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## Priming of central- and peripheral mechanisms with heat and cutaneous capsaicin facilitates secondary hyperalgesia to high frequency electrical stimulation

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*Published in:*  
Journal of Neurophysiology

*DOI (link to publication from Publisher):*  
[10.1152/jn.00154.2021](https://doi.org/10.1152/jn.00154.2021)

*Publication date:*  
2022

*Document Version*  
Accepted author manuscript, peer reviewed version

[Link to publication from Aalborg University](#)

*Citation for published version (APA):*  
Hugosdottir, R., Kasting, M., Mørch, C. D., Kæseler Andersen, O., & Arendt-Nielsen, L. (2022). Priming of central- and peripheral mechanisms with heat and cutaneous capsaicin facilitates secondary hyperalgesia to high frequency electrical stimulation. *Journal of Neurophysiology*, 127(3), 651-659. Advance online publication. <https://doi.org/10.1152/jn.00154.2021>

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# 1 **Priming of central- and peripheral mechanisms with heat and** 2 **cutaneous capsaicin facilitates secondary hyperalgesia to high** 3 **frequency electrical stimulation**

4

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6 Nielsen

7

## 8 **Abstract**

9 Heat/capsaicin sensitization and electrical high frequency stimulation (HFS) are well known model of  
10 secondary hyperalgesia, a phenomenon related to chronic pain conditions. This study investigated whether  
11 priming with heat/capsaicin would facilitate hyperalgesia to HFS in healthy subjects.

12 Heat/capsaicin priming consisted of a 45 °C heat stimulation for 5 min followed by a topical capsaicin patch  
13 (4x4 cm) for 30 minutes on the volar forearm of 20 subjects. HFS (100 Hz, 5 times 1s, minimum 1.5 mA)  
14 was subsequently delivered through a transcutaneous pin electrode approximately 1.5 cm proximal to the  
15 heat/capsaicin application. Two sessions were applied in a crossover design; traditional HFS (HFS-  
16 HEAT/CAP) and heat/capsaicin sensitization followed by HFS (HFS+HEAT/CAP). Heat pain threshold  
17 (HPT), mechanical pain sensitivity (MPS) and superficial blood perfusion were assessed at baseline, after  
18 capsaicin removal, and up to 40 min after HFS. MPS was assessed with pinprick stimulation (128 mN and  
19 256 mN) in the area adjacent to both HFS and heat/capsaicin, distal but adjacent to heat/capsaicin and in a  
20 distal control area. HPT was assessed in the area of heat/capsaicin. Larger sensitivity to 128 mN pinprick  
21 stimulation (difference from baseline and control area) was observed in the HFS+HEAT/CAP session than in  
22 the HFS-HEAT/CAP session 20 and 30 minutes after HFS. Furthermore, sensitivity was increased after  
23 HFS+HEAT/CAP compared to after heat/capsaicin in the area adjacent to both paradigms, but not in the area  
24 distal to heat/capsaicin. Results indicate that heat/capsaicin causes priming of the central- and peripheral  
25 nervous system, which facilitates secondary mechanical hyperalgesia to HFS.

## 26 **New and noteworthy**

27 High frequency electrical stimulation (HFS) and heat/capsaicin sensitization are well known models of  
28 secondary hyperalgesia. The results from the current study indicate that increased sensitivity to 128 mN  
29 pinprick stimulation can be obtained when HFS is delivered following an already established heightened  
30 central hyperexcitability provoked by heat/capsaicin sensitization.

## 31 **Introduction**

32 Chronic pain is a major world-wide problem (Breivik et al. 2006) and effective and individualized diagnosis  
33 and treatment is considered highly attractive but difficult due to partly unknown mechanisms (Woodcock et  
34 al. 2007). Many chronic pain patients (17-35%) experience wide-spread sensitivity (Schliessbach et al. 2013)  
35 and hyperalgesia, which is observed in pain conditions such as postoperative pain (Lavand'homme et al.  
36 2008) and neuropathic pain (Maier et al. 2010). Hyperalgesia, defined by IASP as “increased pain from a  
37 stimulus that normally provokes pain” ([iasp-pain.org](http://iasp-pain.org)), can be observed in the area of stimulation (primary  
38 hyperalgesia) and in surrounding areas (secondary hyperalgesia). There is an extensive amount of evidence  
39 that point to a central origin of secondary hyperalgesia, but peripheral mechanisms cannot be excluded (see  
40 reviews: (Ruscheweyh et al. 2011; Sandkuhler 2009; Treede et al. 1992)). One related mechanism is long-  
41 term potentiation (LTP) of spinal plasticity, but segmental and/or descending inhibitory control may also be  
42 involved (Ruscheweyh et al. 2011). Nociceptive LTP has been induced in spinal synapses of rodents by high  
43 frequency stimulation (HFS) of primary afferent fibers (Ikeda et al. 2003; Liu et al. 1997; Randic et al.  
44 1993). It is therefore believed that LTP may develop after initial strong painful event such as injury or  
45 operation and play an important role in the development of chronic pain in humans (Ruscheweyh et al. 2011;  
46 Sandkühler 2007).

47 The most common and maybe the only way to investigate pain mechanisms in humans is by use of  
48 experimental pain models. The evidence will however be somewhat indirect, but with robust and well-  
49 controlled models, understanding in relation to human pain mechanisms can be gained. Perceptual correlates  
50 of LTP have been observed in humans as increased pain sensitivity after cutaneous high frequency  
51 stimulation (HFS) through special pin electrodes (Van Den Broeke et al. 2011; Klein et al. 2004; Xia et al.  
52 2016). Increased sensitivity has been observed in the area of HFS (e.g. Klein et al. 2004; Lang et al. 2007;  
53 Magerl et al. 2018) and secondary mechanical hyperalgesia has been observed at surrounding sites (Van Den  
54 Broeke et al. 2011; van den Broeke and Mouraux 2014; Klein et al. 2004, 2008; Lang et al. 2007; Xia et al.  
55 2016). Other human experimental models of secondary hyperalgesia involve intradermal capsaicin injection  
56 (Koltzenburg et al. 1992; Lamotte et al. 1991) and heat-burn (Dahl et al. 1993; Werner et al. 2001), inducing  
57 stable and lasting cutaneous sensitization. To avoid discomfort and invasiveness of the capsaicin injection  
58 and skin injury and blisters to the heat-burn, heat/capsaicin sensitization model was developed by combining  
59 two relatively low intensity stimuli (Dirks and Petersen 2003). The combined model did not reveal additive  
60 effect of the two stimuli (Dirks and Petersen 2003).

61 HFS and the heat/capsaicin sensitization models act through somewhat similar mechanisms, but some  
62 differences are also evident. The heat/capsaicin application acts selectively on the TRPV1-positive A- and C  
63 nociceptive fibers (Caterina and Julius 1999; Magerl et al. 2001) and causes primary heat hyperalgesia  
64 (peripheral sensitization) and secondary mechanical hyperalgesia (Dirks and Petersen 2003). The HFS model  
65 acts on all epidermal primary afferent fibers, i.e. both TRPV1-positive and TRPV1-negative A $\delta$ - and C-fibers

66 (Henrich et al. 2015) and has also been shown to induce secondary hyperalgesia. Secondary hyperalgesia  
67 involves both spinal and supraspinal mechanisms (Sandkuhler 2009) and contrary to the fibers involved in its  
68 induction, the pain facilitation is obeyed by the TRPV1-negative A-fibers (van den Broeke et al. 2016;  
69 Magerl et al. 2001; Ziegler et al. 1999). The current study investigated the effectiveness and feasibility of a  
70 human experimental model combined of heat/capsaicin priming of the central nervous system followed by  
71 HFS. The hypothesis was that the combined model would cause facilitated secondary hyperalgesia mediated  
72 through synergistic/additive mechanisms from the two models.

73

## 74 **Materials and Methods**

### 75 **Subjects**

76 Twenty-one healthy volunteers participated in the experiment ((11 male, 10 female ranging from 19-43;  
77 mean age 23 years), which consisted of three experimental sessions. Data from two of the three sessions will  
78 be included in this study. Participants were excluded from the study if they had a history of psychiatric or  
79 neurological disorder, previous drug- or medication abuse, were pregnant or unable to cooperate. One subject  
80 was excluded from the study prior to participation according to the exclusion criteria. All subjects signed an  
81 informed consent form after being informed about the experimental procedure. Approval was obtained from  
82 the local ethical committee (N-20160076).

### 83 **Conditioning stimulation**

#### 84 **High frequency electrical stimulation (HFS)**

85 In both sessions, the participants received trains of 100 Hz (pulse width; 2 ms) for 1 sec. repeated 5 times at  
86 10 sec intervals with an intensity of 10 times perception threshold or a minimum of 1.5 mA. The electrical  
87 stimulator was a DS5 constant current stimulator (Digitimer LTd; Welwyn Garden City, UK). The  
88 stimulation was performed on the volar forearm, approximately 5 cm distal to the cubital fossa with a small-  
89 diameter pin electrode (Klein et al. 2004; Poulsen et al. 2020), which consisted of 15 blunt stainless steel  
90 pins protruding 1 mm from the base with a diameter of .2 mm. The cathodal pins were placed in a circle with  
91 a diameter of 10 mm. A rectangular electrode patch (9x5 cm, Pals Neurostimulation electrode; Axelgaard,  
92 Fallbrook, CA) placed on the dorsal forearm served as an anode.

#### 93 **Heat and Topical Capsaicin application**

94 In one session, the participants were treated with a heat/capsaicin sensitization paradigm prior to the HFS. A  
95 3x3 cm thermode (Pathway, Medoc Ltd., Ramat Yishai, IL) was placed 2.5 cm distal to the center of the  
96 electrode (see Fig. 1) to deliver thermal stimulation of 45 °C for 5 min. Subsequently, a 4x4 cm Qutenza 8%  
97 Capsaicin patch was placed on the same location for 30 minutes. The thermode was placed on top of the

98 capsaicin patch at 32° to control for normal skin temperature. After removal of the patch, a Qutenza cleaning  
99 gel was applied for 1 minute. The remainder of the gel and capsaicin was removed using paper towels.

## 100 **Conditioning- and test stimulation areas**

101 The forearm was divided into areas for applying the different test- and conditioning stimuli, which were  
102 marked on the subjects. The drawing in Fig. 1 illustrates the areas where the two conditioning stimulation  
103 paradigms and pin prick stimulations were applied (A1, A2, A3). A1 was considered as the main test area  
104 where the pin prick sensitivity to either HFS (session ‘HFS-HEAT/CAP’) or the combined HFS and  
105 heat/capsaicin sensitization (session ‘HFS+HEAT/CAP’) could be compared (see protocol below). A2 was  
106 considered to represent an area, which was mainly sensitized due to the heat/capsaicin and A3 served as a  
107 non-conditioning control area.

108

109 *Figure 1 – The areas used for conditioning – and test stimuli. All stimulation was performed on the volar forearm. The areas where*  
110 *high frequency stimulation (HFS) and heat/capsaicin were applied are marked with arrows and the three areas where pin prick*  
111 *stimulation was performed are referred to as A1, A2, and A3. The heat pain threshold was also measured in the area of*  
112 *heat/capsaicin.*

## 113 **Variables measured**

### 114 **Perception threshold**

115 The perception threshold was identified using the method of limits to determine the HFS intensity.  
116 Participants were asked to press a button when they became aware of the presence or absence of a single 2  
117 ms square pulse, which was delivered at 0.5 Hz. After each pulse, the amplitude was slowly increased or  
118 decreased 5% using a custom-made stimulation software (LabVIEW, National Instruments). The staircase  
119 procedure was repeated 3 times and the average of 3 upper and 3 lower values was calculated as the  
120 perception threshold.

### 121 **Pain to HFS**

122 Participants were asked to rate their sensation to each of the five pulse trains of the HFS on a Numerical  
123 Rating Scale (NRS) ranging from 0 (no sensation at all) to 10 (worst pain imaginable) with 5 being the pain  
124 threshold. Participants responded verbally and were free to use integers and decimals.

### 125 **Mechanical pain sensitivity**

126 In order to quantify the amount of secondary hyperalgesia (i.e. the increased sensitivity around the  
127 conditioning stimulation), mechanical pain sensitivity (MPS) of the subjects was evaluated by performing  
128 pin-prick stimulations with 128 mN and 256 mN at baseline (before any conditioning stimulation) and 10,

129 20, 30 and 40 minutes after HFS. Participants were stimulated three times within each area (A1, A2, or A3,  
130 see Fig. 1), after which they reported their average sensation on a NRS ranging from 0 (no sensation at all) to  
131 10 (worst pain imaginable) with 5 being the pain threshold. Participants responded verbally and were free to  
132 use integers and decimals. All measurements were performed twice using a randomized order for both  
133 weight and location. The participants were asked to close their eyes or look away from the arm during the  
134 pin-prick stimulation.

### 135 **Heat-pain threshold**

136 To examine primary heat hyperalgesia (peripheral sensitization) to the heat/capsaicin paradigm, the heat pain  
137 threshold (HPT) was found in the area where the heat/capsaicin was applied at baseline, immediately after  
138 heat/capsaicin, and at 10, 20, 30 and 40 minutes after HFS. An increasing heat stimulus of 1°C/s was  
139 delivered from a baseline temperature of 32°C with the thermode (Medoc Ltd, Israel) until the subjects  
140 pressed a button indicating a change in sensation from warm to painful. This procedure was repeated three  
141 times with a randomized time from 5-20 s in between. The average of the three temperatures was used to  
142 report the HPT.

### 143 **Superficial blood perfusion**

144 The superficial blood perfusion was measured using a Full-Field Laser Perfusion Imaging ('FLPI',  
145 Axminster, Devon, UK). The forearm was placed on a black surface, 35 cm underneath the device. Single  
146 images were obtained at baseline, immediately after the heat/capsaicin and 10, 20, 30 and 40 minutes after  
147 HFS.

### 148 **Protocol**

149 Participants were familiarized with the staircase procedure for determining the perception threshold of the  
150 electrical pulses, the HPT and the pin-prick stimuli in a separate 30 minutes session, at least two days prior to  
151 the experimental sessions. The order of the two experimental sessions was randomized to avoid bias. To  
152 avoid interference of lateral dominance, the order of paradigms and dominant side was balanced across  
153 subjects. Each session started with a brief summary of the methods and time plan used in the upcoming  
154 session. In the beginning, the participants were seated comfortably on a chair, with their lower arm placed  
155 horizontally on a table in front of them. The two experimental sessions will be referred to as session  
156 'HFS+HEAT/CAP' and 'HFS-HEAT/CAP'.

157 Session 'HFS-HEAT/CAP': Each session started with baseline measurements including FLPI imaging, HPT  
158 and pin-prick stimulation. The perception threshold was found and subsequently the HFS was performed. 10,  
159 20, 30 and 40 minutes after the HFS, the test measures were carried out; FLPI imaging, HPT and pin-prick  
160 stimulation.

161 Session ‘HFS+HEAT/CAP’: The same baseline measurements were performed as described in former  
162 session and the perception threshold was afterwards identified. Following that, the heat/capsaicin  
163 conditioning was applied, which lasted 35 minutes. After removal of the capsaicin patch, the test measures  
164 were carried out and following that the HFS was performed. The test measures were performed 10, 20, 30  
165 and 40 minutes after HFS.

166 *Figure 2 – Timeline for the experimental protocol. The gray shaded areas where only performed in session ‘HFS+HEAT/CAPS’,*  
167 *making that session 40 minutes longer. HPT = Heat pain threshold, MPS = Mechanical pain sensitivity, FLPI = Full-Field Laser*  
168 *Perfusion Imaging, HFS = High frequency stimulation*

169

## 170 **Data analysis**

171 The perception threshold and intensity used to deliver HFS were both compared with a paired t-test between  
172 the two experimental sessions. The perceived sensation to the HFS paradigm, pinprick stimuli, and heat-pain  
173 thresholds were analyzed separately using repeated-measures analysis of variance (RM-ANOVA). In case of  
174 violation of sphericity, Greenhouse-Geisser correction was used. Normal distribution of the studentized  
175 residuals was evaluated by the Shapiro-Wilk test of normality ( $p > 0.05$  indicated normal-distribution). Sidak  
176 correction of the p-value was applied for multiple comparisons and p values  $\leq 0.05$  were considered  
177 statistically significant.

## 178 **Pain to HFS**

179 A two-way RM-ANOVA was used to compare the pain ratings to HFS. The model included two within-  
180 subject variables: session (HFS+HEAT/CAPS and HFS-HEAT/CAPS) and stimulation no. (1, 2, 3, 4, 5).

## 181 **Mechanical pain sensitivity**

182 The pinprick stimuli ratings were normalized to baseline and the unconditioned control area (A3) and then  
183 compared between the experimental sessions (HFS+HEAT/CAPS and HFS-HEAT/CAPS) and time (10-, 20-  
184 , 30-, and 40 min after HFS) with a two-way RM-ANOVA for both weights. The ratings to pinprick stimuli  
185 were furthermore compared between areas (A1 and A2) and time (post Caps, 10-, 20-, 30-, and 40 min after  
186 HFS) for the HFS+HEAT/CAPS session using a two-way RM-ANOVA for both weights.

## 187 **Heat-pain threshold**

188 To evaluate the primary hyperalgesia caused by the heat/capsaicin sensitization paradigm, the heat pain  
189 threshold in the area of heat/capsaicin application was compared between baseline, immediately after  
190 capsaicin application and at the four time points after HFS using a one-way RM-ANOVA in the  
191 HFS+HEAT/CAPS session. Same analysis was used for the HFS-HEAT/CAPS session.

## 192 **FLPI**

193 The FLPI variables include a grayscale image, a flux image and a colored image. Using the flux image, the  
194 superficial blood perfusion values (mean flux) were extracted in the area of HFS, i.e. a circular area of 1.5  
195 cm<sup>2</sup> in diameter directly underneath the small diameter pin electrode (Moor FLPI Review). A two-way RM-  
196 ANOVA was used to compare the blood perfusion in the area of HFS (1.5 cm<sup>2</sup>) between session  
197 (HFS+HEAT/CAPS and HFS-HEAT/CAPS) and time (baseline, 10-, 20-, 30-, and 40 min after HFS). The  
198 area of flare was furthermore calculated in the HFS+HEAT/CAPS session at the time point immediately after  
199 removal of capsaicin using a threshold of baseline + twofold standard deviation (Terkelsen et al. 2014).

## 200 **Results**

### 201 **Intensity used for HFS**

202 The perception threshold (mean ± standard error) were not different between the two sessions (119.86 μA±  
203 12.98 μA and 109 μA ± 14.33 μA for session ‘HFS-HEAT/CAP’ and ‘HFS+HEAT/CAP’, respectively). As  
204 the minimum intensity was set to 1.5 mA the average intensities used for HFS were: 1.62 mA ± 0.08 mA and  
205 1.63 mA ± 0.07 mA for session ‘HFS-HEAT/CAP’ and ‘HFS+HEAT/CAP’, respectively (n.s, p = 0.95).

### 206 **Pain to HFS**

207 Pain ratings to HFS (see Fig. 3) were higher when the subjects had received the heat/capsaicin paradigm  
208 prior to HFS (main effect,  $F(1,19) = 5.130$ ,  $p = 0.035$ ). The main effect of time was not significant  
209 ( $F(2.50,47.45) = 2.85$ ,  $p = 0.057$ ).

210

211 *Figure 3 – Sensitivity ratings on a NRS (0-10, 5: pain threshold) to the high frequency stimulation (HFS) in the two experimental*  
212 *sessions. Asterisk indicate significant main effect of paradigm, \* p < 0.05.*

### 213 **Mechanical pain sensitivity**

214 Results for ratings to pinprick stimuli in the area between HFS and heat/capsaicin paradigms (A1) are shown  
215 in figure 4. Analysis of the pinprick ratings to low weight pin prick stimuli, 128 nM, revealed a significant  
216 interaction ( $F(3,54) = 2.77$ ,  $p = 0.05$ ), see Fig. 4. Post-hoc comparisons showed larger ratings to pinprick  
217 stimuli at 20- and 30 minutes after HFS in session ‘HFS+HEAT/CAP’ than in session ‘HFS-HEAT/CAP’  
218 (20 min:  $p = 0.017$ , 30 min:  $p = 0.041$ ).

219 For the 256mN pin prick stimulation, results showed a significant interaction between paradigm and time  
220 ( $F(4,76) = 2.62$ ,  $p = 0.032$ ). No differences were observed between the paradigms, but the interaction can be  
221 explained by higher pinprick ratings in ‘HFS-HEAT/CAP’ at 40 minutes after HFS compared to 20- and 30  
222 minutes after HFS.

223

224



225 *Figure 4 – The ratings (NRS difference from baseline and control area) to pinprick stimulations at 10 min, 20 min, 30 min, and 40*  
 226 *min after high frequency stimulation (HFS) for the two experimental sessions in A1 (area between the paradigms).*  
 227 *HFS+HEAT/CAP: Heat/capsaicin priming before HFS, HFS-HEAT/CAP: HFS without heat/capsaicin priming. Left) 128 mN*  
 228 *pinprick stimulation, right) 256 mN pinprick stimulation. Asterisks indicate differences between paradigms from post hoc*  
 229 *comparison with Sidak correction, \*,  $p < 0.05$ .*

230 The results on pinprick ratings in the HFS+HEAT/CAP session are shown for areas A1 (between  
 231 heat/capsaicin and HFS applications) and A2 (distal to heat/capsaicin application) and time points including  
 232 the ratings immediately after heat/capsaicin and at the four time points after HFS in Fig. 5. For the low  
 233 weight, 128mN, interaction between time and area was observed ( $F(4,76) = 3.03$ ,  $p = 0.023$ ). The interaction  
 234 is explained by larger ratings 20-40 minutes after HFS than immediately after capsaicin for area A1 but not  
 235 for area A2 (see Fig, 5). No differences were found between the areas at the individual time points. No  
 236 statistical effects were observed for the 256 mN pinprick stimulation.

237

238 *Figure 5 – The sensitivity ratings (NRS difference from baseline and control area) to pinprick stimulations in the HFS+HEAT/CAP*  
 239 *session after capsaicin removal (Post caps), and 10 min, 20 min, 30 min, and 40 min after high frequency stimulation (HFS) in areas*  
 240 *A1 and A2. A1: Area between HFS and heat/capsaicin application, A2: area distal to heat/capsaicin application. Left) 128 mN*  
 241 *pinprick stimulation and right) 256 mN pin prick stimulation. Asterisk indicate post hoc differences for area A1 with Sidak*  
 242 *correction, \*  $p < 0.05$ , \*\*  $p < 0.01$ .*

## 243 Heat pain threshold

244 Analysis of HPT (see Fig. 6) in the heat/capsaicin sensitized area revealed a main effect of time  
 245 ( $F(2.52,47.95) = 45.94$ ,  $p < 0.001$ ). Post hoc comparisons showed a decrease in HPT for all post-treatment  
 246 measurements compared to baseline ( $p < 0.001$ ). There was furthermore a tendency for the HPT to increase  
 247 linearly from the moment capsaicin was removed until the end of the session (Fig. 6). No differences were  
 248 observed in HPT in the ‘HFS-HEAT/CAP’ session.

249

250 *Figure 6 – The heat pain threshold (HPT) in the area of heat/capsaicin. Left) HPT in session ‘HFS+HEAT/CAP’ at baseline, directly*  
 251 *after removal of the capsaicin patch (Post caps), and 10, 20, 30 and 40 minutes after high frequency stimulation (HFS). Right) HPT*  
 252 *in session ‘HFS-HEAT/CAP’ at baseline and 10, 20, 30 and 40 minutes after HFS. Asterisk indicate post hoc difference from*  
 253 *baseline, \*\*\*  $p < 0.001$ .*

## 254 Superficial blood perfusion

255 The superficial blood perfusion (the mean flux within the area underneath the HFS electrode) is shown in  
 256 Fig. 7. Analysis revealed a two-way interaction between session and time ( $F(2.59,40.24) = 12.99$ ,  $p < 0.001$ ),  
 257 which is explained by a larger blood perfusion in the ‘HFS+HEAT/CAP’ session 10 and 20 minutes after  
 258 HFS compared to session ‘HFS-HEAT/CAP’. At 30 and 40 minutes after HFS, the average flux had

259 decreased in both sessions, and there were no observed differences between the sessions. The area of flare in  
260 the ‘HFS+HEAT/CAP’ immediately after heat/capsaicin application was  $39.92 \pm 3.40 \text{ cm}^2$ .

261

262 *Figure 7 – The superficial blood perfusion (mean flux) in a  $1.5 \text{ cm}^2$  circular area of high frequency stimulation (HFS) (mean  $\pm$*   
263 *standard error) at baseline and 10, 20, 30, and 40 minutes after HFS for the two sessions. Asterisks indicate significant differences*  
264 *from post hoc comparison with Sidak correction,  $**p < 0.001$ .*

## 265 **Discussion**

266 This study showed that priming the central- and peripheral nervous system with a heat/capsaicin application  
267 increased pain ratings to high frequency electrical stimulation and it further indicated an enhancement of the  
268 amount of secondary hyperalgesia in the area between the application areas of the two paradigms.

## 269 **Mechanisms involved**

270 The mechanisms underlying secondary hyperalgesia, observed in many chronic pain conditions, are believed  
271 to involve facilitated primary afferent input, which causes sensitization of nociceptive neurons in the spinal  
272 cord (Iannetti 2013 – find better ref?). The current study is the first study to the authors knowledge, which  
273 combines heat/capsaicin and HFS experimental models of secondary hyperalgesia where heat/capsaicin was  
274 used to prime the central- and peripheral nervous system, followed by HFS. The two methods are believed to  
275 act through somewhat overlapping mechanisms. Possible mechanisms involved in the induction and  
276 facilitated pathways in the two models and the combined model will be discussed.

## 277 **Heat/capsaicin induced secondary hyperalgesia**

278 The heat/capsaicin application acts selectively on the capsaicin-sensitive (TRPV1-responsive) fibers, which  
279 counts approximately 80% of the peripheral nociceptors (Michael and Priestley 1999) including most C-  
280 fibers (Schmelz et al. 2000) and type II A-fiber mechano-heat nociceptors (type II AMHs) (Ringkamp et al.  
281 2001). Two A-type nociceptors, namely the high-threshold mechanoreceptors (HTMs) and type 1 AMHs  
282 have not been shown to respond to the action of capsaicin (Magerl et al. 2001; Ziegler et al. 1999). A  
283 prolonged application of capsaicin causes desensitization of the TRPV1-responsive fibers (Henrich et al.  
284 2015), which is also used as a treatment of neuropathic pain (Finnerup et al. 2015) but as some afferent  
285 fibers, likely involved in neuropathic pain syndrome, are insensitive to capsaicin, the treatment may be  
286 partially ineffective (Magerl et al. 2001; Sindrup and Jensen 1999). Short-term action of capsaicin causes  
287 sensitization of the capsaicin-sensitive fibers and induces secondary mechanical hyperalgesia as pain ratings  
288 to pin prick stimulation and pin prick evoked potentials are increased in a capsaicin sensitized state (Iannetti  
289 et al. 2013; Lamotte et al. 1991). Secondary hyperalgesia to capsaicin injection was abolished during A-fiber  
290 block, but not during desensitization of capsaicin-responsive fibers (Magerl et al. 2001). Therefore the A-

291 fiber nociceptors, which are not responsive to capsaicin (type 1 AMHs and HTMs) are believed to be  
292 involved in the facilitated pathway (Magerl et al. 2001). The mechano-insensitive C-fibers or „silent C-  
293 fibers“ are also believed to be involved in secondary hyperalgesia to capsaicin (Serra et al. 2004).

294 There are some controversies in the literature regarding heat hyperalgesia to capsaicin. Primary hyperalgesia  
295 to heat is well established (Hughes et al. 2020; Lamotte et al. 1991) as also observed with decreased HPT in  
296 the current study. A 1-2 cm zone of hyperalgesia to heat has been observed in few studies, which may still be  
297 within the primary hyperalgesic area (Lamotte et al. 1991; Torebjörk et al. 1992). One study however  
298 observed desensitization to heat stimuli in the area of injection (Ali et al. 1996), but the area of stimulation  
299 with heat may affect the response, since a small laser was used in the study by (Ali et al. 1996) compared to  
300 the 3x3 contact heat thermode applied in the current study. Secondary heat hyperalgesia has to the author  
301 knowledge not been observed (Ali et al. 1996; Hughes et al. 2020).

302 A recent study observed a drop in electrical pain threshold in primary and secondary area of capsaicin  
303 injection (Hughes et al. 2020) supporting the increased pain to HFS observed in the secondary area in the  
304 current study.

### 305 **HFS induced secondary hyperalgesia**

306 HFS through small diameter pin electrodes acts on both C and A $\delta$  fibers as reduced pain ratings to HFS were  
307 recently shown during block of capsaicin-responsive fibers with long-term capsaicin desensitization and  
308 during A-fiber block (Henrich et al. 2015). HFS causes secondary hyperalgesia (e.g. Van Den Broeke et al.  
309 2011; Klein et al. 2004), which recently was shown to be heavily reduced under block of TRPV1-positive  
310 fibers and reduced to some extent during A-fiber block (Henrich et al. 2015). This indicates HFS acts on  
311 both TRPV1-positive and TRPV1-negative fibers, which both contribute to the development of secondary  
312 hyperalgesia (Henrich et al. 2015). Slightly different from the capsaicin model, where the facilitated pathway  
313 in secondary hyperalgesia is likely mediated by the TRPV1-negative fibers, which include both HTMs and  
314 type 1 AMHs (Magerl et al. 2001), van den broeke and colleagues recently proposed that the secondary  
315 hyperalgesia to pin prick stimulation after HFS was only mediated by the HTMs and not by type 1 AMHs.  
316 This was based on a study where perception to long-lasting heat stimuli was not increased in the area of  
317 secondary hyperalgesia (van den Broeke et al. 2016). They have further proposed that nonnociceptive  
318 somatosensory input could also contribute to the enhanced responses to mechanical pinprick stimuli, since  
319 enhanced vibrotactile event related potentials (ERPs) (van den Broeke and Mouraux 2014) and ERP to  
320 transcutaneous electrical nerve stimulation (TENS) (van den Broeke et al. 2010) have been observed.

321 Similar to the capsaicin model there are some controversies in the literature regarding heat hyperalgesia to  
322 HFS. One study has showed secondary heat hyperalgesia to thermonociceptive laser stimuli after HFS but  
323 thermonociceptive ERPs were unaffected by HFS (van den Broeke and Mouraux 2014). They suggested that  
324 the secondary heat hyperalgesia could be mediated by the quickly adapting, heat-sensitive C-fibers and not

325 type II AMHs (van den Broeke and Mouraux 2014). In the current study, no differences were observed in  
326 HPT in the secondary area of HFS, which is in line with studies where no change in heat sensitivity was  
327 observed (Lang et al. 2007; Xia et al. 2016).

### 328 **Synergistic mechanisms of heat/capsaicin and HFS**

329 The results indicate that greater secondary hyperalgesia is obtained in the combined model than when only  
330 HFS is applied (Fig. 4) and also when only heat/capsaicin is applied as increased sensitivity was observed  
331 after HFS in the area proximal to capsaicin but not in the area distal to capsaicin (Fig. 5). This was however  
332 only shown for 128 mN pinprick stimulation, but results for 256 mN followed a similar trend.

333 In the current study, the largest blood perfusion and drop in HPT were observed immediately after capsaicin  
334 removal. Both of these effects diminished in the following time period opposite to the increased sensitivity to  
335 pinprick in the HFS+HEAT/CAP session, which was maximized 30 minutes after HFS (Fig. 4). A likely  
336 explanation of the difference between the paradigms is that the heat/capsaicin priming caused an increased  
337 sensitivity/activity in the central cells, which is in line with the general belief that secondary hyperalgesia is  
338 centrally mediated (Lamotte et al. 1991; Schmelz et al. 2000).

339 Another possibility is that increased peripheral sensitization following heat/capsaicin facilitates the induction  
340 of secondary hyperalgesia, which maintains for at least 30 minutes despite a concurrent decrease blood  
341 perfusion and increase in HPT. This could be explained by the larger blood perfusion in the  
342 HFS+HEAT/CAP session. Pain ratings to HFS performed shortly after capsaicin removal were furthermore  
343 facilitated in the combined model (Fig. 3). This is likely due to priming of the capsaicin-responsive fibers  
344 with heat and capsaicin, which are also a part of the fibers mediating HFS induced pain (Henrich et al. 2015).  
345 Whether this peripheral priming could have caused delayed central priming cannot be excluded.

### 346 **Stimulation methods/parameters**

347 In this study, two experimental pain paradigms were applied sequentially. As both methods cause moderate  
348 to high discomfort, participant discomfort had to be considered. The participants rated the pain to  
349 heat/capsaicin relatively mild (data not shown) compared to severe pain ratings observed after capsaicin  
350 injection (Lamotte et al. 1991) and it is unknown whether a more robust priming had been caused by  
351 capsaicin injection. Neither was rekindling of the heat/capsaicin method was performed (Dirks and Petersen  
352 2003) and therefore unclear whether maintaining increased peripheral sensitization would have affected the  
353 results. To further limit discomfort, a relatively low intensity for HFS was also applied compared to studies  
354 from Van den Broeke and colleagues (van den Broeke et al. 2016; Van den Broeke et al. 2019; Gousset et al.  
355 2020), which could have affected the amount of secondary hyperalgesia.

### 356 **Implications**

357 The current model is considered to have wide range of potential implications both within experimental and  
358 clinical purpose. It is first of all considered to improve existing models of neuropathic pain causing both

359 primary heat hyperalgesia and facilitated secondary hyperalgesia. In relation to clinical applicability, the  
360 model can simulate a sensitized state of the central nervous system, which is more prone to the HFS-induced  
361 sensitization. The model can therefore be considered to resemble patients in a vulnerable state  
362 (experimentally sensitized by capsaicin) who are more prone to developing long lasting pain after injury  
363 (experimental HFS). Based on these speculations, the current model is considered highly useful within pain  
364 diagnostics, pharmacological testing, or even for prediction of postoperative pain.

## 365 **Conclusion**

366 This study showed the combined model of heat/capsaicin and HFS causes greater mechanical pinprick  
367 sensitivity to 128 mN pinprick stimulation than HFS without priming with heat/capsaicin, and, increased  
368 pinprick sensitivity in A1 (area between the paradigms) was observed after HFS compared to after  
369 immediate removal of capsaicin. This increase is likely explained by the addition of HFS rather than time as  
370 this increase was not observed distally to capsaicin outside the area of HFS induced secondary hyperalgesia.  
371 The two models may therefore cause synergistic peripheral and/or central mechanisms facilitating  
372 hyperalgesia and mimicking widespread increase in pain observed in many chronic pain conditions.

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