Supplementary material

Methods

Nissl Staining and Immunohistochemistry

Under anesthesia with isoflurane (3%) and mixed anesthesia with medetomidine hydrochloride (0.3 mg/kg), midazolam (4 mg/kg), and butorphanol tartrate (5 mg/kg) (i.p.), rats were perfused with PBS followed by 4% paraformaldehyde phosphate buffer (Wako, Osaka). After perfusion fixation, the medulla oblongata containing the trigeminal ganglion and the trigeminal nucleus were extracted and post-fixed at 4°C for 12 h. After dehydration and replacement with sucrose, the samples were embedded in OCT Compound (Sakura Finetek Japan, Tokyo, Japan) and sectioned horizontally at a thickness of 30 μm using a freezing microtome. Sections were stained with Cresyl Violet acetate (MP Biomedicals, Santa Ana, CA, USA) solution for 2 min, dehydrated, permeabilized, and covered and sealed with DPX Mountant (Sigma-Aldrich, St. Louis, MO, USA). Images were acquired using a BX63 digital fluorescence microscope (Olympus Inc., Tokyo, Japan).

Sections on glass slides were washed with PBS and treated with 0.1% Triton X-100 in PBS for 10 min. After washing with PBS and blocking with 3% bovine albumin serum, the sections were incubated with primary antibodies at 4°C for overnight. The following respective antibodies were used to detect neurons, neuronal activity, the $\alpha_2\delta$-1 subunit, microglia, activated astrocytes, and

inhibitory interneurons: guinea pig anti-rat NeuN polyclonal antibody (Sigma-Aldrich; 1:500), rabbit anti-rat c-Fos monoclonal antibody (Cell Signaling Technology, Danvers, MA, USA; 1:500), mouse anti-rat α_2 subunit monoclonal antibody (Sigma-Aldrich; 1:1000), rabbit anti-rat GFAP monoclonal antibody (Cell Signaling Technology; 1:500), mouse anti-rat CD11b monoclonal antibody (Sigma-Aldrich; 1:500), and mouse anti-rat parvalbumin (PARV) monoclonal antibody (Sigma-Aldrich; 1:2000). After washing, the sections were incubated with secondary antibodies labeled with either Alexa Fluor 488, Alexa Fluor 555, or Alexa Fluor 633 (Invitrogen, 1:1000) for 1 h at room temperature and then washed. DAPI staining was performed for 2 min, and then the slides were washed, covered, and sealed. Fluorescent images were acquired using an LSM880 laser scanning confocal microscope with airy scan (Carl Zeiss, Jena, Germany) and analyzed using Imaris high-definition 3D/4D image analysis software (Oxford Instruments, Belfast, UK).

Real-Time Quantitative PCR (qRT-PCR)

Rats were perfused with PBS under inhalation anesthesia with isoflurane (3%), and the area containing the trigeminal ganglion and trigeminal nucleus was immediately sampled and flash frozen at −80°C. The manually crushed samples were then immediately shredded. Total RNA for reverse transcription–polymerase chain reaction (RT-PCR) was extracted using a FastGene™ RNA Premium kit (NIPPON Genetics, Tokyo, Japan). Total RNA was reverse transcribed into cDNA using

SuperScript VILO MasterMix (Invitrogen). For qRT-PCR, KOD SYBR qPCR Mix (TOYOBO, Osaka, Japan) and a 7500 Fast Real-Time PCR System (Applied Biosystems) were used. Gene expression was quantified using specific primers for the genes encoding c-Fos (forward, 5'-TGTGACCTCCCTGGACTTG-3'; reverse, 5'-CACTGGGCCTAGATGATGC-3'), $\alpha_2\delta$-1 subunit (forward, 5'-AAAGCAAGGAGCAGGACATCG-3'; reverse 5'-GCCTCAAGGAGCCGTGGAAA-3'), GFAP (forward, 5'-TGGAGGGCGAAGAAAACCGCATCAC-3'; reverse 5'-TCCTTAATGACCTCGCCATCCCGC-3'), CD11b (forward, 5'-TTATTGGGGTGGGAAACGCCT-3'; reverse, 5'-CTGGAGCTGGTTCCGAATGGT-3'), and GAPDH (forward, 5'-CCATGGAGAAGGCTGGGGCTCAC-3'; reverse 5'-ACATGGGGGCATCAGCGGAAGG-3'). mRNA levels were quantified using GAPDH as an internal control. The relative expression of each gene was calculated using the comparative ΔΔCt method.

Neurodegeneration Assay

After immunohistochemical staining, degenerated neurons were stained with Fluoro-Jade C (FJC; Merck Millipore, Burlington, MA, USA) according to the manufacturer's instructions and covered and sealed with DPX (Sigma-Aldrich). The number of neurons (labeled with NeuN) per

section of the trigeminal nucleus was then determined. Neurons that were NeuN and FJC double positive were considered degenerated. Quantitative analysis was performed using Imaris high-definition 3D/4D image analysis software.

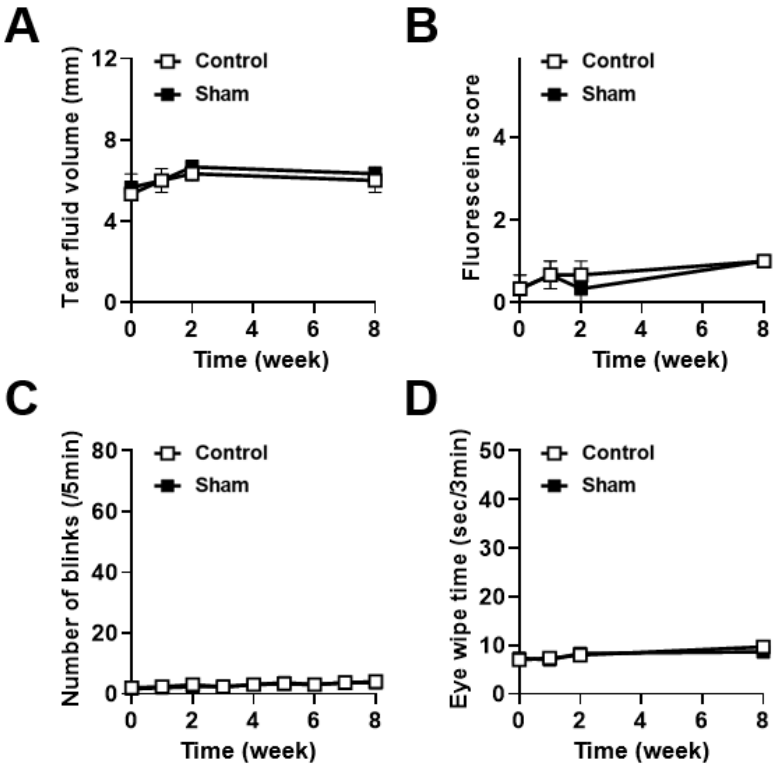

Figure S1

Comparison of phenotypic analysis results between untreated control side and the sham surgery side of control-sham rats. Sham surgery did not affect tear volume, corneal epithelial damage, corneal hypersensitivity, or corneal hyperalgesia. (A) Change in the tear fluid volume in control-sham rats. (B) Time-dependent progression of corneal epithelial damage evaluated by fluorescein staining scoring in control-sham rats. (C) Progression of hypersensitivity in control-sham rats evaluated by the number of blinks. (D) The time course of hyperalgesia progression in control-sham rats evaluated by eye wipe response. Each point represents the mean ± SEM (n=3). There was no significant difference between control side and sham side (Two-way ANOVA).

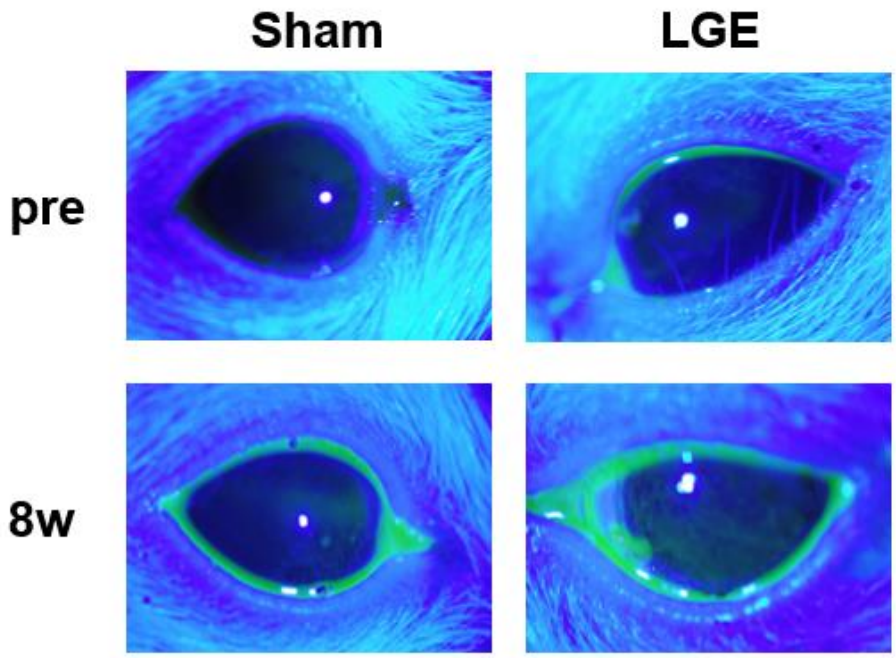

Figure S2

Unilateral LGE surgery caused significant corneal epithelial damage on the LGE side but minimal corneal epithelial damage on the sham side. The cornea was examined using a slit-lamp microscope with cobalt-blue filter after Flores 0.7 mg ophthalmic test paper was applied. Preoperatively, both the sham and LGE sides was scored as a value of almost zero. At 8 weeks after surgery, the Sham side had a score of 1. In contrast, the LGE side had a score of 4.

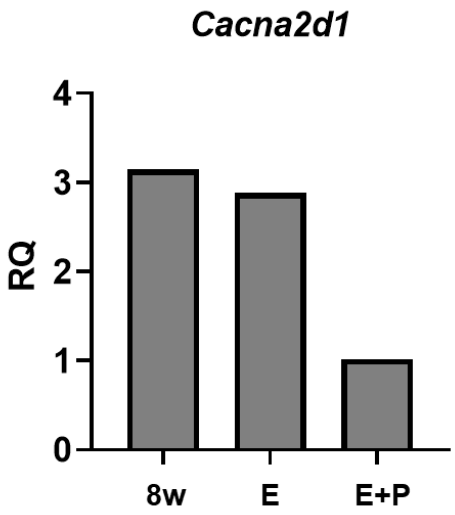

Figure S3

Chronic pregabalin administration normalized the upregulation of $\alpha_2\delta$-1 subunits. The mRNA level of $\alpha_2\delta$-1 in the trigeminal ganglion was quantified at 8w, E, and E+P. The vertical axis shows the RQ value standardized on the Sham side. Each sample consisted of the trigeminal ganglia of three rats.