SUPPLEMENTARY MATERIALS AND METHODS

Pruritogen-evoked alloknesis test

At least 4 days before the test, areas with ~2 cm diameter were shaved on the necks and backs of the mice using a razor. The mice were allowed to acclimatise to the cage for at least 30 min before testing. The mice received a 10 μL intradermal injection of histamine (Sigma-Aldrich, St. Louis, MO; 271 nmol dissolved in saline), or chloroquine (Sigma-Aldrich, 193 nmol dissolved in saline), or serotonin (Sigma-Aldrich, 23.5 nmol dissolved in saline) using a 29-gauge needle attached to a microsyringe (Terumo, Japan) into the neck. After injection, the mice were immediately placed back in the cage, and their scratching behaviour was recorded for 30 min using a video camera. The number of scratch bouts was counted during the recording. For pruritogen-evoked alloknesis, mice received five separate innocuous mechanical stimuli with a von Frey filament (0.008 g bending force, Muromachi Kikai) on the injection site 35 min after the intradermal pruritogen injection. The presence or absence of scratching bouts was recorded after each stimulus. These innocuous stimuli were repeated six times at 5 min intervals (30 minutes in total). The alloknesis score was the total number of positive responses during the 30 min observation period (starting at 35 min after pruritogen injection).

Immunohistochemical staining for epidermal innervation and infiltrating cells

For PGP9.5 immunostaining, skin samples were fixed using 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 4 h at 4°C and immersed in PBS containing 20% sucrose overnight at 4°C. The specimens were embedded and cut into 30 μm sections (CM1850UV; Leica Microsystems, Wetzlar, Germany), and dried. After washing, the sections were incubated in a blocking buffer (Protein Block Serum-Free (Dako, Carpinteria, CA)) and incubated overnight at 4°C with primary polyclonal antibodies against PGP9.5 (clone

Ab27053, Abcam, Cambridge, UK). After washing, the specimens were incubated for 1 h with anti-rabbit secondary antibodies (Ab15077, Abcam) at room temperature and counterstained with propidium iodide. Staining was evaluated under a fluorescence microscope (BZ-X810; KEYENCE, Japan). Images were captured from 12 skin sections from each animal at 20× magnification (4 mice per group). The number of labelled nerve fibres crossing the dermal-epidermal junction was counted. Epidermal nerve fibre density was calculated as the number of epidermal nerve fibres divided by the length of the dermal-epidermal junction. PGP9.5 staining was confirmed using repetitive acetone-induced murine dry skin as a positive control. For the detection of basophils, mast cells, and eosinophils, dorsal skin samples were formalin-fixed and paraffin-embedded. Eosinophils were detected based on their specific morphology by staining with haematoxylin and eosin; mast cells were detected by positive metachromatic staining with toluidine blue; and basophils were detected by positive immunostaining with anti-mMCP-8 antibody (clone TUG8; BioLegend, San Diego).

Reverse transcriptase-PCR analysis

Dorsal skin was collected from B6 mice 4 weeks after ovariectomy/HRT using a sterile 5 mm biopsy punch. The dorsal skin was homogenised, and total RNA was isolated using the NucleoSpin® RNA Midi kit (MACHEREY NAGEL, Duren, Germany). Total RNA (140 ng) was reverse-transcribed using Prime Script RT Master Mix (Perfect Real Time, Takara Bio Inc., Kusatsu, Japan). SYBR® Premix Ex Taq II (Til RNaseH Plus) (Takara Bio Inc.) was used for reverse transcription-PCR. The following steps were used to amplify all genes: 30 s at 95ºC and 55 cycles for 5 s at 95ºC and 34 s at 60ºC. The forward and reverse primer sequences for NGF, semaphorin 3A, TSLP, IL-4, IL-13, IL-31, artemin, aryl hydrocarbon receptor, and β-actin (control) are listed in Supplementary Table 1. Expression analysis was

performed using the 7500 SDS v1.4.1 software (Thermo Scientific, Waltham, MA, USA) and the relative standard curve method.

SUPPLEMENTARY RESULTS

Table S1. Sequences of the primers used for quantitative reverse transcriptase-PCR

Gene	Forward	Reverse
NGF	5′-TGCCAAGGACGCAGCTTTC-3′	5′-TGAAGTTTAGTCCAGTGGGCTTCAG-3′
Sema3A	5′-AGCCCTTATGATCCCAAACTACTGA-3′	5′-CGAAGTCCCGTCCCATGAA-3′
TSLP	5′-AATGACCACTGCCCAGGCTA-3′	5′-TTGTGAGGTTTGATTCAGACAGATG-3′
IL4	5′-ACGGAGATGGATGTGCCAAAC -3′	5′-GCACCTTGGAAGCCCTACAGA-3′
IL13	5′-CGGCAGCATGGTATGGAGTG-3′	5′-ATTGCAATTGGAGATGTTGGTCAG-3′
IL31	5′-AGCCTACCCTGGTGCTGCTT-3′	5′-ACGGCAGCTGTATTGATTCGTC-3′
Artn	5′-GCTTCTGATGGGAGCTTCTGG-3′	5′-AGGGCTAGAACAGCTAGGGTTGG-3′
Hsd11b1	5′-GGGAGCCCATGTGGTATTGA-3′	5′-TGCCAGCAATGTAGTGAGCAGA-3′
Piezo2	5′-ACTCAGCAAAGCAACTACGTGGAC-3′	5′-GAGCCATCCGAAGCAGAATGA-3′
Ahr	5′-GCATGAGGTGTCACCAAGGATTTA-3′	5′-GCCACCATACTCTCAGTGTCCAA-3′
β-actin	5′-CATCCGTAAAGACCTCTATGCCAA-3′	5′-ATGGAGCCACCGATCCACA-3′

Supplementary Figure legends

Figure S1

Levels of mRNA of molecules related to alloknesis and itch sensation in a mouse model of menopause. mRNA levels of thymic stromal lymphopoietin (TSLP), piezo-type mechanosensitive ion channel component 2 (Piezo2), aryl-hydrocarbon receptor (Ahr), hydroxysteroid 11-beta dehydrogenase 1 (Hsd11b1), and artemin (Atrn) in sham-operated (Sham), ovariectomised (OVX), and hormone replacement therapy (HRT) groups of mice were evaluated by quantitative reverse transcription polymerase chain reaction (RT-PCR); the results were normalised to β-actin mRNA levels and are represented as mean ± standard deviation (n = 27–33).

Figure S2

Touch-evoked alloknesis was evaluated using 0.07 *g*, 0.16 *g*, and 0.4 *g* von Frey filaments at indicated time points after intradermal administration of isotype IgG as a negative control for neutralizing antibodies against IL-4 and IL-13 in Sham and OVX mice. Data are from at least two independent experiments. Significant differences between groups are indicated by *P < 0.05, **P < 0.01, and ***P < 0.001.