Title:
Secondary hyperalgesia is mediated by heat-insensitive A-fiber nociceptors

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Key point summary:

- It is believed that secondary hyperalgesia (the increase sensitivity to mechanical nociceptive stimuli that develops after cutaneous tissue injury in the surrounding uninjured skin) is mediated by a subclass of nociceptors: the slowly adapting A-fiber mechano-heat nociceptors (AMH-type I). Here we tested this hypothesis.
- By using intense long-lasting heat stimuli, which are known to activate these slowly adapting AMH-type I nociceptors, we show that the perceived intensity, elicited by these stimuli, is *not* increased in the area of secondary hyperalgesia.
- Moreover, we show that during an A-fiber nerve conduction block the perception elicited by the long-lasting heat stimuli is significantly reduced in a time window that matches the response profile of the AMH-type I nociceptors.

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- To conclude, AMH-type I nociceptors contribute to the perception of sustained heat, but they do not mediate secondary hyperalgesia. Therefore, we propose that secondary hyperalgesia is mediated by high threshold mechanoreceptors.

ABSTRACT

Secondary hyperalgesia refers to the increase in sensitivity to mechanical nociceptive stimuli delivered outside the area of tissue injury. Previous studies suggested that secondary hyperalgesia is mediated by a specific class of myelinated nociceptors: slowly-adapting A-fiber mechano- and heat-sensitive (AMH) Type I nociceptors. Here, we tested this hypothesis by examining, whether long-lasting heat stimuli, which are known to activate AMH-type I nociceptors, elicit enhanced responses when delivered to the area of secondary hyperalgesia induced by high frequency electrical stimulation of the skin (HFS). Before and twenty minutes after HFS, sustained 30-s radiant heat stimuli were delivered to the area of increased mechanical pinprick sensitivity while participants continuously rated intensity of perception using an online visual-analogue scale (0-100 mm). After HFS, no significant enhancement of heat perception was observed in the area of increased pinprick sensitivity. To establish that myelinated nociceptors actually contribute to the perception of sustained heat, we conducted a second experiment in which sustained heat stimuli were presented before and during an A-fiber nerve conduction block, achieved by applying a rubber band with weights which compresses the superficial radial nerve against the radius. During the block, heat perception was significantly reduced 17-33 s after the onset of the heat stimulus (before: mean = 53 mm, during: mean = 31 mm; p = .03), matching the response profile of AMH-type I nociceptors. These results support the notion that AMH-type I nociceptors

contribute to the perception of sustained heat, but also show that these afferents do not mediate secondary hyperalgesia.

ABBREVIATION LIST

AMH, A fiber mechano-heat nociceptor; HFS, High frequency stimulation; HTM, High threshold mechanoreceptor; NRS, numerical rating scale; RT, reaction time; TRPV1, Transient receptor potential, vanilloid subfamily, member 1; VAS, visual analog scale.

INTRODUCTION

Cutaneous tissue injury produces inflammation and hyperalgesia. This hyperalgesia is thought to result from a combination of peripheral and central sensitization of the nociceptive system. Peripheral sensitization refers to the enhanced responsiveness of peripheral nociceptors innervating the injured or inflamed tissues. Central sensitization refers to an increased responsiveness of nociceptive neurons in the central nervous system (Loeser & Treede, 2008).

A key feature of central sensitization is the development of an increased sensitivity to mechanical pinprick stimuli that spreads beyond the site of injury or inflammation, a phenomenon also referred to as *secondary hyperalgesia*. In humans, secondary hyperalgesia can be induced experimentally by activating nociceptors in a sustained and intense fashion, for example, using an intradermal injection of capsaicin to selectively activate the TRPV1

receptor (LaMotte *et al.*, 1991), or using high frequency electrical stimulation of the skin (Klein *et al.*, 2004). With these methods, studies have demonstrated that, contrasting with the marked enhanced sensitivity to mechanical pinprick stimuli, the sensitivity to short-lasting heat stimuli delivered to the area of secondary hyperalgesia is not enhanced (Ali *et al.*, 1996). Studies have also shown that selective blockade of myelinated fibers suppresses secondary hyperalgesia (Ziegler *et al.*, 1999). Furthermore, increased pinprick sensitivity also developed in skin pre-treated with capsaicin to induce a denervation of fibres expressing TRPV1 (Magerl *et al.*, 2001). Based on these results, it is generally accepted that secondary hyperalgesia is mediated by myelinated nociceptors that respond to mechanical pinprick stimuli but do not respond to short-duration heat stimuli and capsaicin (Ali *et al.*, 1996; Ziegler *et al.*, 1999; Magerl *et al.*, 2001).

Based on their response to sustained heat (30 s, 53°C), teased-fibre recordings in monkeys have shown that myelinated nociceptors can be categorized into quickly adapting nociceptors (AMH-type II), slowly adapting nociceptors (AMH-type I) and heat-insensitive nociceptors (Treede *et al.*, 1995; Treede *et al.*, 1998). AMH-type II nociceptors respond immediately at the onset of the sustained heat stimulus (mean heat threshold around 47°C), but their rate of discharge fades out very quickly over time. These nociceptors most likely mediate the “first” pain elicited by a short-lasting heat stimulus (Treede *et al.*, 1995; Treede *et al.*, 1998). In contrast, AMH-type I nociceptors do not respond immediately after the onset of the sustained heat stimulus, and they do not respond to short-lasting 1-s heat stimuli, even at 53°C. When exposed to sustained heat, they start responding after a few seconds, with an average peak latency around 16 or 21 seconds, depending on the studies (Treede *et al.*, 1995; Treede *et al.*, 1998). Finally, heat-insensitive nociceptors do not

respond at all to heat. Because they do respond to mechanical nociceptive stimuli, they are also called high-threshold mechanoreceptors (HTMs).

Treede et al. (1998) showed that, when long-duration heat stimuli are used (e.g. 30 seconds) AMH-type I and AMH-type II nociceptors have similar heat thresholds (Treede *et al.*, 1998).

As most AMH-type I nociceptors respond to mechanical stimuli but not to short-lasting heat stimuli (Treede *et al.*, 1998) or capsaicin (Ringkamp *et al.*, 2001) it is believed that secondary pinprick hyperalgesia is mediated by this class of nociceptors (Iannetti *et al.*, 2013). However, an alternative possibility could be that secondary pinprick hyperalgesia is mediated by HTMs (Magerl *et al.*, 2001).

The aim of the present study was to test whether secondary hyperalgesia is mediated by AMH-type I by examining whether long-duration heat stimuli (30 seconds) delivered at an intensity above the threshold of AMH-type II elicit increased heat pain in the heterotopic area of increased pinprick sensitivity with a similar time course as the response profile of the AMH-type I nociceptors.

In a first experiment, we show that, in the area of increased pinprick sensitivity induced by HFS, the perception of long-lasting heat stimuli is not significantly enhanced. In a second study, we show that, during an A-fiber block, the perception of long-lasting heat is significantly reduced within a time window corresponding to the expected response profile of AMH-type I. Taken together, the results of these two experiments strongly suggest that the AMH-type I nociceptors do not mediate mechanical secondary hyperalgesia.

MATERIALS AND METHODS

Ethical approval. The experiments were conducted according to the Declaration of Helsinki. Approval for the two experiments was obtained from the local Ethical Committee (Hospital and Departmental Ethics Committee, Saint-Luc - Université catholique de Louvain). All participants signed an informed consent form.

Participants. Fourteen healthy volunteers took part in experiment 1 (8 men and 6 women; aged 21-33 years; 25.1 ± 3.6 years [mean ± sd]). Six healthy volunteers took part in experiment 2 (3 men and 3 women; aged 24-38 years; 27.8 ± 5.1 years). The participants in experiment 1 received financial compensation for their participation.

Experiment 1

The design of the experiment is summarized in Fig. 1. During the experiment, participants were comfortably seated in a chair with their arms resting on a table in front of them.

Induction of increased pinprick sensitivity. Transcutaneous high frequency electrical stimulation (HFS) of the left or right volar forearm was used to induce secondary hyperalgesia. Several studies have shown that this technique induces a robust and long-lasting increase in mechanical pinprick sensitivity, extending beyond the area of stimulation (Klein *et al.*, 2004; Klein *et al.*, 2008; van den Broeke & Mouraux, 2014; Henrich *et al.*, 2015). Similar to other commonly used methods to induce secondary hyperalgesia (e.g. intradermal injection of capsaicin), HFS induced secondary hyperalgesia depends on the activation of capsaicin-sensitive fibers (Magerl *et al.*, 2001; Henrich *et al.*, 2015). HFS stimulation was applied to the left or right volar forearm, 10 cm distal to the cubital fossa, and consisted of five trains of 100 Hz (pulse width: 2 ms), each lasting 1 s. The time interval

between each train was 10 s. The intensity of stimulation was individually adjusted to 20x the detection threshold to a single pulse (0.25 ±0.10 mA, [mean ± sd]). The electrical pulses were triggered by a programmable pulse generator (Master-8; AMPI Israel), generated by a constant current electrical stimulator (Digitimer DS7A, Digitimer UK), and delivered to the skin using a specifically designed electrode built at the Centre for Sensory-Motor Interaction (Aalborg University, Denmark). The electrode consists of 16 blunt stainless steel pins (diameter: 0.2 mm) protruding 1 mm from the base. The pins are placed in a 10 mm diameter circle and serve as cathode. A stainless steel anode electrode is concentrically located around the steel pins (inner diameter: 22 mm; outer diameter: 40 mm). To avoid any confounding effect of handedness, the arm onto which HFS was applied (dominant vs. non dominant) was counterbalanced across participants. Handedness was assessed using the Flinders Handedness Survey (Nicholls *et al.*, 2013).

Mapping the area of increased pinprick sensitivity. To map the area of increased mechanical pinprick sensitivity after HFS, a calibrated pinprick stimulator exerting a normal force of 128 mN (MRC Systems, Heidelberg, Germany) was applied perpendicular to the skin, starting at a distance from the conditioning stimulation area and repeated every 1 cm along a proximal-distal and lateral-medial towards the conditioning site. During this procedure, participants did not view the arm. Participants were instructed to indicate the point at which the perception was changed. This point was then indicated on the skin with a marker.

Quantifying changes in mechanical pinprick sensitivity. To assess changes in pinprick sensitivity, the same pinprick stimuli used to map the area of increased pinprick sensitivity were applied three times onto the skin. To avoid sensitization of the stimulated skin, the

target of each pinprick stimulus was displaced after each stimulus. Participants were asked to report the intensity of perception elicited by the pinprick stimulation on a numerical rating scale (NRS) ranging from 0 (no perception) to 100 (maximal pain), with 50 representing the transition from non-painful to painful domains of sensation. Stimuli were applied to the HFS-conditioned arm and the contralateral control arm, before applying HFS (T0) and 20 minutes after applying HFS (T1).

Quantifying changes in sensitivity to long-duration heat stimuli. To assess changes in heat sensitivity, long-duration (30 s) radiant heat stimuli were delivered onto the skin of the HFS-conditioned arm and the contralateral control arm, before applying HFS (T0) and 20 minutes after applying HFS (T1). The heat stimuli were generated by an infrared CO_2 laser stimulator (wavelength 10.6 µm) whose power output is regulated using a closed-loop control of pulse-width modulation based on an online measurement of skin temperature performed using a radiometer whose field of view is collinear with the laser beam (Laser Stimulation Device; SIFEC, Belgium). The stimuli consisted of a 10 ms heating ramp during which the skin was brought to the desired target temperature, followed by a plateau during which skin temperature was maintained at target temperature for 30 seconds. The diameter of the laser beam at the target site was 6 mm. At each stimulation site and time point, a total of five stimuli were delivered. To avoid skin overheating and minimize stimulus-induced sensitization or habituation, the target of the laser beam was displaced manually by the experimenter after each stimulus. In order to rate continuously the perception elicited the by long-duration heat stimulus, a custom made Visual Analogue Scale (VAS) box was used, consisting of a 100 mm vertical slider connected to a computer. The lower extremity of the slider was labelled “no perception” and the upper extremity of the slider was labelled

"maximal pain". An anchor at the middle of the scale marked the transition between non-painful and painful domains of sensation. Participants were instructed to rate continuously the intensity of perception, as soon as they perceived a sensation, by moving the slider. The recording lasted 40 seconds from the onset of the stimulus. During heat stimulation, participants were instructed to keep their gaze on the VAS, and view of the arm was prevented. Before starting the heat stimulation during the post measurement, subjects were explicitly told that although there was increased sensitivity to mechanical pinprick stimulation (as experienced during the mapping procedure), this was not necessarily going to be the case for thermal stimulation.

At the beginning of the experiment, for each participant, the target temperature for the long-duration heat stimuli was defined as follows. The individual thermal detection threshold of quickly-adapting AMH-type II nociceptors was estimated for each arm using an adaptive staircase procedure. Participants were requested to press a button held in the contralateral hand as soon as they detected a short-lasting heat stimulus (10 ms heating ramp followed by a 90 ms plateau). Reaction times (RT) were used as criterion to distinguish between detections mediated by myelinated AMH-type II (RT < 650 ms) vs. unmyelinated C fibers (RT ≥ 650 ms) (Churyukanov *et al.*, 2012). The staircase algorithm started at 46°C. If the stimulus was detected with a RT <650 ms, the temperature of the subsequent stimulus was decreased by 1°C. Conversely, if it was detected with a RT ≥650 ms or if it was undetected, the temperature of the subsequent stimulus was increased by 1°C. The staircase procedure ended after four reversals (when a previous stimulus was undetected and the subsequent one was detected or the reverse). The threshold was defined as the stimulus temperature of the fourth reversal. The target of the laser beam was displaced

manually by the experimenter after each stimulus. During the threshold estimation procedure, the view of the arm was prevented. Considering that previous studies have suggested that, when long-duration heat stimuli are used, the thermal activation thresholds of AMH-type I and AMH-type II are similar (Treede *et al.*, 1998), the actual target temperature used for the long-duration heat stimuli was set to 1°C above the estimated AMH-type II threshold (with a maximum of 50 °C for safety reasons).

Data analysis. The effect of HFS on the intensity of the percept elicited by the mechanical pinprick stimuli was assessed using a repeated measures ANOVA with *time* (T0 and T1) and *side* (control vs. HFS arm) as within-subject factors. The interaction between the two factors was taken as a measure of the effect of HFS. The assumption of sphericity was tested using Mauchly's test. If the data violated the assumption of sphericity, F-values were corrected using the Greenhouse-Geisser procedure. Post-hoc tests were performed using paired-sample t-tests in which *p*-values were Bonferroni corrected for the number of tests. The level of significance was set at $p<.05$ (two-sided). The statistical analyses were conducted using SPSS 18 (SPSS Inc., Chicago, IL, USA).

Analysis of the effect of HFS on the continuous ratings of the percept elicited by the long-lasting heat stimuli was conducted using Letswave6 (http://www.nocions.org/letswave6/). First, we computed the average of the five rating waveforms for each participant and condition. Second, we computed for each participant, *difference waveforms* assessing the change in heat perception at T1 vs. T0 at the HFS arm ($HFS_{T1} – HFS_{T0}$) and at the control arm ($control_{T1} – control_{T0}$). Finally, we performed a temporal non-parametric cluster-based permutation test (Maris & Oostenveld, 2007; Groppe *et al.*, 2011) using the *difference*

waveforms of the two arms, thereby testing the interaction between *time* (T1 vs. T0) and *side* (HFS vs. control arm). The permutation test consisted of the following steps. First, the *difference* waveforms were compared by means of a point-by-point paired-sample *t*-test. Second, samples above the critical t-value for a parametric two-sided test that were adjacent in time were identified and clustered. An estimate of the magnitude of each cluster was then obtained by computing the sum of the absolute *t*-values constituting each cluster (cluster-level statistic). Random permutation testing (2000 times) of the subject-specific *difference* waveforms of the two arms (performed independently for every subject) was then used to obtain a reference distribution of *maximum* cluster magnitude. Finally, the proportion of random partitions that resulted in a larger cluster-level statistic than the observed one (i.e. *p*-value) was calculated. Clusters in the observed data were regarded as significant if they had a magnitude exceeding the threshold of the 95.0^{th} percentile of the permutation distribution (corresponding to a critical alpha-level of .05 for a two-sided test).

Experiment 2

To assess the relative contribution of myelinated fibers to the perception of sustained heat, we delivered long-duration heat stimuli in the innervation territory of the left superficial branch of the radial nerve, before and during an A-fiber conduction block of that nerve.

A-fiber nerve conduction block. In order to block selectively the conduction of myelinated fibers, pressure was applied to the left superficial branch of the radial nerve, at the level of the wrist (Magerl *et al.*, 1999; Nahra & Plaghki, 2003; Mouraux *et al.*, 2010). Participants were comfortably seated in a chair with the left forearm immobilized on an armrest in a

neutral pronation/supination position with a handle bar in their left hand preventing any pronation or supination of the hand and forearm. The pressure was delivered by means of 2 weights of 700 grams each hanging on a 1.5 cm rubber band applied on the forearm proximally to the wrist.

Development of the nerve conduction blockade was monitored by assessing, every 10 minutes, the ability of the participants to detect tactile stimuli (calibrated nylon filament exerting a target force of 5.9 mN; Semmens-Weinstein Von Frey Aesthesiometers, evaluator size 3.84, Stoelting Co, USA) and short-lasting cold stimuli (10°C stimuli applied during 100 ms using a 125 mm^2 surface; custom-built contact probe based on micro Peltier elements; Prof. André Dufour, Université de Strasbourg, France). As soon as the tactile and cold perception disappeared, we then assessed RTs to short-lasting heat stimuli (55°C stimuli lasting 100 ms, delivered using the temperature-controlled infrared CO_2 laser stimulator, laser beam diameter 6 mm) and mechanical pinprick stimuli (custom-made calibrated pinprick stimulator exerting a normal force of 90 mN with a 0.35 mm probe diameter). Such as in Experiment 1, RTs to these stimuli were used to distinguish detections triggered by sensory input conveyed by myelinated A-fibers (RT < 650 ms) and unmyelinated C-fibers (RT ≥650 ms). The area of hypoesthesia was then defined as the area where (1) tactile stimuli and cold stimuli were not detected and (2) both the short-lasting heat stimuli and the pinprick stimuli were either undetected or detected with RTs ≥650 ms. During the assessments the view of the hand was prevented.

Quantifying changes in sensitivity to long-duration heat stimuli. The assessment of heat perception elicited by the long-duration heat stimuli before and during the nerve conduction blockade was performed in the same way as in Experiment 1.

Data analysis. To test the effect of the A-fiber nerve conduction block on the percept elicited by the long-duration heat stimuli, we used the cluster-based permutation testing also used in Experiment 1 (Maris & Oostenveld, 2007; Groppe *et al.*, 2011) to compare the average intensity rating waveforms obtained before vs. during the block. Such as in Experiment 1, clusters were regarded as significant if their magnitude exceeded the threshold of the 95.0th percentile of the permutation distribution (corresponding to a critical alpha-level of .05 for a two-sided test).

RESULTS

Experiment 1

Thermal detection thresholds to short-lasting heat stimuli. The AMH-type II detection threshold estimated at the beginning of experiment was on average (± sd) 47.6 ± 1.0 °C (range 45-49 °C) at the control arm and 47.6 ± 1.2 °C (46-49 °C) at the HFS-treated arm.

HFS-induced change in mechanical pinprick sensitivity. HFS induced a clear increase in the intensity of the percept elicited by mechanical pinprick stimuli delivered to the HFS-treated arm (Figure 2). The repeated-measures ANOVA showed a significant *time × side* interaction ($F(1, 13) = 38.208$, $p < .001$, $\eta^2 = .746$). Post-hoc tests showed that the perceived intensity

after HFS at the treated arm was significantly increased (paired t-test; t (13) = 7.177, p < .001). In contrast, no significant differences were observed for the control arm (p=.468).

HFS-induced change in the sensitivity to long-lasting heat stimuli. None of the participants showed any visible sign of a burn lesion after the long-duration heat stimuli. The group-level average waveforms of intensity of perception are shown in Figure 3. After HFS, the percept elicited by long-lasting heat stimuli delivered to the HFS-treated arm was similar to the percept elicited by stimulation of the control arm, except during the first 5 seconds which tended to show a slight enhancement of perception at the HFS arm. Nevertheless, the permutation test did not reveal any cluster having a p-value smaller than the critical alpha level of .05. This means that, after HFS, the heat perception elicited throughout the long-duration heat stimuli delivered to the HFS-treated arm was not significantly different as compared to baseline and control side.

Experiment 2

Thermal detection thresholds to short-lasting heat stimuli. The AMH-type II detection threshold estimated at the beginning of the experiment was on average (±sd) 47.0 ± 1.5 °C (range 45-49).

Time required to establish the A-fiber nerve conduction block. In five out of six participants, the block was established 110 minutes after the start of the procedure. In one participant the block was established 130 minutes after the procedure.

Effects of the A-fiber nerve conduction block on the perception of brief tactile, heat, pinprick and cold stimuli. Before the start of the block, tactile, pinprick, cold and heat stimuli were all

detected by every participant. Once the block was established, four out of six subjects spontaneously reported a mild burning sensation in their hand. Five out of six subjects did not detect any of the tactile stimuli and one subject detected 1/5 stimuli. Five out of six subjects were no longer able to detect the cold stimuli, and one subject detected 2/5 cold stimuli (Figure 4C). Heat and pinprick stimuli were either not detected or detected with increased reaction times (heat stimuli: 325.5 ± 65.0 ms vs. 958.5 ± 256.8 ms; pinprick stimuli: 261.9 ± 77.2 ms vs. 1065.0 ± 454.4 ms) (Figure 4A and 4B). All participants recovered completely after the release of the nerve conduction block.

Effect of the A-fiber nerve conduction block on the perception of long-lasting heat stimuli. None of the participants showed any visible sign of a burn after the long-duration heat stimuli. On average, ratings were decreased during the block, especially during the second half of the stimulus (Figure 5). The permutation test comparing the rating waveforms before versus during the block revealed one cluster having a *p*-value smaller than the critical alpha level of .05 extending between 17 and 33 seconds after stimulus onset (*p* value = 0.03). During this interval, heat perception was significantly reduced during the block.

DISCUSSION

Although there was a clear increase in the perception of mechanical pinprick stimuli after HFS, no significant change in the perception of long-lasting heat stimuli delivered to the area of secondary hyperalgesia was observed. This indicates that, unlike previously proposed, increased pinprick sensitivity is not mediated by slowly-adapting AMH-type I nociceptors.

The results of our second experiment support the view that myelinated fibers do contribute to sustained heat perception. Indeed, heat perception *during the second half* of the sustained heat stimulus was significantly decreased during the A-fiber nerve conduction block. Interestingly, the perception of long-lasting heat was not completely abolished, suggesting that sustained heat perception also involves *unmyelinated* afferents.

If AMH-type I nociceptors are not the primary mediators of secondary hyperalgesia, then the most likely candidate are HTMs (Treede & Magerl, 2000; Magerl *et al.*, 2001; Meyer & Treede, 2004). Indeed, HTMs are mechano-sensitive but do not respond to short- or long-lasting heat stimuli and capsaicin (Treede *et al.*, 1998; Ringkamp *et al.*, 2001).

Peripheral vs. central sensitization

In rats, Reeh et al. (1987) showed that mechanical injury of the tail skin leads to peripheral sensitization of HTMs, both when the injury is applied within their receptive fields, or closely outside the border of their receptive fields. Most of the sensitized HTMs developed spontaneous activity, expanded their receptive fields, and exhibited lower thresholds to punctate mechanical stimuli (Reeh *et al.*, 1987). These results suggest that peripheral sensitization of HTMs could contribute to the increased mechanical sensitivity surrounding the site of tissue injury. However, it remains unclear whether this peripheral phenomenon can explain the increased mechanical sensitivity commonly observed at a greater distance from the injured site (i.e. the area of secondary hyperalgesia). Moreover, it remains unknown whether peripheral sensitization of HTMs can be induced in the absence of tissue injury.

On the other hand, there is ample evidence that secondary mechanical hyperalgesia results, at least partly, from a facilitation of nociceptive transmission at the level of the spinal cord, i.e. *central sensitization* (Kenshalo *et al.*, 1982; McMahon & Wall, 1984; Woolf & King, 1990; Baumann *et al.*, 1991; LaMotte *et al.*, 1991; Simone *et al.*, 1991). For example, McMahon and Wall (1984) showed in rats that the receptive field of dorsal horn lamina I neurons increases after a burn injury applied outside the receptive field of these neurons. Moreover, activation thresholds to mechanical stimuli delivered within their receptive fields were lower after the burn injury. These lamina I neurons also responded more strongly to transcutaneous electrical stimuli delivered to their cutaneous receptive fields. Because such stimuli bypass peripheral transduction processes, these effects can only be explained by a central mechanism.

The present study shows that, unlike mechanical pinprick perception, long-lasting heat perception is not significantly increased in the area of secondary hyperalgesia. This suggests that the central mechanism underlying increased pinprick sensitivity involves the facilitation of a modality-specific pathway dedicated to noxious mechanical input. Is there any evidence for such a pathway? In cats (Christensen & Perl, 1970; Light & Perl, 1979) and monkeys (Light & Perl, 1979), it was shown that HTMs project to the superficial layer (lamina I) of the dorsal horn. Moreover, the presence of modality-specific nociceptive neurons has been demonstrated in lamina I. For example, Han et al. (1998) showed, in cats, that 18 out of 38 lamina I neurons were nociceptive-specific. Eight of these neurons responded *only* to mechanical nociceptive stimulation (pinch) (Han *et al.*, 1998). In a follow-up study, Andrew and Craig (2002) found that four out of twenty nociceptive-specific lamina I neurons responded only to noxious mechanical stimulation (Andrew & Craig, 2002). Taken together,

these results demonstrate, at least in some animals, the existence of neurons specifically relaying mechanical nociceptive input at the level of the dorsal horn.

At first glance, the lack of increase in perception to long-lasting heat observed in the present study seems at odds with our previous results showing that HFS can enhance the percept elicited by short-lasting heat stimuli delivered to the skin closely surrounding the area onto which HFS is applied (van den Broeke & Mouraux, 2014). In the present study, the heat stimuli were often applied at a greater distance from the site of HFS, corresponding to the area of increased pinprick sensitivity. Possibly, the increase in heat sensitivity reported in our previous study involved a small area not covering the entire area of secondary hyperalgesia.

To conclude, we provide evidence that the increased sensitivity to mechanical pinprick stimulation in the area of secondary hyperalgesia is not conveyed by AMH-type I nociceptors. For this reason, we propose that HTMs are the primary mediators of mechanical secondary hyperalgesia. The lack of increase in perception to heat in the area of secondary hyperalgesia further suggests that the central mechanisms underlying secondary hyperalgesia involves the facilitation of CNS neurons specifically involved in the transmission of mechanical nociceptive input conveyed by HTMs.

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COMPETING INTERESTS

All authors declare that they have no competing interests

AUTHOR CONTRIBUTION

ENvdB, CL and AM were involved in the conception and design of the study. ENvdB and CL collected and analyzed the data. ENvdB, CL and AM interpreted the data and drafted the manuscript. All authors approved the final version of the manuscript, and agreed to be accountable for all aspects of the work. All authors meet the criteria for authorship.

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FIGURE LEGENDS

Figure 1. Time-line of the experiment. Before (T0) and after (T1) applying HFS on the left or right forearm, the sensitivity to mechanical pinprick stimuli and long-lasting heat stimuli was assessed at both arms (control vs. HFS). The area of increased mechanical pinprick sensitivity was mapped immediately before T1.

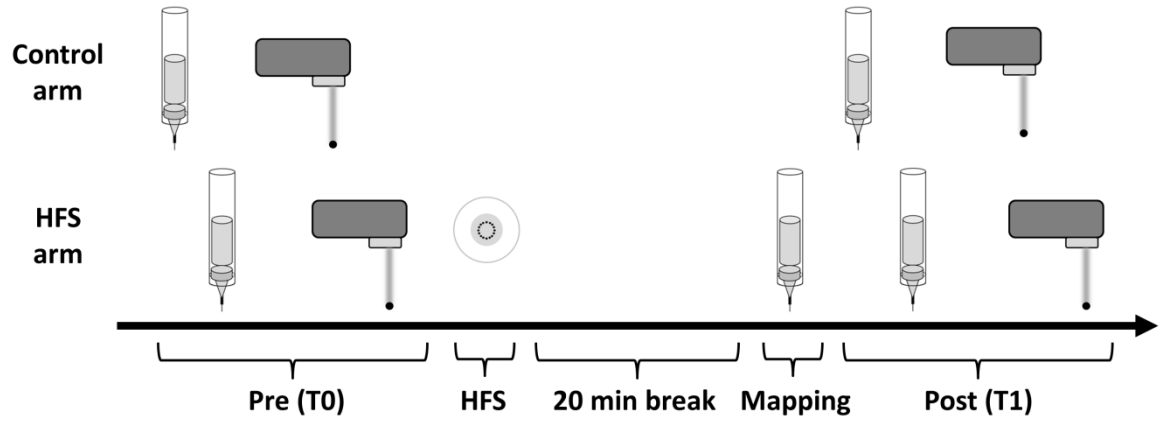


Figure 2. Effect of HFS on mechanical pinprick sensitivity. **A.** Individual (*thin* black lines) and group-level average (*thick* black line) area of increased pinprick sensitivity at the forearm, estimated 20 minutes after applying HFS. **B.** Group-level mean (and SD) intensity of perception elicited by pinprick stimulation of the control arm (grey line) and the HFS arm (black line), before (T0) and twenty minutes after (T1) applying HFS. At the HFS arm, pinprick sensitivity was significantly increased at T1 vs. T0 (***; p <.001).

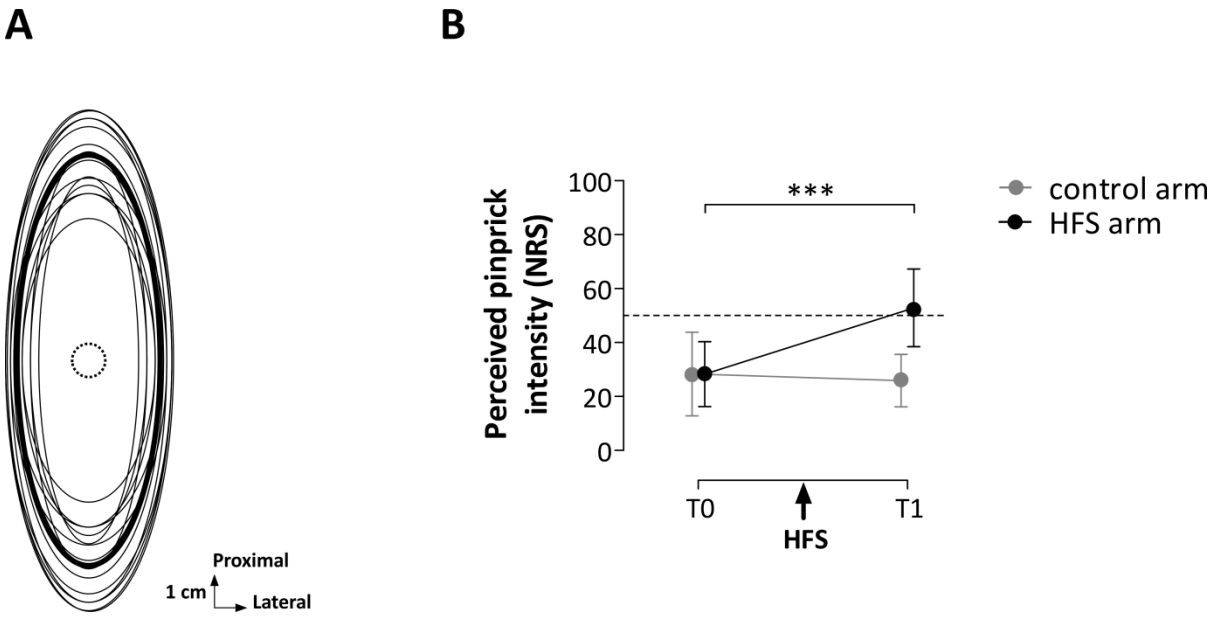
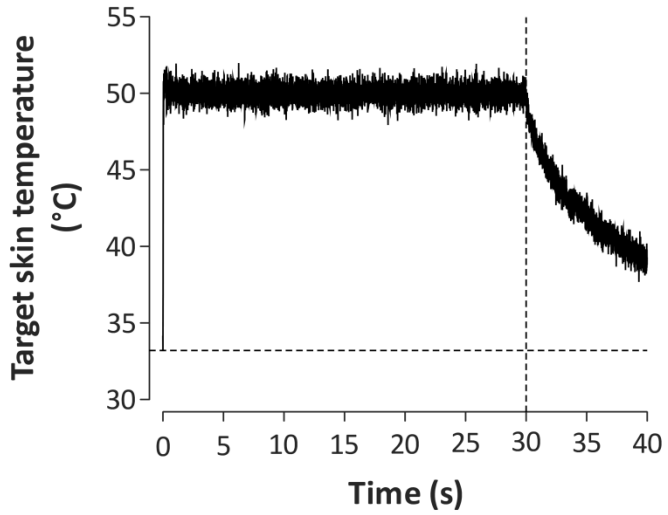


Figure 3. Effect of HFS on the perception of long-lasting heat stimuli. **A.** Example of the skin heating profiles generated by the temperature-controlled laser stimulator and measured by the radiometer collinear with the laser beam. The horizontal dashed line indicates baseline skin temperature. The vertical dashed line indicates the end of the heat stimulation. **B.** Group-level mean rating waveforms obtained during the long-duration heat stimulation for all conditions. The horizontal dashed line indicates the transition from non-painful to painful domains of sensation. The vertical dashed line indicates the end of the heat stimulation. **C.** Group-level average *difference* rating waveforms (T1 minus T0) for both arms (control and HFS). The vertical lines represent error bars expressing standard deviation.

A



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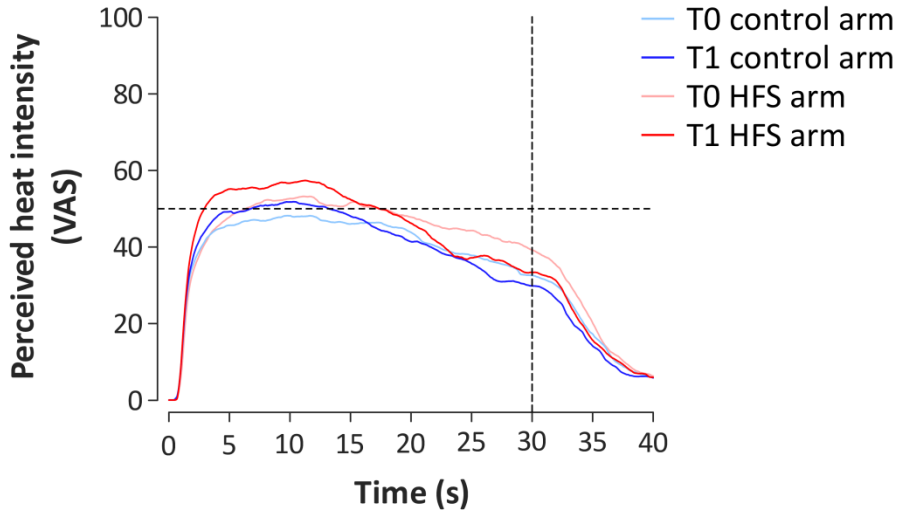

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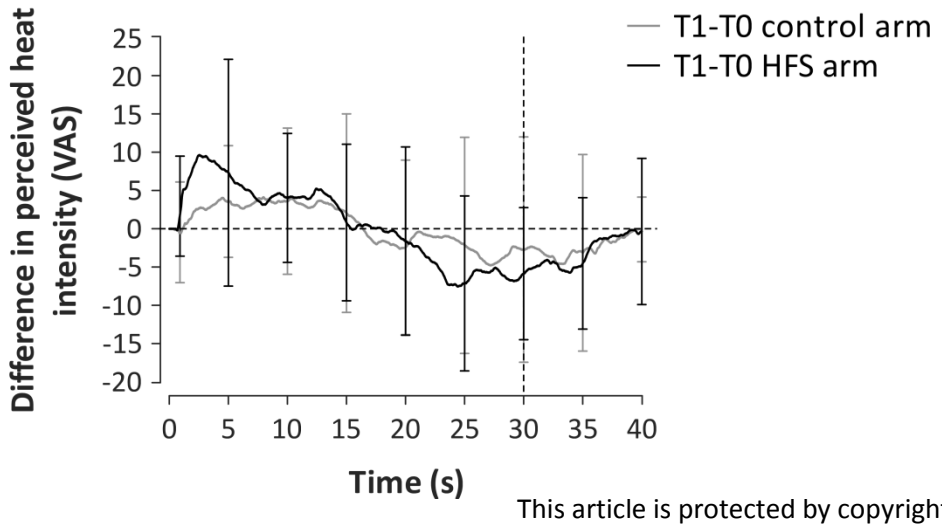
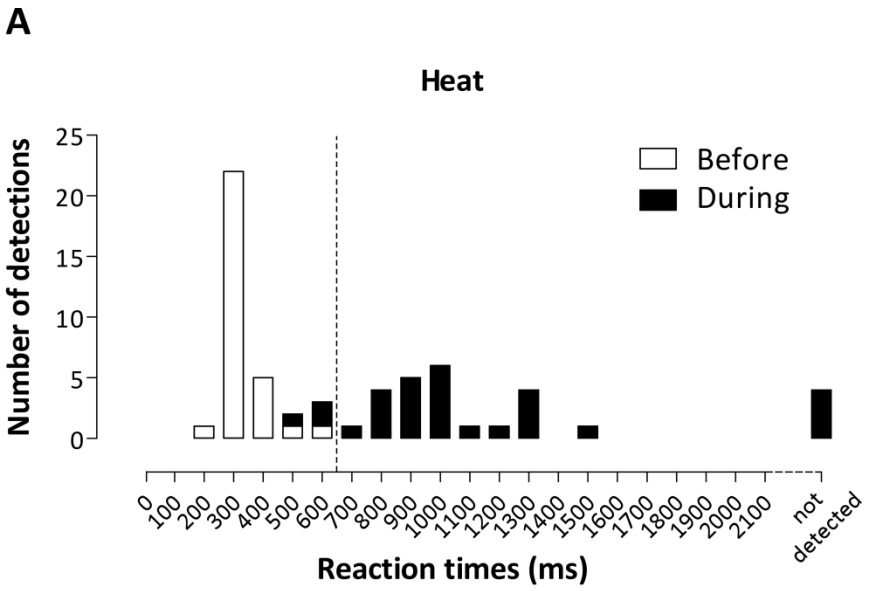
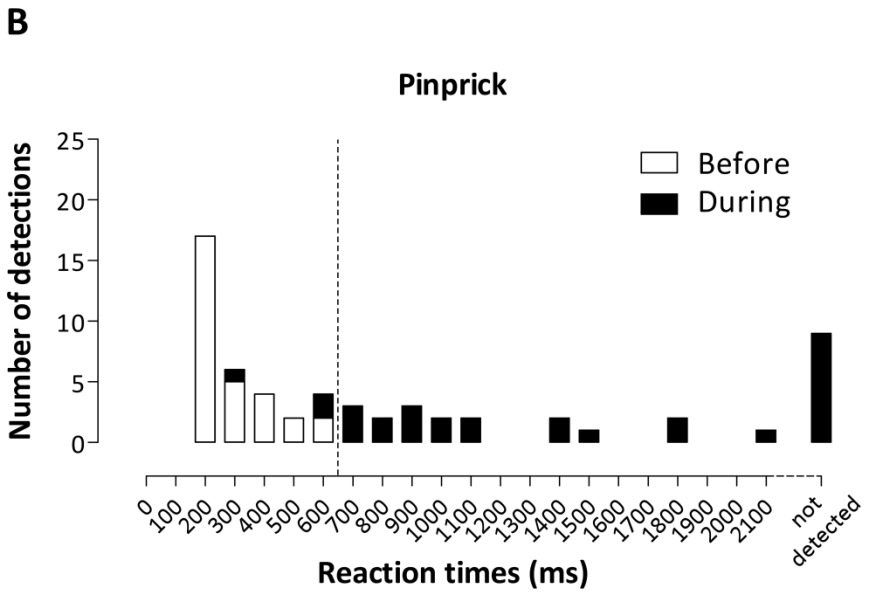


Figure 4. Effect of the A-fiber nerve conduction block on the reaction times to short-lasting heat stimuli (A), the reaction times to mechanical pinprick stimuli (B), and the detection of tactile and cold stimuli (C). **A.** Stacked reaction time distribution for short-lasting (100 ms) 55°C heat stimuli applied on the dorsum of the left hand before and during the A-fiber nerve block. The vertical dashed line represents the cut-off used to distinguish reaction times compatible with the conduction velocities of myelinated vs. unmyelinated fibers. **B.** Stacked reaction time distribution for mechanical pinprick stimulation. Note that during the A-fiber block the reaction times to both short-lasting heat stimuli and mechanical pinprick stimuli are shifted towards the right. **C.** During the A-fiber block, tactile and cold stimuli were, in almost all cases, not detected.





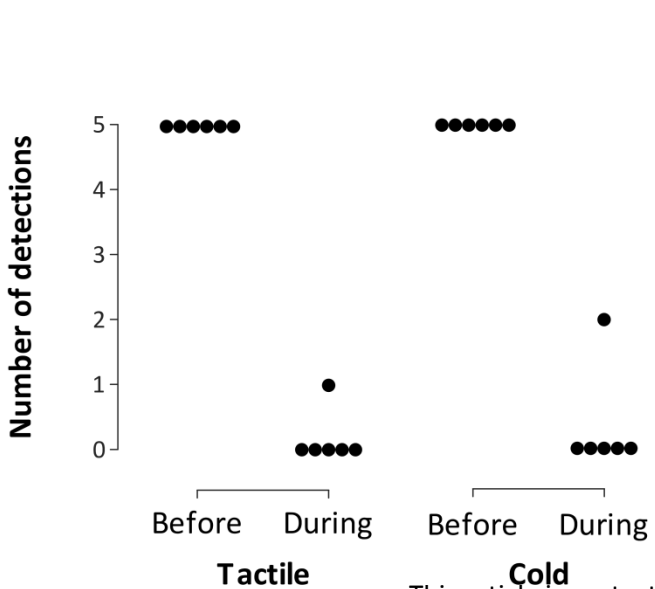
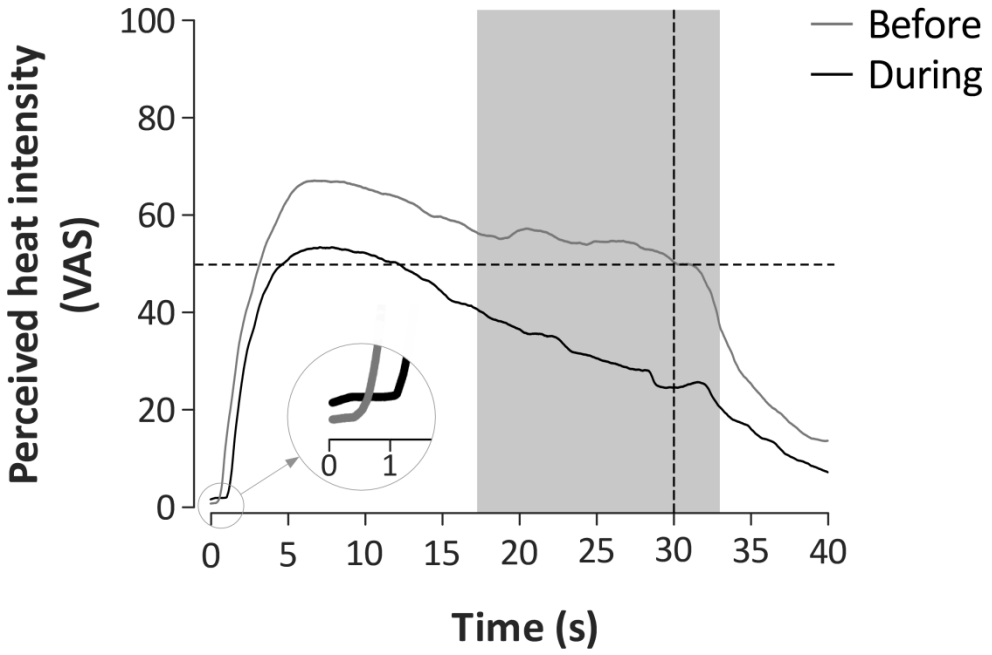


Figure 5. Effect of the A-fiber nerve conduction block on the percept elicited by long duration heat stimuli. **A.** Group-level average rating waveforms obtained before and during the A-fiber never conduction block. During the A-fiber nerve conduction block heat perception was significantly decreased 17-33 seconds after the onset of the 30-s heat stimuli (grey area, $p<.05$). The horizontal dashed line indicates the transition from non-painful to painful domains of sensation. The vertical dashed line indicates the end of stimulation. **Inset:** note that during the block the delay between the increase in skin temperature and the increase in rating was delayed by approximately 1-s, compatible with the blockade of myelinated afferents. **B.** Individual ratings representing the mean calculated within the time window 17-33 s, before and during the A-fiber block.

A



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