

Appendix S1.

METHODS

Recruitment

Recruitment of healthy subjects was conducted by DermScan, a French company specializing in the management of clinical studies (Villeurbanne, France). The protocol was approved by an ethics committee and the French National Agency for Medicines and Health Products Safety (ANSM, registration number 2013-A00023-42). This clinical study was performed on subjects in accordance with Good Clinical Practice (ICH Topic E6 Note for Guidance on GCP CPMP/ICH/135/95, ISO1455 standard), with the French public health law of 9 August 2004, and in accordance with the Declaration of Helsinki and its amendments. Fifty healthy, 30–50-year-old women were recruited according to skin types I–III, as defined by the Fitzpatrick scale, which classifies skin types according to their colour and their tolerance to sunlight (skin types I–III are pale skin types). Exclusion criteria were: the presence of any dermatological or systemic diseases, intensive exposure to sunlight or ultraviolet (UV) rays on the studied area within the previous month, or any topical or systemic treatment for any reason. Skin sensitivity was assessed according to a new questionnaire (with the answer "yes" to at least 5 out of 7 questions) (Table S1¹) associated with a stinging test score greater than or equal to 3; this test was performed on the nasolabial folds, as described by Frosch & Kligman in 1977 (28). Twenty-six subjects were considered to be non-sensitive skin subjects and 24 sensitive skin subjects. All subjects gave their informed, written consent.

Skin biopsy processing

A 4-mm punch biopsy was removed from the neck of each subject, just below the ear, an area close to the face but inconspicuous. Each skin sample was identified by a code number to allow for further blinded histological analyses. Immediately after excision, the biopsies were fixed overnight in the same 4% paraformaldehyde bath. The samples were then preserved in the same phosphate-buffered saline (PBS) – 10% sucrose bath for additional 24 h prior to being frozen and stored at –80°C. The biopsies were further cut using a cryostat into 7-µm- and 30-µm-thick sections. Four 7-µm- or 30-µm-thick sections were included per slide to perform a triplicate analysis and a negative control condition. The 7-µm sections were spaced at 98 µm, and the 30-µm sections were spaced at a minimum of 120 µm to allow the analysis of non-consecutive sections on each slide.

Fluorescent immunolabelling

For each marker evaluation, the 50 slides corresponding to the 50 subjects were processed simultaneously to avoid experimental bias. Test conditions were performed in triplicate and compared with a negative control. After the staining step, the sections were analysed using an AxioStar plus microscope (Carl Zeiss) provided with an AxioCam ICc1 camera (Carl Zeiss) and AxioVision Software (Carl Zeiss). The stainings and the fluorescence evaluations were blindly performed by the same observer (VB).

Antibodies. Primary antibodies were mouse IgG2a antibody to PAR2 (sc-13504, 1/50, Santa Cruz Biotechnology); mouse IgG3 antibody to NFκB p65 (MAB3026, 1/60, Millipore); rabbit IgG antibody to TRPV-1 (ab63083, 1/800, Abcam); rabbit IgG antibody to ASIC-1 (ab87514, 1/400, Abcam); rabbit IgG antibody to GPR32 (ab79516, 1/70, Abcam); rabbit IgG antibody to PGP9.5 (RA95101, 1/800, Ultraclone Limited); rabbit IgG antibody to NF200 (ab8135, 1/1000, Abcam); mouse IgG2a antibody to CGRP (ab81887, 1/100, Abcam);

rabbit antibody to NGF (CLMCNET-011, 1/800, Cedarlane); and rabbit IgG antibody to Semaphorin 3A (ab23393, 1/100, Abcam). Isotype control antibodies were mouse IgG3 antibody (ab91537, 1/6, Abcam), mouse IgG2a (ab91361, 1/25, Abcam) and rabbit IgG (ab27478, 1/200, Abcam). Secondary antibodies were TRITC-conjugated goat antibody to mouse IgG (T5393, 1/500, Sigma Aldrich) for PAR2 and NFκB detection; Chromo 546-conjugated goat antibody to rabbit IgG (ab60317, 1/1000, Abcam) for TRPV-1, ASIC-1, GPR32, NF200, NGF and Semaphorin 3A detection; Chromo 488-conjugated goat antibody to mouse IgG (ab60313, 1/1000, Abcam) for CGRP detection; and FITC-conjugated goat antibody to rabbit IgG (111-095-003, 1/5, Interchim) for PGP9.5 detection.

ASIC-1, GPR32, NFκB, PAR2, TRPV-1, NGF and Semaphorin 3A evaluation. Evaluations were performed on 7µm-thick sections. For ASIC-1, NFκB, PAR2, TRPV-1, NGF and Semaphorin 3A, the overall epidermal fluorescence intensity was scored from 0 (no immunoreactivity) to 3 (high immunoreactivity), and the result was expressed in arbitrary units. The GPR32 immunoreactivity was scored on epidermal basal cells from 0 to 3, and the result was expressed as the percentage of highly immunoreactive epidermal basal cells (scoring from 2 to 3).

Determination of the linear nerve fibre densities. Immunostainings of PGP9.5, NF200 or CGRP were performed on 30-µm-thick sections to detect the small intraepidermal fibres, the Aβ fibres or the peptidergic C fibres, respectively. We used the coarse focus adjustment to count all the spatially distinct immunoreactive fibres. The NF200- and CGRP-positive fibres were counted until 300-µm depth in the dermis. For intraepidermal nerve fibres, we counted fibres or branches that crossed the dermo-epidermal junction or arose from it. Secondary branches or fragments occurring in the epidermis were not counted, as described by Lauria and colleagues (29). In order to have comparable results between the subjects, we determined a linear density using the corresponding dermo-epidermal junction length, as described previously (30): a photograph of the entire section was further taken at 25× total magnification and we used the ImageJ software to assess the length of the dermo-epidermal junction: we drew a segmented line following the junction, and the length of this line was calculated by the software according to the scale of the picture. Thus, the number of the counted fibres was divided by this length to obtain a linear density expressed as number of nerve fibres per mm of dermo-epidermal junction.

Epidermal thickness evaluation

The epidermal thickness was blindly determined by the same observer (VB) on a portion of each section after NFκB staining because this staining highlighted each epidermal cell. The selected portion was the more representative of the entire epidermis on the section and was devoid of hair follicles. A photograph of this portion was obtained (100× total magnification) to determine the length of the dermo-epidermal junction and the area of the epidermis using the ImageJ software. The length of the dermo-epidermal junction was obtained as described previously. The epidermal area was determined using the polygon selection and the measure function of ImageJ, according to the picture scale. The area was then divided by the length to evaluate the epidermal thickness, expressed in µm.

Statistical analysis

The relevance of the double recruitment procedure (self-assessment questionnaire combined with the stinging test) was validated by SOLADIS, a statistical consulting company (Lyon, France). Their analysis associated descriptive statistics and a correlational study between the 2 score sets (Spearman's corre-

lation method) and was performed using the SAS® 9.2 software.

The comparison of the mean ages of both groups was performed using the Student *t*-test because each population had a normal distribution according to the Agostino-Pearson normality test (GraphPad software).

Data of the fluorescent immunostainings are means of the triplicates for each subject. Data for each group are the mean

of the means of the corresponding subjects. Data are expressed as the mean $\pm$ standard error of the mean, except for the linear densities of PGP9.5-, NF200- and CGRP-immunoreactive fibres, which are expressed as the mean $\pm$ standard deviation. Each statistical analysis was performed using the Mann-Whitney's test with the GraphPad software. A *p*-value ≤ 0.05 was considered to be statistically significant.