Full title: "Combining topical agonists with the recording of event-related brain potentials to probe the functional involvement of TRPM8, TRPA1 and TRPV1 in heat and cold transduction in the human skin**"**

Running title : ERP_agonist

Authors:

Arthur S. Courtin[1] & André Mouraux[1]

Affiliation:

1. Institute of NeuroScience, Université catholique de Louvain, Brussels, Belgium

Number of pages : 20

Number of figures : 8

Number of tables : 4

Corresponding authors :

Arthur Courtin

UCLouvain/IoNS/COSY

Avenue Mounier 53/B1.53.04

1200 Woluwe-Saint-Lambert

+32 2 764 54 88

arthur.courtin@uclouvain.be

https://uclouvain.be/fr/repertoires/arthur.courtin

https://www.nocions.org/arthur-courtin

Disclosure: ASC is a FRIA grantee from the Fund for Scientific Research – FNRS (Belgium). The authors have no conflict of interest to report.

Abstract:

TRP channels play a central role in the transduction of thermal and nociceptive stimuli by free nerve endings. Most of the research on these channels has been conducted *in vitro* or *in vivo* in non-human animals and translation of these results to humans must account for potential experimental biases and interspecific differences. This study aimed at evaluating the involvement of TRPM8, TRPA1 and TRPV1 channels in the transduction of heat and cold stimuli by the human thermonociceptive system. For this purpose, we evaluated the effects of topical agonists of these three channels (menthol, cinnamaldehyde and capsaicin) on the event-related brain potentials (ERPs) elicited by phasic thermal stimuli (target temperatures: 10°C, 42°C, and 60°C) selected to activate cold Aδ thermoreceptors, warm sensitive C thermoreceptors and heat sensitive Aδ polymodal nociceptors. Sixty-four participants were recruited, 16 allocated to each agonist solution group (20% menthol, 10% cinnamaldehyde, 0.025% capsaicin and 1% capsaicin). Participants were treated sequentially with the active solution on one forearm and vehicle only on the other forearm for 20 minutes. Menthol decreased the amplitude and increased the latency of cold and heat ERPs. Cinnamic aldehyde decreased the amplitude and increased the latency of heat but not cold ERPs. Capsaicin decreased the amplitude and increased the latency of heat ERPs and decreased the amplitude of the N2P2 complex of the cold ERPs without affecting the earlier N1 wave or the latencies of the peaks. These findings are compatible with previous evidence indicating that TRPM8 is involved in innocuous cold transduction and that TRPV1 and TRPA1 are involved in noxious heat transduction in humans.

Perspective

By chemically modulating TRPM8, TRPA1 and TRPV1 reactivity (key molecules in the transduction of temperature) and assessing how this affected EEG responses to the activation of cold thermoreceptors and heat nociceptors, we aimed at confirming the role of these channels in a functional healthy human model.

Keywords:

A-delta nociceptor ; A-delta cold thermoreceptor ; thermonociception ; TRPA1 ; TRPM8 ; TRPV1 ; capsaicin ; cinnamaldehyde ; menthol ; cold evoked potential ; contact heat evoked potential ; CHEP ; CEP ; EEG

1. Introduction:

TRP channels and thermonociception

Following the discovery of the heat- and capsaicin-sensitive channel Transient Receptor Potential Vanilloid 1 (TRPV1) by Caterina, Schumacher, Tominaga, Rosen, Levine, Julius [10], a number of additional thermosensitive TRP channels were discovered and the fundamental role of these channels in the transduction of thermal stimuli by nociceptive free nerve endings was disclosed.[8, 56]

Most of the research on TRP channels has been conducted *in vitro* or *in vivo* in genetically engineered animals. If these techniques have undisputed interest, translation of these results to humans should be done cautiously because of significant interspecific differences in the response properties of some TRP channels[11, 14, 32] and because their response properties are tuned by their chemical and electrical environment[55], which may be disrupted in some experimental setups. Functional studies conducted in living humans are thus needed to understand the involvement of TRP channels in human thermoreception and nociception.

At least three types of TRP channels are thought to be involved in the thermal activation of heat- and cold-sensitive afferents in humans. TRPV1 is thought to be implicated in the signalling of heat, both in the innocuous and noxious range[55], and the desensitization or deactivation of capsaicin-sensitive afferents using high-concentration topical capsaicin was shown to strongly decrease the ability of human subjects to detect heating stimuli in the innocuous and noxious range.[53] The TRP channel TRPM8 is considered to be the main transducer of innocuous cold and may also be involved in noxious cold transduction.[3, 4, 24] It is thus likely that it plays an essential role in the perception of cold triggered by the activation of cold-sensitive Aδ fibre thermoreceptors.[21] Finally, TRPA1 has been suggested to contribute to cold perception as a noxious cold sensitive channel, even though this claim is still debated.[9, 45, 56] Furthermore, together with the channels TRPV1 and TRPM3, TRPA1 has been suggested to form a TRP channel "trio" necessary for acute noxious heat sensing in mice [54], a fact supported by the burning pain elicited by various TRPA1 agonists such

as carvacrol or cinnamaldehyde.[23, 42, 44] There is evidence that these channels are expressed by human DRG neurons in physiological conditions.[1, 15] A more extensive review of the evidence of the involvement of these TRP channels in human thermonociception can be found in a review by Weyer-Menkhoff, Lotsch [61].

If thermosensitive TRPs can be activated by temperature, most of them are also sensitive to certain chemicals.[9] Most studies in living humans investigating the role of different TRP channels in thermoreception have focused on characterizing the spontaneous sensations elicited by the topical application of such agonists and by evaluating the changes in sensitivity to heat and cold using quantitative sensory testing.[61] In the literature, the most widely used chemical agonists for TRPV1, TRPA1, and TRPM8 are, respectively, capsaicin, cinnamic aldehyde, and menthol.[61]

Effects of TRP agonists on thermal-evoked brain potentials

Treating the skin with topical TRP agonists before applying transient thermal stimuli to record event related brain potentials (ERPs) is another relatively simple way to probe the functional involvement of TRP channels in human thermonociception. Such an approach was used by a few studies aiming at assessing the role of TRPV1 positive fibers in the perception of heat by evaluating the acute effects of capsaicin on contact heat-evoked brain potentials (CHEPs).[12, 29, 42] The authors of one of these studies also assessed the effects of topical cinnamic aldehyde and menthol on CHEPs.[42] All failed to observe clear effects of these products. Conversely, Valeriani, Arendt-Nielsen, Le Pera, Restuccia, Rosso, De Armas, Maiese, Fiaschi, Tonali, Tinazzi [49] showed that laser evoked potentials (LEPs) recorded after stimulation of skin pre-treated with 3% capsaicin cream for one hour were significantly delayed and reduced in amplitude compared to LEPs recorded before capsaicin application or on the untreated hand. This difference in results may come from the low signal to noise ratio of CHEPs recorded in the aforementioned studies, which can be largely ascribed to the low heating ramps allowed by conventional Peltier thermodes compared to laser stimulators (*max.* 70°C/s *vs* >1000°C/s respectively).

Brisk innocuous cooling of the skin has been shown to elicit ERPs selectively reflecting the activation of cold-sensitive Aδ fibre receptors [18], which are usually thought to be specific cold thermoreceptors.[16, 21] However, to date, the effects of TRP channels agonists on these cold evoked potentials (CEPs) have not been assessed.

The ERPs investigated in all these studies had latencies compatible with the conduction velocity of Aδ fibres but it is also possible, under special conditions [34], to record heat evoked potentials in the C-fibre conduction velocity range. Valeriani, Tinazzi, Le Pera, Restuccia, De Armas, Maiese, Tonali, Arendt-Nielsen [50] found that C-fibre LEPs recorded after one hour of pre-treatment with 3% capsaicin cream were delayed and reduced in amplitude compared to those recorded before capsaicin application. However, this time, LEPs elicited by stimulation of skin untreated with capsaicin (stimuli delivered to the contralateral arm) were also reduced in amplitude.

Aim

The aim of the present study was to characterize the contribution of TRPV1, TRPM8 and TRPA1 channels in the function of heat-sensitive Aδ nociceptors, innocuous warm-sensitive C fibre thermoreceptors and innocuous cold Aδ fibre thermoreceptors in humans. For this purpose, ERPs elicited by thermal stimulation preferentially activating these fibres were recorded for skin treated with chemical agonists of these three TRP channels (capsaicin, cinnamic aldehyde, and menthol), diluted in a vehicle solution, and compared to the ERPs elicited by stimulation of skin treated with the vehicle solution only.

2. Methods:

2.1. Subjects:

A total of 64 healthy young volunteers were recruited (see Table 1 for information on the samples characteristics). Each participant was given oral and written information on the experiment. After reading the information leaflet, subjects gave their written informed consent. The study complied with the latest version of the Helsinki Declaration (October 2013), except for study pre-

registration, and was approved by the local Ethical Committee. Exclusion criteria were (1) suffering or having a history of neurological, metabolic, or psychiatric condition, (2) habitual consumption or consumption within the week before the experiment of medication (except anticonception) or recreational drugs, (3) not having slept at least 6h the night before the experiment, (4) suffering of a dermatologic condition, (5) having a lesion or scar on the volar forearm, (6) suffering of chronic pain or being in pain on the day of the experiment, (7) playing volleyball or another sport in which the volar forearm is stimulated extensively, (8) having a history of allergic reaction to chili pepper, mint or cinnamon. One recruited subject was excluded because she did not satisfy the inclusion/exclusion criteria at screening. The data of another subject was discarded because of a dysfunction of the thermal stimulator during the experiment.

2.2. Study groups

Participants were assigned to one of four study groups. The first group (16 participants) was treated with a topical solution of menthol (20%) used as an agonist of TRPM8. The second group (16 participants) was treated with a topical solution of cinnamaldehyde (10%) used as an agonist of TRPA1. The third group (15 participants after excluding one participant because she did not satisfy inclusion criteria) was treated with a topical solution of capsaicin (0.025%) used as an agonist of TRPV1. Finally, because the effects of the 0.025% solution of capsaicin were inconsistent, a fourth group of 15 participants (after excluding one participant because of a malfunction of the stimulator during the experiment) was treated with a topical solution of 1% capsaicin.

All participants were treated with the agonist solution at one of the two forearms, and a vehicle solution at the other forearm, during a single experimental session lasting about 2 hours. The whole experimental sequence was performed with one of the two solutions at one forearm and then repeated with the other solution at the other forearm (approximatively 50 minutes separated the application of the first and second patch). The order of application of the agonist or

vehicle solution, as well as whether it was applied on the dominant or non-dominant arm was balanced between participants.

Participants were not aware of which products they were receiving. Due to the smell of the compounds, most participants receiving patches with menthol or cinnamic aldehyde properly guessed what was applied to that forearm.

2.3. Agonist and vehicle solutions

Solutions were made using either capsaicin (>95% capsaicin; M2028, Sigma-Aldrich, Saint-Louis, The USA), menthol (>99% L-menthol; W266523, Sigma-Aldrich, Saint-Louis, The USA) or cinnamic aldehyde (99% trans-cinnamaldehyde; C80687, Sigma-Aldrich, Saint-Louis, The USA). The compounds were diluted in pure ethanol (>99.8% ethanol, 20821.310, VWR, Radnor, the USA) to obtain solutions of 0.025% and 1% capsaicin (w/v), 20% menthol (w/v) and 10% cinnamic aldehyde (w/v).

Corresponding vehicle solutions were produced by mixing water and ethanol (80%, 90%, 99% ethanol in water). In the case of 0.025% capsaicin, undiluted ethanol absolute was used as corresponding vehicle solution.

2.4. Topical agonist treatment

Capsaicin, menthol or cinnamic aldehyde was applied to the skin of the volar forearm using two 5x5 cm gauze pads (Sterilux ES, Hartmann) set on a transparent self-adhesive film (Opsite Flexifix, Smith and nephew) and soaked with 1 ml of either active or vehicle solution *per* gauze pad.

The site of application of the patch (5 cm distal from the elbow crease; area: 5x10 cm) was marked with four dots. Depilatory cream (Veet Silky Fresh®) was applied inside this area for three minutes. After removal of the cream, three temperature measurements were taken inside and outside (3 to 5 centimetres distally) of the application zone, using an infrared thermometer (Tempett, SENSELab, Sweden). Subjects then rinsed their forearm using tepid water. After the skin was dried, the two soaked gauze pads were applied against the skin during 20 minutes.

During patch application, subjects were asked to report whether they had a sensation at the site of the patch and, if applicable, were asked to rate the intensity of this sensation using a non-graduated 100 mm visual analog scale (VAS; left extremity corresponding to “no sensation” and right extremity to “the most intense imaginable”) and to describe its quality (as many and whichever qualifiers the subjects saw fit). At the end of the 20 minutes, the patch was removed, the area was wiped with a paper towel to remove residual solution, and three temperature measurements were taken inside and outside of the patch area. Finally, the subject was asked to wash the zone using tepid water and regular hand soap.

2.5. Thermal stimulation

After topical treatment of each forearm, a total of 30 high-intensity noxious heat stimuli (60°C), 30 low intensity innocuous heat stimuli (42°C) and 30 cold stimuli (10°C) were delivered to the treated skin in a pseudo random order (random repetition of random sequences of triplets including all target temperatures). After each stimulus, the subject was asked to report the intensity and quality of the elicited sensation using the same procedure used to evaluate the sensation elicited by the patch. The inter-stimulus interval was approximately 12 s. A short break was offered to the subject after 45 stimuli. Approximatively 20 minutes were necessary to deliver these 90 stimulations.

All thermal stimuli were delivered using a contact thermal stimulator (TCS model II.1, QST.Lab, Strasbourg, France). The thermode (model T03) consists of 15 Peltier elements organized onto a 3 cm diameter disk. Each Peltier element has a 7.7 mm^2 surface, resulting in a total stimulation surface of approximately 1.2 cm^2. Target temperature was set to reach 10°C (cold), 42°C (innocuous warm) or 60°C (noxious heat) at a rate of 300°C/s. Stimulus duration was 200 ms (including reaching and holding time but not return to baseline). The stimulator was manually held against the skin by the experimenter and slightly displaced within the treated area after each stimulus.

2.6. EEG recording

The EEG signal was recorded using 32 actively shielded Ag-AgCl electrodes mounted in an elastic electrode cap and arranged according to the International 10– 20 system (WaveGuard 32-channel EEG cap, Advanced Neuro Technologies). It was digitized and amplified with a 1000 Hz sampling rate and an average reference (HS64, Advanced Neuro Technologies). Impedance was kept below 10 kΩ at every electrode site.

The subjects were seated in a comfortable chair. To reduce artefact occurrence, they were asked not to blink, speak, yawn or move during the time of stimulation, signalled at the beginning (approximately 1 s before stimulation onset) and at the end (approximately 2 s after stimulation onset) of each trial by an oral cue. They were also instructed to lock their gaze on a point below the horizontal to reduce eye-blink occurrence.

2.7. EEG analysis

The EEG recordings were analysed offline using Letswave 6 (www.letswave.org). The signal was filtered using a 50 Hz notch filter and then a 0.3-30 Hz bandpass Butterworth filter. The signal was then segmented into epochs ranging from 0.5 s before to 1.5 s after stimulation. Ocular artefacts were removed using the FastICA algorithm (Hyvarinen & Oja, 2000). Epochs with amplitude values exceeding ±100 μV were rejected due to the likeliness of them being contaminated by artefacts. The reference of the signal was changed to generate two versions of the dataset: one referenced to the average of the mastoid electrodes (M1M2) and one referenced to the frontal midline electrode (Fz). Baseline correction was applied to all datasets (reference interval -500 to 0 ms). Lateral electrode coordinates were flipped for all stimuli delivered to the right arm to obtain scalp topographies expressing signal amplitude relative to the stimulated arm (ipsilateral or contralateral). Finally, average waveforms *per* experimental condition and subject and grand averages *per* experimental condition were computed.

Based on previous studies characterizing heat- and cold-evoked ERPs, three peaks were identified in the individual average waveforms of the ERPs elicited by noxious heat and innocuous cold stimulation: a negative-positive complex maximal at the scalp vertex (N2-P2) and an earlier

negative wave maximal over the hemisphere contralateral to the stimulated forearm (N1). Latencies and amplitude of the N2 and P2 peaks were identified at the vertex electrode (Cz) referenced to the mastoids (M1M2). The latency and amplitude of the earlier N1 was identified the temporal electrode contralateral to the stimulated arm (Tc) referenced to the frontal midline electrode (Fz).

Waveforms recorded following 42°C stimulation showed weak and inconsistent responses. It was therefore not possible to identify and characterize latency and amplitude of peaks from these recordings.

2.8. Data analysis and statistics

Statistical testing was conducted using the open-source software JASP (v 0.14). QQ-plots of the residuals were used to check the validity of the distributional assumptions for the ANOVAs. Nonparametric Bayesian paired sample t-tests relied on Wilcoxon signed rank test and a data-augmentation algorithm with 5 chains of 10000 iterations. Convergence of the procedure was assessed using the Gelman-Rubin *R hat* (with values <1.1 considered as indicating that convergence had occurred). All t-test converged.

The groups of 3 temperature measurements were averaged and the change of temperature over patch application ($\Delta=T_{after}-T_{before}$) was computed for measurements taken inside and outside the patch and for each patch. For each active solution group, they were analysed using a Bayesian repeated measures ANOVA (implemented in the open-source statistical software JASP; Wagenmakers, Love, Marsman, Jamil, Ly, Verhagen, Selker, Gronau, Dropmann, Boutin, Meerhoff, Knight, Raj, van Kesteren, van Doorn, Smira, Epskamp, Etz, Matzke, de Jong, van den Bergh, Sarafoglou, Steingroever, Derks, Rouder, Morey [58]) with a factor "vehicle vs active" and a factor "site" (2 levels: inside or outside the treated area).

The VAS intensity ratings were measured (rounded to the mm; "not detected" scored as 0) and then normalized by dividing them by the largest rating given by the subject during the whole

experiment (patch application or stimulation), to account for interindividual differences in the handling of the scale.

For each active solution group, VAS ratings obtained during patch application were analysed using a Bayesian repeated measure ANOVA (implemented in the open-source statistical software JASP) with a factor "vehicle vs active" and a factor "time" (4 levels: 5, 10, 15 and 20 minutes after the beginning of patch application).

The VAS intensity ratings obtained after each thermal stimulation were averaged *per* modality and *per* subject. For each active solution group, they were analysed using a Bayesian repeated measure ANOVA (implemented in the open-source statistical software JASP) with a factor "vehicle vs active" and a factor "target temperature" (3 levels: cold, innocuous warm and noxious heat stimulations). When the ANOVA yielded at least a 3.2 Bayes Factor for the inclusion of the "vehicle vs agonist" factor or the interaction term, post-hoc comparisons were conducted between the ratings obtained from stimulation of the agonist and vehicle arms for the same modality (*i.e.* 10°C agonist *vs* 10°C vehicle, 42°C agonist *vs* 42°C vehicle, and 60°C agonist *vs* 60°C vehicle) using a non-parametric Bayesian paired sample t-test.

As a significant number of 42°C stimulations were not detected, it was decided to also assess whether the detection rate of these stimulations was affected by the agonist solutions. To do so, a non-parametric paired sample Bayesian t-test was used to compare rates obtained from the agonist treated arm *vs* those obtained from the vehicle treated arm.

The N1, N2 and P2 latencies and the N1 and N2P2 amplitudes of cold- and noxious heat-evoked potentials were analysed, within each active solution group, using non-parametric paired sample Bayesian t-tests (implemented in the open-source statistical software JASP)with a factor "vehicle vs active".

The Bayesian statistical procedure used in this article returned Bayes Factor (BF), either for the different terms of the model (ANOVA) or for the difference between two datasets. Bayes Factors correspond to the ratio between the probability of two hypotheses (for the ANOVAs: the factor

should be included or not; for the t-tests: there is a difference or not) and are increasingly recognized as a good alternative to p-values, not suffering from most of its caveats [59]. The BF reported in this paper were computed with H_1 (the factor should be included; there is a difference) in the numerator (BF_{incl} or BF_{10}), therefore, higher values indicate higher probability for an effect. The terminology used to qualify the size of the evidence for H_0 or H_1 is adapted from that proposed by Sir Harold Jeffreys[22], i.e.: BF<0.01 extremely strong evidence for H_0, BF<0.032 very strong evidence for H_0, BF<0.1 strong evidence for H_0, BF<1 trend for H_0, BF>1 trend for H_1, BF>10 strong evidence for H_1, BF>32 very strong evidence for H_1, BF>100 extremely strong evidence for H_1.

Raw data are available upon request to the corresponding author. Averaged waveforms, extracted data, and data analysis conducted in JASP are freely available on the OSF repository of the corresponding author (DOI 10.17605/OSF.IO/VCK6S).

3. Results:

3.1. Patch application

Flare

The 10% cinnamic aldehyde and 1% capsaicin solutions consistently elicited a visible flare of the treated skin. Interestingly, the visible aspect of the flare tended to differ across the two treatments. The shape of the flare elicited by cinnamic aldehyde usually matched the shape of the gauze pad and was uniform in colour. Conversely, the flare elicited by capsaicin tended to spread outside of the area in direct contact with the patch and was less uniform in colour, matching the characteristics of the axon reflex flare classically described after capsaicin application. As these observations are derived from informal visual assessments and not from objective measures (*e.g.* Doppler flowmetry), they must be taken with caution and we cannot exclude that cinnamic aldehyde also induced an axon reflex flare of lesser intensity, as would be expected from previous research.[38-40]. The 20% menthol and 0.025% capsaicin solution sometimes elicited a flare but of lesser strength and in a less consistent fashion. Vehicle patches never elicited a flare.

Temperature

Table 2 shows the results of the two-way repeated measures Bayesian ANOVA used to analyse temperature differences. The left panel of Figure 1 represents differences in temperature measured in each participant. From these results, it appears that the temperature of the treated skin consistently increased as compared to the skin outside the treated area, regardless of the product being applied (moderate or extremely strong evidence for the inclusion of factor "Site" in the model, depending on the agonist solution groups). The presence of 10% cinnamic aldehyde solution on the patch led to a further increase of the temperature (extremely strong evidence for the inclusion of the interaction term in the model) whereas the other solutions did not lead to a clear change of temperature (trend towards an increase for 20% menthol and 1% capsaicin, trend towards an absence of difference for 0.025% capsaicin).

Intensity

Table 3 shows the results of the two-way repeated measures Bayesian ANOVA used to analyse the normalized intensity ratings collected during the application of the patch. The right panel of Figure 1 represents normalized ratings over time for each participant. In the light of these results, all agonist solutions elicited a sensation more intense than their corresponding vehicle solutions (extremely strong evidence for the inclusion of the factor "Agonist vs Vehicle"), which increased over time in the case of 20% menthol, 1% capsaicin, and 0.025% capsaicin (respectively extremely strong, very strong and very strong evidence for the inclusion of the interaction term in the model) but not in the case of 10% cinnamic aldehyde (moderate evidence against the inclusion of the factor "Time" and the interaction term in the model).

Quality

The 20% menthol patch elicited a cold sensation, sometimes with elements of warmness or burning. The 10% cinnamic aldehyde patch elicited a warm/hot sensation, sometimes felt as burning/prickly/itchy. The capsaicin patches elicited a warm/hot sensation with elements of itch, prickle and burning, these later elements being more pronounced for the 1% than for the 0.025% patch.

3.2. Stimulations

Intensity

As can be seen in Figure 2 and Table 4, the different temperatures of stimulation clearly led to different perceived intensities (extremely strong evidence for the inclusion of factor "Target temperature" in the model, for all agonist solutions). The 10°C stimulation was felt as more intense than the 42°C stimulation and less intense than the 60°C stimulation. Except for the 20% menthol solution, the agonists had no impact on the intensity of perception (moderate evidence against the inclusion of the "Agonist vs Vehicle" factor in the model for cinnamic aldehyde and 0.025% capsaicin, trend for its inclusion in the model for 1% capsaicin, moderate evidence for its inclusion

for 20% menthol). The post-hoc comparisons revealed that menthol application led to a decrease in the intensity of the sensation elicited by the 10°C (BF_{10}=13.845, *i.e.,* strong evidence for H_1) and 60°C stimulations (BF_{10}=3.876, *i.e.,* moderate evidence for H_1) but not by the 42°C stimuli (BF_{10}=0.350, *i.e.,* trend for H_0).

Quality

The 10°C stimulations were almost always felt as cold, sometimes with an additional element of wetness, touch, prickle or burning. The 42°C stimuli that were detected were usually felt as warm with sometimes an additional element of prickle or touch. Sometimes, the subject detected them but was not able to describe the quality of the sensation. The 60°C stimuli were almost always felt as hot and burning or pricking, sometimes only as burning or pricking without a clear thermal quality. The agonist solutions did not seem to influence these qualities.

Detection rate

As a significant number of 42°C stimulations were not detected, it was decided to also assess whether the detection rate of these stimulations was affected by the agonist solutions. Individual values as well as BF_{10} for the difference between conditions *vehicle* and *agonist* are shown in Figure 3. The 20% menthol and the 0.025% capsaicin solutions did not affect the detection rate of the 42°C stimulations (moderate evidence for H_0), the effect of the 10% cinnamic aldehyde solution was not clear (trend for H_0) and 1% capsaicin solution seemed to lead to a decrease of the detection rate (moderate evidence for H_1).

Evoked potentials elicited by 42°C stimulations

As can be seen in Figure 4, the 42°C stimulations elicited inconsistent and weak EEG responses, at latencies ranging from the Aδ fibre to the C fibre conduction velocity range. This low signal-to-noise ratio and this inconsistency prevented the extraction of latency and amplitude values and these waveforms were therefore not subjected to statistical analyses.

Cold evoked potentials

The 10°C stimulus elicited a clear cold evoked potential (CEP) in almost every participant. The shape and topographies of the peak was in line with previous recordings of such ERPs [13, 18, 28]. The upper parts of Figures 5 to 8 present the group level average waveforms obtained for each of the agonist solution groups as well as the individual values of latency and amplitude extracted for each peak and condition.

Under these graphs are also reported the BF_{10} obtained for differences between the *vehicle* and *agonist* conditions. The 20% menthol solution led to an increase in latency and a decrease in amplitude of all peaks (with evidence level ranging from moderate to extremely strong for H_1). The 1% capsaicin solution led to a decrease of the amplitude of the N2P2 complex (very strong evidence for H_1) but, unlike menthol, did not affect its latency (trend and moderate evidence for H_0). It did not affect the N1 wave (trend and moderate evidence for H_0). The 0.025% capsaicin and the 10% cinnamic aldehyde solutions had no apparent effect on the latencies and amplitudes of CEP components (evidence for H_0 ranging from a trend to moderate evidence in both cases).

Contact heat evoked potentials

The 60°C heat stimuli elicited a clear contact heat evoked potential (CHEP) in almost every participant. The identification and extraction of N1 waves was, however, notably more difficult than for CEPs. The lower parts of Figures 5 to 8 present the group level average waveforms obtained for each of the agonist solution groups as well as the individual values of latency and amplitude extracted for each peak and condition. In addition to the first ERP in the Aδ fibre range, two additional and more modest positive deflections can be observed at longer latencies (starting around 0.7 s and 1 s after stimulation and peaking around 0.8 s and 1.2 s after stimulation, respectively). Such additional positive waves do not seem to be present in the CEP traces.

As for CEPs, BF_{10} are reported under the linked individual values graphs (corresponding to the peaks of the main CHEP, in the Aδ conduction velocity range) in Figures 5 to 8. In summary, the 20% menthol solution led to an increase in latency of the N1 and N2 waves and a decrease in amplitude of the N2P2 complex (with evidence level ranging from moderate to extremely strong

for H_1). N1 amplitude was apparently not affected (moderate evidence for H_0) and there was only a trend for a difference of P2 latency. No clear effect of the 1% capsaicin solution was noted on the N1 and P2 wave (trend for H_1 or H_0) but it led to an increase of N2 wave latency and a decrease of the N2P2 complex amplitude (evidence ranging from moderate to strong for H_1). The 0.025% capsaicin solution did not seem to affect the CHEPs (evidence ranging from a trend to moderate evidence for H_0). The 10% cinnamic aldehyde solution led to an increase of the N1 wave latency (moderate evidence for H_1) without clearly affecting its amplitude and clearly delayed the N2 wave and decreased the amplitude of the N2P2 complex (strong and very strong evidence for H_1) without really affecting the P2 wave latency (trend for H_0), still leading to a decrease of the overall N2P2 complex amplitude (very strong evidence for H_1).

Residual sensations at the end of the stimulations

For menthol, cinnamic aldehyde and 0.025% capsaicin, 4 participants out of 16 were informally asked at the end of each stimulation block if they still felt something at the site where the patch was applied. For menthol, they all reported a mild fresh/cool sensation at the agonist arm. For cinnamic aldehyde, three participants reported residual sensations at the agonist tested site, respectively “mild warm”, “mild warm/irritated”, and “mild prickle”. For 0.025% capsaicin, two subjects reported a residual mild itchy sensation at the end of the stimulation block. No participant reported residual sensations at the vehicle treated forearm.

For the 1% capsaicin group, all participants were informally asked if they had residual sensations at the end of the stimulation block. Nine reported a residual “hot” sensation at the agonist treated arm, four reported residual “burning hot” sensation and two reported a residual “pricking hot” sensation. Two participants reported a warm residual sensation at the vehicle treated arm.

4. Discussion:

The results obtained during patch application indicate that all three topical treatments penetrated the skin sufficiently to modulate the function of free nerve endings. Menthol modulated both the

latencies and amplitude of cold-evoked ERPs, whereas 1% capsaicin decreased the amplitude of the cold-evoked ERP without affecting its latency, and cinnamic aldehyde had no effect. Menthol, cinnamic aldehyde and 1% capsaicin modulated both the latency and the amplitude of noxious heat-evoked ERPs. The ERPs elicited by 42°C stimulations could not be interpreted but the detection rate of these stimuli was decreased after the application of 1% capsaicin solution.

Interpreting the effects of topical TRP agonists on heat- and cold-evoked ERPs

Chemical agonists decrease the activation threshold of thermosensitive TRP channels and increase the probability of their opening.[57] Sufficient concentrations of such agonists can lead to the activation of thermosensitive primary afferents at baseline skin temperature, as evidenced by the sensations elicited by capsaicin, menthol or cinnamic aldehyde. Furthermore, after agonist application some thermal stimuli may become able to activate some fibre types that are not activated by these same stimuli in the absence of treatment, including silent nociceptors.

The increased firing rate of agonized primary afferents at baseline skin temperature would be expected to alter the characteristics of afferent volleys generated by the presentation of a thermal stimulus. For example, this increased firing could lead to a lesser post-stimulus increase in activity when the stimulus is presented, especially for temperatures already saturating the afferent activity in the absence of agonists. In parallel, the prolonged firing caused by the agonist could lead to habituation or fatigue, *i.e.* the necessity for a larger change in temperature to cause the same effects. Finally, at the time of stimulus presentation, a fraction of the fibres could be in their refractory period, delaying their activation by the thermal stimulus and increasing the temporal jitter of the afferent volley. These three mechanisms could take place at the level of the primary afferent but also at higher-order relays.

Such alterations of the nociceptor afferent volley and its subsequent processing by higher-order neurons could explain the decreased amplitudes and increased latencies that we and others[49, 50] observed when recording the scalp response to brief thermal stimuli delivered to TRP agonised skin. Indeed, somatosensory evoked potentials -such as CEPs, LEPs, and CHEPs- are triggered by

a sudden change in the afferent input received by the brain and the amplitudes and latencies of their peaks partially reflect the temporal dynamics of this change of input. Due to weaker pre-synaptic activity and/or an increased post-synaptic thresholds (habituation), the post-synaptic activation could be weaker (lesser crossing of the threshold), delayed (more time integration necessary to reach the threshold), and more jittered (more variability arise between lines if time integration is needed). This would result in weaker (less activated units, lower firing rates) and slower afferent volleys and, therefore, smaller and delayed ERPs. Furthermore, increased temporal jitter would lead to wider peaks of lesser amplitude and, if the latency jitter distribution is left skewed (as expected for a Poisson process), later latencies.[35]

Importantly, the magnitude of heat evoked potentials has been shown to be strongly dependent on factors influencing the saliency of the stimulus.[19, 27, 36] Because the topical application of TRP agonists can produce an ongoing thermal sensation (caused by the activation of sensitized primary afferents at baseline temperature), a change in ERP amplitude observed during treatment must be interpreted cautiously as it could be due, at least in part, to the fact that this ongoing sensation reduces the salience of cold and/or heat stimuli applied onto the skin.[49] Conversely, manipulating stimulus salience does not seem to affect the latency of heat-evoked ERPs.

Cold-sensitive Aδ fibres.

The effect of menthol on the latency and amplitude of cold-evoked ERPs and the lack of effect of cinnamic aldehyde on these same ERPs are compatible with the notion that cold-sensitive Aδ fibres rely on TRPM8 to encode innocuous cold, and that this transduction process does not involve TRPA1. This is in line with the fact that one of the few cold sensitive Aδ fibres recorded with microneurography was sensitized by menthol.[7]

Topical 1% capsaicin reduced the amplitude of the N2-P2 complex of cold-evoked ERPs without modulating the latency of the N2 and P2 components. This lack of effect on latencies was not due to a lack of power, as the corresponding BFs indicated an absence of difference (moderate evidence and a trend for H_0 in the case of N2 and P2 latencies, respectively). Furthermore, there

was no effect of topical 1% capsaicin on the magnitude and latency of the earlier cold evoked N1 component. This isolated effect of capsaicin on the amplitude of the cold-evoked N2-P2 complex could be explained by the fact that topical capsaicin produces an ongoing burning sensation at the treated skin, continuing for some time after patch removal, which may render the sensation elicited by the transient cold stimuli less prominent and hence less salient. This sustained and prolonged burning pain sensation and the activation of heat nociceptors that underly it is also known to cause central sensitization, a phenomenon that may not only produce hyperalgesia, but also decrease innocuous cold detection based on the findings of one quantitative sensory testing study[26]. This could be another mechanism explaining this effect of capsaicin on cold-evoked ERPs. Finally, it could also be a consequence of an antagonist effect of capsaicin on TRPM8, depressing its reactivity to cold.[46]

Heat-sensitive quickly responding Aδ nociceptors

The finding that both capsaicin 1% and cinnamic aldehyde modulated both the latencies and the amplitudes of Aδ heat-evoked potentials is compatible with the notion that both TRPV1 and TRPA1 are expressed by quickly responding heat-sensitive Aδ nociceptors and play a role in noxious heat encoding by these fibres. These nociceptors are thought to closely resemble the capsaicin-mechano-heat-sensitive quickly adapting AMH type II fibres described in monkeys. [31, 41, 47]

The finding that menthol also modulated the latencies and amplitude of noxious heat-evoked potentials is more surprising. It could be explained by the fact that, at relatively high concentrations, menthol may act as an agonist of TRPA1.[5, 33, 62] This effect could also be a consequence of an antagonistic effect of menthol on TRPV1, depressing its reactivity to heat.[46] Finally, the effect of menthol on heat-evoked responses could reflect a crosstalk between cold and noxious heat sensitive pathways, a mechanism that could also explain why innocuous cooling of the neighbouring skin strongly reduces the sensations and evoked potentials related to the activation of noxious heat sensitive Aδ fibres.[37] These last two mechanisms could also explain why

topical menthol can alleviate the spontaneous pain, hyperalgesia and allodynia caused by cinnamic aldehyde or capsaicin.[2, 42]

Topical capsaicin can also produce some amount of secondary hyperalgesia due to central sensitization. However, previous studies using high-frequency electrical stimulation of the skin to induce such secondary hyperalgesia without agonizing TRPV1 have suggested that this central phenomenon does not significantly affect the ERPs reflecting the activation of heat sensitive Aδ nociceptors[51, 52]. Therefore, it seems unlikely that central sensitization played a role in the observed effects of capsaicin on CHEP latencies and amplitudes.

Heat-sensitive C fibers

The EEG responses to the 42°C stimuli had a low signal-to-noise ratio. Furthermore, between-subject variability of the EEG response to these stimuli was high (both in terms of presence/absence of a response and in terms of latency). Previous studies using CO_2 laser stimuli managed to record innocuous warm C-fibre ERPs with similar target temperatures, shorter pulse durations, and smaller stimulation surfaces.[20, 50] This discrepancy between our results and previous attempts may come from differences between the density of primary afferent subpopulations between the volar forearm and the skin of the back or the perioral region where the laser stimuli were delivered. Differences between the temperature reached at the depth of free nerve endings using an infrared laser or a contact thermode may also be part of the explanation.[17]

Interestingly, application of 1% capsaicin led to a decrease of the detection rate of 42°C stimuli. This could reflect a non-specific distracting effect of the spontaneous burning sensation elicited by capsaicin. Alternatively, it could reflect modulation of the fibers responsible for the warm sensation elicited by these 42°C stimuli.

In the absence of agonist, it seems unlikely that C-nociceptors encoded these innocuous warm stimuli, as the threshold of mechano-heat-sensitive C nociceptors is reported to be somewhere between 40 and 42°C[43, 60, 63] and that of mechano-insensitive C nociceptors are even higher (~48°C).[60] C-warm fibers are a more likely candidate, as they start responding at temperatures

slightly above baseline skin temperatures (32°C) and up to 40°C and maybe more.[25] Another possible candidate to encode these stimuli would be the C2 fibres described by Campero, Baumann, Bostock, Ochoa [7], which responded to cold in the innocuous range and to warmth in the upper innocuous range (median threshold 37.6°C). These two last categories of C fibres are thought to be the main drivers of the sensation of warmth in humans.[30]

Capsaicin modulation of the response of these fibres is expected, as capsaicin "denervation" was shown to strongly disturb the sense of innocuous warmth, suggesting that at least one of these two populations of neurons express TRPV1.[53] The decreased detection rate that we observed after capsaicin application could be explained by agonisation of TRPV1 channels expressed by these fibres, leading to an increased firing rate at baseline temperature and therefore a lesser or absent increase in firing elicited by the 42°C stimuli.

Importantly, after the application of TRP agonists such as capsaicin or cinnamic aldehyde, some fibres may have become responsive to the 42°C stimulus, including C fibre nociceptors and even silent nociceptors. Our results cannot provide evidence for or against this possibility.

Effects of topical agonists in other experimental approaches

The decreased responses to thermal stimuli that we observed in the present study could appear to contradict the results of studies having shown that capsaicin, cinnamic aldehyde and menthol decrease heat pain thresholds (HPT) assessed using quantitative sensory.[61] A likely explanation for this discrepancy is that QST assesses the HPT by slowly ramping temperature by 1°C/s until the subject presses a button. Using such a stimulation protocol, the sensation leading the participant to press the button most probably relates to the overall activity within heat-sensitive afferents which is likely to be increased by the topical agonists. Conversely, transient responses to a short-lasting thermal pulse such as heat-evoked ERPs are determined mainly by the relative increase in activity immediately following the thermal stimulus rather than the total net activity within peripheral afferents. Another explanation could be that quickly responding afferents producing transient responses such as heat-evoked ERPs and slowly-responding afferents that

contribute to the sensations produced by slow thermal ramps such as those used for QST are not similarly modulated by topical agonists. Finally, as the skin acts as a buffer on heat transfer, the relative temperature at different skin depth is certainly different between stimuli reaching their target temperature at 1°C/s or 300°C/s (more uniform across depth in the first case) and such very phasic stimuli may, therefore, fail to recruit primary afferents terminating deeper in the skin.

Limitations of the chosen TRPM8, TRPV1 and TRPA1 agonists

Menthol is often presented as a specific agonist of TRPM8. However, there is a significant amount of evidence that the human TRPA1 is also sensitive to menthol,[5, 33, 62] albeit a higher concentration of this compound is needed to produce the same agonistic effect as cinnamic aldehyde (~20 fold).[5] Menthol has also been reported to have an antagonistic effect on human TRPV1, and capsaicin (usually presented as a selective agonist of TRPV1) may have a symmetrical antagonistic effect on human TRPM8.[46] Further, capsaicin has been reported to have a number of effects that do not depend on TRP channels but could influence thermoreception.[6]

This lack of specificity limits the possibility of using these substances to tease out the specific involvement of TRPM8, TRPV1 and TRPA1 channels in the transduction of thermal stimuli within human free nerve endings. This also applies to our results, even though combined information on the effects of several agonists partially counterbalances this lack of specificity of each separate agonist.

Limitations of using ethanol as solvent

Ethanol is also thought to be an agonist of TRPV1.[48] Its use as a solvent in this experiment may, at first sight, appear as a limitation of our study. However, the design that we used allows to cancel out the effect of ethanol, as it should be equally strong in both the agonist and vehicle conditions and therefore should not influence the paired sample analysis. This holds true only if the effect of ethanol on TRPV1 is weak enough to allow an additional effect of another agonist. Based on the quality of the sensations elicited by the vehicle patch and the absence of flare, it seems that this was the case in our experiment.

Additional deflections in the waveforms recorded after 60°C stimuli

In addition to the classical peaks in the Aδ conduction velocity range elicited by 60°C stimuli, we also observed two positive deflections ranging from approximatively 700 to 800 ms and 1000 to 1200 ms post stimulation. The latter is probably related to the activation of (noxious) heat sensitive C fibres and can be paralleled with numerous recordings of such ultra-late ERPs.[34] However, it is usually accepted that concomitant stimulation of noxious heat sensitive Aδ fibres results in the masking/suppression of this ultra-late response. For some reason, some stimuli may have failed to elicit sufficient Aδ nociceptor activity to elicit an ERP in the late range, leading to the unmasking of an ERP in the ultra-late range, both types of ERPs being pooled together when computing the subject level average waveforms. The deflection ranging from 0.7 to 0.8 s after stimulation could be a small CEP elicited by the active cooling of the skin at the end of the 200 ms stimulation period.

Conclusion

Our results are compatible with the notion that TRPM8 is involved in innocuous cold transduction and that TRPV1 and TRPA1 are involved in noxious heat transduction in human free nerve endings. More specifically, our data are compatible with the notion that small myelinated Aδ cold thermoreceptors are reliant on TRPM8 and that quickly responding heat sensitive Aδ fibre nociceptors are reliant on both TRPV1 and TRPA1.

5. Tables

	20% menthol	10% cinnamaldehyde	0.025% capsaicin	1% capsaicin
n	16	16	15	15
Age (years)	24 [23 - 26]	22 [20.75 - 24]	23 [21.5 - 24]	23 [20 - 24]
Gender (F / M)	9/7	9/7	9/6	9/6
Laterality (RH / LH)	14/2	14/2	14/1	15/0

Table 1: sample characteristics Count variables are presented as sums, continuous variables are presented as *median [25% percentile – 75% percentile]*; F: females, M: males; RH: right handed, LH: left handed

Model component	P_{incl}	P_{excl}	Menthol			Cinnamaldehyde			Capsaicin 0.025%			Capsaicin 1%		
			$P_{incl\|data}$	$P_{excl\|data}$	BF_{incl}	$P_{incl\|data}$	$P_{excl\|data}$	BF_{incl}	$P_{incl\|data}$	$P_{excl\|data}$	BF_{incl}	$P_{incl\|data}$	$P_{excl\|data}$	BF_{incl}
Agonist vs vehicle	0.600	0.400	0.454	0.546	0.555	1.000	3.972e -5	16782.052	0.481	0.519	0.617	0.736	0.264	1.863
Site	0.600	0.400	0.924	0.076	8.138	1.000	9.010e -10	7.399e +8	1.000	5.955e -7	1.120e +6	1.000	1.590e -9	4.192e +8
Interaction	0.200	0.800	0.257	0.743	1.387	0.999	6.779e -4	5896.806	0.126	0.874	0.576	0.345	0.655	2.110

Table 2 Change of temperature during patch application – Results of the Bayesian repeated measures ANOVA

Model component	P_{incl}	P_{excl}	Menthol			Cinnamaldehyde			Capsaicin 0.025%			Capsaicin 1%		
			$P_{incl\|data}$	$P_{excl\|data}$	BF_{incl}	$P_{incl\|data}$	$P_{excl\|data}$	BF_{incl}	$P_{incl\|data}$	$P_{excl\|data}$	BF_{incl}	$P_{incl\|data}$	$P_{excl\|data}$	BF_{incl}
Agonist vs vehicle	0.600	0.400	1.000	0.000	∞	1.000	7.883e -15	8.457e +13	1.000	4.766e -7	1.399e +6	1.000	0.000	∞
Time	0.600	0.400	1.000	5.281e -6	126232.752	0.266	0.734	0.241	1.000	2.128e -6	313214.818	0.981	0.019	33.648
Interaction	0.200	0.800	0.997	0.003	1533.503	0.031	0.969	0.128	0.949	0.051	75.042	0.918	0.082	44.669

Table 3 Intensity ratings during patch application – Results of the Bayesian repeated measures ANOVA

Model component	P_{incl}	P_{excl}	Menthol			Cinnamaldehyde			Capsaicin 0.025%			Capsaicin 1%		
			$P_{incl\|data}$	$P_{excl\|data}$	BF_{incl}	$P_{incl\|data}$	$P_{excl\|data}$	BF_{incl}	$P_{incl\|data}$	$P_{excl\|data}$	BF_{incl}	$P_{incl\|data}$	$P_{excl\|data}$	BF_{incl}
Target temperature	0.600	0.400	1.000	0.000	∞	1.000	2.554e -15	2.611e +14	1.000	0.000	∞	1.000	2.665e -15	2.502e +14
Agonist vs vehicle	0.600	0.400	0.842	0.158	3.541	0.218	0.782	0.186	0.201	0.799	0.167	0.608	0.392	1.033
Interaction	0.200	0.800	0.459	0.541	3.398	0.048	0.952	0.200	0.027	0.973	0.112	0.099	0.901	0.442

Table 4 Intensity ratings of the stimuli – Results of the Bayesian repeated measures ANOVA

6. Figures

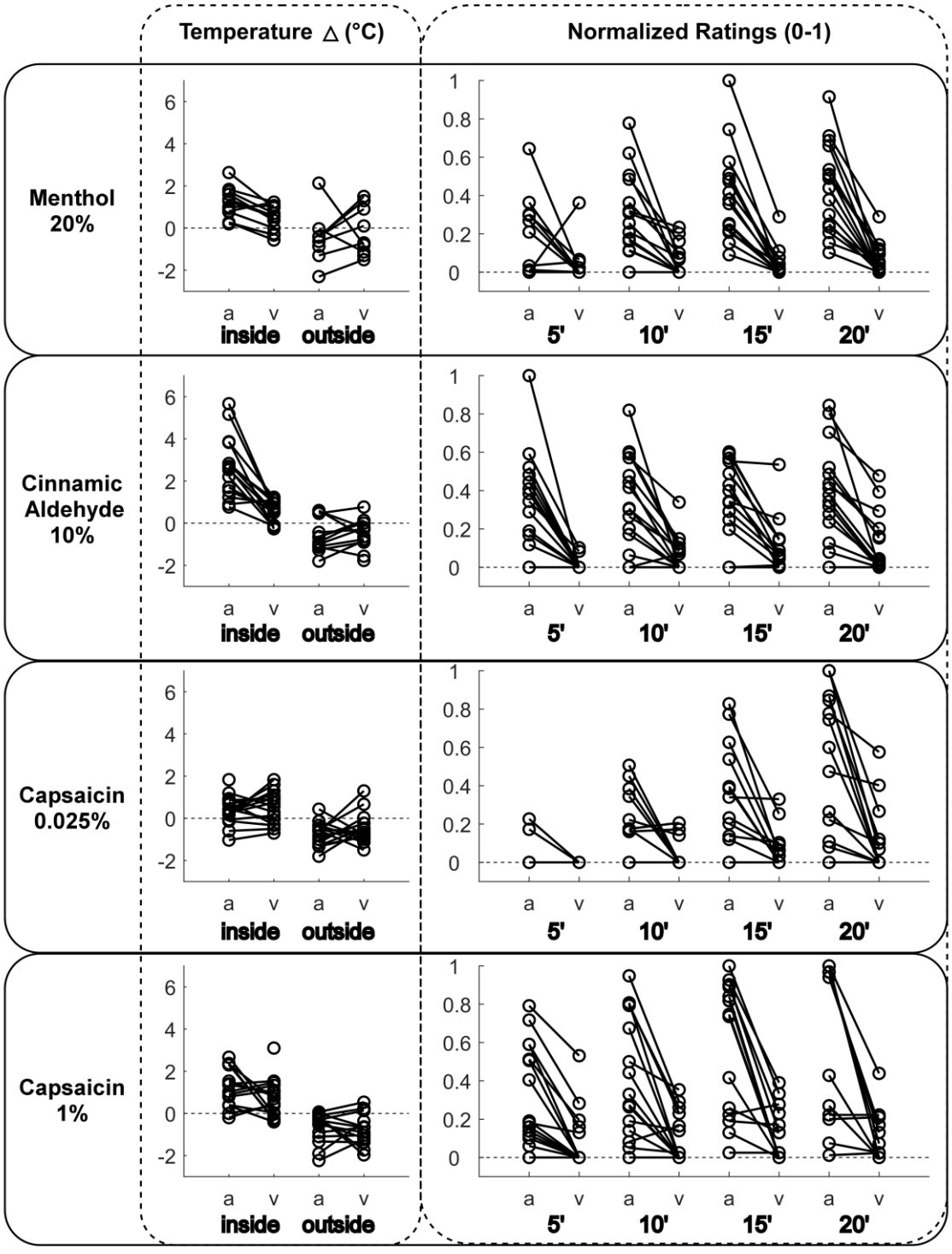


Figure 1 Effects of patch application on skin temperature and perception For each agonist solution group (rows), the individual values of temperature difference inside and outside of the patch (left column) and normalized intensity rating (right column) taken at different time points during patch application (5', 10', 15', and 20' after patch application) are represented. For each

subject, the observations obtained during the active (a) patch application and that obtained during the vehicle (v) patch application are linked.

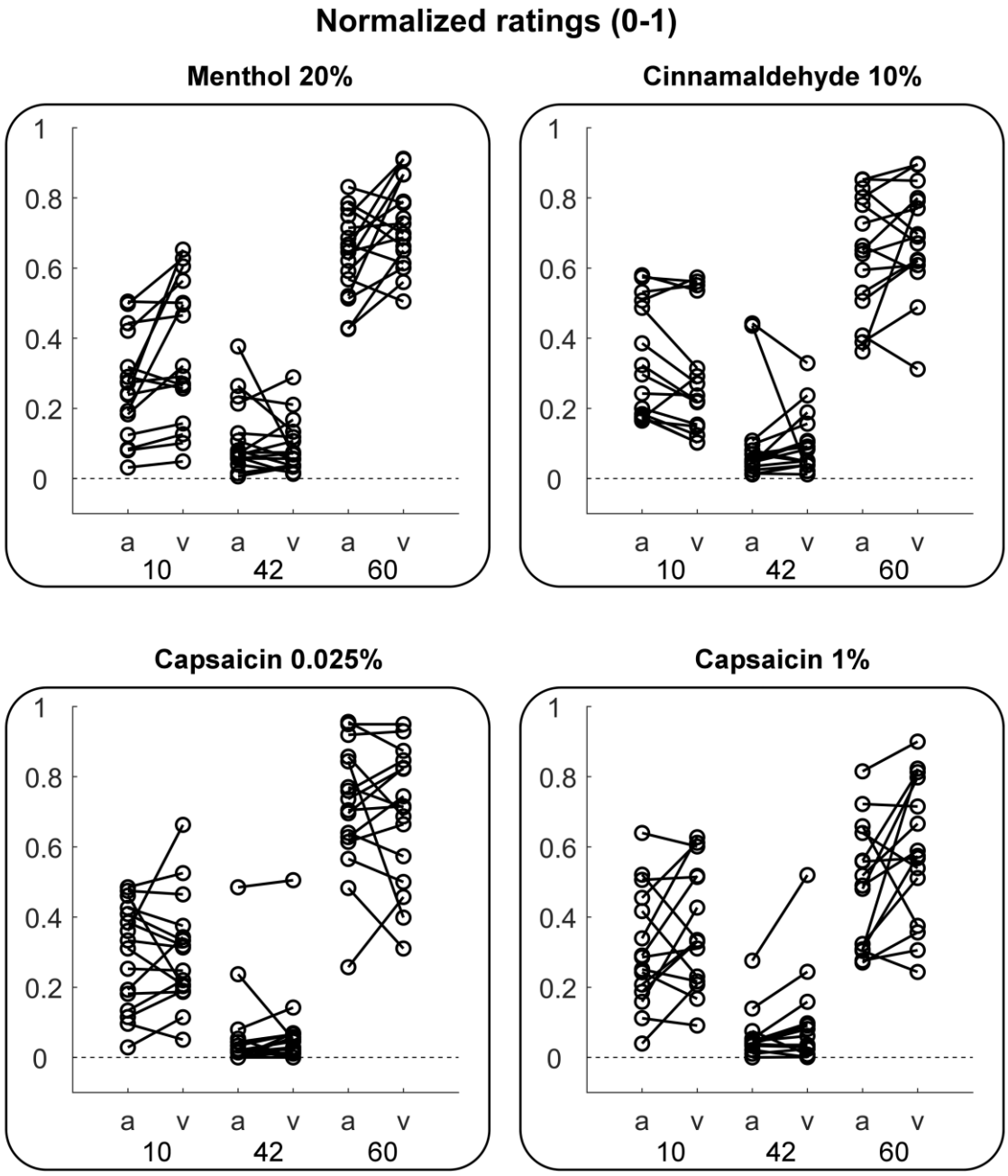


Figure 2 Normalized intensities of the sensations elicited by the thermal stimulations For each agonist solution group, the averaged normalized intensities are plotted per subject and per target temperature (10°C, 42°C, and 60°C) as linked observation for the active (a) patch and the vehicle (v) patch arms.

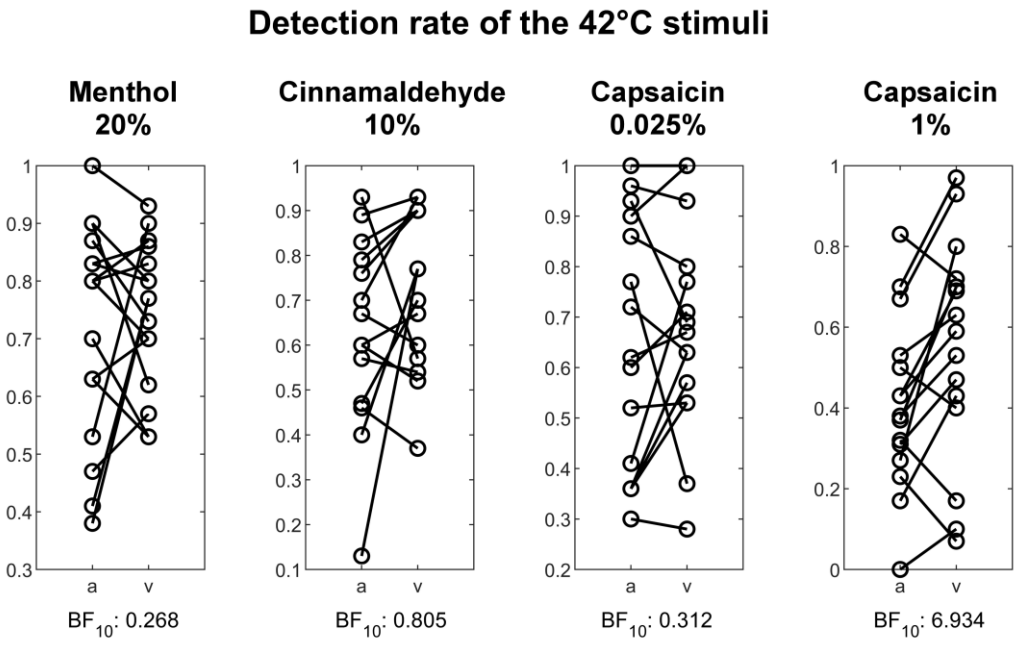


Figure 3 Detection rate for the 42°C stimulation For each agonist solution group, individual detection rates of stimuli are plotted as linked observations for the active patch (a) and vehicle patch (v) arms. The BF_{10} for the paired sample non-parametric Bayesian t-test conducted on these data is reported under each graph.

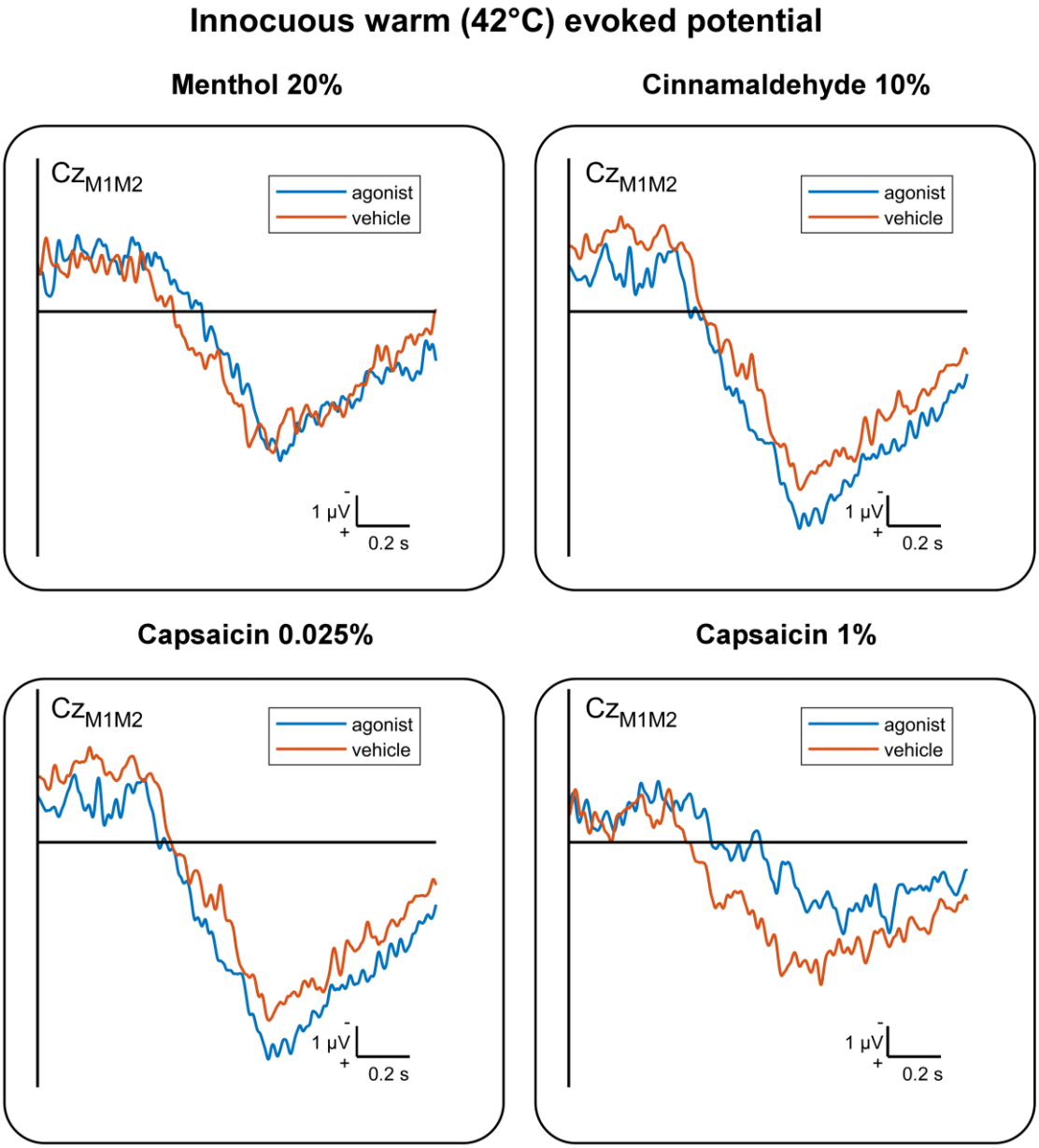
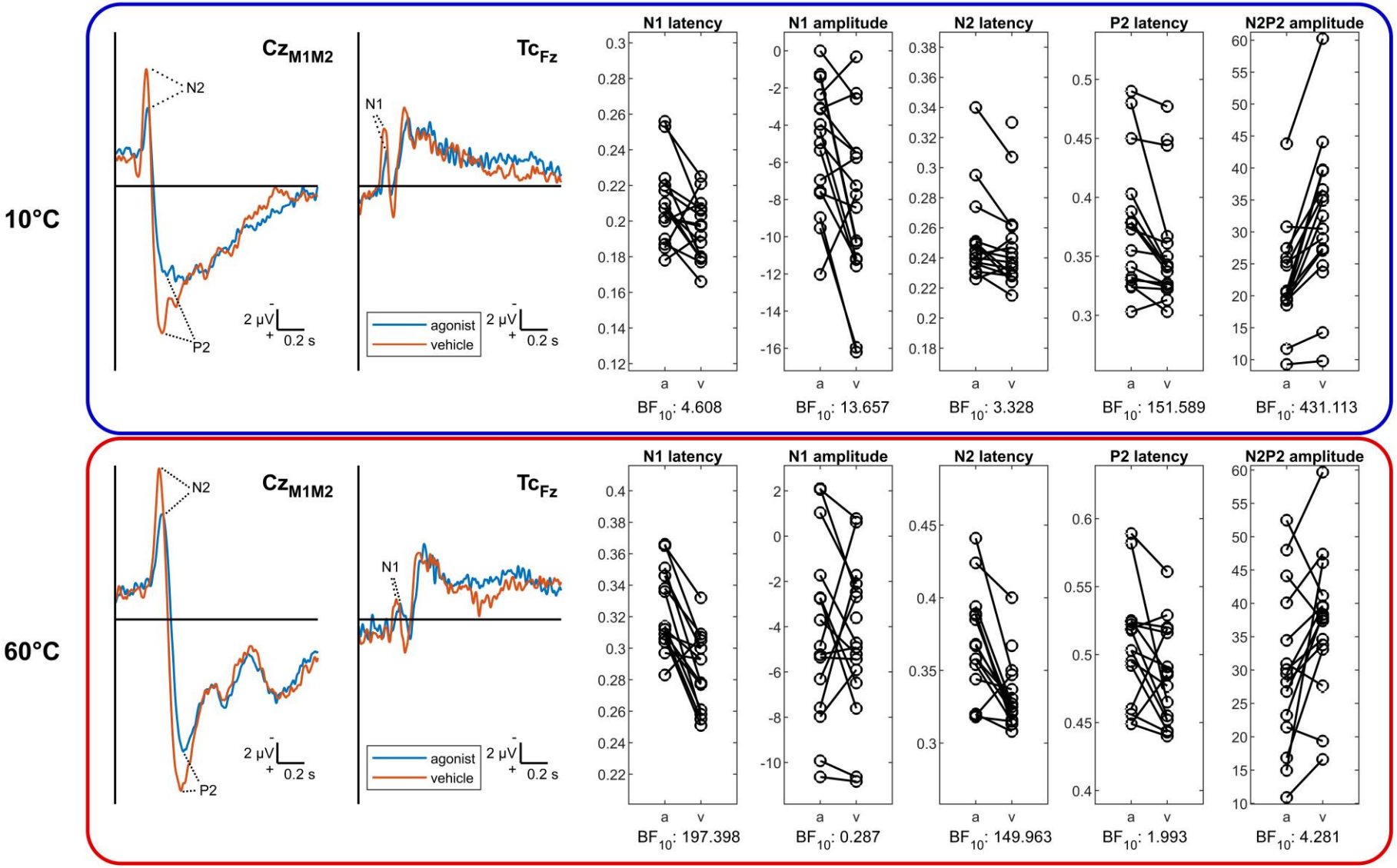
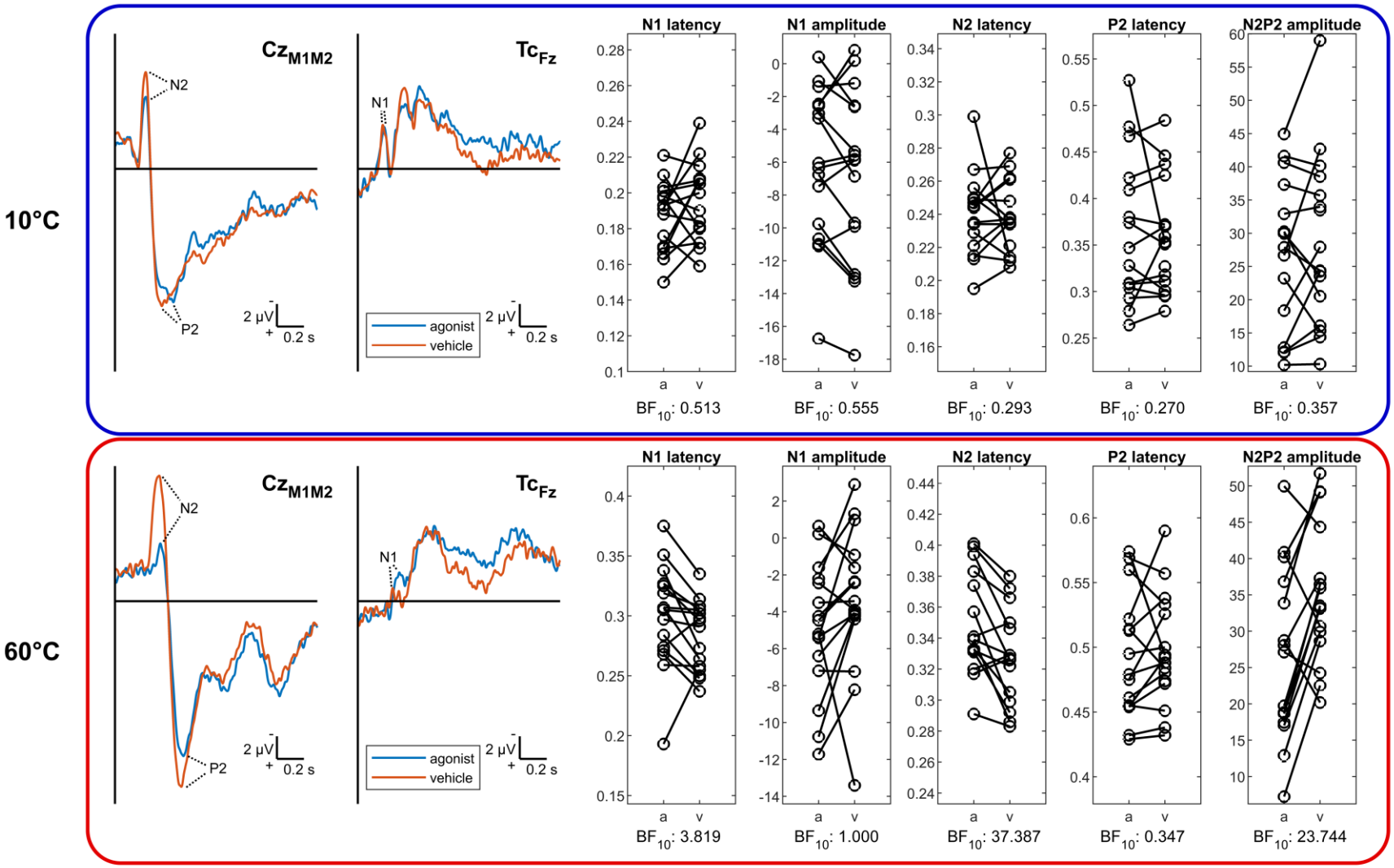


Figure 4 Group level grand average waveforms recorded at the vertex electrode (referenced to the mastoid electrodes) following the 42°C stimulation Waveforms obtained from the agonist patch arm (blue) and the vehicle patch arm (orange) are plotted separately.

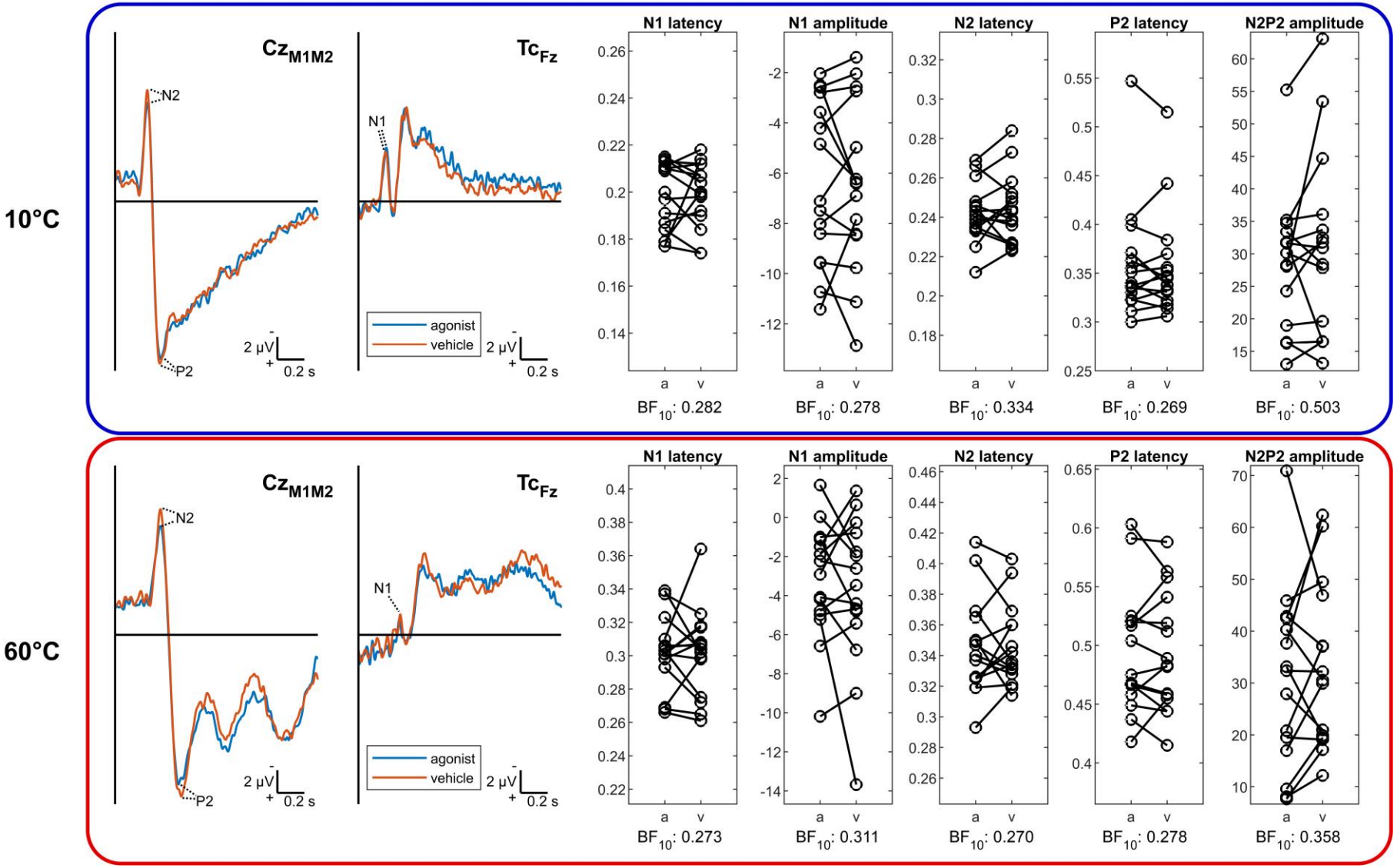
Menthol 20%


10°C
60°C
Cz M1M2
Tc Fz
N1
N2
P2
2 µV
0.2 s
agonist
vehicle
N1 latency
N1 amplitude
N2 latency
P2 latency
N2P2 amplitude
a
v
BF10: 4.608
BF10: 13.657
BF10: 3.328
BF10: 151.589
BF10: 431.113
BF10: 197.398
BF10: 0.287
BF10: 149.963
BF10: 1.993
BF10: 4.281

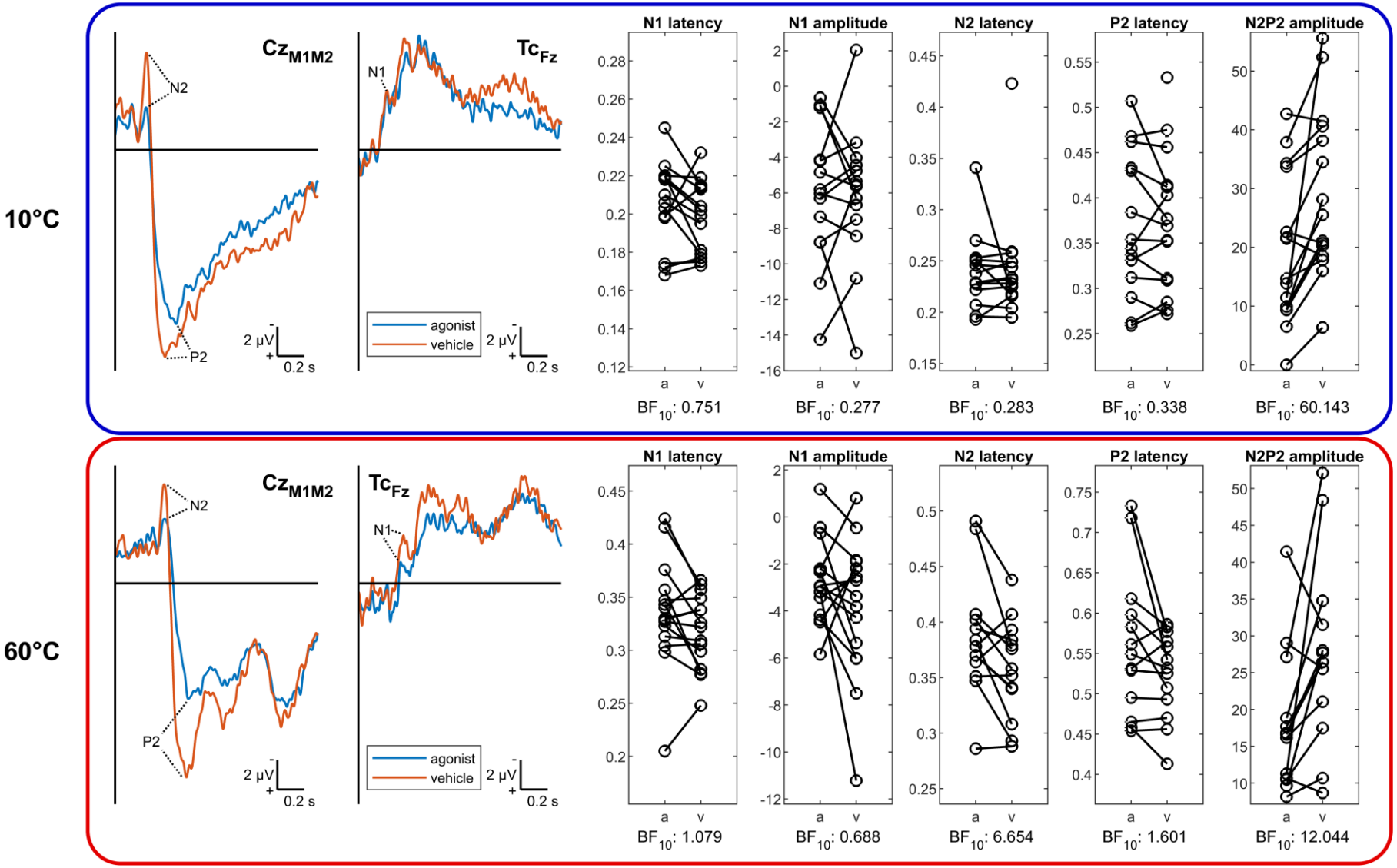
Cinnamic aldehyde 10%



Capsaicin 0.025%



Capsaicin 1%


10°C
60°C
Cz M1M2
Tc Fz
N1
N2
P2
2 µV
0.2 s
agonist
vehicle
N1 latency
N1 amplitude
N2 latency
P2 latency
N2P2 amplitude
a
v
BF10: 0.751
BF10: 0.277
BF10: 0.283
BF10: 0.338
BF10: 60.143
BF10: 1.079
BF10: 0.688
BF10: 6.654
BF10: 1.601
BF10: 12.044

Figures 5/6/7/8 Effects of 20% menthol/10% cinnamic aldehyde/0.025% capsaicin/1% capsaicin patch on CEPs (upper row) and CHEPs (lower row) In the left part of the graph, grand average waveforms recorded at the vertex (reference: mastoid electrodes) and at the contralateral temporal electrode (reference: frontal midline electrode) are displayed. Waveforms obtained from stimulation of the active (blue) and vehicle (orange) patch arms are plotted separately. In the right part of the graph, the latencies and amplitudes extracted from the average waveforms per subject and per condition (a=active patch arm, v=vehicle patch arm) are displayed as linked observations (per subject). The BF_{10} for the paired sample non-parametric Bayesian t-test conducted on these data is reported under each graph.

7. References

1. Anand U, Otto WR, Facer P, Zebda N, Selmer I, Gunthorpe MJ, Chessell IP, Sinisi M, Birch R, Anand P. TRPA1 receptor localisation in the human peripheral nervous system and functional studies in cultured human and rat sensory neurons. *Neuroscience letters.* 438:221-227, 2008

2. Andersen HH, Gazerani P, Arendt-Nielsen L. High-Concentration L-Menthol Exhibits Counter-Irritancy to Neurogenic Inflammation, Thermal and Mechanical Hyperalgesia Caused by Trans-cinnamaldehyde. *The journal of pain : official journal of the American Pain Society.* 17:919-929, 2016

3. Babes A, Ciobanu AC, Neacsu C, Babes RM. TRPM8, a sensor for mild cooling in mammalian sensory nerve endings. *Current pharmaceutical biotechnology.* 12:78-88, 2011

4. Bautista DM, Siemens J, Glazer JM, Tsuruda PR, Basbaum AI, Stucky CL, Jordt SE, Julius D. The menthol receptor TRPM8 is the principal detector of environmental cold. *Nature.* 448:204-208, 2007

5. Bianchi BR, Zhang XF, Reilly RM, Kym PR, Yao BB, Chen J. Species comparison and pharmacological characterization of human, monkey, rat, and mouse TRPA1 channels. *The Journal of pharmacology and experimental therapeutics.* 341:360-368, 2012

6. Braga Ferreira LG, Faria JV, Dos Santos JPS, Faria RX. Capsaicin: TRPV1-independent mechanisms and novel therapeutic possibilities. *Eur J Pharmacol.*173356, 2020

7. Campero M, Baumann TK, Bostock H, Ochoa JL. Human cutaneous C fibres activated by cooling, heating and menthol. *The Journal of physiology.* 587:5633-5652, 2009

8. Caterina MJ. Transient receptor potential ion channels as participants in thermosensation and thermoregulation. *American journal of physiology. Regulatory, integrative and comparative physiology.* 292:R64-76, 2007

9. Caterina MJ, Pang Z. TRP Channels in Skin Biology and Pathophysiology. *Pharmaceuticals (Basel, Switzerland).* 9, 2016

10. Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, Julius D. The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature.* 389:816-824, 1997

11. Chen J, Kang D, Xu J, Lake M, Hogan JO, Sun C, Walter K, Yao B, Kim D. Species differences and molecular determinant of TRPA1 cold sensitivity. *Nature communications.* 4:2501, 2013

12. Chiang H, Chang KC, Kan HW, Wu SW, Tseng MT, Hsueh HW, Lin YH, Chao CC, Hsieh ST. Physiological and pathological characterization of capsaicin-induced reversible nerve degeneration and hyperalgesia. *European journal of pain (London, England).* 22:1043-1056, 2018

13. De Keyser R, van den Broeke EN, Courtin A, Dufour A, Mouraux A. Event-related brain potentials elicited by high-speed cooling of the skin: A robust and non-painful method to assess the spinothalamic system in humans. *Clinical neurophysiology : official journal of the International Federation of Clinical Neurophysiology.* 129:1011-1019, 2018

14. de la Roche J, Eberhardt MJ, Klinger AB, Stanslowsky N, Wegner F, Koppert W, Reeh PW, Lampert A, Fischer MJ, Leffler A. The molecular basis for species-specific activation of human TRPA1 protein by protons involves poorly conserved residues within transmembrane domains 5 and 6. *The Journal of biological chemistry.* 288:20280-20292, 2013

15. Facer P, Casula MA, Smith GD, Benham CD, Chessell IP, Bountra C, Sinisi M, Birch R, Anand P. Differential expression of the capsaicin receptor TRPV1 and related novel receptors TRPV3, TRPV4 and TRPM8 in normal human tissues and changes in traumatic and diabetic neuropathy. *BMC neurology.* 7:11, 2007

16. Filingeri D. Neurophysiology of Skin Thermal Sensations. *Comprehensive Physiology.* 6:1429, 2016

17. Frahm KS, Gervasio S, Arguissain F, Mouraux A. New Insights into Cutaneous Laser Stimulation - Dependency on Skin and Laser Type. *Neuroscience.* 448:71-84, 2020

18. Hullemann P, Nerdal A, Binder A, Helfert S, Reimer M, Baron R. Cold-evoked potentials - Ready for clinical use? *European journal of pain (London, England).* 20:1730-1740, 2016
19. Iannetti GD, Hughes NP, Lee MC, Mouraux A. Determinants of laser-evoked EEG responses: pain perception or stimulus saliency? *Journal of neurophysiology.* 100:815-828, 2008
20. Iannetti GD, Truini A, Romaniello A, Galeotti F, Rizzo C, Manfredi M, Cruccu G. Evidence of a specific spinal pathway for the sense of warmth in humans. *Journal of neurophysiology.* 89:562-570, 2003
21. Janig W. Peripheral thermoreceptors in innocuous temperature detection. *Handbook of clinical neurology.* 156:47-56, 2018
22. Jeffreys H: The theory of probability, OUP Oxford, 1998.
23. Klein AH, Carstens MI, Carstens E. Eugenol and carvacrol induce temporally desensitizing patterns of oral irritation and enhance innocuous warmth and noxious heat sensation on the tongue. *Pain.* 154:2078-2087, 2013
24. Knowlton WM, Bifolck-Fisher A, Bautista DM, McKemy DD. TRPM8, but not TRPA1, is required for neural and behavioral responses to acute noxious cold temperatures and cold-mimetics in vivo. *Pain.* 150:340-350, 2010
25. Konietzny F. Peripheral neural correlates of temperature sensations in man. *Human neurobiology.* 3:21-32, 1984
26. Lang S, Klein T, Magerl W, Treede RD. Modality-specific sensory changes in humans after the induction of long-term potentiation (LTP) in cutaneous nociceptive pathways. *Pain.* 128:254-263, 2007
27. Legrain V, Mancini F, Sambo CF, Torta DM, Ronga I, Valentini E. Cognitive aspects of nociception and pain: bridging neurophysiology with cognitive psychology. *Neurophysiologie clinique = Clinical neurophysiology.* 42:325-336, 2012
28. Leone C, Dufour A, Di Stefano G, Fasolino A, Di Lionardo A, La Cesa S, Galosi E, Valeriani M, Nolano M, Cruccu G, Truini A. Cooling the skin for assessing small-fibre function. *Pain.* 2019
29. Linde LD, Haefeli J, Jutzeler CR, Rosner J, McDougall J, Curt A, Kramer JLK. Contact Heat Evoked Potentials Are Responsive to Peripheral Sensitization: Requisite Stimulation Parameters. *Frontiers in human neuroscience.* 13:459, 2019
30. Ma Q. Labeled lines meet and talk: population coding of somatic sensations. *The Journal of clinical investigation.* 120:3773-3778, 2010
31. Magerl W, Ali Z, Ellrich J, Meyer RA, Treede RD. C- and A delta-fiber components of heat-evoked cerebral potentials in healthy human subjects. *Pain.* 82:127-137, 1999
32. Moparthi L, Kichko TI, Eberhardt M, Hogestatt ED, Kjellbom P, Johanson U, Reeh PW, Leffler A, Filipovic MR, Zygmunt PM. Human TRPA1 is a heat sensor displaying intrinsic U-shaped thermosensitivity. *Scientific reports.* 6:28763, 2016
33. Moparthi L, Survery S, Kreir M, Simonsen C, Kjellbom P, Hogestatt ED, Johanson U, Zygmunt PM. Human TRPA1 is intrinsically cold- and chemosensitive with and without its N-terminal ankyrin repeat domain. *Proceedings of the National Academy of Sciences of the United States of America.* 111:16901-16906, 2014
34. Mouraux A: Electrophysiological techniques to study the supraspinal responses to nociceptive input in human. In: Pain persistence after surgery and altered central processing: science and clinical practice.(O. H. G. Wilder-Smith, L.A.-N., D. Yarnitski & K. Vissers Ed.), IASP Press, Philadelphia, 2015, pp. 119-136.
35. Mouraux A, Iannetti GD. Across-trial averaging of event-related EEG responses and beyond. *Magnetic resonance imaging.* 26:1041-1054, 2008
36. Mouraux A, Iannetti GD. Nociceptive laser-evoked brain potentials do not reflect nociceptive-specific neural activity. *Journal of neurophysiology.* 101:3258-3269, 2009
37. Nahra H, Plaghki L. Innocuous skin cooling modulates perception and neurophysiological correlates of brief CO2 laser stimuli in humans. *European journal of pain (London, England).* 9:521-530, 2005
38. Namer B, Kleggetveit IP, Handwerker H, Schmelz M, Jorum E. Role of TRPM8 and TRPA1 for cold allodynia in patients with cold injury. *Pain.* 139:63-72, 2008

39. Namer B, Seifert F, Handwerker HO, Maihofner C. TRPA1 and TRPM8 activation in humans: effects of cinnamaldehyde and menthol. *Neuroreport.* 16:955-959, 2005
40. Olsen RV, Andersen HH, Moller HG, Eskelund PW, Arendt-Nielsen L. Somatosensory and vasomotor manifestations of individual and combined stimulation of TRPM8 and TRPA1 using topical L-menthol and trans-cinnamaldehyde in healthy volunteers. *European journal of pain (London, England).* 18:1333-1342, 2014
41. Ringkamp M, Peng YB, Wu G, Hartke TV, Campbell JN, Meyer RA. Capsaicin responses in heat-sensitive and heat-insensitive A-fiber nociceptors. *The Journal of neuroscience : the official journal of the Society for Neuroscience.* 21:4460-4468, 2001
42. Roberts K, Shenoy R, Anand P. A novel human volunteer pain model using contact heat evoked potentials (CHEP) following topical skin application of transient receptor potential agonists capsaicin, menthol and cinnamaldehyde. *Journal of clinical neuroscience : official journal of the Neurosurgical Society of Australasia.* 18:926-932, 2011
43. Schmidt R, Schmelz M, Ringkamp M, Handwerker HO, Torebjörk HE. Innervation territories of mechanically activated C nociceptor units in human skin. *Journal of neurophysiology.* 78:2641-2648, 1997
44. Schwarz MG, Namer B, Reeh PW, Fischer MJM. TRPA1 and TRPV1 Antagonists Do Not Inhibit Human Acidosis-Induced Pain. *The journal of pain : official journal of the American Pain Society.* 18:526-534, 2017
45. Story GM, Peier AM, Reeve AJ, Eid SR, Mosbacher J, Hricik TR, Earley TJ, Hergarden AC, Andersson DA, Hwang SW, McIntyre P, Jegla T, Bevan S, Patapoutian A. ANKTM1, a TRP-like channel expressed in nociceptive neurons, is activated by cold temperatures. *Cell.* 112:819-829, 2003
46. Takaishi M, Uchida K, Suzuki Y, Matsui H, Shimada T, Fujita F, Tominaga M. Reciprocal effects of capsaicin and menthol on thermosensation through regulated activities of TRPV1 and TRPM8. *J Physiol Sci.* 66:143-155, 2016
47. Treede RD, Meyer RA, Raja SN, Campbell JN. Evidence for two different heat transduction mechanisms in nociceptive primary afferents innervating monkey skin. *The Journal of physiology.* 483 (Pt 3):747-758, 1995
48. Trevisani M, Smart D, Gunthorpe MJ, Tognetto M, Barbieri M, Campi B, Amadesi S, Gray J, Jerman JC, Brough SJ, Owen D, Smith GD, Randall AD, Harrison S, Bianchi A, Davis JB, Geppetti P. Ethanol elicits and potentiates nociceptor responses via the vanilloid receptor-1. *Nature neuroscience.* 5:546-551, 2002
49. Valeriani M, Arendt-Nielsen L, Le Pera D, Restuccia D, Rosso T, De Armas L, Maiese T, Fiaschi A, Tonali P, Tinazzi M. Short-term plastic changes of the human nociceptive system following acute pain induced by capsaicin. *Clinical neurophysiology : official journal of the International Federation of Clinical Neurophysiology.* 114:1879-1890, 2003
50. Valeriani M, Tinazzi M, Le Pera D, Restuccia D, De Armas L, Maiese T, Tonali P, Arendt-Nielsen L. Inhibitory effect of capsaicin evoked trigeminal pain on warmth sensation and warmth evoked potentials. *Experimental brain research.* 160:29-37, 2005
51. van den Broeke EN, Lenoir C, Mouraux A. Secondary hyperalgesia is mediated by heat-insensitive A-fibre nociceptors. *The Journal of physiology.* 594:6767-6776, 2016
52. van den Broeke EN, Mouraux A. High-frequency electrical stimulation of the human skin induces heterotopical mechanical hyperalgesia, heat hyperalgesia, and enhanced responses to nonnociceptive vibrotactile input. *Journal of neurophysiology.* 111:1564-1573, 2014
53. van Neerven SGA, Mouraux A. Capsaicin-Induced Skin Desensitization Differentially Affects A-Delta and C-Fiber-Mediated Heat Sensitivity. *Frontiers in Pharmacology.* 11, 2020
54. Vandewauw I, De Clercq K, Mulier M, Held K, Pinto S, Van Ranst N, Segal A, Voet T, Vennekens R, Zimmermann K, Vriens J, Voets T. A TRP channel trio mediates acute noxious heat sensing. *Nature.* 2018
55. Voets T. Quantifying and modeling the temperature-dependent gating of TRP channels. *Reviews of physiology, biochemistry and pharmacology.* 162:91-119, 2012

56. Voets T. TRP channels and thermosensation. *Handbook of experimental pharmacology.* 223:729-741, 2014
57. Voets T, Droogmans G, Wissenbach U, Janssens A, Flockerzi V, Nilius B. The principle of temperature-dependent gating in cold- and heat-sensitive TRP channels. *Nature.* 430:748-754, 2004
58. Wagenmakers EJ, Love J, Marsman M, Jamil T, Ly A, Verhagen J, Selker R, Gronau QF, Dropmann D, Boutin B, Meerhoff F, Knight P, Raj A, van Kesteren EJ, van Doorn J, Smira M, Epskamp S, Etz A, Matzke D, de Jong T, van den Bergh D, Sarafoglou A, Steingroever H, Derks K, Rouder JN, Morey RD. Bayesian inference for psychology. Part II: Example applications with JASP. *Psychonomic bulletin & review.* 25:58-76, 2018
59. Wagenmakers EJ, Marsman M, Jamil T, Ly A, Verhagen J, Love J, Selker R, Gronau QF, Smira M, Epskamp S, Matzke D, Rouder JN, Morey RD. Bayesian inference for psychology. Part I: Theoretical advantages and practical ramifications. *Psychonomic bulletin & review.* 25:35-57, 2018
60. Weidner C, Schmelz M, Schmidt R, Hansson B, Handwerker HO, Torebjork HE. Functional attributes discriminating mechano-insensitive and mechano-responsive C nociceptors in human skin. *The Journal of neuroscience : the official journal of the Society for Neuroscience.* 19:10184-10190, 1999
61. Weyer-Menkhoff I, Lotsch J. Human pharmacological approaches to TRP-ion-channel-based analgesic drug development. *Drug discovery today.* 2018
62. Xiao B, Dubin AE, Bursulaya B, Viswanath V, Jegla TJ, Patapoutian A. Identification of transmembrane domain 5 as a critical molecular determinant of menthol sensitivity in mammalian TRPA1 channels. *The Journal of neuroscience : the official journal of the Society for Neuroscience.* 28:9640-9651, 2008
63. Yarnitsky D, Simone DA, Dotson RM, Cline MA, Ochoa JL. Single C nociceptor responses and psychophysical parameters of evoked pain: effect of rate of rise of heat stimuli in humans. *The Journal of physiology.* 450:581-592, 1992