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Exploration of the Conditioning Electrical Stimulation Frequencies for Induction of Long-term Potentiation-like Pain Amplification in Humans

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Abstract

Background: Spinal nociceptive long-term potentiation (LTP) can be induced by high or low frequency conditioning electrical stimulation (CES) in rodent preparations in vitro. However, there is still sparse information on the effect of different conditioning frequencies inducing LTP-like pain amplification in humans. In this study we tested two other paradigms aiming to explore the CES frequency effect inducing pain amplification in healthy humans.

Methods: Cutaneous LTP-like pain amplification induced by three different paradigms (10 Hz, 100 Hz, and 200 Hz CES) was assessed in fifteen volunteers in a cross-over design. Perceptual intensity ratings to single electrical stimulation at the conditioned site and to mechanical stimuli (pinprick and light stroking) in the immediate vicinity were recorded; superficial blood flow was also measured. The short-form of the McGill Pain Questionnaire (SF-MPQ) was used for characterizing the perception induced by CES.

Results: Compared with the control session, pain perception to pinprick stimuli and area of allodynia significantly increased after all three CES paradigms. In the 10 Hz and 200 Hz sessions, the superficial blood flow 10 min after CES was significantly higher than in the control session reaching a plateau after 20 min and 10 min, respectively; for the 100 Hz paradigm a stable level was found without significant differences compared with CES and control sessions. 10 Hz CES caused a lower SF-MPQ score than 100 Hz.

Conclusions: High frequency (200 Hz) and low frequency (10 Hz) paradigms can induce heterotopic pain amplification similar to the traditional 100 Hz paradigm. The 10 Hz paradigm can be an appealing alternative paradigm in future studies due to its specific association with low-level discharging of C-fibers during inflammation.

Key words: pain facilitation; plasticity; central sensitization; hyperalgesia; conditioning electrical stimulation

1. Introduction

Long-term potentiation (LTP), an important feature of synaptic plasticity in the central nervous system, is considered to contribute to pain amplification in spinal nociceptive pathways (Sandkühler, 2009, Pfau et al., 2011). Spinal LTP plays an important role in the induction of central sensitization which is thought to be involved in acute postoperative pain and in several forms of chronic pain which develop from an initial painful event, e.g., peripheral inflammation or neuropathy (Sandkühler, 2000; Ji et al., 2003; Ruscheweyh et al., 2011). Despite extensive research, an effective pain treatment strategy remains a challenge. Therefore, further understanding of the pain plasticity mechanisms is required to improve therapies for chronic pain and for the prevention of pain chronification.

LTP of the first synaptic connections to superficial spinal dorsal horn neurons receiving afferent C-fiber input can be induced by noxious conditioning electrical stimulation (CES) (Sandkühler, 2009). It has been shown that high frequency CES (more than 2 bursts at 100 Hz) (Liu and Sandkühler, 1997; Benrath et al., 2005), intermediate frequency CES (10 Hz for 1 sec repeated 12 times at 10 sec interval; 10 Hz for 10 sec) (Terman, 2001; Kim et al., 2015) and even low frequency CES (1~2 Hz for several minutes) (Ikeda et al., 2006; Drdla and Sandkühler, 2008; Kim et al., 2015) of primary afferent C-fibers can induce LTP at the first nociceptive synapses *in vivo* and *in vitro*. However, in human subjects the most frequently used paradigm for inducing similar LTP-like pain amplification including homotopic pain-LTP and heterotopic pain-LTP (secondary hyperalgesia) is 1 ms square pulses presented at 100 Hz for 1 sec, repeated 5 times with 10 sec intervals using a special epicutaneous electrode (Klein et al., 2004, 2006; Hansen et al., 2007; Lang et al., 2007; Pfau et al., 2011; van den Broeke et al., 2014 a,b). In contrast, the very low frequency (1 Hz) induced perceptual LTD (long-term depression) rather than LTP (Klein et al., 2004; Rottmann et al., 2010). High frequency CES mimics a burst mode of nociceptive input, e.g., discharge at these high rates at the beginning of a noxious mechanical stimulus or injury (Handwerker et al., 1987). C-fibers may also instantaneously discharge at rates up to 200 Hz in humans (Weidner et al., 2002). However, in normal conditions C-fibers do not fire except for a few spikes at the high frequencies required in experimentally induced LTP-like pain amplification (Ji et al., 2003). Finally, it is important to note that in most neuropathic and inflammatory pain conditions hyperalgesia is caused by sustained discharge of C-fibers at low frequencies (around 1~10 Hz) (Puig and Sorkin, 1996; Han et al., 2000; Xiao and Bennett, 2007; Drdla and Sandkühler, 2008). Therefore, it is difficult for CES to completely mimic the irregularly discharging patterns of nociceptors and still be a reproducible human model. Moreover, different electrical stimulation patterns may cause different patterns of neurotransmitter release (Lever et al., 2001). These substances are important for the induction and maintenance of central sensitization and inflammatory pain states (Sandkühler, 2009).

The frequency range of CES for inducing LTP-like plasticity in human pain pathways

has not been fully investigated and, furthermore, there is a lack of evidence whether high or low frequency CES is more efficient in activating nociceptors for inducing pain LTP in humans. The aim of this study was to compare different CES paradigms (10 Hz, 100 Hz, and 200 Hz) in a randomized cross-over design in an effort to explore the frequency effect to the induction of LTP-like pain amplification in a healthy human model. It was hypothesized that 10 Hz CES closer to the discharging frequency of C-fibers during natural pain conditions might be superior for induction of LTP-like pain amplification. If this is the case, it may help us to understand the mechanisms behind LTP-like pain amplification by peripheral nerve CES in healthy humans.

2. Methods

2.1 Subjects

The experiments were performed on fifteen subjects (7 females and 8 males: 24-47 years; mean age 28 years) after obtaining approval from the local ethical committee (N-20120046). All subjects participated in a training session and four experimental sessions with different CES paradigms. Exclusion criteria were prior or current skin disease, neurological disease, any history of chronic pain as well as drug abuse or suffering from ongoing pain. All subjects gave their written informed consent prior to their inclusion in the study. The study was performed according to the Declaration of Helsinki.

2.2 Conditioning Electrical Stimulation (CES)

Cutaneous electrical stimulation from a constant current stimulator (DS5; Digitimer Ltd; Welwyn Garden City, UK) was applied to the right forearm 7 cm distal to the cubital fossa. The stimulations were applied with an epicutaneous pin electrode (EPE) consisting of a circular array (diameter: 10 mm) of fifteen cathodal electrodes each with diameter of 0.2 mm, protruding 1 mm from the base, and a large circular stainless steel plate served as an anode with an inner diameter of 20 mm and an outer diameter of 40 mm placed concentrically around the cathodes (Fig. 1A) (Biurrun Manresa et al., 2010). This electrode has been verified to induce a sensation of pain at a lower stimulation intensity compared with conventional cutaneous nerve stimulation because the diameter of the cathodes is smaller so a high current density is achieved in the epidermal layers where the nociceptive Aδ- and C fibers terminate (Kaube et al., 2000; Katsarava et al., 2006; Mouraux et al., 2010; Mørch et al., 2011). The individual electrical detection threshold (DTh) was determined according to the method of limits: a series of electrical pulses increasing and decreasing at step sizes of 3% of the stimulation intensity was repeated three times in each session.

Three different paradigms of CES were used for the induction of LTP-like pain amplification: (1) a continuous frequency of 10 Hz lasting 50 sec, (2) a frequency of 100 Hz lasting 1 sec, repeated five times with 10 sec intervals, and (3) a frequency of 200 Hz lasting 0.5 sec, repeated five times with 10 sec intervals. Therefore, all CES processes lasted 50 sec and consisted of 500 rectangular 1 ms square pulses applied at

10 × DTh evoking a clearly painful sensation. In the control session the electrode was just placed on the forearm for 50 sec with no CES applied.

2.3 Experimental Protocol

Each subject participated in five sessions. The first session (training) aimed at familiarizing the subjects with the different stimulus modalities and gaining experience with rating the test stimuli. The data obtained during the training session were not analyzed further. The remaining four sessions (10 Hz, 100 Hz, 200 Hz, and control) were scheduled randomly in four different days for each subject with at least one week intervals in a single-blinded cross-over design. A set of assessments was applied at the right forearm three times before and six times after the CES with a 10 min interval (Fig. 1*B*). The measurements consisted of neurogenic inflammation imaging using blood-flow imagery and thermography, assessments of pricking pain intensity, area of allodynia surrounding the conditioned site, perceived pain intensity to homotopic electrical stimulation at the conditioned site, and heat pain threshold.

2.4 Perception of CES

The subjects were asked to rate continuously the magnitude of pain from the CES using a visual analogue scale (VAS) anchored from 0 (no pain) to 10 (the most intense pain imaginable) using a handheld VAS device. The ratings were stored continuously on a computer allowing later analysis. The same VAS scale was used to rate the pinprick stimuli and the single electrical stimulation (see below).

Further, the subjects were asked to describe the quality of the CES in a short-form McGill Pain Questionnaire (SF-MPQ) in each conditioning session. The SF-MPQ consists of sensory and affective dimensions of pain, evaluative overall intensity of total pain experience and present pain intensity (PPI) index of the standard MPQ. All rating scores were added together to get a total quantitative value for each conditioning stimulus frequency (Melzack, 1987).

2.5 Neurogenic Inflammation Imaging

To assess the possible excitation of peptidergic nerve fibers and observe the temporal changes of cutaneous blood flow in the area of the conditioned site, Full-Field Laser Perfusion Imager (FLPI) was used to assess the superficial blood flow index (MoorFLPI; Moor Instruments Ltd, Axminister, UK). Changes in the skin temperature of the conditioned site were measured using infrared thermography (Thermovision A40; FLIR; Danderyd, Sweden). The superficial blood flow and average temperature of the skin were measured in a round area of about 630 mm^2, concentric to the conditioned site.

2.6 Area of Allodynia

A Q-tip fixed on a flexible plastic mount was used as light stroking stimuli (~100 mN) to map the area of allodynia. The stroking was performed in four directions from the periphery towards the center of the conditioning electrodes at 1 cm intervals (Fig. 1*A*).

The subject indicated when the stroking was unpleasant or painful. The area of allodynia was calculated according to the four recorded positions. A small value (0.1) instead of zero was added to all raw data of allodynia to avoid losing values in the subsequent statistical analysis of data when performing $\log_{10}$-transforms (Magerl et al., 1998).

2.7 Pinprick Perception

Mechanical pinprick-evoked perception was assessed by two custom-made weighted pinprick stimulators (SMI®, Aalborg University; 12.8 g, 30 g; rounded tip, 0.2 mm in diameter) which were randomly applied on different locations adjacent to the conditioned site (i.e., 15~20 mm distant to the border of the cathodal electrodes) (Fig. 1*A*). The subjects indicated the perceived intensity on the VAS scale.

2.8 Heat Pain Threshold

The heat pain threshold was measured using a thermode which covered both the homotopic as well as heterotopic skin (Pathway; 30×30mm ATS; Medoc Ltd.; Ramat Yishai, Israel). The baseline temperature was 32℃ and the temperature was increased at a rate of 1℃/sec. The subject pressed a response button to terminate the stimulus when heat pain was detected. Subsequently, the temperature returned to the baseline at a rate of 8℃/sec. An average of three tests was used as the heat pain threshold.

2.9 Homotopic Electrical Stimulation

A single rectangular constant-current electrical stimulation (intensity: 10×DTh) was applied as a homotopic electrical test stimulus using the same conditioning electrode placed at the conditioned site. The subjects rated the perception intensity on the VAS scale.

2.10 Data Evaluation and Statistics

The highest VAS rating for each 10 second period was chosen to demonstrate the perceived pain intensity during the CES process (i.e., five VAS ratings throughout the 50 sec conditioning period). Data were tested for normality (Kolmogorov-Smirnov test). The area of allodynia, the blood flow index and the SF-MPQ scores of CES were logarithmically transformed to obtain the log-normal distribution. For the remaining data the statistical analysis was performed on the raw data set which presented a normal distribution. The area of allodynia was shown using raw data. To assess the psychophysical changes after CES between sessions, only the post conditioning data (three conditioning and one control sessions) were analyzed using two-way repeated measures analysis of variance (two-way RM-ANOVA; SPSS v. 21.0) (time and conditioning frequencies were within-subject factors). VAS ratings during CES were also analyzed by two-way RM-ANOVA (time and conditioning frequencies were within-subject factors). A three-way RM-ANOVA was used for pinprick ratings (time, conditioning frequencies, and weights were within-subject factors). One-way RM-ANOVA was used to determine differences between the

SF-MPQ scores of three CES. The Greenhouse-Geisser method was used for correction of non-sphericity. The Bonferroni-Holm adjustment was used for multiple comparisons. All data are presented as mean values ±SEM (standard error of the mean). *P*-values<0.05 were considered statistically significant.

3. Results

3.1 Pre-conditioning Measures

The average CES intensity was 3.26±1.43 mA (10×DTh, mean±SD, n=60). This intensity was perceived as painful (4 out of 10) as shown in the single electrical stimulation (preCES, n=60). No significant differences were found between any of the four sessions before conditioning stimulation for any of the outcome measures (RM-ANOVA, Bonferroni-Holm) indicating all subjects have the same starting in all sessions.

3.2 Perception of CES

No visible skin injuries occurred following the CES in any of conditioning sessions. There was an interaction effect between conditioning frequencies and time for the VAS ratings during the conditioning stimulation process ($F=3.048$, $p<0.05$; Fig. 2). The 100 Hz conditioning stimulation induced an increasing pain intensity during the conditioning period, i.e., pain rating during the first 10 sec stimulation was lower than the third (20-30 sec) ($p<0.05$, Bonferroni-Holm) and the fourth (30-40 sec) ($p<0.05$, Bonferroni-Holm), whereas for 10 Hz and 200 Hz CES, a stable VAS score was detected over time (Fig. 2). Pain ratings induced by 200 Hz CES were lower than for 100 Hz CES for the first 10 sec stimulation ($p<0.05$, Bonferroni-Holm; see figure 2 *A*). The total pain score of SF-MPQ for 10 Hz CES was lower than for 100 Hz CES ($p<0.01$, Bonferroni-Holm) (Fig. 2 *B*).

3.3 Superficial Blood Flow

A significant interaction effect was found between conditioning frequencies and post-conditioning time points ($F=4.471$, $p<0.05$). In the 10 Hz and 200 Hz sessions, a higher blood flow was observed within 10 min compared with the control session ($p<0.05$, Bonferroni-Holm) with a significant increase of 8.5% (10 Hz) and 6.9% (200 Hz) above the control session. However, the blood flow in the 100 Hz session was not different from the control session after CES (Fig. 3 *A*). No differences were detected between any of the sessions after 10 min until the end of the post-conditioning period. No temporal changes were found during the post-conditioning period for the control and 100 Hz sessions. In the 10 Hz session the assessment within 10 min after CES was significantly higher than the later time points ($p<0.01$, Bonferroni-Holm) and the 20 min assessment was higher than the 60 min ($p<0.01$, Bonferroni-Holm). In the 200 Hz session the assessment within 10 min after CES was significantly higher than all later time points ($p<0.05$, Bonferroni-Holm) (Fig. 3 *A*).

3.4 Skin Temperature

No differences in the average skin temperature were found between the control

session and any of the conditioning stimulation sessions (F=0.953, p=0.419). No temporal changes were observed through the observation period in any of the sessions (F=1.431, p=0.256) (Fig. 3 *B*). No interaction effect was observed between sessions and post-conditioning time points (F=1.311, p=0.268) (Fig. 3 *B*). The average skin temperature was kept constant at 34±0.06℃ through all sessions.

3.5 Area of Allodynia

Compared with the control session all sessions with CES induced a larger area of allodynia (F=10.505, p<0.01, frequency effect) which lasted until the end of the observation period with an average increase of 320% (10 Hz), 360% (100 Hz) and 310% (200 Hz) above the control session in the post-conditioning period (Fig. 4). In 29 sessions (out of 60) a small area of allodynia was also present before the CES (area smaller than the square area encompassed by the electrode at four stroking directions, i.e., $5\sqrt{2}\times5\sqrt{2}=50$ mm^2, the black square area in Fig. 1*A*) and in the control session when stroking the electrical stimulation region. However, no significant differences were found for the changes of allodynia over time by the Bonferroni-Holm test even though the time main effect was observed (F=5.541, p=0.017). No interaction effect was found between conditioning frequencies and post-conditioning time points (F=1.012, p=0.404).

3.6 Pinprick Perception Adjacent to the Conditioned Site

The three-way RM-ANOVA showed a significant difference for pinprick sensory perception between sessions after the CES (F=13.942, p<0.01, frequency effect). Compared with the control session all sessions with CES induced a significant increase in the pinprick sensory intensity in the post-conditioning period around the conditioned site with 27% (10 Hz), 49% (100 Hz), and 37% (200 Hz) above the average rating found in the control session (p<0.01, Bonferroni-Holm) (Fig. 5), but no statistical differences were found between the conditioning frequencies. The pinprick sensory intensity increased during the post-conditioning period (F=11.881, p<0.01, time effect). 30 minutes after the conditioning stimulation, the pinprick sensory intensity increased to a plateau and remained stable over the rest of the observation period, i.e., the pain ratings within 10 min were lower than 30 min, 40 min, 50 min, and 60 min (p<0.05, Bonferroni-Holm), and 20 min were lower than 30 min and 50 min (p<0.05, Bonferroni-Holm) (Fig. 5). 30 g pinprick stimulation induced a higher sensory intensity than the 12.8 g pinprick stimulation (p<0.01, Bonferroni-Holm, weight effect). No second or third order interactions were observed.

3.7 Heat Pain Threshold

No significant differences were found between the control session and the CES sessions for the heat pain threshold (F=0.786, p=0.461). No temporal changes were observed over the post-conditioning period (F=0.499, p=0.646) (Fig. 6) and no significant interaction effect was observed between conditioning frequencies and time (F=0.750, p=0.563).

3.8 Pain Intensity Evoked by Single Electrical Stimulation at the Conditioned Site

The pain intensity to single electrical stimulation after CES was not significantly different between any of the four sessions ($F=2.238$, $p=0.114$, frequency effect). Temporal changes were observed during the post observation period indicating the development of hyperalgesia ($F=7.0$, $p<0.01$, time effect), i.e., the pain perception at 10 min was lower than at 30 min ($p<0.05$, Bonferroni-Holm), 40 min ($p<0.05$, Bonferroni-Holm), 50 min ($p<0.05$, Bonferroni-Holm), and 60 min ($p<0.01$, Bonferroni-Holm) (Fig. 7). No interaction effect was found for conditioning frequencies and time ($F=1.406$, $p=0.227$).

4. Discussion

The present findings suggest that all three CES paradigms (10 Hz, 100 Hz and 200 Hz) can induce heterotopic LTP-like pain amplification, i.e., enhanced sensory intensity to pinprick stimuli in the skin area surrounding the conditioned site (secondary mechanical hyperalgesia) and allodynia lasting at least one hour. However, unlike the study by Klein et al. (2004) none of the conditioning frequencies induced higher pain perception to single electrical stimulation compared with the control session. This means that CES did not induce homotopic pain-LTP to single electrical stimulation in the present study. The superficial blood flow at and around the conditioned site was increased and exhibited significantly different changes between conditioning sessions indicating that they have different effects on activating peptidergic nerve endings. Compared with 100 Hz CES, the 10 Hz CES caused less pain during the conditioning process, and lower conditioning pain intensity ratings at the first 10 sec stimulation were also found for 200 Hz CES.

4.1 Conditioning Stimulation Frequency

The 100 Hz CES caused an increasing pain perception during the conditioning process, which was in line with Klein's findings in 2004 (see Fig. 2*A*), whereas 10 Hz and 200 Hz CES showed no significant changes of pain sensation over time. The results of the SF-MPQ during the conditioning process showed that the 10 Hz CES was less painful than the 100 Hz CES which may be related to the report that high frequency CES to a higher degree activates mechanosensitive nociceptors and A-δ fibers (Dusch et al., 2007). The pain ratings induced by 200 Hz CES were lower than 100 Hz CES for the first 10 sec stimulation. A possible reason could be a conduction failure as the 100 impulses were conducted within a much shorter time for 200 Hz compared with 100 Hz. Therefore, there might be a decreased signal transmission due to the nerve refractory periods leading to reduced efficiency of the transmitter release following the 200 Hz stimulation (interstimulus interval of 5 ms) (Randić et al., 1993). In 2002 Weidner et al. found that the total time for the absolute refractory period (ARP) and the relative refractory period (RRP) was about 5~10 ms while the maximum discharge frequency of C-fibers could reach 190 Hz with an entrainment interval (ARP+RRP) of 5.3 ms. Therefore, the 200 Hz with 5 ms intervals in the present study might potentially be associated with less efficiency of afferent input to the spinal neurons compared with the lower frequencies. Compared with A-fiber

nociceptors, C-nociceptors are not capable of following the high frequency electrical stimulation (100 Hz and 200 Hz) used in the present study leading to direct conduction failure (Raymond et al., 1990;Weidner et al., 1999;Serra et al., 2012).

Electrical stimulation of sensory nerves at C-fiber intensity can cause spinal release of glutamate activating the NMDA-type glutamate receptors which is very important for the induction of LTP (Randić et al., 1993; Sandkühler, 2009). In addition, LTP at synapses between peptidergic C-fibers and lamina I neurons in the spinal cord, which express the neurokinin 1 receptor for substance P (SP), is a potential mechanism behind certain forms of pain amplification in behaving animals and perhaps humans (see review by Sandkühler, 2009). Based on previous studies, the stimulation frequency is likely to play an important role in the release of specific neurotransmitters and the stoichiometrical proportion. Hence, peptidergic neurotransmitters seem to require a higher frequency stimulation (frequency >2Hz) (Bartfai et al., 1986) whereas the release of neurotrophins depends on the stimulation patterns (Lever et al., 2001). SP and glutamate have been found to be released in relation to the 100 Hz paradigm and intermediate frequency stimulation (IFS) pattern (30 Hz, 1800 pulses, 1 min) whereas most likely only SP is released for continuous lower frequency stimulation patterns (1Hz, 480 pulses, 8min); simultaneously, another burst type of electrical stimulation (300 pulses in 75 trains, 100 Hz) has also been shown to be associated with brain-derived neurotrophic factor (BDNF) release (Lever et al., 2001). No previous studies have shown which substances are released with a 200 Hz CES. In essence, different neurotransmitters seem to be involved in the induction of pain LTP for the different conditioning frequency paradigms.

4.2 Homotopic LTP-like Pain

A recent study showed that the TRPV1-positive C-fiber nociceptors made the largest contribution to induction of homotopic pain-LTP while TRPV1-negative C-fibers also induced homotopic self-facilitation in the paradigm of high frequency CES. Furthermore, the induced homotopic pain-LTP was not affected by a complete or selective blockade of A-fiber conduction (Henrich et al., 2015). In the present study all three CES paradigms induced a higher neurogenic blood flow indicating the activation of peptidergic afferents, most likely C-fibers. The pain ratings to single electrical stimulation (primary hyperalgesia) were found to significantly increase from 30 min and then reach a plateau until the end of the observation period indicating induction of LTP at the central level as the electrical stimulations bypass peripheral nerve-endings. However, the pain ratings of single electrical stimulations in the post-conditioning period at the conditioned site were not significantly different between sessions. This is contrary to Klein's findings (Klein et al., 2004) that high frequency CES resulted in a significant increase in the pain perception compared with the control electrode. The different findings of the present study might reflect that electrical pain intensity ratings in the study of Klein et al. were measured at 2 minutes intervals whereas in the present study the time interval was 10 minutes so there is a chance of missing temporary effects. Moreover, a decreased reported pain intensity

was found after HFS in both conditioned and unconditioned skin sites in van den Broeke's study despite with the coexistence of enhanced event-related potentials in the central nervous system (van den Broeke et al., 2012). In addition, the heat pain threshold remained unchanged after application of all CES paradigms which is in agreement with the observations by Lang and colleagues for 100 Hz conditioning stimulation (Lang et al., 2007). A possible reason for the unclear homotopic pain LTP could be a conflict between LTP and LTD (long-term depression) mechanisms. Indeed, in the rat spinal cord selective high frequency CES of Aδ-nociceptors induced LTD in nociceptive synaptic transmission rather than LTP (Randić et al., 1993; Liu et al., 1998). Electrical stimulation is less specific in activating specific nerve fibers, e.g., Aδ- or C-fibers (Nilsson and Schouenborg, 1999; Inui et al., 2002; Mørch et al., 2011). In addition, both C-fiber and Aδ-fiber pathways are found to be involved in the hyperalgesia at the conditioned site after high frequency CES (Hansen et al., 2007). Therefore, CES may inevitably activate both A-δ- and C-fiber nociceptors that are concurrently involved in homosynaptic depression and homosynaptic facilitation mechanisms with unknown net effects (Pfau et al., 2011). Other possible reasons could be due to habituation or fatigue resulting from repetitive electrical stimulations in the same area (Rankin et al., 2009) or hypoesthesia which could have been induced by continuous 20 Hz CES at C-fiber intensity (De Col & Maihöfner, 2008). Another technical reason for the present study is that the conditioning electrode was removed and placed on the original conditioned site every 10 minutes for assessments of psychophysical changes. This may have resulted in slight variations in which afferents that in fact were activated were masking the homotopic pain-LTP that might have been present. A recent animal cytology study showed that LTP can be induced at spinothalamic neurons by both high (100 Hz, 10 Hz) and low frequency (1 Hz, 2 Hz) CES on primary afferents whereas the same stimulations induced LTD at GABAergic neurons confirming that LTP induction is more dependent on cell specific conditions than on stimulation parameters (Kim et al., 2015). Furthermore, the absence of heat pain hyperalgesia might indicate that the heat pain threshold is not sufficiently sensitive compared to painful suprathreshold heat stimuli for testing heat hyperalgesia (Yucel et al., 2002; van den Broeke et al., 2014b).

4.3 Heterotopic LTP-like Pain

It has been shown that repetitive activation of primary nociceptive C-fibers leads to a synaptic strengthening of nociceptive transmission which may also induce facilitation of non-nociceptive Aβ-fiber and nociceptive Aδ-fiber pathways resulting in dynamic mechanical allodynia and mechanical hyperalgesia, respectively (Klein et al., 2004; Hansen et al., 2007; van den Broeke et al., 2014 a,b). In the present study pain ratings to pinprick stimuli around the conditioned site were also found to significantly increase from 30 min; most likely as a result of heterosynaptic facilitation of Aδ-pathways. This may involve several mechanisms: convergence of facilitated mechanosensitive A-fiber input and the conditioning C-fiber input on nociceptive spinal neurons; simultaneous activation of glutamatergic excitatory interneurons contributing to the sensitization of nociceptive projection neurons in the spinal cord

(Santos et al., 2007); and serotonergic descending facilitation deriving from the rostral ventro-medial medulla (RVM) of the brain stem which can maintain central sensitization by a spino-bulbo-spinal loop (Pertovaara, 1998; Suzuki et al., 2004). In addition, conditioned and surrounding mechanical insensitive "silent" nociceptors might be activated by the CES due to changes in pH and other inflammatory factors related to tissue damage (Serra et al., 2002; Hendry and Hsiao, 2012). This might be a potential element in the induction of secondary mechanical hyperalgesia. Peripheral sensitization has also been proposed as a potential mechanism contributing to the secondary hyperalgesia involving neurogenic vasodilation which causes the release of inflammatory mediators such as SP and calcitonin-gene related peptide (CGRP) (Lewis, 1927; Sumikura et al., 2003). Other mediators provoking the sensitization of nociceptors, such as cytokines and nerve growth factor (NGF), could also be released in the area of vasodilation (Oprée and Kress, 2000; Kidd and Urban, 2001.) which may have contributed to the secondary hyperalgesia. Furthermore, diffusible neuropeptides (SP or CGRP) reaching extrasynaptic receptors can facilitate the mechanical pathway in adjacent neurons (Liu et al., 1994). In the present study, hyperalgesia to mechanical stimuli were present outside the area of increased blood flow for which reason peripheral vascular reactions are not likely to be the explanation for the increased pinprick perception. Instead, the hyperalgesia to pinprick stimuli and the allodynia must be due to central plastic changes (Magerl et al., 2001; Lang et al., 2007). A small area of allodynia was found before CES and in the control session which may due to the irritated skin caused by the process of finding the detection threshold and repeated application of single electrical stimulation. It has been reported that the TRPV1-positive C-fibers (major contribution) and TRPV1-positive A-fibers (minor contribution) are the main inducers of heterotopic pain-LTP (Henrich et al., 2015). Moreover, TRPV1-negative A-fibers are the main afferents for mediating secondary pinprick hyperalgesia without taking effect in the induction of heterotopic LTP-like pain amplification (Henrich et al., 2015). All three CES paradigms used in the present study induced clear heterotopic pain-LTP. Therefore, one possibility is that they have the same effect on TRPV1-positive A-fibers despite the absence of homotopic pain-LTP. Alternatively, each CES paradigm is capable of activating TRPV1-positive C-fibers leading to heterotopic pain-LTP despite the lack of clear homotopic pain-LTP.

4.4 Neurogenic Inflammation

The three different CES paradigms were shown to induce a visible vasodilatation due to axon reflex effects involving peptidergic nociceptive afferents (Low and Westerman, 1989; Klein et al., 2004). However, the stimulation frequencies may have different effects on activating peptidergic afferents resulting in different levels of vascular reactions. Spreading vasodilatation is correlated to the activation of peptidergic afferents, mainly C-fiber afferents (Brain and Williams, 1988), which can release neuropeptides such as SP or CGRP that reach extrasynaptic receptors (Liu et al., 1994). Moreover, a small population of Aδ-fibers is also peptidergic and is involved in neurogenic inflammation and thereby vasodilation (McCarthy and

Lawson, 1990). It has been reported that mechano-insensitive C-fibers activated by high intensity electrical stimulation play an important role in the development of the axon reflex erythema rather than polymodal C-units (Schmelz et al, 2000). In this study, the mechano-insensitive C-fibers could also be recruited by the EPE. 10 Hz CES induced a higher and longer duration of blood flow changes compared with the other two conditioning paradigms which is in agreement with Dusch et al., 2007, who observed a greater flare response following low frequency electrical stimulation due to additional recruitment of mechano-insensitive C-fibers. Moreover, the peptidergic C-fiber nociceptors were found prone to be activated and conduct signals at lower frequencies (below 10 Hz) and the response latencies were increased with higher frequencies stimulation (Raymond et al., 1990). Therefore, the 10 Hz CES seems to recruit more C-nociceptors than the 100 Hz and the 200 Hz paradigms. The magnitude of the skin vasodilation induced by trains of brief transcutaneous electrical stimulation was also strongly related to the number of conditioning pulses (Magerl et al., 1987). Furthermore, neurogenic inflammation caused by release of neuropeptides in the skin has been reported to reflect peripheral mechanisms (Groetzner and Weidner, 2010) rather than appearing as a consequence of a dorsal root reflex (Cervero and Laird, 1996; Willis, 1999). The blood flow had a small increase 20 min before CES in all sessions due to the process of finding the detection threshold by a series of electrical pulses with increasing and decreasing stimulation intensity. This might be due to activation of the peptidergic part of the Aδ-fibers (Mouraux et al., 2010). The skin temperature remained unchanged after application of all CES paradigms. From the present observations, it can be concluded that the average skin temperature is not a sensitive indicator for assessing the neurogenic inflammation caused by CES.

5. Conclusions

The present study found two alternative paradigms for induction of LTP-like pain amplification to reflect the nociceptive plasticity in healthy humans. Hence, high frequency (200 Hz) and low frequency (10 Hz) CES can induce heterotopic pain-LTP similar to the traditional 100 Hz conditioning frequency but in particular the 10 Hz paradigm was associated with less pain during the conditioning process. The pain intensities to single electrical stimuli at the conditioned area, however, did not increase after any of the CES paradigms compared to the control session. In addition the afferent barrage in the 10 Hz paradigm to a larger degree resemble the firing pattern in inflammatory and neuropathic pain conditions. Therefore, the 10 Hz model is recommended in future studies exploring potential analgesic drug effects in humans.

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Author contributions

The measurements were performed by W. Xia. The analysis was performed by O.K. Andersen, C.D. Mørch and W. Xia. The manuscript was written by W. Xia and revised by C.D. Mørch and O.K. Andersen. All authors discussed the results and commented on the manuscript.

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Figure legends

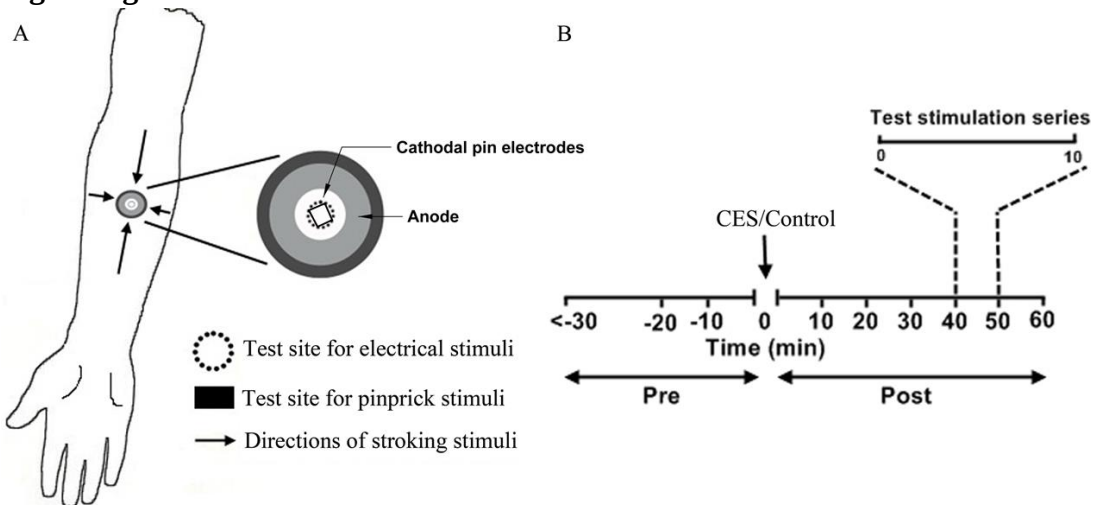

Figure 1. Experimental setup. (A) Conditioning electrical stimulation (CES) (10 Hz, 100 Hz, and 200 Hz) was applied at the volar forearm 7 cm distal to the cubital fossa via a circular array of pin electrodes. The single electrical stimulation was applied at the conditioned site by the same pin electrodes and the light stroking and pinprick stimuli were applied in the surrounding skin area. The black square area stands for the area encompassed by the electrode at four stroking directions. (B) Neurogenic inflammation imaging, area of allodynia, pain to pinprick stimuli, heat pain threshold and homotopic pain perception to single electrical stimulation were assessed with 10 min intervals three times before (pre-conditioning period) and six times after the CES (post-conditioning period).

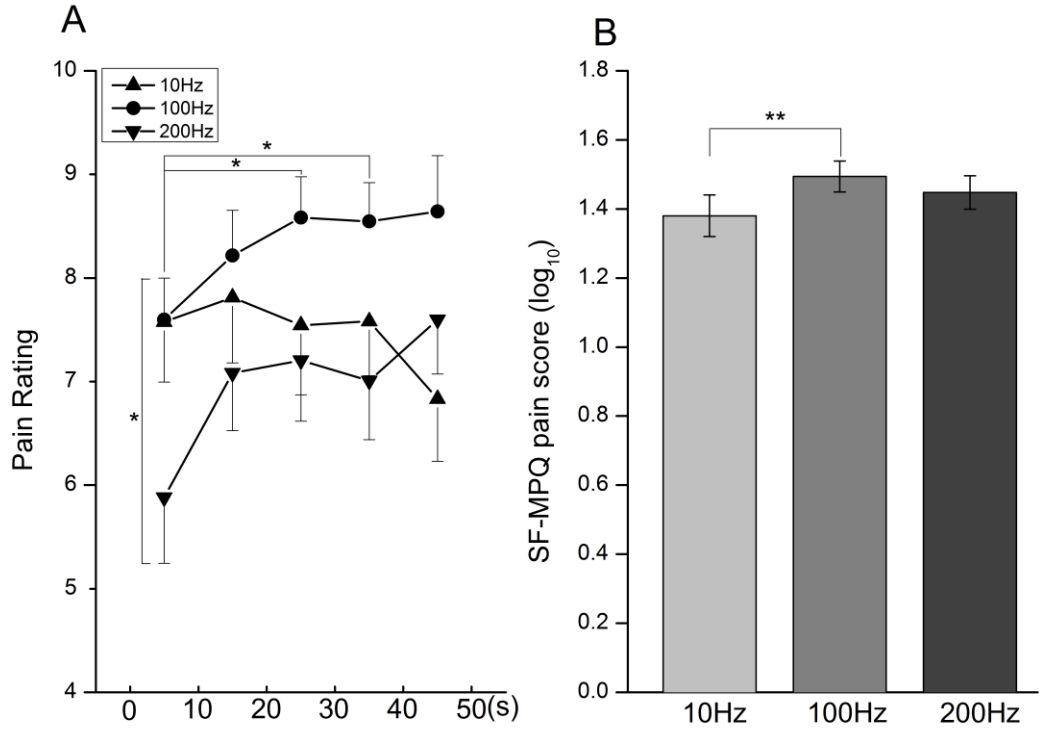

Figure 2. Pain experience induced by CES. A. Temporal changes during the conditioning process. 100 Hz CES induced increasing pain intensity whereas 200 Hz

and 10 Hz CES ratings were not significantly different over time. Pain ratings at the first 10 sec stimulation of 200 Hz CES were lower than the pain ratings at the first 10 sec of 100 Hz CES. B. Depiction of total SF-MPQ scores for CES. 10 Hz CES induced a lower SF-MPQ score than 100 Hz CES. * $p < 0.05$, ** $p < 0.01$.

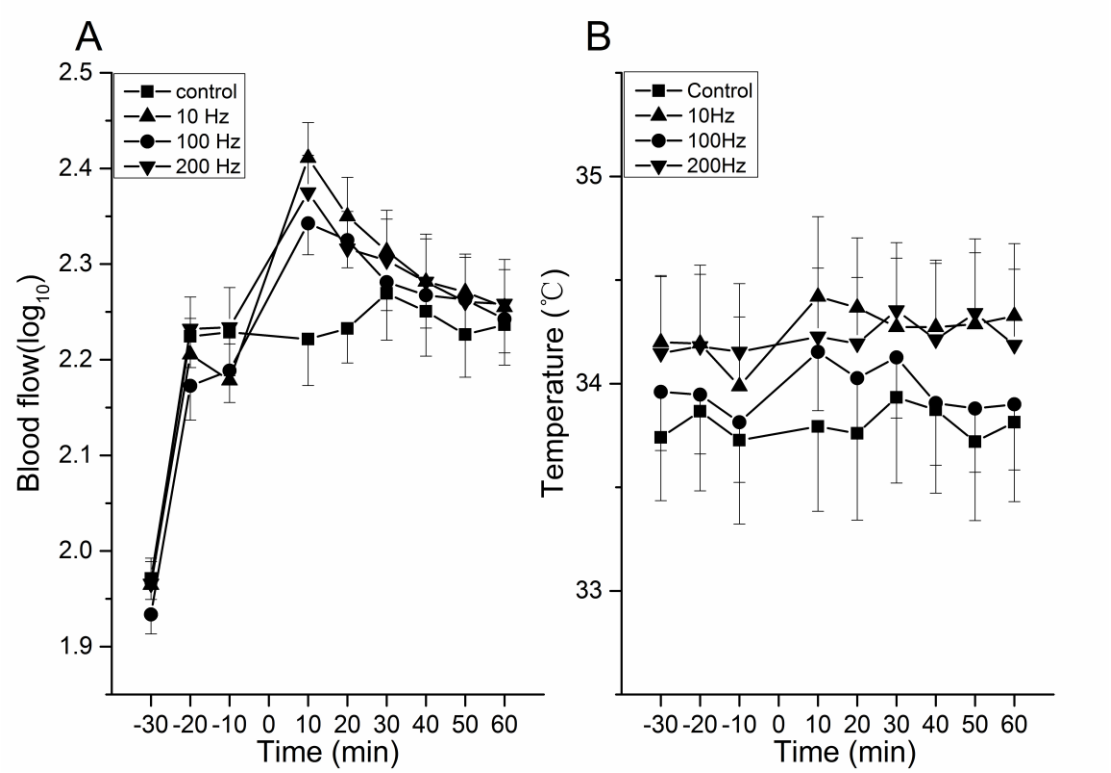

Figure 3. Neurovascular responses to CES. A. Superficial blood flow changes caused by different CES. A significant increase of superficial blood flow occurred within 10 min after CES in the 10 Hz and 200 Hz sessions, which was higher than the control session. After this it declined. The superficial blood flow in the 100 Hz session showed no temporal changes. B. Local average skin temperature at and around the conditioned site. No differences in the skin temperature were observed during the post-conditioning period between any of sessions.

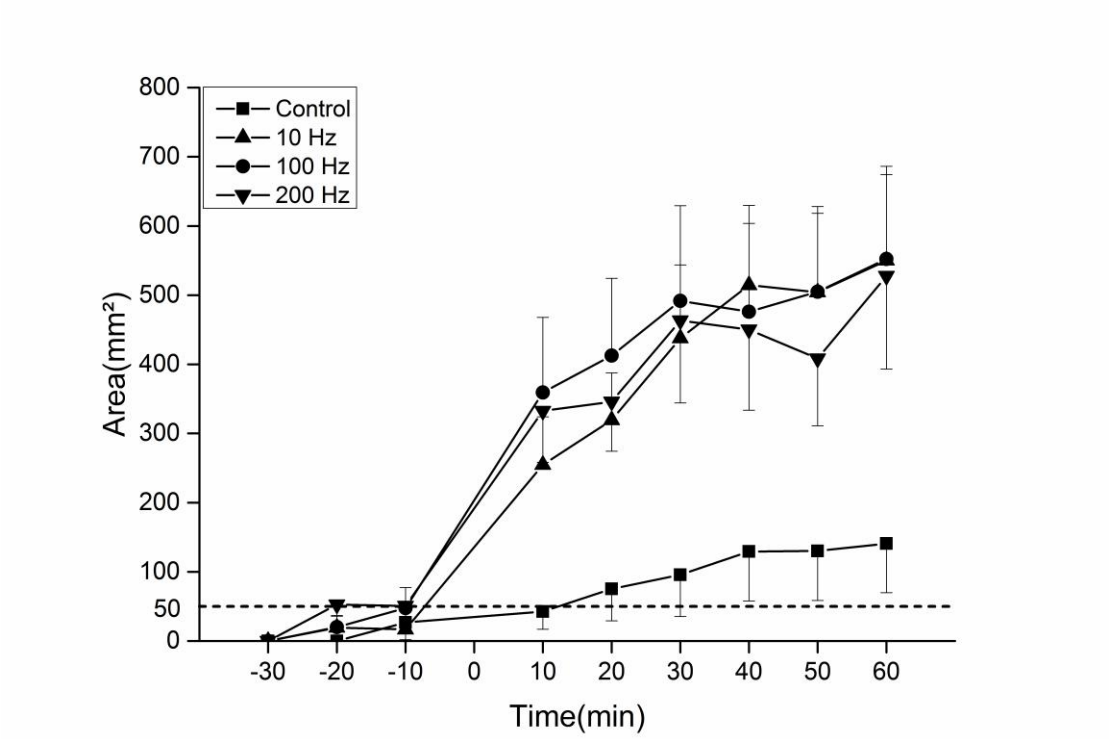

Figure 4. Area of allodynia induced by different CES paradigms. The dash line indicates the area of conditioning pin electrodes. The area of allodynia induced by stroking stimuli significantly increased after all CES paradigms compared with the control session and remained stable until the end of the observation period.

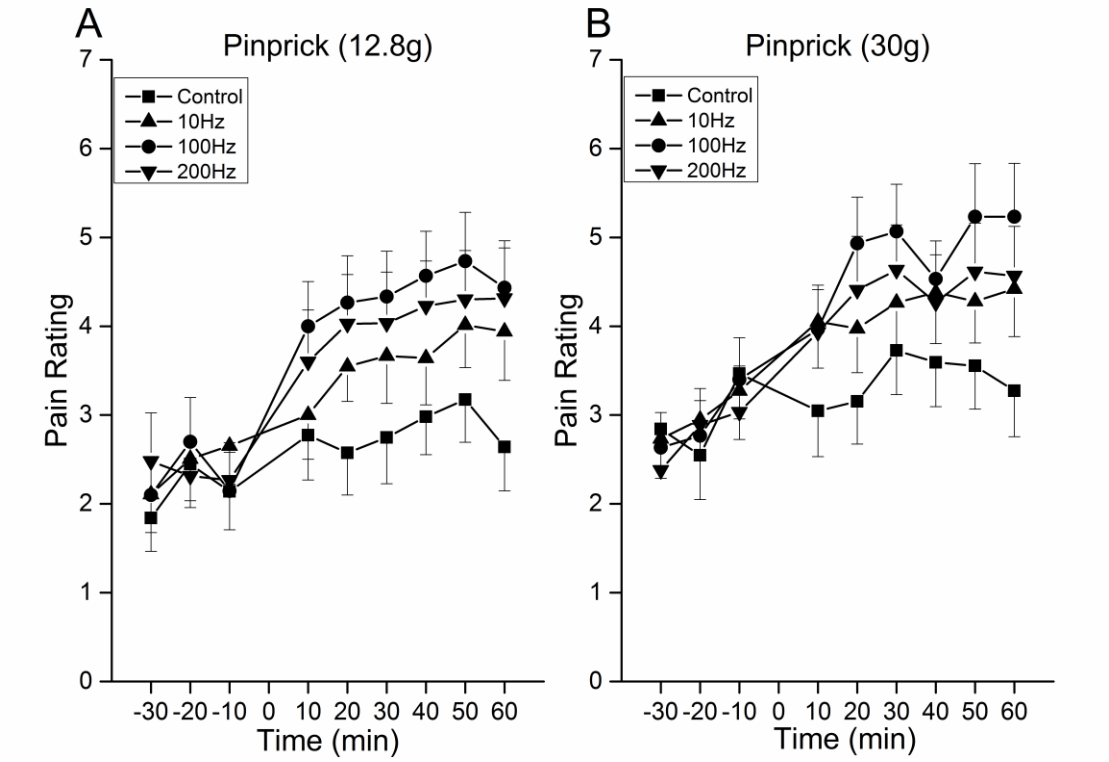

Figure 5. Heterotopic effects of different CES paradigms on different pinprick stimulations. Pinprick-evoked pain after all CES paradigms was significantly higher

than at the control session increasing to a peak around 30 min after the conditioning stimulations. Pain ratings to 30 g stimuli were higher than 12.8 g stimuli.

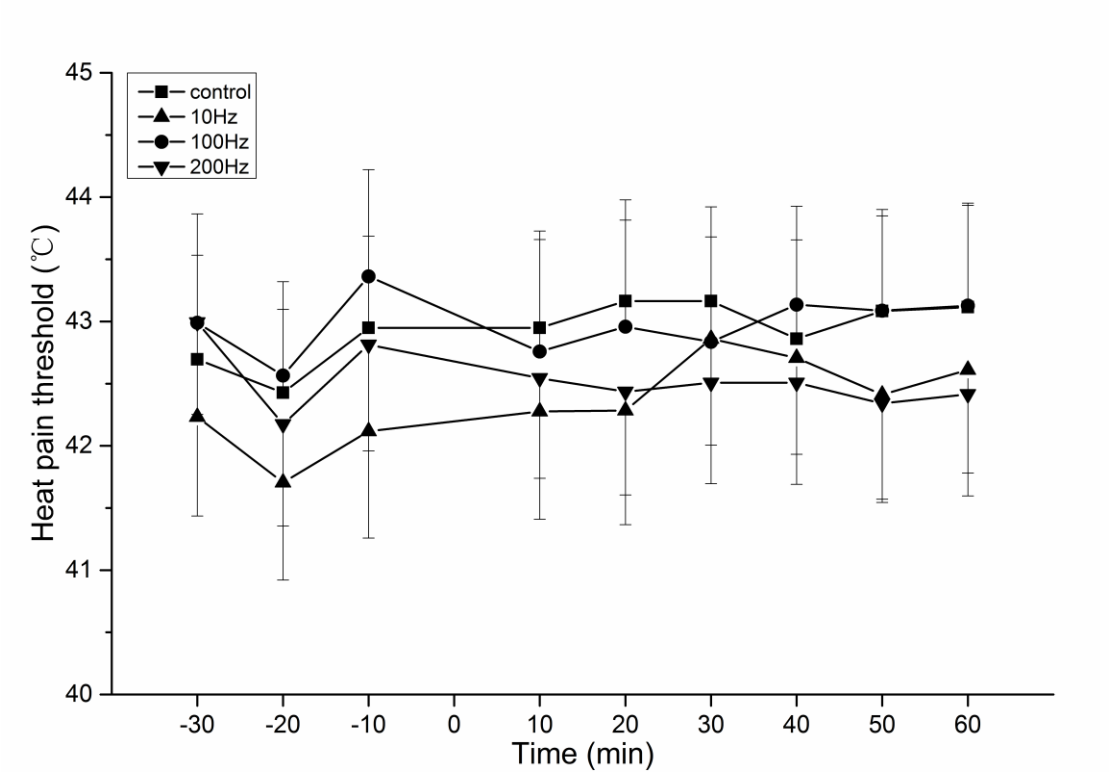

Figure 6. Heat pain threshold following CES. The heat pain threshold was measured three times before and six times after CES. No differences were found between the sessions or over time.

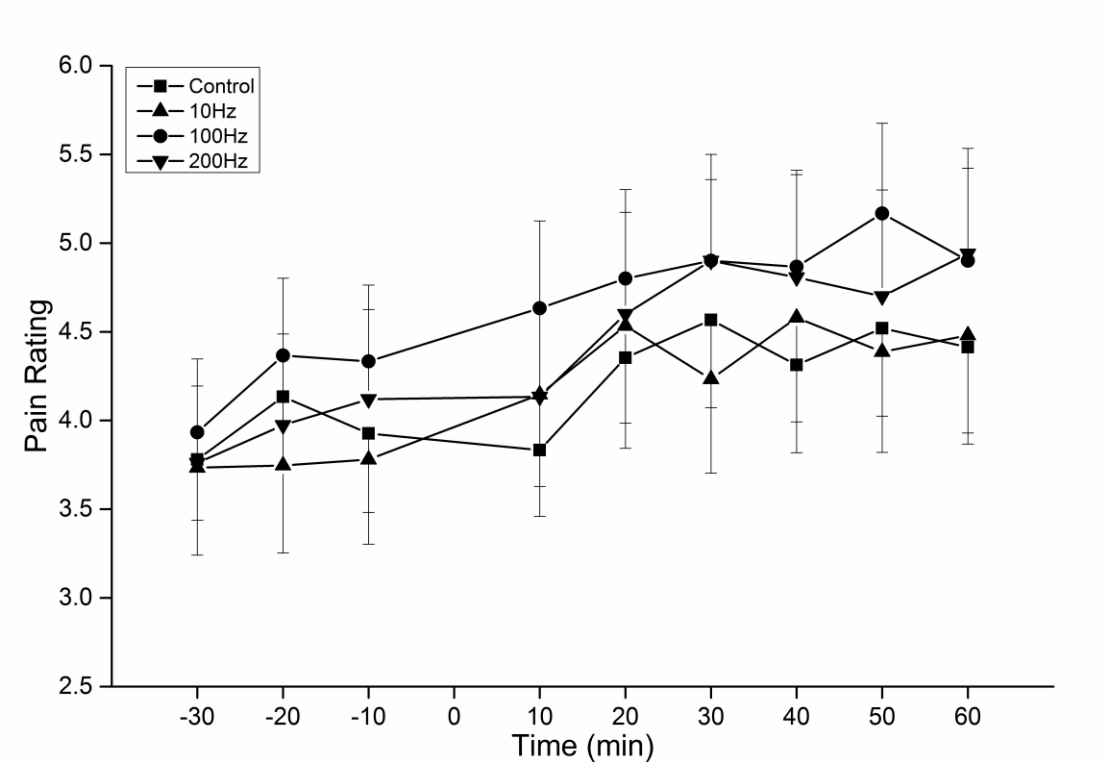

Figure 7. Pain ratings to single electrical stimulation. Pain ratings at 10 min were

significantly lower than at 30 min, 40 min, 50 min and 60 min.