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Psychophysical and vasomotor evidence for interdependency of TRPA1 and TRPV1 nociceptive responses in human skin: an experimental study

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Running title: TRPA1 and TRPV1 interactions in human skin

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Abstract

The TRPA1 and TRPV1 receptors are important pharmaceutical targets for antipruritic and analgesic therapy. Obtaining further knowledge on their roles and inter-relationship in humans is therefore crucial. Preclinical results are contradictory concerning co-expression and functional interdependency of TRPV1 and TRPA1 but no human evidence exists. This human experimental study investigated whether functional responses from the subpopulation of $TRPA1^+$-nociceptors could be evoked following defunctionalization of $TRPV1^+$-nociceptors by cutaneous application of high-concentration capsaicin. Two quadratic areas on each forearm were randomized to pretreatment with an 8% topical capsaicin patch or vehicle for 24h. Subsequently, areas were provoked by transdermal 1% topical capsaicin (TRPV1 agonist) or 10% topical allyl-isothiocyanate ('AITC', a TRPA1-agonist), delivered by 12mm Finn chambers. Evoked pain intensities were recorded during pretreatments and chemical provocations. Quantitative sensory tests were performed before and after provocations to assess changes of heat pain sensitivity. Imaging of vasomotor responses was used to assess neurogenic inflammation after the chemical provocations. In the capsaicin-pretreated areas both the subsequent 1% capsaicin- and 10% AITC-provoked pain intensities were inhibited by 92.9±2.5% and 86.9±5.0% (both: $P<0.001$), respectively. The capsaicin-ablated skin areas showed significant heat hypoalgesia at baseline ($P<0.001$) as well as heat antihyperalgesia, and inhibition of neurogenic inflammation evoked by both 1% capsaicin- and 10% AITC provocations (both: $P<0.001$). Ablation of capsaicin-sensitive afferents caused consistent and equal inhibition of both TRPV1 and TRPA1-provoked responses assessed psychophysically and by imaging of vasomotor responses. The present study suggests that TRPA1 nociceptive responses in human skin strongly depend on intact capsaicin-sensitive, $TPRV1^+$ fibers.

Key words: Capsaicin, AITC, Mustard oil, TRPV1, TRPA1, Neurogenic inflammation, Hyperalgesia

Article category: Original research manuscript

1. Introduction

A variety of receptors are expressed by cutaneous nociceptors (C- and Aδ-fibers). Key transducers include the transient receptor potential vanilloid 1 (TRPV1) and ankyrin 1 (TRPA1), both of which are members of the TRP ion-channel superfamily [45]. TRPA1 and V1 are activated by various algogens and noxious stimuli, and much GPCR-detected nociceptive and pruriceptive signaling co-opts these channels. In particular, TRPA1 is required for non-histaminergic itch, as multiple histamine-independent itch transduction pathways involve TRPA1 co-activation [88]. TRPA1 is also involved in pain as well as inflammation of the skin, airways and gastrointestinal tract, acting both as an inflammatory instigator and a detector of various inflammatory mediators [19,67]. TRPA1-signalling has been implicated in a diverse range of diseases including migraine [28], diabetic neuropathy [48] as well as atopic dermatitis, where lesional skin areas exhibit highly increased expression [60,63].

While capsaicin activates TRPV1, allyl isothiocyanate (AITC), also known as mustard oil (MO) activates TRPA1 [10]. TRPV1 is evidently more densely expressed in rodent dorsal root ganglion (DRG) nociceptors than TRPA1 but the two receptors do exhibit substantial co-expression. However, it remains unclear whether a functionally significant subpopulation of $TRPA1^+$ but $TRPV1^-$ nociceptors exists. Following the initial discovery of TRPA1, rodent studies showed an almost complete DRG co-expression of TRPV1 and TRPA1 mRNA [81]. Using calcium imaging in rat trigeminal ganglia, AITC-responsiveness was shown in 35% of the neurons, while capsaicin exited 55%, including all of the AITC-responsive cells [41]. Contrasting these findings, several recent rodent studies using, e.g., back-labeling of cutaneous afferents and unbiased single cell RNA sequencing have suggested that $TRPA1^+$ nociceptors, which do not express TRPV1 are much more common than previously assumed [37,51,56,82,85]. As such the expressional patterns of TRP-channels in nociceptive DRG neurons and on peripheral nociceptors and in particular, the functional overlap between TRPA1 and V1 in the nociceptive system of rodents remain unknown. Moreover, despite substantial inter-specie differences in somatosensory processing [22], no attempts in have been conducted to assess TRPA1/V1 interactions human skin and in particular whether TRPA1-induced responses are predominantly TRPV1-dependent. Due to the significant pathophysiological implications of TRPA1 and TRPV1, e.g., in pain and itch conditions, development of selective TRPA1 and V1-antagonists are being actively pursued for instance as novel analgesics and antipruritics. Acquiring further knowledge regarding the functional interdependency of TRPA1/V1 is therefore important for drug development, early phase testing, and potential evaluation of disease indications.

Administration of 8% topical capsaicin (Qutenza™) can drastically defunctionalize human TRPV1$^+$ nociceptive cutaneous afferents [4,9,43,61], thus enabling investigation of sensory and vasomotor responses in absence of this significant proportion of nociceptors [9,35]. This randomized, double-blinded, vehicle-controlled study aimed to evaluate the extent of the functional overlap between the TRPA1 and TRPV1 in healthy human skin, by comparatively assessing pain, heat pain sensitivity, and neurogenic inflammation evoked by capsaicin and AITC in skin areas pretreated with topical 8% capsaicin. Based on recent rodent studies, we hypothesized that prolonged 8% capsaicin-pretreatment would result in a complete abolition of TRPV1-evoked responses, but only a moderate reduction in TRPA1-evoked responses, reflecting a substantial, but incomplete, functional overlap between the two nociceptor populations in human skin.

2. Methods

2.1. Participants and study design

Eighteen healthy subjects (9M/9F, aged 28 ± 7 years (mean ± SD)) were recruited. Subjects were pain-free, without previous known dermatological, allergic, musculoskeletal, neurological, or psychiatric disorders. Subjects were instructed to abstain from alcohol and medication 24 h prior to all sessions. Before participating in the study, all subjects signed a statement of informed consent in accordance with the 2013 Helsinki Declaration. The regional ethics committee approved the experimental protocol (study no. N-20170018). The study was carried out in a vehicle-controlled, double-blinded manner with balanced randomization of the placement of pretreatment (vehicle vs. 8% capsaicin), provocation compounds (10% AITC and 1% capsaicin) as well as the order of provocation tests. The study was conducted in three sessions with intersession intervals of 24 h (see Fig. 1A). In session 1, patches were applied, in session 2 patches were removed and in session 3, provocation compounds were applied, and sensory as well as vasomotor responses were assessed. The investigator conducting the psychophysical tests in session 3 was blinded with respect to treatment. Therefore, Investigator A conducted session 1 and 2, and Investigator B conducted session 3 or vice versa. All sessions were conducted using a standardized script, in order to minimize information/observer bias.

2.2. Application of 8% capsaicin and vehicle patch (pretreatment)

A total of four squared areas (A1-A4), measuring 4x4cm, were marked on the volar forearms of all subjects (see Fig. 1B). Each area was treated in a block-randomized manner with a patch (4x4 cm) containing either 8% capsaicin (Qutenza, Astellas Pharma A/S, Kastrup, Denmark) or vehicle (Qutenza Demo patch, Astellas Pharma). The patches were placed 4 cm apart to blind the subjects (taking advantage of the poor spatial resolution of cutaneous chemesthesis (previously estimated to be

approximately 15 cm on the longitudinal axis of the volar forearm in healthy controls) while ensuring that neurogenic inflammatory reactions evoked in the 3rd session would not overlap. Furthermore, unblinding caused by identification of primary and secondary capsaicin-evoked neurogenic inflammation, were avoided by masking patches using non-transparent medical tape. This approach has been applied in a previous study using a similar 8% capsaicin ablation method [9]. Following the application of patches in session 1, subjects rated the pain intensity on each arm, once every hour, for six hours. This was done using a numerical rating scale (NRS_{0-10};"no pain" = 0 to "worst imaginable pain" = 10). Then, 24 h later, subjects rated the average and peak pain scores experienced during the 24 h. Robust defunctionalization of $TRPV1^+$ epidermal fibers is known to be induced by 24 h application of 8% capsaicin [9]; hence, patches were left for 24 h before being removed during session 2. After patch removal, the participants were asked if they could identify the active patch area, to get an estimate of the successfulness of the blinding procedure.

2.3. Quantitative sensory testing (QST)

In session 3, heat pain thresholds (HPT) and suprathreshold heat pain sensitivity (SHPS) were assessed using a Medoc Pathway (Medoc Ltd, Ramat Yishay, Israel) equipped with a 3x3 cm stimulator probe with the aim of assessing the development of heat hyperalgesia. This was done to assess changes in heat pain sensitivity evoked by the pretreatment and provocation compounds. All sensory tests were performed as three consecutive stimuli to the treated areas (A1-A4, Fig. 1B). The provocation order and anatomical locations were randomized. Hence, if A1 was determined to be the first area for provocation compound application, this was also the first area in which sensory testing were performed. Sensory testing was performed in all 4 areas immediately before moving on to provocation compound administration. This was done in order to ensure that all measurements had the same time from pre-HPT/STHP to substance administration (provocation).

Heat pain thresholds were measured using a ramping stimuli of 1°C/s from a baseline temperature of 32°C, with a cut-off of 52°C and with 5s inter-stimulus intervals based on standardized QST protocols [69,70]. As soon as the subjects sensed the warmth sensation becoming painful, they pressed a stop button resulting in a return to the baseline temperature. For SHPS, subjects verbally rated the pain intensities (same NRS_{0-10} as applied for 8% capsaicin pretreatment) following each heat stimulus. A stimulus went from a 32°C baseline to a 3 seconds plateau at 46°C and with ramping of 5°C/s. Inter-stimuli intervals were 10 seconds. Subjects were unable to observe the probe temperatures during the assessments. For SHPS, short 46°C stimuli were applied to induce mild to moderate pain without evoking discernable, prolonged neurogenic inflammation (evoked around 48°C [87]), which could interfere with subsequent

chemically evoked vasomotor responses. Additionally, the stimulus intensity was chosen considering that it would be tolerable in all subjects and to avoid induction of sensitization. While HPT is thought to be encoded predominantly by mechano-heat-sensitive C-fibers ('CMH', possibly the quickly adapting subtype), the SHPS assessment was conducted to more broadly activate heat-sensitive nociceptors, including CMH, C-mechano-insensitive (CMi), and possibly type-II Aδ-fibers [23,40,50,84,89]. Both HPT and SHPS, were calculated as means of the three consecutive stimuli. ΔHPT and ΔSHPS were defined as the average difference before vs after provocations. For correlation analyses, ΔHPT_2 and $\Delta SHPS_2$ were used, defined as the average difference between vehicle and ablated areas at baseline.

2.4. Application of chemical provocations

After the initial sensory testing, a solution containing either 10% allyl isothiocyanate (hereafter referred to as 'AITC10%') or 1% capsaicin (hereafter referred to as 'CAP1%') was applied to the pretreated areas. The AITC (Sigma Aldrich, Brøndby, Denmark) was dissolved in 99% pharmaceutical grade paraffin (Løve Apoteket, Aalborg, Denmark) at a concentration of 10% AITC (vol/vol). This concentration was determined from previous studies [49,77], including a recently published dose-response study [10]. Capsaicin was dissolved in a solution of 30% deionized water and 70% ethanol at a concentration of 1% (10mg/mL; Skanderborg Apotek, Denmark). For both AITC10% and CAP1%, a 50 µl solution was dispensed onto filter disc placed in a 12mm Finn chamber attached with BSN medical tape (Fixomull Stretch, BSN Medical AB, Billdal Sweden). Because AITC penetrates into the epidermis and evokes pain more rapidly than capsaicin, the AITC chamber was left on for 5 minutes while the capsaicin chamber was left on for 20 minutes in accordance with previous studies [49,65]. Following application of provocation compounds, subjects rated the pain intensity on a digital VAS ($VAS_{0\text{-}100}$; "no pain" = 0 to "worst imaginable pain"= 100) for 6 minutes (AITC10%) or 25 minutes (CAP1%). This was done using eVAS software (Aalborg University, Denmark) installed on a 10.1" Samsung tablet computer (Samsung Electronics, Seoul, Korea), and with pain intensity sampled at 0.2 Hz, which allowed for a continuous recording of pain intensity. Chemically evoked pain intensity was regarded as the primary outcome of the study.

2.5. Neurogenic inflammation assessed by superficial blood perfusion

Immediately after removing the Finn chambers, superficial blood perfusion was measured using a Full-Field Laser-speckle Perfusion Imaging instrument (FLPI-1, Moor Instruments Ltd, Axminster, UK). Measurements were performed at a distance of 35 cm between the FLPI-lens and the skin surface. Exposure time was set to 8.3 ms and gain to 160 units. The data were analyzed using MoorFLPI Review V4.0 software (Moor Instruments Ltd, Axminster, UK). Increase in average and peak superficial blood perfusion, within the marked areas, was used as a measure of the primary neurogenic inflammation intensity. The axon-reflex-flare size, evoked by AITC10% and CAP1%, was calculated as the area

exhibiting a >50% increase in superficial blood perfusion, compared to the background baseline perfusion (i.e. the individual baseline capillary perfusion in areas unaffected by any skin provocation). The size was quantified in cm^2 by relative comparison to the known size of the 4x4 cm area, resulting in an estimate of the size of the secondary neurogenic inflammation or "axon-reflex-response". A line-approach was used to evaluate the spatial characteristics of the neurogenic inflammatory reaction. An 8 cm line, was marked longitudinally, centered through the administration area. Hereafter, superficial perfusion along this line was quantified and averaged in 0.33 cm increments. These methods have been used in a series of previous studies [7,8,10,64].

2.6. Statistics

Data handling and calculation of descriptive statistics were carried out using Microsoft Excel (Microsoft, NM, USA), while statistical comparisons were performed using SPSS 25.0 (IBM NY, USA). Sample size estimation was conducted using the approached outlined for similar crossover designs [59,64]. All obtained data are presented as arithmetic means ± the standard error of mean (SEM), unless otherwise stated. The collected data were tested for normality by inspecting Q-Q plots and when needed by Shapiro-Wilk normality test. For combined reporting of statistically significant effects, the lowest F-value was reported. Average and peak pain intensities (NRS and VAS-recordings) were calculated and compared. The primary data analyses were conducted using the repeated measures analysis of variance (RM-ANOVA) with the factors: *Pretreatment* (2 levels; 8% capsaicin ablation and vehicle) and *Provocation* (2 levels; AITC10% and CAP1%). For HPT and SHPS, an additional level of *Time* (2 levels; before and after provocation) was added to the RM-ANOVAs. To assure that the randomized order of stimuli performed in session 3 did not constitute a bias, additional RM-ANOVAs were conducted wherein 'order' and 'anatomical location' were added as between-subjects factors. Moreover, to comparatively assess the achieved inhibition of AITC and capsaicin-evoked pain %-reductions were calculated and compared using Wilcoxon signed rank Test. Z-score changes evoked by the 8% capsaicin pretreatment were calculated allowing for cross-parameter comparisons relative to outcome variability. The formula: $Z = [(\mu_{treatment} - \mu_{baseline}) / \sigma_{baseline}]$ was used. For all tests, Bonferroni post hoc test was used to compensate for multiple comparisons. Correlational analyses between selected parameters were performed by Pearson's coefficient analysis and corrected by the Holm-Sidak method. A P-value ≤ 0.05 was considered significant. Asterisks for all figures: * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$.

3. Results

Nineteen subjects were enrolled and 18 completed the study sessions. One subject was excluded because of premature self-removal of the patches due to intense pain (drop-out). There were no unexpected side effects from applying either AITC or CAP. No significant differences were observed related to arm dominancy for any of the outcomes. It was specifically assessed and confirmed in all subjects that the 8% capsaicin-evoked pain had completely subsided prior to the beginning of session 3 (24 h after the patch removal), which is in line with a previous study using similar ablation technique [9]. The statistical assessment of a potential effect of provocation order, anatomical location, and interference between stimuli revealed no significant results.

3.1. Pain evoked by capsaicin-ablation treatment

During the 24 h topical administration of 8% capsaicin patches, mean application pain plateaued at 3.8 ± 0.5 (NRS_{0-10}) for the right forearm and 3.7 ± 0.6 for left the forearm. There were no differences in pain between the right and left forearms ($F_{1,17}$ = 0.292, P=0.596). Retrospectively rated average and peak pain intensities during 24 h application reached 3.4 ± 0.3 and 5.5 ± 0.5, respectively (Fig. 2). Subjects were able to correctly localize the active capsaicin patch site from the vehicle patch in 80.6% of the cases, with 50% being the 'by chance'. No visible skin reaction or erythema was present when the patches were removed after 24 h application. Hence, unblinding in a subset of participants was based purely on localization of the evoked pain during application. Subjects reported that pain subsided within 0.5-2 hours after removal of the patches.

3.2. Pain intensities following chemical provocations

There was a significant effect of *pretreatment*, on both CAP1% and AITC10% mean and peak pain intensities (lowest test F-value ($F_{1,17}$ = 30.9, P<0.001; mean pain, Fig. 3A and B)). CAP1%-evoked peak pain intensity decreased from 22.7 ± 4.7 (VAS_{0-100}) in vehicle-treated areas to 2 ± 0.8 in ablated areas, i.e. a 92.9 ± 2.5% pain reduction. The very limited CAP1%-evoked pain remaining in the ablated skin areas in 6/18 subjects indicate an almost complete defunctionalization of cutaneous capsaicin-receptiveness. For AITC, evoked peak pain decreased from 27.7 ± 5.2 (VAS_{0-100}) observed in vehicle-treated areas, to 5.6 ± 2.4 in ablated areas, i.e. an 86.9% ± 5.0% pain reduction (Fig. 3C and D). Similarly, robust reductions in the ablated areas were observed for mean pain intensity (Fig. 3E and F).

There was no main effect between type of *provocation* in mean and peak pain (lowest test F-value: $F_{1,17}$ = 1.8, P=0.202; peak pain), signifying that the CAP1% and AITC10% provocations evoked similar peak and mean pain intensities. Moreover, the *pretreatment x provocation* interaction was insignificant for mean and peak pain (lowest test F-value: $F_{1,17}$ = 0.1, P=0.757; peak pain) indicating that the pretreatment-

evoked desensitization inhibited subsequent CAP and AITC-evoked pain to a similar extent. Even with isolated testing, the ablation-induced pain reductions for AITC10% and CAP1%-exposed areas, were not different (P=0.508).

3.3. Thermal sensory sensitivity

There was a significant interaction effect in *pretreatment x provocation x time* in heat pain thresholds (HPT) ($F_{1,17}$=9.4, P=0.007). Subsequent post hoc testing showed that capsaicin-ablated areas had significantly higher heat pain thresholds (average temperature across all HPT assessments in the pretreated skin areas: 47.9 ± 0.5°C), than did vehicle-pretreated areas (average temperature across all HPT assessments in the pretreated skin areas:40.6 ± 0.6°C, P<0.001). This was the case both before and after provocations (P<0.001), i.e., the 24 h 8% capsaicin-ablation established significant heat hypoalgesia even after the chemical provocations. In vehicle-pretreated areas, provocation with CAP 1% decreased HPT more robustly than AITC 10% (P<0.001), while in ablated areas, the HPT values did not differ between provocations (P=0.8, Fig. 4A), constituting an anti-hyperalgesic effect.

A *pretreatment x time* interaction was found for SHPS ($F_{1,17}$ = 30.4, P<0.001). Post hoc testing showed lower heat pain sensitivity in ablated areas (avg. $NRS_{0\text{-}10}$; (1.2 ± 0.3) than vehicle-pretreated areas (4.5 ± 0.5) both before and after chemical provocations (P<0.001), indicating heat hypoalgesia. There was a significant increase in SHPS in vehicle area subsequent to both provocations, signifying development of heat hyperalgesia, which did not occur in ablated areas (P<0.001). The 8% capsaicin-ablation induced an average post-provocation decrease in suprathreshold heat pain ratings of 74.9% ± 7.9 for CAP1% and 74.5% ± 7.7 for AITC10% (Fig. 4B), signifying very robust antihyperalgesic effects.

For ΔHPT, a *pretreatment x provocation* interaction was evident ($F_{1,17}$=9.4, P=0.007). The ΔHPT was significantly reduced in vehicle-pretreated (-5.2 ± 0.7°C) compared with the ablated skin (-1.0 ± 0.2°C, P<0.001), for both types of provocations (CAP1%: P<0.001, AITC 10%: P=0.043), with CAP1% prompting a more pronounced HPT drop than AITC10% only in vehicle-treated areas (P<0.001, see Fig. 4C). In ablated areas, no differences were found for ΔHPT between provocations with CAP1% and AITC10% (P=0.932). For ΔSHPS, there was a significant effect of *pretreatment* ($F_{1,17}$= 30.4, P<0.001). Ablated areas showed lower changes in ΔSHPS following provocations (0.2 ± 0.2) than did vehicle-pretreated areas (2.3 ± 0.3, P<0.001, Fig. 4D). For ΔSHPS there were no differences between the two types of provocations (P=0.534).

3.4. Neurogenic inflammatory response

A *pretreatment x provocation* interaction was found for both mean and peak perfusion (lowest test F-value: $F_{1,17}$=4.861, P=0.042, mean perfusion). In ablated areas, no significant differences were found between provocations (mean P=0.435, peak P=0.183), corresponding to almost entirely indiscernible reactions in most subjects (see Fig. 5). In vehicle areas, CAP1% produced a significantly larger increase in both mean and peak perfusion compared to AITC10% (mean P=0.023, peak P=0.021). For axon-reflex-flare there was a significant effect of *pretreatment* ($F_{1,17}$ = 141.7, P<0.001). The capsaicin-ablation caused a decrease in flare size from 7.5 ± 0.7 cm^2 in vehicle-treated areas to 0.5 ± 0.5 cm^2 in ablated areas for CAP1% and similarly for AITC10% from 8.0 ± 0.8 cm^2 to 0.5 ± 0.4 cm^2 (P<0.001, see Fig. 6A and B). No significant effect of *provocation* was found on axon-reflex flare size ($F_{1,17}$ = 0.213, P=0.650) suggesting that the AITC10% and the CAP1% provocations evoked comparable neurogenic inflammatory responses. The line analysis (Fig. 6C) did not reveal any significant differences in the spatial distribution of the neurogenic inflammatory reaction, in neither the vehicle- nor capsaicin-ablated areas. However, a trend towards a more dispersed, but less homotopically intense AITC10%-induced neurogenic flare was evident (smallest P=0.11). Similarly, an insignificant trend towards slightly increased in perfusion was observed for AITC10% capsaicin-ablated areas restricted only to the provocation administration area (Fig. 6C).

3.5. Sex-related differences

For peak pain, an interaction effect was found between *pretreatment x provocation x sex* ($F_{1,16}$ =4,7, P=0.045). Subsequent post hoc showed that females had significantly higher peak pain scores in vehicle-pretreated areas following CAP1% (34.2 ± 7.3, $VAS_{0\text{-}100}$) than did males (11.2 ± 2.6, P=0.009). This was not the case for AITC10% (P=0.350). For mean perfusion, a *pretreatment x sex* interaction was found ($F_{2,16}$ =4.703, P=0.046). Post hoc tests showed that in female subjects, vehicle-pretreated areas exhibited a higher increase in mean perfusion as compared with that of males. This was true both for CAP1% (P=0.007) and to a lesser extent for AITC10% induced reactions (P=0.05) .

3.6. Correlations

A positive correlation was found between 8% capsaicin-ablation evoked pain and CAP1%-evoked pain in vehicle-pretreated skin (r=0.676, P=0.027, see Table 1). Similarly, both 8% capsaicin-ablation evoked pain and CAP1%-evoked pain correlated strongly with baseline SHPS (lowest: r=0.702, P=0.001), which was not the case for AITC10%-evoked pain and SHPS (P=0.29). No significant correlation was found between mean capsaicin-ablation evoked pain (24 h) and the obtained difference between SHPS in vehicle and ablated areas at baseline ($\Delta SHPS_2$, r=0.577, P=0.094). In vehicle areas, a strong positive

correlation was found for neurogenic flare between CAP1% and AITC10% (mean r=0.756, P<0.001, peak r=0.704, P=0.001). No correlation was found between heat sensitivity or CAP1% and AITC10%-evoked pain (r=0.475, P=0.282), suggesting that sensitivity to provocations by CAP1% does not confer sensitivity to AITC10%.

3.7. Z-scores

Fig. 7 provides a comparative overview of the capsaicin-ablation evoked changes when accounting for the natural variability within each variable. When assessing the efficacy of the different sensory and vasomotor responses for CAP1%, the inhibition of heat hyperalgesia (ΔHPT) showed the highest Z-score, indicating that this parameter most robustly detected the ablation responses. Notice that compared to AITC10%, this effect of the ablation was exclusively driven by CAP1%'s more robust elicitation of heat hyperalgesia (as assessed by HPT) in the vehicle-pretreated areas. For AITC10%, changes in mean axon-reflex flare size exhibited the highest Z-score and this parameter was the 2nd most robust for CAP1%.

4. Discussion

High-concentration capsaicin ablation almost completely abolished both AITC- and capsaicin-evoked pain, heat hyperalgesia, and neurogenic inflammation. For both AITC10% (TRPA1 agonist) and CAP1% (TRPV1 agonist) provocations, the pain intensities were similarly reduced by around 90% by the ablation of capsaicin-sensitive nociceptors. Hence, no differences in the desensitization efficacy of the capsaicin-ablation were observed for the two different TRP-provocations, suggesting that TRPA1-nociceptors in human skin are uniformly TRPV1^{+}.

4.1. Capsaicin ablation

Topical high-concentration capsaicin causes defunctionalization of capsaicin-sensitive fibers resulting in profound reduction of contact and laser-evoked heat pain sensitivity [9,35,43,54,61] properties ascribed to the function of superficial TRPV1^{+} nociceptors [4,21,62]. It is unclear whether nociceptor activation *per se* is a crucial aspect of this desensitization process. Some clinical studies have asserted that the use of local anesthetics do not reduce the efficacy of the capsaicin-ablation [44,68,83]. Experimental studies have found an association between the pain experienced during patch application and the efficacy of the desensitization [9,57,79]. The present study did not show a positive correlation between capsaicin ablation-induced pain and $\Delta SHPS_2$ (difference between vehicle and ablated areas) although a trend was evident (corrected P=0.09) suggesting that the vigorousness of the nociceptive barrage during the ablation and the resultant desensitization are not strictly aligned.

4.2. Capsaicin 8% pre-treatment inhibits both TRPV1 and TRPA1-evoked responses

Previous studies using similar chemical provocation techniques have found pain intensity curves and neurogenic inflammation for both AITC and capsaicin, which were comparable to the present findings [10,49,65,77]. Currently, profound reductions in both mean and peak pain were seen in capsaicin-ablated areas when subsequently exposed to AITC10% or CAP1% with no significant differences between the two provocations. This suggests that the defunctionalization of TRPV1-expressing cutaneous fibers robustly abolishes TRPA1-evoked nociceptive responses and implies that no functionally significant subpopulation of nociceptors in human skin are $TRPA1^{+}/TRPV1^{-}$. These functional human data support animal studies suggesting that TRPA1 receptors are almost completely co-expressed with TRPV1 receptors [41,46,81] (see section 4.3). Strong correlations were present between heat pain sensitivity and capsaicin-evoked pain in vehicle-treated area. On the contrary, a complete lack of correlations was observed between AITC-evoked pain versus heat or capsaicin-evoked pain. With our main conclusion in mind, AITC thus produces pain in a distinct manner compared to heat or capsaicin. Since the prolonged capsaicin pretreatment is thought to defunctionalize and ablate fiber branches and this practically abolished AITC-reactivity, it is highly conceivable that AITC-evoked pain is distinct at the receptors level (i.e. engaging TRPA1) while it appears to rely on $TRPV1^{+}$-fibers.

Mechanistically, development of primary heat hyperalgesia involves sensitization of both Aδ- and C-fibers [78] and it is well documented to occur following topical application of capsaicin [61] and AITC [10,77]. Notably, and distinct from e.g. allodynia, primary heat hyperalgesia can be evoked without spontaneous preceding or ongoing pain (e.g. following UVB irradiation) [35]. In the present study both short-term topical AITC10% and CAP1% provocations generated substantial heat hyperalgesia in vehicle-pretreated skin. However, following a prolonged high-concentration capsaicin ablation, the development of heat hyperalgesia was entirely abolished and replaced by stabile heat hypoalgesia. This signifies that no sensitization of heat-sensitive nociceptors occurred and that no sensitization of a potential heat-insensitive $TRPA1^{+}/TRPV1^{-}$ nociceptor subpopulation resulted in *de novo* heat pain receptiveness. In this context, it has been suggested that rodent $TRPV1^{-}$ heat-insensitive nociceptors may develop heat pain sensitivity during partially TRPA1-mediated inflammation [30] , but these fibers alone are insufficient for the establishment of heat hyperalgesia [47]. This notion is corroborated by the present study. It is very likely that TRPA1-mediated heat hyperalgesia is caused by peripheral sensitization of nerve fibers co-expressing TRPV1, although the exact molecular mechanism(s) for such heat sensitization is unclear [26,36,86]. Presently, the defunctionalization of such nerves markedly inhibited the effect of AITC. The stronger heat hyperalgesia observed for HPT in vehicle-pretreated skin following CAP1% could signify preferential effect on a subset of heat-sensitive nociceptors, which express TRPV1, but not TRPA1.

Based on evidence from primate microneurography it could be speculated that the drastic HPT decreases observed in vehicle-pretreated skin is mediated predominantly by CMH-fibers while a more considerable contribution from additional sensitized heat-nociceptor populations likely contributes to the elevated SHPS [23,40,50,84,89]. Following ablation, essentially all heat sensitive units (CMH, CMi and Aδ-fibers) activated in the TRPV1 activation range would be defunctionalized and thus the heat pain sensitivity diminishes (HPT and SHPS) and no heat hyperalgesia following AITC10% or CAP1% can be mounted.

The axon-reflex-flare response is mediated predominantly by CMi-fibers through release of vasoactive peptides [73–75]. In vehicle-pretreated skin, AITC10% and CAP1% induced similar neurogenic flare reactions suggesting that the same substrate is engaged by both provocations and that differences in transdermal penetration were reasonably well accounted for. At the receptor-level, AITC has been proposed to induce neurogenic inflammation in a TRPV1-independent manner in rodents [15]. However, there are notable differences between neurogenic inflammatory characteristics in human and rodent skin [75]. Presently, the 24 h capsaicin ablation resulted in an almost complete inhibition of the axon-reflex-flare, and robustly reduced increases in mean and peak perfusion after chemical provocations. The inhibitory effect of capsaicin-induced defunctionalization on neurogenic flare evoked by various irritants is well established [25,33,35,55,73]. As the inhibition of the axon-reflex-flare caused by the ablation was similar for both areas treated with CAP1% and AITC10%, the induced flares presumably depend entirely on $TRPA1^+$/$TRPV1^+$ CMi-fibers neurons. This is corroborated by microneurography studies in humans showing that CMi-units are almost always capsaicin-sensitive [72,76]. Lastly, considering the different quantification methods, the observed neurogenic inflammatory reactions (7.35 cm^2; AITC10% and CAP1% averaged) correspond well with previously reported receptive field sizes for human CMi-fibers assessed by microneurography (range: 1.1–14.2 cm^2, median; 5.34 cm^2) [71].

4.3. TRPV1 and TRPA1 co-expression

Story *et al.* (2003) found TRPA1 mRNA expressed in all $TRPV1^+$ rodent DRG neurons [81]. Likewise, when functionally assessed, $TRPA1^+$ rat trigeminal neurons were shown to be $TRPV1^+$ too, i.e., responding to AITC as well as capsaicin [41]. Similar findings have been reported in several studies [18,46]. Contrasting these initial studies, Malin *et al.* (2011) demonstrated that skin afferents express TRPV1, TRPA1 or both more rarely than previously assumed, i.e., 22% expressed TRPV1 mRNA, 6% expressed TRPA1, but only 10% expressed both TRPV1 and TRPA1 [56]. Another study on mice DRG-neurons showed that 49.7% of units solely expressed TRPV1, 43% TRPA1, but only 99 of 149 $TRPA1^+$-neurons co-expressed TRPV1 [82] which is aligned approximately with recent results from rat trigeminal neurons [37]. Finally, RNA-sequencing in mice DRG-neurons have shown a subpopulation of nociceptors expressing

TRPA1 and MrgprD, but not TRPV1 [85,90]. Distinct innervation of various tissues and differences in DRG and axonal mRNA expression may, in part, explain the contradictory findings [24,56]. The present study used a capsaicin-ablation intervention to estimate the functional co-expression of TRPA1 and TRPV1 in human epidermal nerve fibers and findings support the notion of TRPA1 being expressed in a subset of $TRPV1^+$ fibers as described in early rodent studies. In this context, it should be highlighted that substantial interspecies differences are well documented within somatosensory afferent neurophysiology and transducer expression [29,52]. This study adds information about the interdependency of TRPA1 and TRPV1-evoked responses in humans, where prior evidence is scarce. *in vitro* responses of sensory neurons indicate transient homologous and heterologous desensitization mechanisms for TRPA1 and TRPV1 when stimulated with AITC and capsaicin [1], while some *in vivo* data contradict this notion [15]. Data by Simons *et al.* (2003) using administration to the oral mucosa of capsaicin and AITC in humans, also suggested rapid self- and cross-desensitization between the two agonists [80]. However, AITC-evoked responses following a prolonged capsaicin-induced desensitization have not previously been studied in humans and TRPA1-agonists do not appear to induce desensitization in human skin. The discrepancies between data from oral mucosa and skin probably are related to different study designs, including diverse concentrations and exposure times, as well as tissue-related differences in e.g. sensory sensitivity, penetration, and clearance (all of which are remarkably higher in the oral mucosa compared to the skin). Notably, many of the pain and itch conditions where TRPA1 and TRPV1 antagonists have been proposed to be of potential clinical utility, involve skin targeting (e.g. inflammatory skin diseases and peripheral neuropathies [12,30,34,48]).

4.4. Clinical implications

Development of systemic and topical antagonists for TRPA1 and TRPV1 is currently ongoing and several lead compounds have been, and are being, tested in clinical trials [42,58,66]. The efficacy of selective TRP-antagonists have unfortunately so far not shown strong clinical effects in chronic pain and, e.g., TRPV1 antagonists have shown considerable adverse effects such as hyperthermia [58]. To achieve better efficacy developing dual TRP-antagonists (e.g. targeting both TRPV1 and TRPA1) might be an option to increase the therapeutic window. Just as selective activation of TRPV1 and TRPA1 is performed in animal drug profiling studies, it is possible to test, e.g. TRPV1-antagonists for effect and target engagement in early clinical drug trials [13,34] but this requires reliable provocation models. The profound responsiveness observed for FLPI-measured neurogenic inflammation supports monitoring this outcome in response to TRPA1 or TRPV1 agonist provocation as a suitable and sensitive target engagement biomarker in humans [13,20,34]. Chemical activation of TRPV1 and TRPA1 may also be used for sensory profiling purposes in patients with, e.g., peripheral neuropathic pain conditions and adds to the existing QST

platforms as a way to translate pre-clinical findings into patients [16]. Such receptor profiling may provide additional information on loss- or gain-of-function for these specific receptor populations and similar mechanistic phenotyping has been shown to predict the effect of certain analgesics [27]. The current receptor-specific provocation models may also be used for testing, e.g., novel TRPA1-antagonists and recently clinical studies have been initiated provoking TRPA1/TRPV1-responses in patients with, for instance allergies or inflammatory dermatoses, to investigate itch, pain and inflammatory responses [2,5,6,39].

4.5. Limitations and future perspectives

While topical high-concentration capsaicin could potentially evoke effects on non-neuronal tissues, such as endothelial cells or unspecifically desensitize nerve terminals independently of TRPV1, for instance through mitochondrial respiratory distress or off-target effects related to the induced inflammation, such effects are probably marginal. TRPA1-modulation by inflammation generally appears to enhance TRPA1-expression and function [31,38,60], while capsaicin-induced ablation is thought to rely on Ca^{2+}-influx overload through TRPV1 as well as mismatch between high energy expenditure (given the prolonged vigorous firing induced by TRPV1-activation coupled with dysfunctional mitochondrial metabolism) [4,62]. This relative TRPV1-specificity of the applied ablation, is supported by the fact that no lingering inflammation is present when 8% capsaicin patches are removed, and the endothelial reactivity assessed by wheal responsiveness (an entirely non-neurogenic reflex) is normal in ablated skin [9]. Moreover, psychophysical evidence suggests that only warmth and heat sensitivity are very robustly decreased in skin following 8% capsaicin pretreatment [35,54]. The fact that unblinding occurred in a subset of subjects, has limited impact both because testing occurred 24 h after identification (which was never revealed to the subject) but also because the primary analyses simply compared the achieved relative inhibition for the two chemical provocations. A key premise was TRPV1 and TRPA1 activation by capsaicin and AITC, respectively. The selectivity of the two TRP-agonists, AITC in particular, has been questioned [3,32,56], but overall a large amount of evidence support their relative specificity [11,14,17,41,53,71]. More importantly in this context, even if a proportion of the nociceptive response of AITC was mediated by direct TRPV1-activation, the basic result interpretation would have remained unchanged. Similarly, even if prolonged capsaicin exposure causes heterologous desensitization of TRPA1-channels that would still only apply to fibers expressing TRPV1 and would thus leave a potential population of $TRPV1^-$ and $TRPA^+$-fibers unaffected. Because a robust correlation was present between SHPS or HPT and CAP1%-induced pain but not AITC10% pain, such unspecific ATIC-engagement of TRPV1 seems unlikely. Lastly, the present study did not assess to which extent capsaicin-sensitivity is maintained following a TRPA1-assoicated nerve fiber defunctionalization and thus cannot evaluate the significance and function of $TRPV1^+$, but $TRPA1^-$ cutaneous nociceptors. Such a subpopulation is uniformly reported in rodents and thus likely exists in

human skin [46,85]. Unfortunately, a parallel method for TRPA1-associated defunctionalization in human skin does not yet exist and prolonged topical application of TRPA1-agonists is generally associated with more prolonged and extensive skin inflammation than TRPV1-agonists [10].

5. Conclusion

Ablation of capsaicin-sensitive cutaneous fibers using high-concentration capsaicin strongly inhibited both AITC and capsaicin-evoked responses, including spontaneous pain, heat hyperalgesia, and neurogenic inflammation. The inhibition was consistent across all parameters and of similar efficaciousness for both chemical provocations. Thus, normal nociceptive AITC-responses are robustly inhibited when capsaicin-sensitive cutaneous nerve fibers are defunctionalized. This suggests that in human skin, $TRPA1^+$ primary cutaneous afferents belong to a subpopulation of $TRPV1^+$ nociceptors.

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Figure legends

Figure 1: Flowchart of study procedures and treatment areas. A) Sensory and vasomotor responses were conducted according to the sketch. Heat pain thresholds and suprathreshold heat pain sensitivity for all areas (A1-A4) were measured before provocation, while assessments were made individually after each provocation. Notice that both the order of the 8% capsaicin and vehicle patches (pretreatment) and compound application (provocation) (AITC 10% and capsaicin 1%) were randomized. **B)** Illustration of treatment areas on the volar aspects of the forearms (example). Note that areas were randomized. Abbreviations: AITC = Allyl isothiocyanate, CAP = Capsaicin, FLPI = Full-Field Laser-speckle Perfusion Imaging, HPT = Heat pain threshold, NRS = Numerical rating scale (pain intensity), SHPS = Suprathreshold heat pain sensitivity, VAS = Visual analogue scale (pain intensity).

Figure 2: Evoked pain during administration of 8% capsaicin patch. Evoked pain during the first 6 h of application is shown for each forearm. Dashed lines indicate 24 h retrospectively rated average (lower) and peak (upper) pain intensities. Abbreviations: NRS = Numerical rating scale. Means ± SEMs are shown.

Figure 3: Pain evoked by provocations with CAP 1% and AITC 10%. Mean pain intensities are shown over time for **A)** CAP 1% and **B)** AITC 10%**.** Dashed lines represent mean and peak pain intensities. 8% capsaicin induced sensory desensitization for CAP 1% and AITC 10% on **C)** peak**,** and **E)** mean pain intensities. %-changes in **D)** for peak pain and **F)** for mean pain intensities following capsaicin-ablation for CAP 1% and AITC 10%. Individual subjects (white dots) and mean (grey dots). Notice that **C-F** share legends. Abbreviations: AITC = Allyl isothiocyanate, CAP = Capsaicin, VAS = Visual analog scale. Means ± SEMs are shown. Asterisks: *** $P < 0.001$.

Figure 4. Heat pain sensitivity *before* and *after* provocation by CAP 1% and AITC 10% in vehicle- and capsaicin-ablated areas. A) Heat pain thresholds in capsaicin-ablated (grey) and vehicle-pretreated (white) skin area for both CAP 1% and AITC 10%. **B)** Suprathreshold heat pain sensitivity in capsaicin-ablated (grey) and vehicle-pretreated (white) skin area for both CAP 1% and AITC 10%. **C)** Change in HPT following application of CAP 1% and AITC 10%. **D)** Change in SHPS following application of CAP 1% and AITC 10%. Abbreviations: AITC = Allyl isothiocyanate, CAP = Capsaicin, NRS =

Numerical rating scale. Means ± SEMs are shown (**A** and **B**) and means (**C** and **D**). Asterisks: ** P < 0.01 and *** P < 0.001.

Figure 5. Representative superficial blood perfusion readouts. Four images from 5 different subjects following provocation by CAP 1% and AITC 10% in vehicle- and capsaicin-ablated skin areas. Notice almost complete inhibition of neurogenic inflammation, with only very slight increases in perfusion corresponding to the Finn chamber application area in two subjects shown in the upper panels. Black quadrant in lower left corner marks the treatment area while the black circle marks the provocation administration area. Abbreviations: AITC = Allyl isothiocyanate (10%), Arb. = Arbitrary, CAP = Capsaicin (1%).

Figure 6. Neurogenic inflammation and axon-reflex-flare evoked by CAP 1% and AITC 10% in vehicle- and capsaicin-ablated skin areas. A) Mean and peak superficial blood perfusion in capsaicin pretreated (grey) and vehicle-pretreated (white) skin areas. **B)** Axon-reflex-flare size. **C**) Spatial extent of of superficial blood perfusion following chemical provocations in capsaicin-ablated (dark grey and black) and vehicle-pretreated skin (white and light grey). X-axis depicts the longitudinal distribution of the flare reaction. Abbreviations: AITC = Allyl isothiocyanate, CAP = Capsaicin, arb = Arbitrary, cm = Centimeter. Means ± SEMs are shown. Asterisks: * P < 0.05 and *** P < 0.001.

Figure 7. Z-score plot for mean and peak evoked pain, heat pain, antihyperalgesia, and axon-reflex flare size for CAP 1% and AITC 10%. Negative values represent desensitization effect (i.e. reduced responses in treated skin). Note that the two chemical provocations only differ with respect to HPT (driven by differences in vehicle-treated skin area). HPT = Heat pain threshold, SHPS = Suprathreshold heat pain sensitivity. Means ± SEMs are shown.

Table legends

Table 1. Correlational analysis. Significant correlations are marked in grey. V= vehicle area, A= ablated area. P-values are multiplicity corrected by the Holm-Sidak method. An isolated correlational analysis of mean perfusion data was performed to retain power. Asterisks: * P < 0.05, ** P < 0.01, *** P < 0.001.

	1. Mean pain cap 8% (24h)	2. Mean pain CAP 1% (V)	3. ΔCAP 1% pain (V – A)	4. Mean pain AITC 10% (V)	5. ΔAITC 10% pain (V – A)	6. Mean pre-SHPS (V)		8. Mean perf. CAP 1% (V)
2. Mean pain CAP 1% (V)	*r* = 0.676 * *P* = 0.027						**9. Mean perf. AITC 10% (V)**	*r* = 0.756 *** *P* < 0.001
3. ΔCAP 1% pain (V – A)	*r* = 0.665 * *P* = 0.029	*r* = 0.989 *** *P* < 0.001						
4. Mean pain AITC 10% (V)	*r* = 0.367 *P* = 0.293	*r* = 0.475 *P* = 0.284	*r* = 0.441 *P* = 0.293					
5. ΔAITC 10% pain (V – A)	*r* = 0.369 *P* = 0.293	*r* = 0.470 *P* = 0.284	*r* = 0.433 *P* = 0.293	*r* = 0.984 ****P* < 0.001				
6. Mean pre-SHPS (V)	*r* = 0.849 *** *P* < 0.001	*r* = 0.702 * *P* = 0.016	*r* = 0.669 * *P* = 0.029	*r* = 0.386 *P* = 0.293	*r* = 0.435 *P* = 0.293			
7. ΔSHPS (V – A)	*r* = 0.577 *P* = 0.094	*r* = 0.642 * *P* = 0.040	*r* = 0.625 * *P* = 0.049	*r* = 0.416 *P* = 0.293	*r* = 0.427 *P* = 0.293	*r* = 0.722 ** *P* = 0.01