Appendix S1

METHODS

Test subjects and study design

Thirteen healthy test subjects (mean age 22.8 ± 3 years), 8 males and 5 females, participated in and completed the study. The subjects received written and oral information about the experimental procedure and provided written consent prior to the participation. The regional ethics committee of Northern Jutland approved the study protocol (N-20140078) and its adherence to the principles of the Declaration of Helsinki (39). None of the test subjects had chronic itch or pain, or perfume contact allergy. Exclusion criteria were: pregnancy, drug addiction (cannabis, opioids, etc.), previous neurological, musculoskeletal or mental illnesses, decreased communication or cooperation skills, any dermatological disorders, and tattoos on the forearms. Participants were instructed to abstain from analgesics, antihistamines, and/or other antipruritic medications 24 h prior to the experiment. All test subjects participated in 2 90-min sessions with a minimum 24 h interval between sessions. The study was conducted in randomized and single-blinded manner with order of experimental interventions and arm dominancy randomized, thus histamine challenges and counter-irritation stimuli always alternated between the right and the left arm, ensuring approximately 28 min before reapplication to a new site on the same arm. Histamine provocations and thermal counter-irritation with 32°C were performed at each session, acting as control conditions for chemical and thermal experimental interventions, respectively. In total, 6 histaminergic itch provocations and 6 randomized counter-irritation stimuli (thermal/chemical) or control conditions were performed in each of the 2 experimental sessions (Fig. 1). Rescue precautions were taken prior to the experiment to treat adequately any allergic reaction and anaphylactic shocks.

Quantitative sensory thermal tests

Prior to induction of itch in the first of the 2 experimental sessions, individual cold sensitivity was established using quantitative sensory testing (QST). Cold detection threshold (CDT) and cold pain threshold (CPT) were assessed in accordance with the standard quantitative sensory testing (QST) protocol proposed by the German Research Network on Neuropathic Pain (DFNS) (40). CDT and CPT were performed with a baseline temperature of 32°C and ramp stimuli decreasing at a rate of 1°C/s. The final CDT and CPT for each subject were calculated as the arithmetic mean of 3 consecutive measurements. When the subjects pressed a button denoting CDT or CPT, the temperature returned to the baseline temperature at a rate of 5°C/s. Test subjects were unable to observe both when the ramp stimuli were initiated and their results.

Itch induction

Itch was induced using a solution of 1.0% histamine dihydrochloride (Allergopharma, Reinbek, Germany), introduced by standard shouldered 1-mm tip skin prick test (SPT) lancets (Allergopharma, Reinbek, Germany). A drop of histamine solution was applied to the centre of a 3×3 cm square on the skin of the forearm and a custom-made SPT lancet-mount (Aalborg University, Denmark) attached to a SENSEbox was used to perform and control the prick through the outer epidermal layer. By using an electronic von Frey (EvF) pressure transducer (Somedic, Hörby, Sweden) a consistent SPT lancet pressure of ≈200 g was applied for 2–3 s (41). Itch was induced 6 times at each session, 3 times on each arm and always alternating between arms, while keeping a minimum 6 cm distance from previous injection sites.

Thermode-induced cold counter-irritation

Thermal stimulations and thermal threshold assessments of cold sensitivity (CDT and CPT) were conducted using the same 3 × 3 cm Thermal Stimulator Probe (thermode) (Medoc, Ramat Yishai, Israel) attached to a Medoc Main Station (MMS). The MMS was programmed to apply predetermined thermal counter-irritation at 6 temperatures: 37, 32, 28, 22, 12 and 4°C. The counter-irritation was commenced 1 min and 55 s post-introduction of histamine, since initial pilots and previously published literature shows this to be close to the peak itch intensity (10, 42). Thermal counter-irritation was ceased 5 min after the introduction of histamine. The 32°C was chosen as a control stimulus and applied in both sessions to adjust for the influence of the mechanical pressure introduced by the weight of the thermal probe.

Chemically induced cold ("cold-like") counter-irritation

Chemical counter-irritation was induced with 40% L-menthol (> 99.9%, TRPM8-agonist) and 10% CA (> 99%, TRPA1-agonist), (both substances: Sigma Aldrich, Broendby, Denmark). Since these receptors are tightly linked to the somatosensation of innocuous and painful cold, the application or their agonists for counter-stimulatory purposes are herein referred to as chemical "cold-like" stimuli. These were dissolved in 96% ethanol and topically applied to the skin using a 3 × 3 cm cotton pad on a plastic sheet, which was attached to the arm by medical tape (see further on the application method in (27)). They were attached to the skin 7 min before itch induction to achieve a high level of counter-irritation during the application period. Lastly, 5% doxepin (Region Hovedstadens Apotek, Copenhagen, Denmark) was applied to the skin under occlusion at least 60 min before the histamine injection as a positive control (being a well-studied antihistamine, known to drastically reduce experimentally induced histaminergic itch (43)).

Quantitative psychophysical assessments

Test subjects were told to express the itch intensity they experienced on a 10-cm visual analogue scale ($VAS_{0–10}$) during each itch provocation, with 0 being "no itch" and 10 being "worst imaginable itch". Subjects were told to disregard potential pain from counter-irritation stimuli and to move a virtual button (lever) to express the itch intensity score continuously. The VAS was sampled 4 times/min for the first 5 min during each application. Itch was defined as the sensation causing a desire to scratch. Area under the curve (AUC) was calculated at 0–2 min (before thermode-induced cold counter-irritation) and 2–5 min (during cold-counter stimulation). A total AUC of 0–5 min was calculated and statistically compared for the conditions with chemical interventions, while the 2–5 min AUC data were compared for the thermal interventions (i.e. itch intensity data from the moment the thermal counter-stimuli were initiated at 2 min to the end of the monitoring at 5 min). Five min after each interventional condition, subjects were asked to rate the mean pain experienced as a consequence of the counter-irritation. Similarly to the rating of the itch intensity, the pain rating was performed on a $VAS_{0–10}$, with 0 being labelled as "no pain" and 10 being labelled as "worst imaginable pain".

Neurogenic inflammation (superficial blood flow) and wheal reactions to histamine

To visualize changes in the cutaneous perfusion as a proxy for neurogenic inflammation, a speckle contrast imager (Moor FLPI, Moor Instruments, Axminister, UK) was used 6 min after each histamine application. The imager was placed with a 50 cm distance

to the application area, and the images analysed with proprietary software (MoorFLPI Review version 4.0, Moor Instruments, Axminister, UK). The line histogram tool was used to analyse the intensity and dispersion of neurogenic inflammation through a 9-cm proximal-to-distal line. As a result of the intradermal histamine injection, a wheal occurred around the injection site. It was measured, horizontally and vertically, 9 min after every application. Statistical analysis for neurogenic inflammation was performed by comparison of the mean perfusion intensity in a 4-cm segment surrounding the site of histamine introduction. Infrared thermography (A40, FLIR systems, Wilsonville, OR, USA) was conducted to ensure that the control condition of 32°C was within an appropriate temperature range, i.e. close to the normal physiological temperature of the subject. Thermography was conducted at a 45-cm distance to the area of application, with an exposure time of 18 ms. Images were analysed using the manufacturer's software (ThermaCAM Researcher Pro 2.10, FLIR Systems, Wilsonville, OR, USA).

Statistical analysis

Basic data handling and descriptive statistics were performed in Excel (Microsoft, Redmond, WA). All statistical analyses were performed using SPSS version 22 (IBM, New York, USA). The sample size calculation was conducted based on test–retest reliability data (41) in combination with an α-level of 0.05, a power of 80%, and a least relevant difference of a 50% itch reduction. Data from the present study were confirmed to be parametric by visual inspection and complementary Shapiro–Wilk tests. The primary outcome was considered to be the intensity of itch 2–5 or 0–5 min after 1% histamine injections with/without counter-stimuli or control interventions, and the resulting data was expressed as itch intensity/min (itch AUC) and analysed with a RM-ANOVA test using the Sidak *post hoc* correction for multiple paired comparisons. Comparisons were made between experimental interventions and their respective controls; in particular, the thermal counter-stimuli were statistically compared with the 32°C control condition to control for the pressure of the probe. Secondary outcome parameters were the induced wheal and neurogenic inflammation, which were analysed similarly to the primary outcome. Furthermore, correlational analyses were performed between the individually experienced itch inhibition for each the thermal interventions and the CDT and CPT obtained using the Pearson product-moment correlation coefficient. The statistical test results were considered significant at $p < 0.05$ and highly significant at $p < 0.01$. All data are presented as the arithmetic means ± SEM.