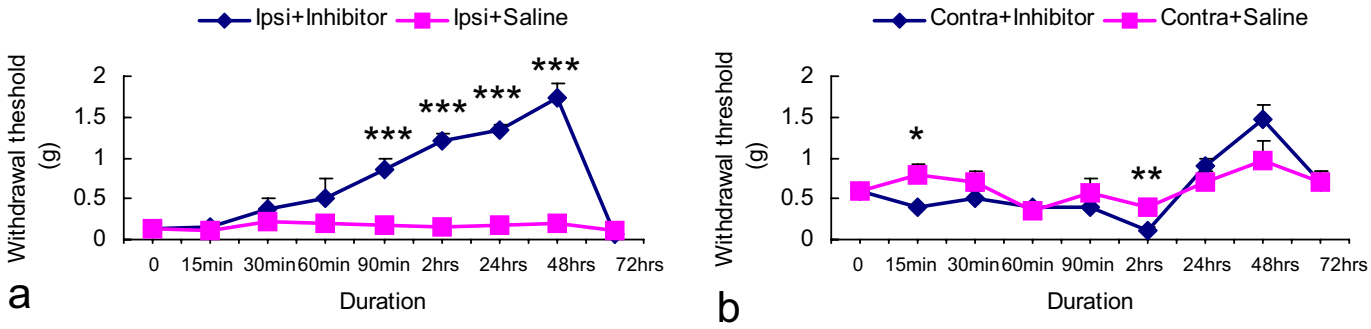

Fig. S1. Effects of U73122 (30 mg/kg) after SNI. (*a*) Eighteen days after SNI, a single dose of U73122 causes a long lasting, ipsilateral increase in mechanical threshold compared with saline-treated mice. (*b*) The transient effects of inhibitor on mechanical threshold is also seen in the contralateral paw compared with saline-treated group 18 days after SNI ($n = 6$ per group). Data are expressed as mean ± SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared with the vehicle-treated group.

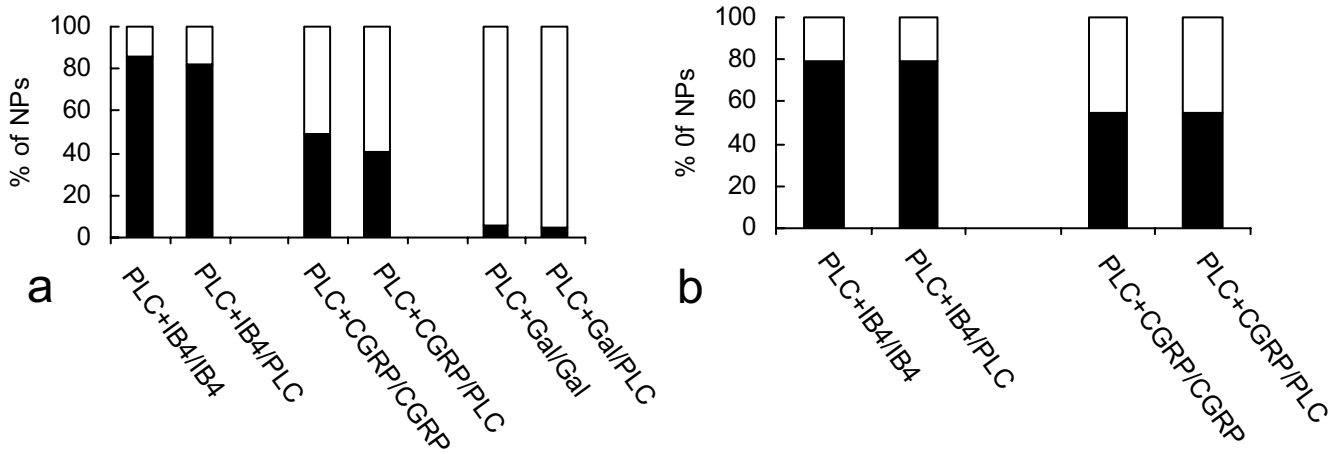

Fig. S2. Expression of PLCβ3 in several neuronal subpopulations within the DRGs. (*a*) A summary of the quantitative evaluation of coexistence between PLCβ3 and IB4 (control), CGRP (control) or galanin (gal) 2 weeks after SNI, respectively. (*b*) Quantitative evaluation of coexistence between PLCβ3 and IB4 and CGRP (both 2 weeks after SNI), respectively. More than 200 NPs were counted in each group.

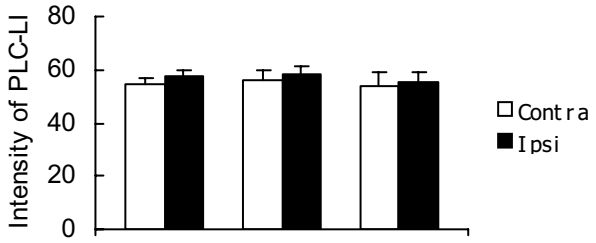

Fig. S3. Expression of PLCβ3 in spinal cord. Quantitative evaluation of PLCβ3-IR levels (intensity of immunofluorescence) in spinal dorsal horn indicates no significant difference between ipsi- and contralateral dorsal horn 15 min (left columns), 1 h (center columns), or 3 days (right columns) after peripheral inflammation induced by carrageenan injection, respectively (*, $P > 0.05$ compared with contralateral side).