

MATERIALS AND METHODS

Subjects and study design

Healthy volunteers (12 females, median age 21.5 years; 12 males, median age 23.5 years) were included. Female subjects were non-pregnant, non-breast-feeding, and without dysmenorrhoea. Subjects were excluded if they had any chronic or current pain, itch, neurological or dermatological diseases, history of allergy, history of drug addiction, musculoskeletal or mental illnesses. Subjects with tattoos within the test areas of the forearms were also excluded. In addition, subjects were instructed to avoid alcohol, nicotine, caffeine, and any analgesics 24 h before the experiment. All subjects received written and oral information about the experiment before giving their written consent. The study protocol was approved by the regional ethics committee (N-20140030) and conducted in accordance with the Declaration of Helsinki. The same investigator (CRH) performed all experiments in a quiet environment at the Laboratory for Cutaneous Pain Research, Aalborg University, in a time-span of 3 weeks. Fig. S1 illustrates the experimental timeline and the punched-map template used to assess allodynia (i.e. itch evoked by light touch) and hyperknesis (i.e. itch evoked by pinprick stimuli).

Upon arrival, subjects were asked to rest for 15 min. Cotton pads (3 × 3 cm) soaked with 1 ml of vehicle (ethanol 70%) or trans-cinnamaldehyde (5%) were applied for 10 min to the volar aspects of the forearms in altogether 24 healthy subjects (Fig. S1b). In addition, a soft plastic template, consisting of 8 tracks of 5 cm radially arranged around the centre of the application area, was used to mark stimulation points on the skin (Fig S1b). The stimulation points were separated by 1 cm, given a total of 40 stimulation points. Similar methodology has already been used for determination of hyperalgesia (27) or mapping dysesthetic areas (28) in the human skin.

Throughout the experiment, subjects sat in a comfortable chair where their forearms rested on a table with the elbow in a semi-flexion position. Trans-cinnamaldehyde was applied on the non-dominant volar forearm of all participants. The dominant arm (self-reported) served as a control with topical application of the vehicle. It is not clear whether hand dominance affects responsiveness to itch; however, this factor has been considered in psychophysical studies of pain (29). The order of application of the 2 substances was randomized and the assessments were conducted in a single-blinded design, in which the participants were unaware of the substance applied.

Application of trans-cinnamaldehyde and vehicle

Trans-cinnamaldehyde (99% Sigma-Aldrich, Germany) was dissolved in 70% ethanol to yield a 5% solution (≈ 0.38 M).

Pilot experiments were conducted on 3 young male volunteers, with trans-cinnamaldehyde at concentrations of 1%, 2%, 5% and 10%, which showed that a concentration of 5% induces the highest intensity of itch while evoking no or minimal hot/burning pain with no wheal reaction. Therefore, this concentration was chosen for further investigation. A 1 ml volume of this 5% solution was added to a 3 × 3 cm cotton pad and placed on the volar forearm for 10 min. The cotton pad was fixed with surgical tape and covered with an adhesive film to prevent the applied solutions from evaporating. After 10 min, the pad was removed and the remaining solution was wiped off. A similar process was followed for application of the vehicle on the contralateral arm (1 ml, 70% ethanol), which served as the control.

Assessment of itch intensity and area

Itch was defined as the sensation evoking a desire to scratch. During the application of trans-cinnamaldehyde and ethanol (10 min), the subjects were asked to rate the itch intensity every min on a visual analogue scale (VAS 0–10 cm), where 0 represented no itch and 10 the maximum imaginable itch. After removal of the cotton pad, each volunteer mapped the area where they perceived itch on a standard A4 arm-chart. This chart was covered with transparent film, which was later used for analysis of the pattern and size of the area. The area (cm²) of itch perception was calculated by Vista Metrix (version 1.38, Skill Crest LLC). To assess the temporal profile of the itch intensity during prolonged exposure to cinnamaldehyde a small additional experiment was performed on 4 participants (2 males and 2 females). During the additional experiment trans-cinnamaldehyde was applied, as described above, but not removed until the participants no longer reported itch. Itch intensity was monitored with a Co-VAS (used only in the sub-experiment to gain a higher resolution of the temporal profile) at a resolution of ≈ 10 s between recordings.

Assessment of flare, skin blood flow and temperature

After removal of the cotton pad, the flare area was drawn on transparent sheets for later analysis of the pattern and size. The flare area (cm²) was calculated by Vista Metrix (version 1.38, Skill Crest LLC). The skin blood flow (presented in arbitrary units; a.u.) and skin temperature (°C) were measured by a moor FLPI Speckle Contrast Imager (FLPI: full field blood perfusion imaging, Moor Instruments, Devon, UK) and an infrared thermography (FLIR systems, A40, Täby, Sweden), respectively. The head of the FLPI device and the infrared thermo-camera were placed at distances of 39 cm and 57 cm, respectively, and perpendicular to the surface of the skin. The distances were determined in the preliminary tests for an opti-

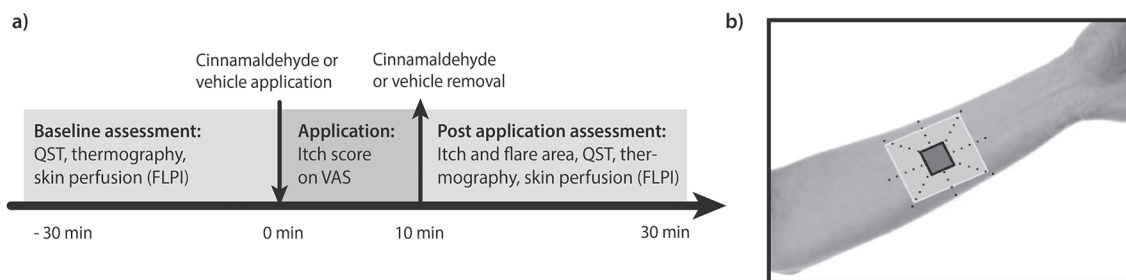


Fig. S1. (a) Overview of the experimental protocol and the timeline of the recordings. Before and after the application, sensory and vasomotor reactions were recorded. FLPI: full field blood perfusion imaging, Thermo: thermography, VAS: visual analogue scale, QST: quantitative sensory test. (b) Area of trans-cinnamaldehyde and vehicle application and the region of interest (ROI) for recording of sensory and vasomotor reactions on each forearm. Trans-cinnamaldehyde and vehicle were applied within the area of 3 × 3 cm in the middle of forearm. This area and an extended area of 5 × 5 cm (around the application area) were considered for blood flow and temperature measurements. The dotted map was drawn to detect the area of allodynia and hyperknesis.

mal imaging condition for these 2 techniques. The blood flow and temperature at the application area were measured once before and then after the application of trans-cinnamaldehyde or vehicle. Two regions of interest (3×3 cm at the application site, and 5×5 at the surrounding area) were marked on the captured images for later analysis with Laser Speckle software (MoorFLPI Review V4.0, Moor Instruments) and the software for infrared thermography (ThermaCAM Researcher Pro, V2.1, FLIR Systems, Täby, Sweden). The regions of interest were kept similar for all images. Extracted data were expressed as the mean of perfusion flux (a.u.) and temperature ($^{\circ}\text{C}$) in each region of interest (3×3 cm and 5×5 cm) for further statistical analysis.

Quantitative sensory testing

Mechanical stimuli by brush, von Frey and pinprick were applied to investigate whether areas of hyperknesis and alloknesis were developed following topical application of trans-cinnamaldehyde or vehicle. Alloknesis was defined as the dysesthetic state in which innocuous mechanical stimulation (light touch, von Frey) is capable of generating itch, while hyperknesis was defined as the state in which a normally pruritic stimulus elicited an increased duration and/or magnitude of itch (30). To avoid the introduction of bias related to the order of the test on subjects' responses, randomization was used for application of the mechanical stimuli. Alloknesis and hyperknesis assessments were performed immediately after removal of trans-cinnamaldehyde and vehicle.

Mapping the area of hyperknesis to pinprick. To map the area of hyperknesis, weight-calibrated pinpricks were used. The set consisted of metallic mechanical stimulators with a blunt tip 0.6 mm in diameter and weights of 0.8, 1.6, 3.2, 6.4, 12.8, 25.6, 50.1 and 60.0 g (custom-made, Aalborg University). One pinprick stimulator was selected based on the mechanical pain threshold of each subject before the application of trans-cinnamaldehyde or vehicle. Stimuli were applied from 8 different paths (Fig. S1b) around the application area in increments of 1 cm, at intervals of 2 s. Stimulation started from the furthest-most point on each track (Fig S1b) towards the centre of the application area. The stimulation point where the volunteer reported evoked or increased pricking-evoked itch was then marked. The points marked on the skin were connected to draw a polygon, which was transferred on a transparent film for later analysis of the area by Vista Metrix (version 1.38, Skill Crest LLC).

Mapping the area of alloknesis to von Frey. A process similar to that of hyperknesis to pinprick was followed for alloknesis using von Frey hair stimulators. The set consisted of 20 filaments (Touch Test[®] Sensory Evaluators, target force in gram from 0.008 to 300, North Coast Medical and Rehabilitation Products, Gilroy, USA). The same template map as described

above for the pinprick (Fig. S1b) was also used here. The von Frey hair stimulator (the filament just above the detection threshold of each subject) was applied perpendicular to the skin on each dot, pressed gently for 1 s until it bowed. An interval of 2 s was considered between each stimulus. The subjects were asked to report when the normal "light touch" or "tingling" sensation changed into "itch" sensation, and these points were marked. The defined area was then transferred to a transparent film and the area of alloknesis was calculated using Vista Metrix (version 1.38, Skill Crest LLC).

Mapping the area of dynamic brush-evoked alloknesis. Response to light brushing was tested with a standard brush (SENSELab Brush-05, Somedic, Hörby, Sweden). It is generally considered that 150–250 mN/cm² pressure is applied to the skin by using a soft brush (3.5 cm width) (31). A single stimulus of a 3-cm stroke with a velocity of approximately 5 cm/s was applied for each stimulation point. Moving towards the application area, the subjects were instructed to report if any sensation of itch occurred. The defined area was then transferred to a transparent film and the area (cm²) of brush-evoked alloknesis was calculated by Vista Metrix (version 1.38, Skill Crest LLC).

Statistical analysis

To assess potential sex-related responses, 12 males and 12 females were included. Three of the 24 subjects only experienced burning pain in response to application of trans-cinnamaldehyde and were excluded from subsequent analyses, yielding 11 male and 10 female participants.

All data in the text and graphs are presented as mean values \pm standard errors of the mean (SEM) unless otherwise stated. Normality testing was performed in all data subsets using D'Agostino's K-squared test, and statistical tests were chosen in accordance with determined data distribution. Comparisons between 2 groups were performed using an unpaired *t*-test or Mann-Whitney *U* test for non-paired data and a paired *t*-test or Wilcoxon signed-rank test for paired data. For multiple comparisons a 2-way repeated measures analysis of variance (RM-ANOVA) was used when parametric assumptions were met, this was followed by Holm-Sidak *post-hoc* analysis. Wherever assumptions for ANOVA was not met, sex differences were analysed using an unpaired *t*-test or Mann-Whitney *U* test and treatment differences using a paired *t*-test or Wilcoxon signed-rank test (according to distribution). Fisher's exact test was used in the analysis of categorical data. Correlations between responses were tested by Pearson or Spearman's correlation analyses, in accordance with normal or non-normal data distribution, respectively. Statistical analysis was performed using SigmaPlot (version 12.5, Systat Software, Inc., San Jose, USA). Graphs were prepared using GraphPad Prism (version 6, GraphPad Software Inc., USA). *p*-values < 0.05 were considered significant.