TITLE

Heterosynaptic facilitation of mechanical nociceptive input is dependent on the frequency of conditioning stimulation

RUNNING TITLE

Frequency of stimulation and secondary hyperalgesia

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NEW AND NOTEWORTHY

Burst-like electrical conditioning stimulation applied to human skin induces an increase in pinprick sensitivity in the surrounding unconditioned skin (a phenomenon referred to as secondary hyperalgesia). Here we show that the development of the increase in pinprick sensitivity is dependent on the frequency of the burst-like electrical conditioning stimulation.

KEYWORDS

High frequency stimulation; frequency; pinprick; secondary hyperalgesia

ABSTRACT

High-frequency burst-like electrical conditioning stimulation (HFS) applied to human skin induces an increase in mechanical pinprick sensitivity of the surrounding unconditioned skin (a phenomenon known as secondary hyperalgesia). The present study assessed the effect of frequency of conditioning stimulation on the development of this increased pinprick sensitivity in humans. In a first experiment we compared the increase in pinprick sensitivity induced by HFS using monophasic non-charge-compensated pulses and biphasic charge-compensated pulses. High-frequency stimulation, traditionally delivered using non-charge-compensated square-wave pulses, may induce a cumulative depolarization of primary afferents and/or changes in pH at the electrode-tissue interface due to the accumulation of a net residue charge after each pulse. Both could contribute to the development of the increased pinprick sensitivity in a frequency-dependent fashion. We found no significant difference in the increase in pinprick sensitivity between HFS delivered using charge-compensated and non-charge-compensated pulses, indicating that the possible contribution of charge accumulation when non-charge-compensated pulses are used is negligible. In a second experiment, we assessed the effect of different frequencies of conditioning stimulation (5, 20, 42 and 100 Hz) using charge-compensated pulses on the development of increased pinprick sensitivity. The maximal increase in pinprick sensitivity was observed at intermediate frequencies of stimulation (20 and 42 Hz). It is hypothesized that the stronger increase in pinprick sensitivity at intermediate frequencies may be related to the stronger release of substance P and/or neurokinin-1 receptor activation expressed at lamina I neurons following C-fiber stimulation.

INTRODUCTION

Long-term potentiation (LTP) refers to a long-lasting increase in synaptic efficacy and was discovered following repetitive stimulation in the rabbit hippocampus (Lømo, 2003; Bliss and Lømo, 1973; Bliss and Gardner-Medwin, 1973 Bliss and Lømo, 1973). LTP is thought to be a crucial mechanism involved in memory formation (Neves, Cooke and Bliss, 2008; Bliss and Collingridge, 1993). Animal studies have shown that LTP can also be induced in spinal nociceptive pathways. Indeed, it has been shown that high frequency burst-like stimulation (HFS; several burst of 100 Hz for 1 s) of primary C-fiber nociceptors triggers LTP between the peripheral C-fiber terminals and spinal lamina I neurons projecting to the parabrachial area of the brainstem (Ikeda et al., 2003; 2006). Moreover, Kronschläger et al., (2016) showed that HFS also activates glial cells in the spinal cord which, via the release of d-serine and tumor necrosis factor (TNF), trigger LTP at remote or nearby C-fiber synapses. LTP at synapses that were active during conditioning stimulation (homosynaptic LTP) may contribute, besides peripheral sensitization, to primary hyperalgesia; i.e. the increase in pain at the site of tissue injury or inflammation (Sandkühler and Gruber-Schoffnegger, 2013). LTP at remote synapses (heterosynaptic LTP) could contribute to the phenomenon of secondary hyperalgesia; i.e. the increase in pain sensitivity that develops surrounding the site of tissue injury (Kronschläger et al., 2016; Ruscheweyh et al., 2011).

In humans, HFS (5 trains of 100 Hz for 1 s, repeated at 10 s intervals) delivered to the skin induces a pronounced and long-lasting increase in mechanical pinprick sensitivity in the surrounding skin, a phenomenon reminiscent of secondary hyperalgesia (Klein et al., 2004; Vo and Drummond, 2013; Henrich et al., 2015; van den Broeke et al., 2016; Xia et al., 2016).

We also showed that after HFS, the perception elicited by small-spot laser stimuli selectively activating C-fiber nociceptors is enhanced when the stimuli are delivered inside the surrounding area of increased pinprick sensitivity, although the effect of HFS on these laser stimuli was less pronounced than the effect on pinprick stimuli (Lenoir et al., 2018). The effect of HFS on the perception elicited by the C-fiber laser stimuli could be a perceptual correlate of the "gliogenic" heterosynaptic LTP at C-fiber synapses identified by Kronschläger et al. (2016) in animals. However, a peripheral origin cannot be presently excluded.

Previous studies using intradermal capsaicin injection to induce increased pinprick sensitivity surrounding the site of injection in humans have shown that the increase in pinprick sensitivity is mediated by A-fiber nociceptors rather than C-fibers (Ziegler et al., 1999). Moreover, by recording the activity of nociceptive neurons in the primate spinal cord before and after intradermal capsaicin injection, Simone et al. (1991) showed that, after the injection, both high-threshold (HT) neurons in lamina I and wide-dynamic-range (WDR) neurons in lamina V respond more strongly to mechanical pinprick stimuli delivered to the skin surrounding the injection site. The same group also recorded the activity of peripheral A-fiber and C-fiber nociceptors but their activity was unchanged (Baumann et al., 1991), confirming that the increase in responsiveness of spinal neurons results from a facilitation at spinal level. Torsney (2011) found that inflammation of the hindpaw of rats by complete Freunds adjuvant increases the incidence and magnitude of monosynaptic A-fiber input to lamina I neurons expressing the NK1 receptor. It was hypothesized that this novel monosynaptic A-fiber input results from normally "silent" synapses and that this may contribute to secondary hyperalgesia (Torsney, 2011). It is, however, presently not known

144 whether spinal LTP also affects A-fiber mediated synaptic transmission (Ruscheweyh et al.,
145 2011).

146 Henrich et al. (2015) showed that when HFS is delivered to skin pre-treated with capsaicin to
147 induce a denervation of TRPV1-expressing nociceptors, HFS does not induce any increase in
148 pinprick sensitivity in the surrounding skin. They also showed that both A- and C-fiber
149 nociceptors contribute to the induction of increased pinprick sensitivity, but the
150 contribution of C-fiber input is greater than that of A-fibers (Henrich et al., 2015). Taken
151 together, these results suggest that mainly TRPV1-expressing C-fiber nociceptors are
152 involved in the HFS-induced enhancement of pinprick sensitivity (Henrich et al., 2015).

153 It is thought that the activation of mechano-insensitive “silent” C-fiber nociceptors is crucial
154 for the induction for secondary mechanical hyperalgesia (Sauerstein et al., 2018; Schmelz et
155 al., 2000). However, in pig skin this subclass of nociceptors shows conduction failure at high
156 frequencies of stimulation (Obreja et al., 2013; Werland et al., 2015), which raises the
157 question to what extent this subclass of C-fibers contributes to the induction of secondary
158 mechanical hyperalgesia by HFS. However, in rats, some C-fiber nociceptors are able to
159 follow HFS (Adelson et al., 2009).

160 Not much is known about the effect of frequency of the conditioning stimulation on the
161 development of the increased pinprick sensitivity in humans. Xia et al. (2016) investigated
162 the effect of three frequencies of electrical conditioning stimulation (10 Hz, 100 Hz and 200
163 Hz) on the averaged magnitude of the increase in pinprick sensitivity in the surrounding skin.
164 Although the magnitude of the increase in pinprick sensitivity was not the same between
165 the different frequencies (100Hz>200Hz>10Hz), no statistically significant differences were
166 observed. In that study, the authors matched the 10 Hz and 100 Hz regarding the total

number of stimuli, pulse duration and total duration of the protocol. As a consequence, the pattern of stimulation was not the same which makes it difficult to compare the two conditions. Indeed, whereas the 100 Hz condition consisted of five trains of 100 Hz that lasted for 1 s and were repeated in a 10 s interval, the 10 Hz condition consisted of continuous stimulation. Another previous study comparing 20 Hz continuous stimulation versus 20 Hz stimulation for 1 s repeated using a 2 s inter-train interval found that continuous stimulation induces *hypo*algesia to pinprick stimulation, whereas burst stimulation induces *hyper*algesia to pinprick stimulation (de Col et al., 2008).

Furthermore, in the study by Xia et al. (2016), and other studies exploring how frequency influences the after-effects of the conditioning stimulation, they used square-wave electrical pulses. Because square-wave electrical pulses are not charge-compensated, a net residue charge may accumulate after each pulse. This accumulation can be expected to be stronger when the frequency of pulse delivery is high, leading to a stronger cumulative depolarization of the membrane potential of afferent fibers (Grover et al., 2009) and/or tissue damage (Piallat et al., 2008) or inflammation related to changes in pH at the electrode-tissue interface.

Therefore, the present study followed two aims. The first aim was to assess whether the increase in pinprick sensitivity induced by HFS is dependent on cumulative depolarization of the membrane potential and/or inflammation related to changes in pH at the electrode-tissue interface. To test this we compared the increase in pinprick sensitivity induced by HFS delivered using non-charge-compensated versus charge-compensated electrical pulses (Experiment 1). The second aim was to explore whether the development of the increase in pinprick sensitivity depends on the frequency of the conditioning stimulation (Experiment

2). If indeed mechano-insensitive “silent” C-fiber nociceptors play a crucial role one would expect a stronger increase in pinprick sensitivity induced by low frequencies of stimulation compared to high frequencies of stimulation. In this second experiment, four frequencies were tested (5, 20, 42 and 100 Hz) using charge-compensated electrical pulses, keeping constant both the total number of pulses and the stimulation pattern (1 second trains separated by a 10 s inter-train interval).

MATERIALS AND METHODS

Participants

Fifteen healthy volunteers took part in Experiment 1 (7 men and 8 women; aged 21 – 27 years; 23.5 ± 1.6 years [mean ± sd]). In this experiment, participants took part in two experimental sessions separated by at least one week, during which they were exposed to either charge-compensated 100 Hz HFS or non-charge-compensated 100 Hz HFS. The order of two sessions was counterbalanced across participants.

Sixty participants took part in Experiment 2 (31 men and 29 women; aged 18 – 40 years; 23.4 ± 4.3 years); Fifteen participants per condition (5 Hz, 20 Hz, 42 Hz or 100 Hz). For the 100 Hz group, this included the data of the seven participants of Experiment 1 that had received 100 Hz charge-compensated HFS in the first experimental session. All participants were naïve regarding HFS.

The experiments were conducted according to the declaration of Helsinki (except preregistration of the trial). Approval for the experiments was obtained from the local Ethical Committee (*comité d'éthique hospitalo-facultaire des Cliniques universitaires Saint-*

212 *Luc-UCLouvain)* of the Université catholique de Louvain (UCLouvain) (B403201316436). All
213 participants signed an informed consent form and received financial compensation for their
214 participation.

215

216 **Experimental design**

217 In both experiments, the electrical conditioning stimulation was applied to the dominant or
218 non-dominant volar forearm, counterbalanced across participants (10 cm distal to the
219 cubital fossa) (Fig. 1). Handedness was assessed using the Flinders Handedness Survey
220 (Nicolls et al., 2011). Pinprick sensitivity of the skin was assessed by applying mechanical
221 pinprick stimuli (128 mN) before applying the conditioning stimulation ('pre') and 20
222 minutes after the end of the conditioning stimulation ('post'), to the skin surrounding the
223 site where the conditioning stimulation was delivered ('pinprick test area') and to the
224 corresponding skin area of the contralateral arm serving as control. In Experiment 1, we
225 compared in a cross-over design, the increase in pinprick sensitivity induced by 100 Hz HFS
226 delivered using either biphasic charge-compensated pulses or monophasic non-charge-
227 compensated pulses (Fig. 2). In Experiment 2, we compared in a between-subject design,
228 the change in pinprick sensitivity induced by 100 Hz HFS to the change in pinprick sensitivity
229 induced by 5, 20, and 42 Hz conditioning stimulation. In this second experiment, all stimuli
230 were delivered using biphasic charge-compensated pulses.

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234 **Conditioning stimulation**

235 All stimuli were delivered to the forearm skin using a constant-current electrical stimulator
236 (Digitimer DS5, Welvyn Garden City, UK) and a specifically designed electrode built at the
237 Centre for Sensory-Motor Interaction (Aalborg University, Denmark). The electrode consists
238 of 16 blunt stainless-steel pins (diameter: 0.2 mm) protruding 1 mm from the base. The pins
239 are placed in a 10 mm diameter circle and serve as cathode. A stainless-steel anode
240 electrode is concentrically located around the steel pins (inner diameter: 22 mm; outer
241 diameter: 40 mm).

242 Monophasic non-charge-compensated electrical pulses were square-wave pulses having a 2-
243 ms pulse width (Fig.2B). Biphasic charge-compensated electrical pulses consisted in the
244 same 2-ms square-wave pulse followed, after a 0.1 ms delay, by a 4-ms compensation pulse
245 of opposite polarity having half the intensity of the first pulse (Fig. 2B).

246 In all conditions, the intensity of conditioning stimulation was individually adjusted to 20x
247 the detection threshold to a single non-charge-compensated monophasic pulse (pulse
248 width: 2 ms). The detection threshold was determined after the pre measurements using a
249 staircase procedure.

250 A total of 500 electrical pulses were delivered as 1-s trains separated by a stimulation-free
251 interval lasting 9 seconds. For 100 Hz HFS, five trains were delivered, each including 100
252 pulses (total duration: 50 seconds). For 5 Hz stimulation, a total of 100 trains were
253 delivered, each including 5 pulses (total duration: ≈17 minutes). For 20 Hz stimulation, 25
254 trains were delivered, each including 20 pulses (total duration: ≈4 minutes). For 42 Hz
255 stimulation, 12 trains were delivered, 8 including 42 pulses and 4 including 41 pulses (total

duration: ≈2 minutes). The 42 Hz stimulation was chosen, instead of 40 Hz (the double of 20), because it is able to deliver the same total number of stimuli as the 5 Hz, 20 Hz and 100 Hz.

The electrical pulses were triggered by a National Instruments digital-analogue interface (NI, National Instruments, Austin, Texas, USA), controlled by custom Matlab code (Matlab 2014B, Mathworks, USA).

Quantifying changes in the perceived intensity of mechanical pinprick stimuli

To assess changes in pinprick sensitivity, a calibrated pinprick stimulator exerting a normal force of 128 mN using a 0.25 mm probe (MRC Systems, Heidelberg, Germany) was applied perpendicular to the skin. Before applying the conditioning stimulation and 20 minutes after having applied the conditioning stimulation a total of three pinprick stimuli were applied inside the pinprick test area of the conditioned arm and the contralateral control arm. 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. For the statistical analysis, the mean of the three pinprick ratings was calculated for each arm and time point.

Mapping the area of increased pinprick sensitivity

The same pinprick stimulator was used to map the area of increased mechanical pinprick sensitivity after conditioning stimulation. Mechanical pinprick stimuli were applied to the skin along eight axes, each separated by an angle of 45 degrees. Along each axis, testing started far outside the skin showing increased pinprick sensitivity and moved towards the centre of the conditioning site in steps of 1 cm. Participants were instructed to indicate the point at which the pinprick perception changed. This point was then indicated on the skin with a marker. Then, the distance between each mark and the centre of the conditioning stimulation was measured. Finally, the area was drawn on millimetre paper and the surface (cm^2) was calculated using the open-source platform Fiji (Schindelin et al., 2012).

Statistical analysis

Statistical analyses were performed using SPSS Statistics 24 (IBM Corp., Armonk, NY, USA). In Experiment 1, the change in perceived pinprick intensity induced by non-charge-compensated monophasic pulses and charge-compensated biphasic pulses was compared using a repeated measures ANOVA with three within-subject factors: 'time' (pre vs. post), 'arm' (conditioned vs. control) and 'condition' (charge-compensated vs. non-charge-compensated). Post-hoc paired t-test were performed comparing the 'post' minus 'pre' change in perception intensity at the conditioned vs. control arm. To compare the size of the area of increased pinprick sensitivity after charge-compensated vs. non-charge-compensated HFS, we performed a paired t-test on the individual area sizes (cm^2).

In Experiment 2, the change in intensity of pinprick perception after conditioning stimulation using four frequencies of stimulation was compared using a mixed ANOVA with two within-subject factors, 'time' (pre vs. post) and 'arm' (conditioned vs. control); and one between-subject factor, 'condition' (5, 20, 42 and 100 Hz). Tukey post-hoc tests were performed comparing the 'post' minus 'pre' change in pinprick intensity ratings at the conditioned arm vs. the control arm. The size of the area of increased pinprick sensitivity was compared across the four frequencies of stimulation (5 Hz, 20 Hz, 42 Hz and 100 Hz) using a one way-ANOVA. A Tukey post-hoc test was performed to identify which comparisons were significantly different.

Finally, to test whether the electrical detection thresholds to a monophasic non-charge-compensated pulse differed in the two experimental sessions of Experiment 1, the individual detection thresholds were compared using paired t-test. To test if in Experiment 2 the detection thresholds differed between the four different groups (5, 20, 42 and 100 Hz), the individual detection thresholds were compared using a one-way ANOVA. In all tests, the level of significance was set at $p<.05$.

RESULTS

Detection thresholds

The electrical detection thresholds to a single monophasic non-charge-compensated pulse in experiment 1 were 0.29 ± 0.13 mA (mean ± sd) for the non-charge-compensated condition and 0.32 ± 0.11 mA for the charge-compensated condition. The electrical detection thresholds in experiment 2 were 0.25 ± 0.09 (5 Hz), 0.27 ± 0.10 (20 Hz), 0.29 ±

0.09 (42 Hz) and 0.29 ± 0.12 (100 Hz). No significant difference in electrical detection thresholds was observed between the charge-compensated and non-charge-compensated condition of Experiment 1, and between the four groups in Experiment 2.

Experiment 1

Intensity of pinprick perception

The means and standard deviations of the intensity of perception elicited by pinprick stimuli delivered before and after HFS at both arms (control vs. conditioned) in both conditions (charge-compensated vs. non-charge-compensated pulses) are shown in Figure 3A. The repeated-measures ANOVA revealed a significant time x arm interaction ($F(1,14)=54.684$, $p<.001$, $\eta 2=.796$). This means that, after HFS, the intensity of perception elicited by pinprick stimulation of the HFS arm was higher compared to pinprick stimulation of the control arm, across the two conditions (charge-compensated and non-charge-compensated stimulation) (Fig 3A). No significant time x arm x condition was observed ($F(1,14)=1.392$, $p=.258$, $\eta 2=.090$) suggesting that there was no difference in the enhancement of pinprick sensitivity after HFS delivered using charge-compensated and non-charge-compensated pulses (Fig 3B).

Area size

The mean and standard deviations of the area of increased pinprick sensitivity after charge-compensated and non-charge-compensated HFS are shown in Figure 3C. The paired t-test

comparing area sizes revealed no significant difference between charge-compensated and non-charge-compensated pulses ($t(14)=-.738$, $p=.472$). Figure 4 shows scatter plots of the individual changes in pinprick perception and area size after HFS delivered with a charge compensated pulse versus non-charge compensated pulse.

Experiment 2

Intensity of perception

The means and standard deviations of the intensity of pinprick perception before and after conditioning stimulation at both arms (conditioned vs. control) in all four groups (5, 20, 42 and 100 Hz) are shown in Figure 5. The mixed ANOVA revealed a significant time x arm interaction ($F(1,56)=179.621$, $p<.001$, $\eta2=.762$), compatible with an increase in pinprick perception at the conditioned forearm in all four groups (5 Hz, 20 Hz, 42 Hz and 100 Hz). Most importantly, there was a significant time x arm x condition interaction ($F(3,56)=8.493$, $p<.001$, $\eta2=.313$), indicating that the strength of the increase of pinprick perception at the conditioned arm differed across the four frequencies of stimulation.

To assess whether the increase in pinprick sensitivity was significant in all four groups, we then performed, for each group of participants, separate repeated-measures ANOVAs with the factors 'time' and 'arm'. For all four frequencies of stimulation, there was a significant time x arm interaction (5 Hz: $F(1,14)=26.846$, $p<.001$, $\eta2=.657$; 20 Hz: $F(1,14)=58.031$, $p<.001$, $\eta2=.806$; 42 Hz: $F(1,14)=86.701$, $p<.001$, $\eta2=.861$; 100 Hz: $F(1,14)=20.459$, $p<.001$, $\eta2=.594$).

Tukey post-hoc tests performed on the 'post' minus 'pre' change in pinprick perception at the conditioned arm vs. the control arm revealed a significant difference between 5 and 20 Hz stimulation ($p=.007$), between 5 and 42 Hz stimulation ($p<.001$), and between 42 and 100 Hz ($p=.005$) (Fig. 6A).

Area size

The means and standard deviations of the area of increased pinprick sensitivity after 5, 20, 42 and 100 Hz conditioning stimulation is shown in Figure 6B. The one-way ANOVA revealed a statistically-significant difference between the different frequencies ($F(3,59)=7.781$, $p<.001$). Tukey post-hoc tests revealed a significant difference between 5 and 42 Hz stimulation ($p<.001$) and between 42 and 100 Hz stimulation ($p=.006$)(Fig. 6A).

DISCUSSION

The present study yields two important findings. First, there is no significant difference in the intensity and area size of the increase in pinprick sensitivity induced by 100 Hz HFS delivered using charge-compensated and non-charge-compensated pulses. This result indicates that HFS is able to induce increased pinprick sensitivity even when the conditioning pulses are charge-compensated, and that the possible contribution of cumulative depolarization of sensory afferents and/or tissue lesion or inflammation induced by charge accumulation when non-charge-compensated pulses are used is negligible.

Second, we show that the increase in pinprick sensitivity, which is thought to result from spinal heterosynaptic facilitation, is dependent on the frequency of conditioning

384 stimulation. Indeed, with a constant number of electrical pulses delivered using the same
385 pattern of stimulation (1-second trains delivered every 10 seconds), intermediate
386 frequencies of stimulation (20 and 42 Hz) induce a stronger increase in pinprick sensitivity as
387 compared to both high-frequency stimulation (100 Hz) and low-frequency stimulation (5
388 Hz).

389 At present, one can only speculate about the possible mechanism(s) underlying the
390 frequency-dependence of HFS-induced increase in pinprick sensitivity. One possibility could
391 be that the frequency-dependent increase in pinprick sensitivity is related to spinal
392 neurokinin-1 (NK1) activation through the release of substance P following primary afferent
393 peptidergic A- and C-fiber nociceptor stimulation. Both the release of substance P as well as
394 the activation of the NK1 receptor are frequency-dependent (Adelson et al. 2009; Go and
395 Yaksh, 1987). Indeed, Go and Yaksh (1987) showed in cats that the release of substance P
396 following sciatic nerve stimulation at 2, 5, 10, 20, 50 and 200 Hz was largest at 20 and 50 Hz
397 and then decreased. Furthermore, Adelson et al. (2009) showed in rats that NK1 receptor
398 activation was maximal when C-fibers are stimulated at frequencies between 30-100 Hz.
399 Moreover, Substance P can diffuse at a considerable distance from its site of release (Liu et
400 al., 1994), and may be able to activate extrasynaptic NK1 receptors (Klein et al., 2008).
401 Moreover, animal studies have shown that spinal lamina I neurons expressing the NK1
402 receptor play a pivotal role in central sensitization and mechanical hyperalgesia (Khasabov
403 et al., 2002; Nichols et al., 1999; Mantyh et al. 1997; Abbadie et al., 1997).

404 That high frequency stimulation induces a similar increase in pinprick sensitivity than low
405 frequency stimulation is somewhat surprising, as the aforementioned studies have shown
406 that high frequency stimulation results in a greater release of substance P and more NK-1

activation than low frequency stimulation (Go and Yaksh, 1987; Adelson et al., 2009). One possibility is that HFS triggers LTP at GABAergic synapses of spinal lamina I neurons that receive monosynaptic A- or C-fiber input (Fenselau et al., 2011) which may influence the net output (less facilitation) of these lamina I neurons. Second, HFS may recruit more strongly descending inhibitory pathways that may interact with the development of increased pinprick sensitivity. In animals, intense nociceptive stimulation recruits diffuse inhibitory noxious controls (DNICs), which can inhibit the activity of wide-dynamic range (WDR) neurons of the dorsal horn (LeBars, 2002). Simone et al. (1991) showed in primates that both high threshold (HT) neurons (in the superficial laminae) and WDR neurons (in deeper lamina) show increased responses to pinprick stimulation when these pinprick stimuli are applied after intradermal capsaicin injection to the surrounding skin, suggesting that WDRs also contribute to the increase in pinprick sensitivity, at least after capsaicin. That a DNIC-like mechanism can interfere with the development of increased pinprick sensitivity has been shown recently by Xia et al. (2017). In that study they showed that 10 Hz conditioning stimulation of the forearm skin, delivered just after the application of conditioned pain modulation (CPM) to the foot, which is believed to recruit a DNIC-like mechanism (Yarnitsky, 2010; Bannister and Dickenson, 2017), induces a smaller increase in pinprick sensitivity compared to a control condition not preceded by CPM.

In summary, our results show that the induction of increased pinprick sensitivity by repeated burst-like electrical stimulation of cutaneous nociceptors is not significantly dependent on charge accumulation within the stimulated tissues, and that the induced increased pinprick sensitivity is significantly dependent on the frequency of the burst stimulation, being maximal at intermediate frequencies of stimulation.

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GRANTS

EvdB is supported by the Fonds de Recherche Clinique (FRC) provided by Université catholique de Louvain (UCLouvain), Belgium. AM, AS and LL are supported by the ERC "Starting Grant" (PROBING PAIN 336130). SvN is supported by the MoveIN-Louvain fellowship provided by the Université catholique de Louvain (UCLouvain), Belgium.

DISCLOSURE

The authors declare no conflict of interest.

FIGURE LEGENDS

Fig. 1. Experimental design. **A.** Conditioning stimulation is applied to the dominant or non-dominant volar forearm. Pinprick stimulation (128 mN) was applied to the skin surrounding the area onto which conditioning stimulation was applied ("pinprick test area") as well as to the same skin area on the contralateral control arm. **B.** Characteristics of the conditioning electrode. **C.** Time-line of the experiment. The perceived intensity elicited by the pinprick stimulation was assessed at two different time-points: before conditioning stimulation ("Pre") and twenty minutes after applying conditioning stimulation ("Post"). At the end of the experiment the area of increased pinprick sensitivity at the conditioning arm was mapped.

Fig. 2. A. Example of a train of 100 Hz (1 s) delivered with a non-charge-compensated pulse (top) and a charge-compensated pulse (bottom). Shown is the actual current is delivered to the skin (stimulation intensity: 5 milliampere). **B.** First two stimuli of each train in A. Non-charge-compensated pulses left and charge-compensated pulses right.

Fig. 3. A. Intensity of perception elicited by the mechanical pinprick stimulation (128 mN) before and twenty minutes after applying HFS using a monophasic non-charge-compensated pulse (left) and a biphasic charge- compensated pulse (right). Shown are the group-level average and standard deviation of the numerical rating scale (NRS) scores. **B.** Group-level average and standard deviation increase in NRS compared to baseline and control site. **C.** (left) Group-level average and standard deviation area size of the increase in pinprick sensitivity. (right) Group-level average areas of increased pinprick sensitivity.

Fig. 4. A. Scatter plot (and linear regression line) showing the individual changes in pinprick ratings after HFS (compared to baseline and control site) delivered using charge-compensated pulses (y-axis) and non-charge-compensated pulses (x-axis). **B.** Scatter plot (and linear regression line) showing the individual area sizes of increased pinprick sensitivity after HFS delivered using charge-compensated pulses (y-axis) and non-charge-compensated pulses (x-axis).

Fig. 5. Intensity of perception elicited by the mechanical pinprick stimulation (128 mN) before and twenty minutes after applying HFS using a biphasic charge- compensated pulse for all frequencies of conditioning stimulation (5 Hz, 20 Hz, 42 Hz and 100 Hz). Shown are the group-level average and standard deviation of the numerical rating scale (NRS) scores.

Fig. 6. A. Group-level average and standard deviation increase in NRS compared to baseline and control site. **B.** (left) Group-level average and standard deviation area size of the increase in pinprick sensitivity. $P<.05$ refers to the significant comparisons of the post-hoc Tukey test. (right) Group-level average areas of increased pinprick sensitivity.