

Appendix S1

## SUPPLEMENTARY MATERIALS AND METHODS

### *Antibodies*

The following antibodies were used in this study: rabbit anti-protein gene product 9.5 (PGP9.5) (1:600 dilution; Enzo Life Science, Inc., Farmingdale, NY, USA), rat anti-substance P (SP) (1:100 dilution; Chemicon, Temecula, CA, USA), and Guinea pig anti-calcitonin gene-related peptide (CGRP) (1:800 dilution; Acris Antibodies GmbH., Herford, Germany). Secondary antibodies conjugated with Alexa Fluor dye (1:300–500 dilution) were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA) and Jackson ImmunoResearch, Inc. (West Grove, PA, USA).

### *Immunohistochemical analyses*

Skin samples were fixed in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4) for 4 h at 4°C. After washing with phosphate-buffered saline (PBS, pH 7.4), skins were immersed successively in PBS containing 20% sucrose overnight at 4°C. The samples were then embedded in optimal cutting temperature (OCT) compound (Sakura Seiki Inc., Tokyo, Japan) and frozen on

dry ice. Cryosections 20 µm thick were cut using a CM1850 cryostat (Leica, Wetzlar, Germany) and mounted on silane-coated glass slides. After blocking in PBS with 5% normal donkey serum, 2% bovine serum albumin, and 0.2% Triton X-100, the cryosections on the glass slides were incubated with primary antibodies overnight at 4°C. The next day, the primary antibodies were washed with PBS containing 0.05% Tween 20 (PBS-T), and incubated with secondary antibodies for 1 h at room temperature. The sections were washed again with PBS-T and mounted in Vectashield® mounting medium. The immunoreactivity then was assessed by confocal laser scanning microscopy (DMIRE2; Leica).

### *Semi-quantitative measurements*

To semi-quantify the number of epidermal or dermal nerve fibres, 9 specimens in each skin biopsy were stained with the above-mentioned primary antibodies, and optical sections 0.9-µm thick were scanned through the z-plane of the stained specimens by confocal microscopy. The images were reconstructed in 3 dimensions using Leica Confocal Software (Leica). For measurement of the number of epidermal or dermal nerve fibres, we analysed at least 8 confocal images per specimen. The number of epidermal or dermal nerve fibres in each  $1.6 \times 10^5 \mu\text{m}^2$  in the images was counted manually. All values represent means  $\pm$  standard deviations.