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BY
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PH.D. THESIS

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Dissertation submitted

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PREFACE

The present Ph.D. thesis is partly based on the below three research papers. The experiments have been accomplished at the Integrative Neuroscience Group, SMI, Department of Health Science and Technology, Faculty of Medicine, Aalborg University, Denmark, in the period from 2012 to 2016. The results in details of the three experimental studies are documented in the three papers.

1. Xia, W.[1], Mørch, C.D.[1], & Andersen, O.K[1]. (2016) Exploration of the conditioning electrical stimulation frequencies for induction of long-term potentiation-like pain amplification in humans. Exp. Brain Res. (Epub ahead of print)
1. Center for Neuroplasticity and Pain (CNAP), SMI, Department of Health Science and Technology, Aalborg University, Aalborg, Denmark.
DOI:10.1007/s00221-016-4653-1

2. Xia, W. [1], Mørch, C.D. [1], & Andersen, O.K[1]. (2016) Test-retest reliability of 10 Hz conditioning electrical stimulation inducing long-term potentiation-like pain amplification in humans. (accepted for publication in PLOS ONE)
1. Center for Neuroplasticity and Pain (CNAP), SMI, Department of Health Science and Technology, Aalborg University, Aalborg, Denmark.
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3. Xia, W. [1], Mørch, C.D. [1], Matre, D.[2], & Andersen, O.K[1]. (2016) Exploration of conditioned pain modulation on long-term potentiation-like pain amplification in humans. (submitted to European Journal of Pain)
1. Center for Neuroplasticity and Pain (CNAP), SMI, Department of Health Science and Technology, Aalborg University, Aalborg, Denmark.
2. National Institute of Occupational Health, Department of Work Psychology and Physiology, Oslo, Norway.

ENGLISH SUMMARY

Chronic pain is a worldwide health problem. It not only lowers the life quality of the patients, but also takes a lot of social economic cost. However, chronic pain treatments now are still not ideal and developing an efficient analgesic therapy is persistent challenge. Therefore, exploring the mechanisms behind pain chronification and endogenous pain modulation is still the medical priority. Long-term potentiation (LTP) is an important feature of synaptic plasticity in the central nervous system. Spinal nociceptive LTP induced by high frequency conditioning electrical stimulation (CES) has been considered to be a potential mechanism underlying central sensitization demonstrated as hyperalgesia and allodynia in clinical patients. However, it is still questionable on the biological significance of high frequency CES paradigm as actually the low frequency discharging of nociceptors plays the critical role inducing central sensitization. Conditioned pain modulation (CPM) is a kind of endogenous pain inhibitory modulation. A better understanding of the balance between CPM inhibition and pain facilitation is crucial to understand the importance of endogenous pain inhibition in the prevention of LTP-like pain amplification in humans.

The aims of the present Ph.D. project were to: investigate the different CES paradigms to induce pain LTP in healthy humans in order to find a paradigm more like inflammatory/neuropathic pain conditions (study I); show the reliability of the corresponding measurements indicating LTP-like pain amplification and inflammation responses, and calculate sample sizes for potential drug testing studies (study II); assess the pain inhibitory effect of CPM on the development of pain LTP (study III).

In study I, 10 Hz CES induced heterotopic pain amplification like the high frequency (100, 200 Hz) CES but associated a less pain experience during the CES process, however, homotopic pain amplification were absent in all paradigms. In study II, the test-retest reliability results for 10 Hz CES paradigm showed that superficial blood flow is reliable indicator for neurogenic inflammation response; painful pinprick and light stroking stimuli are reliable indicators for measuring heterotopic perception amplification. In study III, CPM induced by cold pressor conditioning stimulus inhibited the development of heterotopic perception amplification to non painful mechanical stimuli but no effect was observed to heterotopic pinprick painful stimulus, homotopic electrical stimuli and peripheral inflammation responses.

In conclusion, the present work has provided more information on pain LTP induction in healthy human models and recommends 10 Hz CES paradigm in potential analgesic studies because of the biological significance. The endogenous

pain inhibition effect of CPM may play a role in modulating pain amplificatory system.

DANISH SUMMARY

Kronisk smerte er et verdensomspændende sundhedsproblem. Det handler ikke kun om lavere livskvalitet for patienterne, men også om en række social-økonomiske omkostninger. Behandlingen af kroniske smerter er i dag ikke optimal og udviklingen af effektive smertestillende terapier er en vedvarende udfordring. Udforskning af de underliggende mekanismer bag hvorfor smerte kan ende med at blive kroniske og en bedre forståelse af endogen smerte modulation har derfor stor prioritet. Long Term Potentiation (LTP) er en vigtig mekanisme i den synaptiske plasticitet i centralnervesystemet. Spinal LTP i det nociceptive system induceret er anset for at være en potentiel underliggende mekanisme bag central sensibilisering som manifesterer sig som hyperalgesi og allodyni hos patienter. LTP i smertesystemet kan induceres af højfrekvent konditionerende elektrisk stimulation (CES). Anvendelse af højfrekvent CES paradigmet til inducering af central sensibilisering er dog fysiologisk problematisk, idet nociceptorer i reglen fyrer ved lave frekvenser (få aktionspotentialer per sekund). Conditioned Pain Modulation (CPM) igangsætter endogen smerte hæmmende modulation. En bedre forståelse af balancen mellem CPM inhibering og smerte sensibilisering er afgørende for at forstå betydningen af endogen smerte inhibering i forebyggelse af LTP-lignende smerte sensibilisering hos mennesker.

Formålet med nærværende ph.d. projekt var 1) at undersøge forskellige CES paradigmer til at inducere smerte LTP i raske forsøgspersoner for at finde et brugbart paradigme, der i højere grad minder om inflammatoriske/neuropatiske smertetilstande (studie I); 2) vise pålideligheden af de valgte effektmål for LTP-lignende smerte sensibilisering og for måling af vaskulære reaktioner efterfulgt af teoretiske beregninger af stikprøvestørrelser for fremtidige potentielle kliniske afprøvninger (studie II); 3) vurdere den smerte-inhibitoriske virkning af CPM på udviklingen af smerte LTP (studie III).

I studie I inducerede 10 Hz CES heterotopisk smerte sensibilisering lige så effektivt som højfrekvent (100, 200 Hz) CES, dog forbundet med en mindre smerte oplevelse under CES processen. Homotopisk smerte sensibilisering var fraværende i alle tre paradigmer. I studie II viste test-retest pålideligheden for 10 Hz CES paradigmet, at den superficielle blodgennemstrømning er en pålidelig indikator for neurogen inflammation og at smertefulde nåleprik og let berøring er pålidelige indikatorer for heterotopisk smerte sensibilisering. I studie III hæmmede CPM udløst ved cold-pressor smerte udviklingen af heterotopisk smerte sensibilisering til ikke-smertefulde mekaniske påvirkninger (allodyni). Der var dog ingen virkning på heterotopisk nåleprik, homotopiske elektriske stimulationer eller på målinger af perifere vaskulære reaktioner .

Dette ph.d. arbejde har givet ny viden om induktionen af LTP i smertesystemet i raske forsøgspersoner. Samlet set anbefales et 10 Hz CES paradigme i fremtidige eksperimentelle studier grundet den tættere knytning til normal patofysiologi. Endeligt kan det konkluderes, at den endogene smerte hæmmende effekt af CPM kan spille en rolle i smerte sensibiliseringsmekanismer.

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Weiwei Xia, August, 2016, Aalborg, Denmark

ABBREVIATIONS

BDNF: Brain-derived neurotrophic factor

CES: Conditioning electrical stimulation

CGRP: Calcitonin-gene related peptide

CPCS: Cold pressor conditioning stimulus

CPM: Conditioned pain modulation

CPT: Cold pressor test

CV: Coefficient of variation

DNIC: Diffuse noxious inhibitory control

DTh: Detection threshold

EPE: Epicutaneous pin electrode

HFS: High frequency stimulation

HPT: Heat pain threshold

ICC: Intraclass correlation coefficient

LTP: Long-term potentiation

LTD: Long-term potentiation

NS: Nociceptive specific

SBF: Superficial blood flow

SES: Single electrical stimulation

SF-MPQ: Short form McGill Pain Questionnaire

SP: Substance P

ST: Skin temperature

TRPV1: Transient receptor potential vanilloid type 1

VAS: Visual analogue scale

WDR: Wide dynamic range

1. INTRODUCTION

1.1. BACKGROUND

- **Central Sensitization and Pain-LTP**

According to the definition made by International Association for the Study of Pain (IASP), pain is "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage". Pain is ubiquitous in our daily life, but the underlying mechanism is quite complicated and unclear. There are a huge number of people suffering from a variety of pain conditions lowering their quality of life, both in mental and physical aspects. Pain has a protective function for the body and is necessary to promote healing after injury. However, if pain persists with the absence of ongoing nociceptive input or pain exceed the normal response to ongoing nociceptive stimuli, pain is considered to be maladaptive; especially in chronic pain states, such as neuropathic and inflammatory dysfunctional pain, as pain no longer has a protective function. (Costigan *et al.*, 2009).

Chronic pain has become a serious worldwide public health problem, and this takes a lot of social and family economic costs (McCarberg & Billington, 2006). However, the outcomes of the treatments for chronic pain are still unsatisfactory and effective pain medications are persistent challenging (Ossipov & Porreca, 2005; Backonja *et al.*, 2006; Finnerup *et al.*, 2010). Therefore, improving chronic pain treatments is obviously a significant medical priority. A better understanding of the mechanisms underlying pain chronification and pain amplification, as well as those underlying the endogenous pain modulation systems, will help to further know the human pain system. Hopefully, this may help to develop novel medical treatments against chronic pain states.

As seen in inflammation or nerve injury conditions, persistent low frequency discharging of peripheral nociceptors (below 10 Hz; mainly C-fiber type) could lead to an enhanced response of spinal dorsal horn neurons to incoming afferent stimuli, a condition that is termed "central sensitization", i.e., enhanced pain perception to noxious stimuli (hyperalgesia) and pain perception induced by normally non-noxious stimuli (allodynia) (Woolf, 1983; Basbaum & Julius, 2006; Woolf & Ma, 2007; Costigan *et al.*, 2009). Many chronic pain patients show features of central sensitization (Banic *et al.*, 2004; Nijs *et al.*, 2010). Central sensitization is characterized by facilitation of pain transmission pathways which involves increases in the spontaneous activity of nociceptors, evoked responses and enlarged receptive field, and lowered response thresholds of wide dynamic range (WDR) and nociceptive specific (NS) dorsal horn neurons (Suzuki *et al.*, 2002,

2004; Suzuki & Dickenson, 2005; Basbaum & Julius, 2006; Woolf & Ma, 2007; Costigan *et al.*, 2009).

Long-term potentiation (LTP) is an important feature of synaptic plasticity in the central nervous system and was considered to be involved in learning and memory formation in the hippocampus (Bliss & Lomo, 1973; Blanchard *et al.*, 2010). LTP was presented as a long-lasting enhancement of synaptic transmission efficiency in the central nervous system (Bliss & Collingridge, 1993). Moreover, it has been shown that a form of LTP is also involved in the nociceptive pathways in the spinal synapses, and an enhanced responsiveness of spinal dorsal horn neurons were present in both slice preparations *in vitro* and animal experiments *in vivo* (Randić *et al.*, 1993; Sandkühler, 2000; Ikeda *et al.*, 2003). An important underlying mechanism has been considered to be involving LTP of the C-fibre synapses mediating nociceptive transmission located at the superficial and deep spinal dorsal horn (Randić *et al.*, 1993; Ikeda *et al.*, 2003). Therefore, LTP of nociceptive synaptic transmission has been considered to be one important mechanism underlying central sensitization, i.e., its perceptual correlate of neurogenic pain amplification (hyperalgesia and allodynia) (Ji *et al.*, 2003; Klein *et al.*, 2004).

- **Pain-LTP Induction Methodologies**

Many experimental models of pain facilitation have been used for exploring the underlying mechanisms or evaluating analgesic agents in healthy humans, including 1) electrical nerve stimulation: high frequency stimulation (HFS) at 100 Hz (1~2 ms pulse duration) for 1 s, repeated 5 times with 10 s intervals (Klein *et al.*, 2004, 2008; Hansen *et al.*, 2007; van den Broeke & Mouraux, 2014a; Study I) or continuous intermediate frequency electrical stimulation at 5 or 10 Hz (0.5~1 ms pulse duration) (Chizh *et al.*, 2004; Koppert *et al.*, 2004; Bandschapp *et al.*, 2010; Study I, II, III); 2) natural noxious stimulation: skin incision (Kawamata *et al.*, 2002), chemical injury, such as capsaicin, mustard oil or formalin (LaMotte *et al.*, 1991; Treede *et al.*, 1992; Pertovaara, 1998), and thermal injury, such as heat or UVB burn (Modir & Wallace, 2010a, 2010b); 3) pharmacological stimulation: opioid withdrawal during intermediate frequency electrical stimulation or after capsaicin injection (Angst *et al.*, 2003; Hood *et al.*, 2003) or acute opioid withdrawal (Compton *et al.*, 2003). In addition to the induced homotopic hyperalgesia at the site of injury (primary hyperalgesia), these models also produce heterotopic mechanical hyperalgesia to pinprick stimulation (secondary hyperalgesia) and allodynia to light touch stimulation in the surrounding area without injury.

LTP can be induced by high frequency conditioning electrical stimulation (CES) in the brain (Bliss & Lomo, 1973). Similarly, conditioning electrical stimulation of peptidergic nociceptive C-fibre afferents can lead to LTP of synaptic transmission in the spinal cord at high frequency (100 Hz), low frequency (1~2 Hz), or intermediate frequency (10 Hz) *in vivo* and *in vitro* (Randić *et al.*, 1993; Yang *et al.*,

2014; Kim *et al.*, 2015). In healthy humans, the most typical paradigm inducing pain LTP is conditioning HFS using the special epicutaneous pin electrodes (EPE) which can selectively activate C-fibre nociceptors compared to conventional patch electrode (Klein *et al.*, 2004, 2008; Hansen *et al.*, 2007; van den Broeke & Mouraux, 2014a; Study I, II, III). In contrast, CES at low frequency (1 Hz, 1000 pulses) induced perceptual LTD (long-term depression) rather than LTP in humans (Klein *et al.*, 2004; Rottmann *et al.*, 2010). Therefore, compared with animal and slice preparation studies, there are some opposite findings in human studies. In most neuropathic or inflammatory pain conditions, however, hyperalgesia is caused by persistent low frequency discharging of C-fibre nociceptors (i.e., 0.3~10 Hz) which is far different from the electrical HFS paradigm used in experiments to produce LTP-like pain amplification (Puig & Sorkin, 1996; Han *et al.*, 2000; Xiao & Bennett, 2007a; Drdla & Sandkuhler, 2008). HFS may mimic the high frequency discharge of nociceptors at the beginning of injury (Handwerker *et al.*, 1987). Therefore, it is still difficult for CES to fully resemble the irregular discharging of nociceptors and still be reproducible in humans. However, frequencies closer to the firing frequencies of nociceptors under pain conditions still need to be explored for inducing a similar LTP-like pain amplification in humans.

- **Pain-LTP Measurements**

Psychological measurements of central neuronal sensitization are focusing on observing the reduction of pain threshold/increased pain response at the injured sites and spread of pain to uninjured sites. The assessments of cutaneous CES-induced LTP-like pain amplification include pain perception ratings at the conditioned sites stimulated by EPE (homotopic) and in the immediate vicinity to the EPE (heterotopic). The homotopic pain amplification was assessed using single electrical stimulation at the conditioned sites and this is thought to share characteristics of homosynaptic LTP (Klein *et al.*, 2004; van den Broeke & Mouraux, 2014b). The heterotopic pain amplification was assessed by mechanical stimuli, such as punctate probes, soft brush or cotton swab which is thought to share characteristics of heterosynaptic LTP (Klein *et al.*, 2004; van den Broeke *et al.*, 2011). The thermal modalities, e.g., heat pain threshold, are important measuremenst of hyperalgesia involved in animal and human models (Hargreaves *et al.*, 1988; Lang *et al.*, 2007; Sumikura *et al.*, 2003). The TRPV1 receptors in spinal dorsal horn have been shown to contribute to spinal LTP induction and development (Yang *et al.*, 2014). Activation of afferent fibers by heat may involve the TRPV1 ion channel which helps to produce heat pain perception (Caterina *et al.*, 1997). Heat stimulation has been used to assess peripheral sensitization in CES model. However, there were still controversies about the presence of heat pain hyperalgesia in previous studies on CES human model (Lang *et al.*, 2007; van den Broeke & Mouraux, 2014b).

After being activated by nociceptive CES, axon reflex occurs on peptidergic C-fiber nerve endings which can release neuropeptides, such as substance P (SP), calcitonin gene-related peptide (CGRP) (Sauerstein *et al.*, 2000), resulting in neurogenic vasodilatation, plasma and protein extravasation, attraction of macrophages, or degranulation of mast cells, which was termed "neurogenic inflammation" (Jancsó *et al.*, 1967; Lynn, 1996; Schaible *et al.*, 2005; Schaible, 2007). The neurogenic inflammation can be assessed by measuring the superficial blood flow (SBF) and temperature of the skin which can be representatives of the peripheral processes (Magerl & Szolcsányi, 1987; Klein *et al.*, 2004; Study I, II, III).

- **Conditioned Pain Modulation (CPM)**

A conditioning painful stimulus can inhibit the nociceptive response evoked by a test stimulus located at a remote extra-segmental body area which was named "diffuse noxious inhibitory control (DNIC)" (Le Bars *et al.*, 1979b). Afterwards, the term "conditioned pain modulation (CPM)" has been introduced involving a broader description of "pain inhibits pain" phenomenon in humans. CPM effect refers to that perceived pain intensity caused by a test stimulus can be inhibited by a conditioning painful stimuli applied at a remote location of the body (Yarnitsky *et al.*, 2010). CPM is considered to be an important manifestation of endogenous descending inhibitory modulation (Yarnitsky *et al.*, 2010). The CPM effect has been shown to inhibit the activity of spinal neurons involved in nociceptive pathways resulting in decreased hyperalgesia and nociceptive responsiveness in animals (Bouhassira *et al.*, 1992) and pain perception in humans (Meeus *et al.*, 2008; Villanueva, 2009; Roussel *et al.*, 2013). The mechanisms underlying CPM is thought to involve multiple pain modulatory pathways, such as the activation of cortical (anterior cingulate cortex, orbitofrontal cortex and amygdala) and brainstem (periaqueductal gray and medulla) structures mediating nociception via descending inhibitory serotonergic and noradrenergic systems leading to inhibition of wide dynamic range (WDR) neurons located in deep spinal dorsal horn (Le Bars *et al.*, 1979b; Bouhassira *et al.*, 1992; Le Bars, 2002; Pichéet *al.*, 2009; Nir *et al.*, 2011; Sprenger *et al.*, 2011). Furthermore, CPM efficiency has been suggested as a biomarker of central endogenous descending inhibition integrity in patients with chronic pain. The CPM effect has been shown to be less efficient in these patients (van Wijk & Veldhuijzen, 2010; Chalaye *et al.*, 2014; Corrêa *et al.*, 2015), suggesting that the central endogenous inhibitory system is dysfunctional (Yarnitsky, 2015). Interestingly, populations with deficient CPM effect could be at risk for having the chronic postoperative pain (Yarnitsky *et al.*, 2008; Wilder-Smith *et al.*, 2010). Chronic pain can be the consequence of the imbalance of pain inhibition and pain facilitation systems. In human studies, the cold pressor conditioning stimulus (CPCS) is most used to induce CPM effect because of its better reliability compared with pressure pain or tourniquet pain methods (Oono *et al.*, 2011; Lewis *et al.*, 2012). Until now, the endogenous pain modulation

mechanisms on pain facilitation are still not clear, and an effective chronic pain treatment strategy is a persistent challenge.

1.2 AIMS AND HYPOTHESIS

Based on the background information above, three studies were designed and performed (Fig. 1) and the hypothesis and aims of this Ph.D. project are presented as below:

In study I: We hypothesized that 10 Hz CES closer to the discharging frequency of C-fibres during natural pain conditions might be superior for induction of LTP-like pain amplification. This experimental protocol in humans aimed to explore the conditioning electrical stimulation frequencies standing on a biological significance to induce cutaneous long-term potentiation (LTP)-like pain amplification in humans.

In study II: According to the results of study I that 10 Hz CES could be used as an alternative paradigm for inducing heterotopic LTP-like pain amplification in humans, but no studies have been done to do the test-retest reliability of 10 Hz CES-induced inflammatory and sensory outcomes. The primary aim of this study was to quantify and evaluate the test-retest reliability of long-term potentiation (LTP)-like pain amplification and inflammatory responses induced by 10 Hz CES with cross-over and parallel study designs in humans. By calculating hypothetical sample sizes, it will be helpful for researchers when using this model for future pharmacological testing studies.

Study III: We hypothesised that CPM might have an inhibitory effect to decrease the sensitization of central nervous system which might prevent the induction of LTP-like pain amplification by 10 Hz CES in healthy humans. The aim of this study was to assess the endogenous perceptual inhibitory modulation on LTP-like pain amplification in humans.

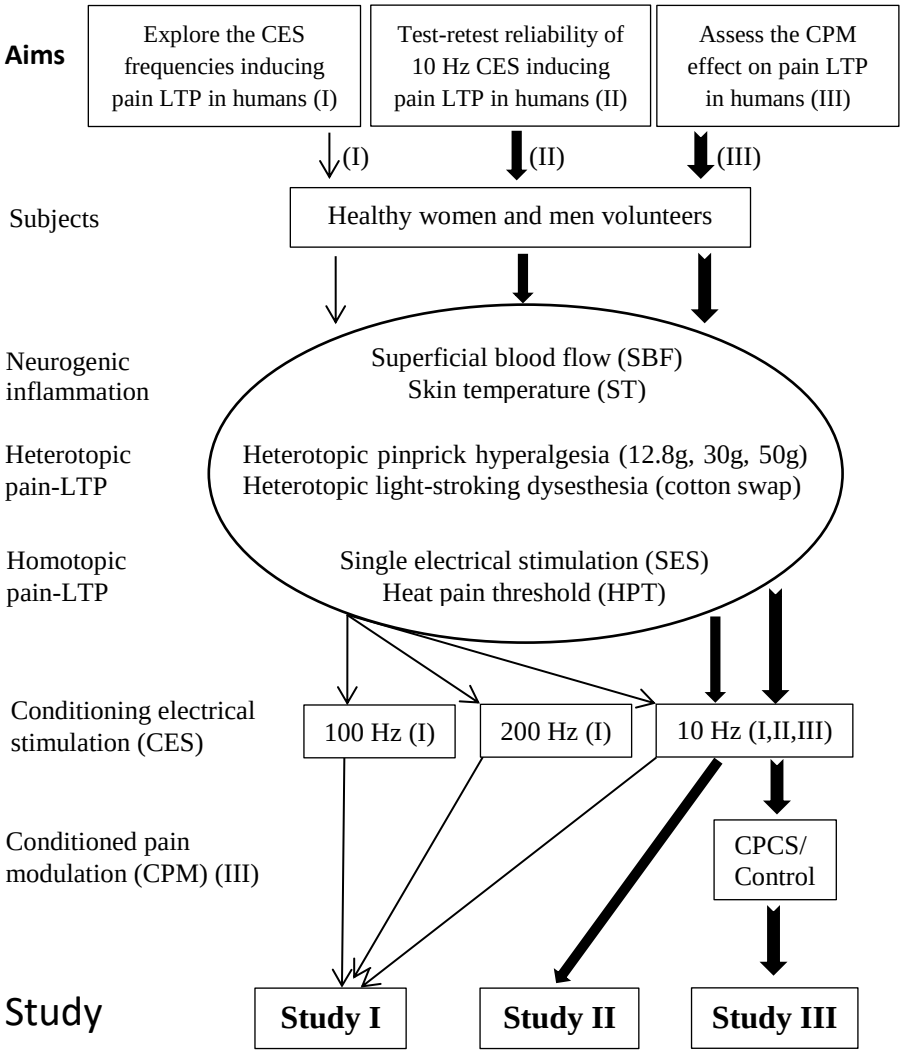

Fig. 1 Ph.D. project aims and design.

The 100 Hz CES (500 pulses, pulse width at 1 ms) was given as five 1-s trains with 10 s intervals. The 200 Hz CES was given as five 0.5-s trains with 10 s intervals (500 pulses, pulse width at 1 ms). The 10 Hz CES was given as continuous train for 50 s (500 pulses, pulse width at 1 ms). Arrow: Study I; bold arrow: Study II; swallowtail arrow: Study III. CPCS: cold pressor conditioning stimulus.

2. OVERVIEW OF THE PH.D. WORK

2.1. EXPERIMENTAL STUDIES

Based on the hypothesis, three experimental studies were designed in this Ph.D. project (Fig. 1):

In study I, three CES paradigms (10 Hz, 100 Hz and 200 Hz) were applied to induce pain LTP in three sessions and observe the corresponding neurogenic inflammation responses in fifteen healthy subjects (7 females). The three paradigms include 1) 10 Hz lasting 50 sec, 2) 100 Hz lasting 1 sec repeated five times with 10 sec intervals, 3) 200 Hz lasting 0.5 sec repeated five times with 10 sec intervals. All CES process in each CES paradigm lasted 50 sec and consisted 500 rectangular pulses (pulse width: 1 ms) with stimulation intensity at 10 × detection threshold (DTh). The three CES paradigms and the control session without applying any CES were randomly arranged on four separate days with at least one-week interval (Fig. 2*B*). In study II, only the 10 Hz paradigm which was the same 10 Hz paradigm used in study I was applied to induce pain LTP and the corresponding neurogenic inflammation responses were observed in twenty healthy subjects (8 females). This paradigm was repeated applied for each subject on two separate days with at least one week interval (Fig. 2*C*). In study III, the LTP-like pain amplification was induced using the 10 Hz paradigm by the EPE. Conditioned pain modulation effect was induced by the cold pressor test (CPT) applied as the conditioning stimulus at a remote body location (left foot) from the CES stimulated area (right forearm). Two experimental sessions (CPCS/control) were randomly scheduled for twenty subjects (6 females) on two separate days with at least one-week interval in a cross-over design. Half of the subjects experienced CPCS session as the first experimental session, and the other half of the subjects experienced control session as the first experimental session. This was aimed to reduce the bias because of the sequence of the two sessions. A training session was arranged for each subject before experimental sessions in three studies to be familiar with the different stimulus modalities and to know how to rate perception intensities to these test stimuli using the visual analogue scale (VAS). All the perception intensity measurements were done by the same tester.

2.2. EXPERIMENTAL METHODOLOGY

2.2.1. LONG-TERM POTENTIATION (LTP)-LIKE PAIN MODEL

The epicutaneous pin electrode (EPE) was used in this project to induce pain LTP by conditioning electrical stimulation (CES). The EPE has a circular of fifteen cathodal pin electrodes (diameter: 10 mm; area: 79 mm^2) with diameter of 0.2 mm

protruding from the base; the anode is a large circular stainless steel with an inner diameter of 20 mm and an outer diameter of 40 mm placed concentrically around the pin cathodes (Fig. 2*A*) (Biurrun Manresa *et al.*, 2010). Compared with the conventional patch electrode, the EPE could induce pain/stinging sensation with lower electrical stimulation intensity because the diameter of the cathodes is smaller leading to a high current density in the epidermal layers where the nociceptive Aδ- and C-fibers terminate (Nilsson *et al.*, 1997; Mouraux *et al.*, 2010; Mørch *et al.*, 2011). The EPE was placed on the right forearm 7 cm distal to cubital fossa (i.e., conditioned sites). The EPE was connected to the constant current stimulator (DS5; Digitimer Ltd; Welwyn Garden City, UK) which generates the 1 ms square pulses electrical stimulation. The method of limits was used to determine the individual electrical detection threshold (DTh): three series of electrical pulses with increasing and decreasing stimulus intensities at a step size of the 3% present stimulation intensity. In each series of stimulation pulses, all subjects terminated the electrical stimulation with increasing intensities when detecting the electrical pulse, and then terminated the electrical stimulation with decreasing the intensities when the electrical pulse became insensible. This process was repeated three times. The DTh was recorded using the electrical stimulus intensity they terminated the electrical stimulation each time. The geometric mean of the six DThs measured in three series of assessments was used as the final DTh.

2.2.2. PAIN PERCEPTION MEASUREMENTS

The visual analogue scale (VAS) was used to evaluate perceived stimulation intensities. In study I, the VAS was anchored from 0 (no pain) to 10 (the most intense pain imaginable) with a custom made slide ruler. In study II and III, the VAS was anchored from 0 (no sensation) to 100 (the most intense pain imaginable) where 30 indicated the pain threshold. According to the experience in study I, the VAS used in study II and III is likely a more reasonable way, as sometimes the subjects may still slide a small number even the stimulation is just close to becoming painful. 30 indicated the pain threshold means all subjects should rate non-painful stimulation under 30 and rate painful stimulation above 30 on this scale. This is a way of merging two rating scales (with pain threshold in the middle) for painful and non-painful stimulation which actually activate different sensory nerve endings. Two rating scales used in one study may confuse the subjects when rating the perception intensities to different intensities of stimuli. There are some other studies involving healthy humans and clinical patients which also used a similar pain rating scale (Matre *et al.*, 2006; Biurrun Manresa *et al.*, 2010; Dawson *et al.*, 2012; Mørch *et al.*, 2013). The reason we changed the 10-scale is that 100-scale may help to do possible $\log_{10}$ conversion of raw data in the statistic analysis.

In study I, II, and III, the perception intensity during the CES process (i.e., 50 s) in each paradigm in each session was rated by the subjects using a hand-held VAS-device. The recorded pain ratings were sampled in a computer. Then the highest

pain rating in each 10 s stimulation was chosen to observe and compare the temporal changes within and between sessions. The short-form McGill Pain Questionnaire (SF-MPQ) was used to further describe the pain intensity and quality of the CES process in each experimental session. The SF-MPQ consists of sensory and affective dimensions of pain, evaluative overall intensity of total pain experience and present pain intensity index of the standard MPQ. The present pain intensity is the average pain rating subjects gave to the whole conditioning process after conditioning stimulation. All rating scores were added together to get a total quantitative value for comparison between different CES paradigms (Melzack, 1987).

The heterotopic non-painful tactile mechanical perception was assessed by a cotton swab stroking surrounding the conditioned sites. The cotton swab has a flexible plastic mount which can exert ~100 mN when slightly bent. The perception was measured by a light-stroking stimulus with a distance of 1 cm moving at a speed of 1-2 cm/s each time. In study I, the area of allodynia was measured by light stroking stimulus moving from outside to the centre of the circular pin electrodes in four directions (Fig. 2*A*). The area was calculated by connecting the four recorded positions where the stroking perception became painful/unpleasant. In study II, instead of measuring area of allodynia, we measured the perception intensity to the light stroking stimulus which stopped at 1 cm to the border of circular pin electrodes. The stoking speed was the same with study I. An average of the four tests by a VAS scale in four sites was used as the final perception intensities for light-stroking stimuli. The method used to measure perception intensities to light stroking stimuli in study III was the same with the method used study II (Fig. 2*A*).

The heterotopic mechanical pinprick perception was measured by a set of custom-made weighted pinprick stimulators (SMI®, Aalborg University, round tip, 0.2 mm in diameter, contact time 1~2 s) applied surrounding the conditioned sites (i.e. 15~20 mm to the circular pin electrodes) (Fig. 2*A*). In study I, two weighted pinprick stimulators (12.8 g and 30 g) were used. In study II, three weighted pinprick stimulators (12.8 g, 30 g and 50.1 g) were used. We employed a heavier weight pinprick stimulator (i.e., 50.1 g) in study II because we aim to perform the test-retest reliability, therefore a more broad range of weighted stimulators is needed to compare and confirm the more reliable pinprick stimulator for measuring the mechanical pinprick LTP-like perception amplification. In study III, The heterotopic mechanical perception was measured by three custom made weighted pinprick stimulators (12.8 g, 30 g, 50.1 g) which were the same with the stimulators used in study II.

The homotopic perception to single electrical stimulation (SES) was assessed at the conditioned sites using the same EPE (Fig. 2*A*). SES used single electrical square pulse stimulus (pulse duration: 1 ms) with stimulation intensity at 10× DTh. An average value of three SES tests with 10 s intervals was used as the final perception

intensity to SES. The methods for measuring homotopic perception to SES were the same in Study I, II, and III.

The heat pain threshold (HPT) was measured for observing thermal perception changes by a heat stimulator with a contact thermode (Pathway; 30×30 mm ATS; Medoc Ltd.; Ramat Yishai, Israel) placed on the forearm concentric to the circular pin electrodes. The stimulated area covered both conditioned and surrounding skin areas as the area of the contact thermode is bigger than the area of circular pin electrodes. The baseline temperature was 32℃. The temperature increased at a speed of 1℃/s and returned to the baseline at a speed of 8℃/s. All subjects pressed a response button to terminate the ramping heat stimulation when the heat pain was detected. An average value of three tests was used as the final HPT. The methods for measuring HPT were the same in study I, II, and III.

2.2.3. INFLAMMATION MEASUREMENTS

The inflammation response at the skin after CES was assessed by observing the superficial blood flow (SBF) and skin temperature (ST) with imaging recordings in study I, II, and III. Full-Field Laser Perfusion Imager (FLPI) (MoorFLPI; Moor Instruments Ltd, Axminister, Uk) was used to observe the possible excitation of peptidergic nerve fibres (mainly C-fibres) and assess the temporal changes of SPF (i.e., neurogenic inflammation) in the area surrounding the conditioned sites. The infrared thermography (Thermovision A40; FLIR; Danderyd, Sweden) was used to measure the ST surrounding the conditioned site. The SBF and ST were assessed in the same round area with a diameter of 15 mm concentric to the circular pin electrodes which however did not cover the area of pinprick stimuli.

2.2.4. CONDITIONED PAIN MODULATION (CPM)

In study III, subjects immersed the left foot in the circulated cold ice water (i.e., cold pressor test) at the ankle level for CPM induction. There was a steel mesh in the bucket to prevent the direct contact between ice and foot. The temperature of the cold water bath was 4℃±1℃. All subjects could withdraw the foot from the water bucket if the cold water pain became intolerable. However, they were encouraged to place back the foot as soon as they could. A warm, comfortable water bath (32℃) was used in the control session. The CPCS/ control water bath lasting two minutes was applied immediately before 10 Hz CES in each session (Fig. 2*D*).

2.2.5. DATA ANALYSIS

In study I, two-way repeated measures (RM)-ANOVA was done on the pre-conditioning data to determine any significant differences before CES between four sessions (control, 10 Hz, 100 Hz and 200 Hz) in any of the outcome measures. To

strengthen the statistical analysis, only post-CES measurements were included in the RM-ANOVA to identify hyperalgesia in comparison with the control condition and to compare the three conditioning frequencies. RM-ANOVA was done on the whole data (pre- and post-conditioning period) in study II and III. In study III, the normalized data of pain perception measurements were used for analysis whereas study I and II used raw data, as 1) pain intensity increments (percentage of baseline data) can be used for presenting pain perception changes intuitively and better present the sensation changes on different days to the same stimulation equipment for each subject; 2) according to study II, subjects may have different sensitivities on different days due to different conditions of the body (i.e., giving different responses to the same stimulation), so normalization will strengthen analysis as the difference in the baseline data might lead to a wrong proposed difference between two sessions. In all three studies, Greenhouse-Geisser method was used for correction of non-sphericity. Bonferroni-Holm adjustment was used for multiple comparisons. In study II, the reliability was assessed using intra-class correlation coefficient (ICC), coefficient of variation (CV) and Bland-Altman analysis. Sample sizes were calculated for potential crossover and parallel study designs. The calculation formulas were: $N_{parallel}=(15.6\times\sigma^2)/E^2$; $N_{crossover}=(15.6\times\sigma^2\times(1-ICC))/E^2$. The clinical relevant effect (E) was set at 30% of the difference between the average baseline value and the average value of three time points (10, 20, 30 min) after CES. σ is the average value of the standard deviation of post-CES values at three time points. ICC was calculated in within sessions. This was also considered to be a valid alternative approach for assessing reliability (Mørch *et al.*, 2013; Biurrun Manresa *et al.*, 2014).

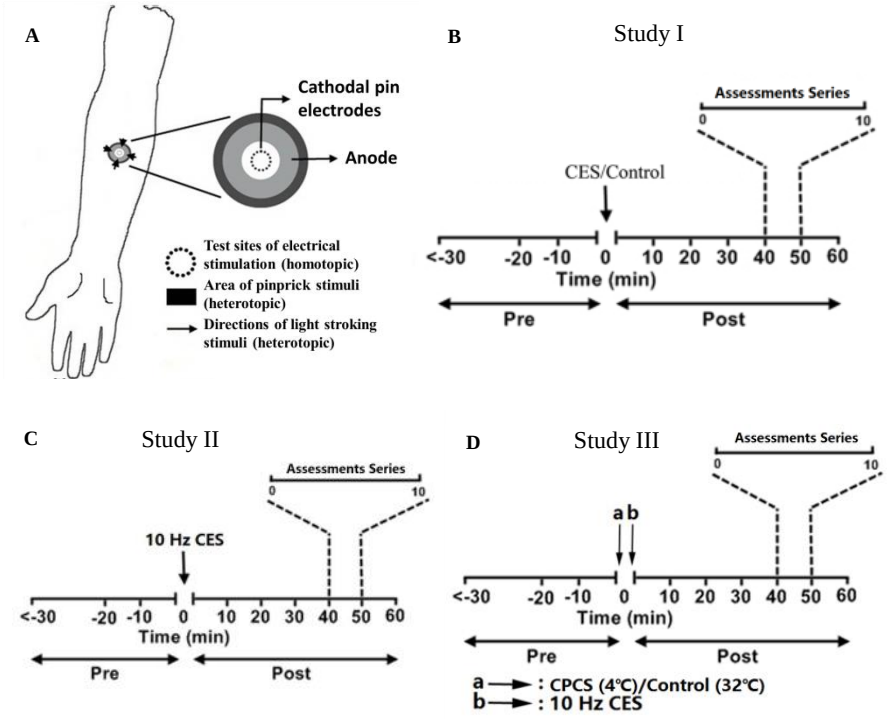

Fig. 2 Experiment setup for Study I, II, and III . A. The EPE was placed on the right forearm 7 cm to the cubital fossa. The homotopic pain ratings to SES were tested at the conditioned sites after CES using the same EPE. The heterotopic perception intensity to pinprick and light-stroking stimuli were tested in the area surrounding the circular pin electrodes. B. Study I procedure. C. Study II procedure. D. Study III procedure. B,C,D. A series of assessments including pain ratings to SES, pinprick and light-stroking stimuli, HPT, neurogenic inflammation (SBF,ST) was repeated three times before and six times after CES with 10 min intervals in each session. D. Study III procedure. CPCS and control water bath lasting for 2 min on the left foot was applied immediately before 10 Hz CES in each session.

2.3. PERCEPTION FEATURES OF CES

- **Main results**

In study I, we found that the perception intensities during the 100 Hz CES process increased whereas no temporal changes were found for 10 Hz and 200 Hz CES process. 100 Hz CES caused a higher pain rating than 200 Hz CES in the first 10 s stimulation. The SF-MPQ score for 10 Hz CES process was lower than 100 Hz CES process indicating less pain during the 10 Hz process. In study II, the pain ratings declined during the CES process in both sessions and session one induced higher pain ratings than session two. In study III, the pain perception ratings declined during the train of 50 s 10 Hz CES process. Pain perception ratings in the CPCS session were found to be lower than perception ratings in the control session.

- **Discussion**

In study I, the pain perception intensity increased during the 100 Hz CES process which is in agreement with Klein's study (Klein *et al.*, 2004). The 100 Hz CES caused higher pain intensities than 200 Hz for the first 10 sec stimulation which may be due to conduction failure of the impulses. The interstimulus interval for transferring the same amount of impulses was shorter in 200 Hz CES (5 ms) compared to 100 Hz CES (10 ms). It has been reported that C-fibres could reach the maximum discharge frequency of 190 Hz with the entrainment interval (i.e., absolute refractory period+relative refractory period) of 5.3 ms indicating that 200 Hz CES is likely to be associated with less efficiency of afferent input to the spinal neurons compared with the lower frequencies (Weidner *et al.*, 2002). Alternatively, the potentially decreased pain transmission might be due to the nerve refractory periods leading to the decreased efficiency of the neurotransmitter release following the 200 Hz stimulation (Randić *et al.*, 1993). In study II, the pain ratings declined during the 10 Hz process which may be due to habituation or fatigue of nociceptors when the stimulus is repeatedly applied and lasting long time (Rankin *et al.*, 2009; van den Broeke *et al.*, 2012). The pain ratings in session two were lower than session one which was most probably considered to be due to learning effect, i.e., subjects get less pain in session two as a consequence of the previous pain experience in session one (Hopkins, 2000). The pain experience of 10 Hz CES process was lower than 100 Hz CES process which supports the finding that increase of pain at increasing frequencies as mechanosensitive nociceptors and A-δ fibres are prone to follow high-frequency electrical stimulation (Dusch *et al.*, 2007). During neurogenic or inflammatory pain conditions, persistent low-frequency C-fiber nociceptive input to the central nervous system may lead to hyperalgesia (Puig & Sorkin, 1996; Han *et al.*, 2000; Xiao & Bennett, 2007b; Drdla & Sandkühler, 2008). In fact, C-fiber nociceptor discharging tends to follow low frequency (1~10 Hz) electrical stimulation but not the high frequencies electrical stimulation used to induce LTP-like pain amplification in experiments (Raymond *et al.*, 1990). In

addition, different patterns of electrical stimulation are considered to act differently on nociceptive nerve endings resulting in different release of neuropeptides and inflammatory mediators which are important for inducing central sensitization, such as substance P (SP), bradykinin, calcitonin gene-related peptide (CGRP), glutamate, brain-derived neurotrophic factor (BDNF), etc. (Khan *et al.*, 1992; Sauerstein *et al.*, 2000; Lever *et al.*, 2001). A relative higher frequency electrical stimulation (frequency >2 Hz) is likely to play an important role in the release of peptidergic neurotransmitters (Bartfai et al., 1986) whereas the stimulation patterns are important for the neurotrophins releasing (Lever et al., 2001). Therefore, the different pain sensation between sessions may be related to different neuro-mediators involved in different CES paradigms.

In study III, the decreased pain ratings to CES in CPCS session reflected the effect of CPM. This indicated that the endogenous CPM inhibition depressed the pain transmission of CES. CPM activated by a conditioning noxious stimulus applied to a remote location of the body can inhibit the activity of convergent WDR neurons receiving both A- and C-fibre input; hence, the C-fibre response to noxious transcutaneous electrical stimulation could be depressed (Le Bars *et al.*, 1979b). However, CPM showed a lack of inhibition effect on nociceptive specific neurons (Le Bars *et al.*, 1979a). The peptidergic C-fibre nociceptive pathway might be selectively activated by the EPE according to the neurogenic vasodilation flare induced by axon reflex. The facilitation at superficial dorsal horn nociceptive specific and deep dorsal horn WDR neuron connections are important cellular mechanisms underlying central sensitization (Svendsen *et al.*, 1999; Bester *et al.*, 2000; Ikeda *et al.*, 2003). Therefore, the CPM inhibition on WDR neurons most probably is the underlying mechanism for the decreased pain ratings during the CES process in the CPCS session. Furthermore, the decreased pain ratings during the CES process indicated that the endogenous inhibition system might decrease the sensitization or the population of sensitized spinal cord neurons. In addition, the SF-MPQ scores during the CES process were not different between two sessions indicating that the overall pain experience could not be affected by the CPCS. Hence, it can be speculated that CPM plays a major role depressing the pain intensity without showing any effect affecting the pain quality.

2.4. INFLAMMATION RESPONSES

- **Main results**

In study I, no significant difference was found for the SBF between 100 Hz session and control session in the post-conditioning period. SBF was significantly increased within 10 min after 200 Hz CES sessions compared to the control session, after this, it declined. In study I, II, and III, SBF kept at a high level within 10~20 min after 10 Hz CES, then gradually declined. The increased SBF lasted to the end of the observation period (i.e., 60 min) in every CES paradigm.

In study I, ST was not found to be different between all three CES paradigm sessions and the control session. No temporal changes were found for ST in all sessions. In study II, ST was found to decline throughout the observation period. However, no difference was found between the ST 10 min preCES, 10 min postCES, and 20 min postCES. No difference was observed between sessions. However, in study III, ST increased after CES in both sessions which lasted to the end of the observation period.

In study II, no difference was found for SBF and ST between sessions. According to the ICC values within session (ICCwi) and between sessions (ICCbt) (ICCwi=0.79, ICCbt=0.62) and sample sizes estimation for crossover (Ncr) and parallel (Np) study designs (Ncr=3, Np=13), SBF showed acceptable reliability for measuring the neurogenic inflammation when employing 10 Hz CES healthy human model. If the drug effect were hypothesized to have a 30 % recovery of SBF after 10 Hz CES, three subjects would be needed for crossover studies and thirteen subjects would be needed for parallel studies. The Bland-Altman plot of SBF showed that the level of equality (i.e., the mean difference is zero) was within the confidence interval of the mean difference indicating no significant systematic difference was observed between two sessions which further confirmed the results of RM-ANOVA. The differences between two sessions were within the limits of agreement.

In study III, no difference was found between CPCS and control sessions for both SBF and ST.

- **Discussion**

The CES-induced neurogenic inflammation was highly related to neuropeptides released from the peripheral nociceptors (Holzer, 1998). In study I and II, SBF significantly increased after 10 Hz and 200 Hz CES paradigms most probably indicating the activation of peptidergic C-fibre nociceptors leading to axon reflex effects (Low & Westerman, 1989; Klein *et al.*, 2004). However, the stimulation frequencies may act differently on peptidergic afferents leading to different levels of neurogenic vasodilatation. In study I, higher frequency CES (100 and 200 Hz) did not induce a higher blood flow compared with lower frequency (10 Hz) CES. This is supposed to be due to that nociceptors responsible for neurogenic vasodilation could not transfer all impulses delivered at high-frequency electrical stimulation, but had a conduction failure (Dusch *et al.*, 2007). Moreover, compared to high-frequency stimulation, low-frequency stimulation showed a better effect to activate mechanical insensitive “silent” nociceptors which are important for the axon reflex flare and mechanical secondary hyperalgesia (Serra *et al.*, 2004; Dusch *et al.*, 2007; Hendry & Hsiao, 2012). In addition, it has been shown that SP mainly induces protein extravasation whereas CGRP is responsible for neurogenic vasodilatation (Holzer, 1998). In humans, SP and CGRP were found to be released

after electrical stimulation which caused a large flare reaction (Sauerstein *et al.*, 2000). In study II, the increased SBF which lasted for one hour after CES was reliable in both crossover and parallel study designs. This makes SBF assessment a reliable indicator for peripheral neurogenic inflammation to be measured in potential drug testing studies using 10 Hz CES human model. However, skin temperature was not an ideal indicator for recording inflammation responses induced by CES as no differences were found after CES compared to the control session in study I. Furthermore, ST appears to be easily affected by the surrounding circumstances as shown in the different changes in study I and study II. ST could be a reflection of the temperature in both superficial and deep tissues, whereas SBF was focusing on the blood flow changes in superficial skin layers. This also makes SBF a better choice as the circular pin electrodes work on the nociceptive nerve endings which also locate in superficial skin layers. A significant increase of SBF was found before CES in study I, II and III which were due to the process of determining DTh by a series of increasing and decreasing electrical pulses with low stimulation intensities. This process may activate the peptidergic Aδ-fibers (about 20% of Aδ-fibers) and a small proportion of C-fiber nociceptors by the stimulation intensity below pain threshold (Beitel & Dubner, 1976; Georgopoulos, 1976; McCarthy & Lawson, 1989; Mouraux et al., 2010).

In study III, the inflammation responses, including SBF and ST, were not different between CPCS session and control session, which indicated that both sessions could involve the same level of peripheral peptidergic nociceptors activation. This indicated that the inflammation responses were not affected by CPCS. Therefore, it is speculated that CPM effect could not affect the vasodilatation mediators released from peripheral nociceptive nerve endings. This further confirms that the CPM inhibitory effect on pain LTP may take effect at the central nervous system rather than at the peripheral organs. Furthermore, the same neurogenic inflammation findings (i.e., SBF) most likely indicated the similar amount of nociceptive input might be transmitted into the central nervous system in both sessions. The cardiovascular response (i.e. blood pressure) to the conditioning stimulus (i.e. cold water bath) was reported positively associated with the magnitude of CPM effect in healthy controls (Chalaye et al., 2013). However, the SBF was not influenced by CPM in the present study indicating only the systemic circulations but not superficial microvascular circulation is affected by CPCS.

2.5. HOMOTOPIC PAIN RESPONSES

- **Main results**

In study I, the pain intensities to SES were found to increase and reach the plateau at 30 min in the post-conditioning period of all four sessions. However, no difference was found between any two sessions. In study II, the pain intensities to SES, however, were not found to change after the 10 Hz CES and no difference was

found between two sessions. The measurement of perception intensity to SES was reliable after reliability analysis (ICCwi=0.94, ICCbt=0.85). However, a large number of subjects (Ncr=634, Np=11310) would be needed if a 30% of recovery of perception intensity to baseline in potential crossover and parallel drug testing studies. Most of the differences between two sessions were within the limits of agreement. Furthermore, in study III, the pain perception intensities were not found to increase after CES in both sessions either. No difference was found for perception intensities to SES between CPCS and control sessions.

- **Discussion**

The homotopic LTP-like pain amplification is a rather complex phenomenon. In contrast to Klein's study (Klein *et al.*, 2004) which showed an increasing homotopic pain after high-frequency CES, the homotopic pain amplification was absent in all CES paradigms in study I, II and the control session in study III. However, this is in agreement with some other studies without finding a similar homotopic pain hyperalgesia either (van den Broeke *et al.*, 2012; Matre *et al.*, 2013). These three studies showed that the homotopic pain amplification was absent when comparing with control session (study I) and baseline values (study II and III). In animal experiments and slice preparations studies, both 100 Hz bursts of CES and a continuous train of 10 Hz CES were found to induce LTP at the spinal delay of neuro-connections (Terman *et al.*, 2001; Kim *et al.*, 2015). The homotopic pain amplification was thought probably to be a perceptual correlate of nociceptive homosynaptic LTP in spinal dorsal horn (Klein *et al.*, 2004). The TRPV1 receptors in spinal dorsal horn, especially located at superficial layers, have been shown to be involved in spinal LTP induction and development (Yang *et al.*, 2014). It has been reported that the TRPV1-positive C-fiber nociceptors are the main contributors for the induction of homotopic pain LTP, and TRPV1-negative C-fibers could induce a homotopic self-facilitation in the high-frequency CES paradigm (Henrich *et al.*, 2015). In addition, the heat pain hyperalgesia was absent in three studies which are in agreement with the study by Lang and colleagues (Lang *et al.*, 2007). Primary thermal hyperalgesia was reported to be mainly due to sensitization of peripheral nociceptors, and it is an important feature of primary hyperalgesia (Treede *et al.*, 1992). The absence of heat pain hyperalgesia indicated that a lack of peripheral sensitization would be present in CES-induced pain LTP models or heat pain threshold is not a sensitive indicator for measuring heat pain hyperalgesia compared with suprathreshold heat pain stimuli (Yucel *et al.*, 2002; van den Broeke & Mouraux, 2014b). The thermode with a large contact area may concomitantly activate mechanical low threshold afferent fibers when thermo-nociceptive laser stimuli can selectively activate Aδ- and C-fiber nociceptors (Plaghki & Mouraux, 2003). The lack of homotopic pain amplification could be due to several reasons: 1) counter effects of LTP and LTD (long-term depression) resulting from activation of C-fiber and A-δ fiber nociceptive pathways by CES (Pfau *et al.*, 2011); 2) habituation or fatigue because of repeated electrical stimulation, e.g., the fatigue of

C-fiber nociceptors after stepped noxious stimuli with interstimulus intervals less than 150 s (Slugg *et al.*, 2000; Rankin *et al.*, 2009); 3) hypoesthesia which could be induced by continuous 20 Hz CES at C-fiber strength (De Col & Maihöfner, 2008); 4) the time interval is 10 min which may miss the temporal changes as shown in Klein's study using 2 min interval, or 5) the technical reason that the EPE need to be removed and placed back to the original position which may change the impedance between EPE and skin. This may influence the intensity of SES. Reposition of the EPE could also bring variations which could mask the homotopic pain LTP that might be present. In addition, the intensity of single electrical stimulation might not be strong enough, or the pin electrode might not be thin enough to efficiently activate nociceptors when using a single pulse electrical stimulation. In study II, a minor effect of 10 Hz CES was observed to induce homotopic pain amplification which would require a large number of subjects to detect a 30% return to the baseline in potential crossover and parallel drug testing studies. This indicates that further studies are needed to explore the complex underlying mechanisms for homotopic pain amplification in humans.

In study III, the homotopic pain ratings were not found to be inhibited by CPM. CPM induced an inhibitory effect on the pain LTP induction process which however did not affect the efficiency to develop pain amplification at the homotopic skin sites. As CPM seems to take less inhibition effect on nociceptive superficial spinal dorsal horn neurons which play an important role in the induction of nociceptive LTP (Le Bars *et al.*, 1979a; Ikeda *et al.*, 2003), therefore, the homotopic pain LTP might not be completely inhibited by CPM. However, the lack of pain amplification in the control session makes it hard to speculate the effect of CPM on homotopic pain responses. A similar absence of pain LTP to homotopic single electrical stimulation was also presented in the study from van den Broeke EN and colleagues whereas observing increased event-related potentials (van den Broeke *et al.*, 2012). Therefore, the CPM inhibition on homotopic pain amplification could be reflected on the event-related potentials rather than on pain perceptions. This needs further research in the future.

2.6. HETEROTOPIC PAIN RESPONSES

- **Main results**

In study I, the perception intensities to heterotopic pinprick stimuli (12.8 g, 30 g) increased after all three CES paradigms (10 Hz, 100 Hz and 200 Hz) compared to the control session. The increased perception intensities reached to the plateau 30 min in the post-conditioning period. No difference was found between any two of the CES paradigms.

In study II, the pain intensities to higher weight heterotopic pinprick stimuli (30 g and 50.1 g) were found to increase within 10 min after 10 Hz CES in both sessions

whereas the perception intensity to lower weight pinprick stimulus (12.8 g) was found to increase 30 min after 10 Hz CES. The perception intensities in session one were higher than session two for 12.8 g and 30 g pinprick stimulators, whereas no difference was found for the perception intensity to 50.1 g pinprick stimulator between sessions. From the reliability results, the higher weight pinprick stimulator showed higher reliability based on ICC values and a higher weight pinprick stimulator required fewer subjects to detect a 30% return to the baseline (i.e., drug effect) in potential crossover and parallel drug testing studies (see more details in paper II).

In study III, the time effect was found for both 30g and 50g pinprick stimulators. The perception intensity to 50.1 g pinprick stimulator increased after CES which lasted to the end of the observation period in both sessions. However, in 30 g pinprick stimulator testing, the perception intensities were not found to increase after CES in multiple comparisons with Bonferroni-Holm adjustments. The perception intensity to 12.8 g pinprick stimulator was not found increase after CES. The pain rating increments after CES were depressed only in 12.8 g pinprick testing at 40 and 50 min in the post-conditioning period (conditioning stimulus effect) of the CPCS session compared with the control session. The heterotopic stroking perception intensity increased after CES which lasted to the end of the observation period in both sessions. The heterotopic perception intensity increments to light stroking stimuli were found to be lower in the CPCS session compared with the control session.

- **Discussion**

In study I and II, all three (10, 100, 200 Hz) CES paradigms induced heterotopic perception intensity amplificatory states to pinprick (activate A-δ-fibre endings) and light stroking stimuli (activate A-β-fibre endings), however, without showing any significant difference between each paradigm. This indicated that all paradigms presented a similar effect to induce the facilitated surrounding unconditioned A-δ- and A-β-fibre pathways (Lang *et al.*, 2007). It has been reported that pinprick pain is mainly mediated by TRPV1-negative A-fibres whereas LTP of TRPV1-positive C-fibres (major contribution) and A-fibres (minor contribution) neurotransmission is the main cause for heterotopic pinprick hyperalgesia (Ziegler *et al.*, 1999; Henrich *et al.*, 2015). Therefore, a potential interaction is speculated to happen between the two perception transmission pathways. The cutaneous pain amplification at the unconditioned skin site (secondary hyperalgesia) is considered to depend on central sensitization of spinal nociceptive neurons (Simone *et al.*, 1991). This is also in accordance with the study I and II that heterotopic perception amplification was most probably due to the central mechanisms rather than the peripheral mechanisms. First, the sites for pinprick stimuli are outside of neurogenic vasodilation flare which largely eliminates the potential influence of peripheral processes (Sumikura *et al.*, 2003). Secondly, the increasing pinprick and

light stroking perception intensities did not follow the decreased peripheral SBF in the post-conditioning period indicating most probably heterotopic pain LTP comes from the central changes. One cause for heterotopic hyperalgesia could be due to mechanical insensitive "silent" nociceptors which could be activated by the CES (Serra *et al.*, 2004; Hendry & Hsiao, 2012). Moreover, the convergence of A-fiber and C-fiber mediated nociceptive input in spinal dorsal horn WDR neurons provide the anatomic basis for heterotopic pain amplification. After CES applied on peripheral nociceptors, the release of diffusible neurogenic mediators, such as SP and CGRP, at the central terminals of peptidergic nociceptive fibers may expand to facilitate the nearby spinal neurons receiving mechanical A-fibers input (Liu *et al.*, 1994; Sumikura *et al.*, 2003). The simultaneous LTP at heterosynaptic GABAergic synapses which was mediated by activating metabotropic glutamate receptors located at spinal lamina I neurons through glutamate released from primary afferents (Fenselau *et al.*, 2011). In study II, the heterotopic pinprick perception amplification measurement showed better reliability for heavier weight pinprick stimulators and the heavier weight pinprick method need fewer subjects to detect a 30% recovery to baseline in the 10 Hz paradigm. Moreover, this pinprick perception amplification lasted at least one hour which allowed a time course for potential drug testing. Strictly speaking, the pinprick perception amplification for 12.8 g and 30 g stimulator can not be labelled hyperalgesia as the stimulus should be painful before CES and becomes more painful after CES (Loeser & Treede, 2008). The light stroking perception amplification can not be labelled as allodynia either as the stimulus should not be painful before CES and should become painful after CES, but it is not the case either (Loeser & Treede, 2008). The possible reasons may be due to 1) the VAS scale in study II has a non-painful rating range that is different from study I and other studies which may miss the assessment to non-painful stimuli and the subjects probably also give a small number even it is not painful; or 2) the pinprick stimulators are different from the stimulators used in other studies in nature. Additionally, the learning effect could have a less influence on perception intensities to heavier weight pinprick stimulators between sessions. This will help to choose a more reliable and validated painful pinprick stimulator when assessing the mechanical punctate hyperalgesia in future studies.

The heterotopic perception amplification after CES to non-painful mechanical stimuli including pinprick and light stroking stimuli was found to be depressed in the CPCS session compared with the control session in study III. In addition, in all three pinprick stimulators testing, a lower perception intensities always seemed to be present in CPCS session compared to the control session. This tendency of pinprick sensory changes indicated that the endogenous inhibition (i.e., CPM) might have inhibited the sensitization or population of sensitized spinal cord neurons which hence decreased the development of heterotopic pain amplification. Moreover, the decreased pain amplification to non-painful pinprick stimulus took place 40 min after CES whereas the reduction of light-stroking evoked perception intensities occurred 10 min after CES. This indicated that a slow desensitization

process might be involved after the induction of CPM. CPM inhibition could, at least partly, decrease the facilitation of mechanical stimuli transmission. Moreover, the different time courses of non-painful pinprick and light-stroking stimuli suggest their modulation by CPM is mediated by distinct mechanisms. In human studies, conditioning cold pressor test reduced the capsaicin-induced pain intensity and brush-evoked pain intensity (i.e., allodynia) but not the area of allodynia (Witting et al., 1998). This indicates CPM effect working on the magnitude and area of allodynia is selective but not general. LTP of WDR convergent neurons receiving nociceptive (C-fibers) and non-nociceptive stimuli (A-β fibers) which locate in deep dorsal horn play an important role in developing secondary hyperalgesia (Willis, 1993; Svendsen *et al.*, 1997, 1999). It has been shown that LTP can be induced after continuous 10 Hz CES at spinothalamic convergent neurons (Le Bars *et al.*, 1979a; Giesler *et al.*, 1981; Kim *et al.*, 2015). In addition, DNIC was able to modulate the activity of spinothalamic convergent neurons (Dickenson & Le Bars, 1983). Therefore the CPM inhibitory effect on nociceptive WDR neurons could be a potential mechanism for the decreased heterotopic mechanical perception amplification. It can be speculated that CPM could decrease the sensitization level of the WDR neurons or prevent a part of WDR neurons from being sensitized.

CPM showed no inhibition effect on pain amplification to strong, painful pinprick stimulus. Moreover, CPM effect on CES process could not completely prevent the development of perception intensity amplification as it was still found in the CPSC session. It can be speculated that other potential mechanisms contributing to heterotopic pain amplification may not be affected by CPM: 1) The expansion of diffusible neuropeptides such as substance P or CGRP released from the central terminals of peptidergic fibers may cause facilitation of nearby A-δ and A-β neuropathways (Liu *et al.*, 1994); 2) Glutamatergic excitatory interneurons could be activated by CES which may lead to the increased responsiveness of spinal nociceptive projection neurons (Santos *et al.*, 2007); 3) Serotonergic descending facilitation deriving from the rostral ventromedial medulla of the brain stem may cause release of serotonins which could act on central terminals of Aδ-fibers or on NK1-receptor positive neurons in lamina I of the spinal dorsal horn (Pertovaara, 1998; Suzuki *et al.*, 2002; Zeitz *et al.*, 2002). Therefore, to a certain extent, CPM inhibition system could be able to prevent heterotopic perception amplification to non-painful mechanical stimuli in cutaneous CES human model. The endogenous inhibitory pain modulation could help, at least in part, to relieve central sensitization in humans.

2.7. PAIN-LTP IN CHRONIC PAIN PATIENTS

- **Discussion**

The time course of LTP-like pain amplification phenomena demonstrated in the human CES model corresponds to the early phase of LTP demonstrated in animal

models which primarily involves the post-translational modifications like phosphorylation of NMDA- and AMPA-receptors (Klein *et al.*, 2006). The post-translational effects lead to an enhancement of postsynaptic currents boosting synaptic efficacy which play an important role in the maintenance of sensitization of spinal cord neurons (Lynch, 2004). The long-lasting and enlarged area of secondary hyperalgesia is often involved in the development of chronic pain after surgery in clinical studies (De Kock *et al.*, 2001, 2005; Lavand'homme *et al.*, 2005; Wilder-Smith *et al.*, 2010). Moreover, hyperalgesia to mechanical and electrical stimuli is a typical feature of various chronic pain states, including fibromyalgia (Lautenbacher *et al.*, 1994), rheumatoid arthritis (Leffler *et al.*, 2002), osteoarthritis (Kosek & Ordeberg, 2000), low back pain (O'Neill *et al.*, 2007), irritable bowel syndrome (Verne & Price, 2002), headache (de Tommaso *et al.*, 2008), gallstones (Giamberardino *et al.*, 2005), and pancreatitis (Dimcevski *et al.*, 2007). Inflammatory and neuropathic conditions resulting in amplified nociceptive input and spontaneous afferent discharging could lead to enhanced nociceptive barrage into the spinal dorsal horn (Jensen & Baron, 2003). This process may be similar to the CES-induced pain LTP process. Spinal nociceptive LTP has been proposed to be a potential cellular mechanism that can be considered to partially explain the long lasting pain amplification in chronic pain states. Therefore, an effective way to prevent LTP induction or reverse the established pain LTP states is promising in preventing or treating chronic pain states. In human volunteer models, a variety of pharmacological interventions have been found to be able to inhibit the stimulus-induced secondary hyperalgesia and allodynia. First, reduce synaptic transmission at the first nociceptive synapses, such as the classical opioid receptor agonists (Eisenach *et al.*, 1997; Wallace, Ridgeway, *et al.*, 2002; Wang *et al.*, 2008). Second, directly interfere with the NMDA receptor activation, such as ketamine, a non-specific NMDA receptor agonist (Warncke *et al.*, 2000; Wallace, Barger, *et al.*, 2002; Wallace, Ridgeway, *et al.*, 2002). Third, interfere with additional sources of activity-dependent intracellular Ca^{2+} rise, such as gabapentinoids, a voltage-gated calcium channel modulator (Chizh *et al.*, 2007). Therefore, there are many pharmacological interventions can be tested for inhibiting the heterotopic pain amplification by 10 Hz CES paradigm which could be more resemble the sustained low frequency discharging of C-fiber nociceptors during inflammatory/neuropathic pain conditions. This provides a better understanding of CES-induced pain LTP in human models which will help to explore pain alleviating interventions in clinical studies.

2.8. CPM IN CHRONIC PAIN PATIENTS

- **Discussion**

CPM has been shown to be in dysfunction (i.e. less efficient) in most chronic pain patients compared with healthy controls (Lewis *et al.*, 2012), such as osteoarthritis and muscle pain (Kosek & Ordeberg, 2000), whiplash associated disorders (Daenen

et al., 2013; Ng *et al.*, 2013), irritable bowel syndrome (Chang, 2005), fibromyalgia (Kosek & Hansson, 1997; Lautenbacher & Rollman, 1997), temporomandibular disorder (Maixner *et al.*, 1995) and migraine and tension-type headache (Sandrini *et al.*, 2006). The decreased CPM effect was supposed to be due to two possible reasons: 1) the long-term exhausting of endogenous pain inhibition function during the persistent chronic pain or 2) a less inhibitory CPM might be present already at the beginning to predispose pain (Yarnitsky, 2015). However, it is still difficult to clarify this question until now. It has been reported that only brain structure lesions but without ongoing pain can not lead a decreased efficiency of CPM; this indicates that the presence of clinical pain may be a necessary contributor to dysfunctional endogenous inhibition in chronic pain patients (Willer *et al.*, 1990; Perrotta *et al.*, 2012). However, CPM antinociceptive pathway could be modified after pain-relieving surgery (Kosek & Ordeberg, 2000). It has been suggested that the efficiency of CPM before surgery could be used as a biomarker to predict chronic post-surgical pain; a less efficient CPM pre-surgery could be at risk developing chronic pain (Yarnitsky *et al.*, 2008; Landau *et al.*, 2010; Wilder-Smith *et al.*, 2010). In addition, the efficiency of CPM could predict the drug effect for alleviating pain; as a less efficient CPM could have a stronger drug effect for pain relief (Yarnitsky *et al.*, 2012). In animal study, efficient endogenous descending inhibition could protect against the development of chronic neuropathic pain (De Felice *et al.*, 2011). In study III, the decreased pain ratings during CES process after CPCS would indicate that CPM effect should be efficient in the process to induce pain facilitation. The decreased heterotopic perception amplification to non-painful mechanical stimuli indicated that pain LTP as a state of pronociception and efficient CPM as a state of antinociception have a counteracting effect between each other. In clinical studies, cold pressor test also depressed dynamic mechanical allodynia in neuropathic pain patients whereas a lack of inhibition when using ischemic conditioning painful stimulus has been observed (Witting *et al.*, 2003; Tuveson *et al.*, 2007). This also indicates that the endogenous pain modulating system, to a certain extent, can alter chronic neuropathic allodynia. The clinical implication from study III is that the endogenous pain inhibition may counteract the pain facilitated process in the central nervous system at the early phase of pain chronification which may intend to inhibit the development of allodynia in patients.

3. CONCLUSIONS AND PERSPECTIVES

The CES-induced perception intensity amplification in healthy humans has been used as a surrogate model for hyperalgesia and allodynia in persistent inflammatory or neuropathic pain patients (Klein *et al.*, 2004; van den Broeke *et al.*, 2012; Matre *et al.*, 2013). The EPE used in this model can selectively activate superficial nerve endings which are from unmyelinated C- and myelinated A-fibre nociceptor in the skin (Klein *et al.*, 2004, 2008; Hansen *et al.*, 2007; van den Broeke & Mouraux, 2014a). Therefore, this model can be used to mimic the discharging frequency of nociceptors irritated by nociceptive stimuli in certain inflammatory and neuropathic pain conditions. The peripheral CES may change the properties of central nociceptive neurons in the spinal dorsal horn which facilitates ascending nociceptive pathway. A better understanding of this model can help to explore the mechanisms underlying pain chronification and pain modulation. Moreover, from a clinical point of view, these results are important for future pharmacological testing studies when applying this healthy human model. A further exploration of the counteraction between endogenous pain inhibition and pain facilitation will help to better understand the mechanisms behind pain modulation.

From study I, we found that both relative continuous low frequency (10 Hz) and bursts of high frequency (200 Hz) CES were able to induce heterotopic pain-LTP like the traditional bursts of 100 Hz CES whereas with an absence of homotopic pain-LTP in all CES paradigms. However, it is still difficult for CES paradigm to completely mimic the irregular discharging pattern of nociceptors. Therefore, 10 Hz CES which is specially closer to the rather low frequency discharging of nociceptors during inflammatory and neuropathic pain conditions can be a better alternative paradigm inducing pain-LTP in humans. High-frequency CES probably caused more conduction failure of electrical stimulation impulses into the central nervous system. From study II, we found that 10 Hz CES paradigm was still able to induce heterotopic pain-LTP but without showing the homotopic pain-LTP. The 10 Hz CES-induced neurogenic inflammation and heterotopic pain-LTP to painful pinprick and non-painful light stroking stimuli showed acceptable reliability. From study I and II, the pain perception amplification at the homotopic site may involve a far more complex pain transmission and modulation process, or the methodology differences between other studies may result in the absence of homotopic pain-LTP. As a counterpart of pain facilitation system, the pain inhibitory system may counteract with the facilitated pathways which may decrease the efficiency of pain amplification. As WDR neurons located at deep dorsal horn playing an important role in nociceptive LTP can be depressed by CPM demonstrated by a “pain inhibits pain” phenomenon. Therefore, both nociceptive LTP and CPM have the same

working point in the spinal cord. Based on this theory, in study III, we found a decreased pain perception intensity during the CES process and decreased heterotopic perception intensity amplification to non-painful mechanical stimuli indicating that the endogenous pain inhibition system may play a role in preventing pain facilitation.

- **Conclusions:**

1. Both low-frequency (10 Hz) and high-frequency (200 Hz) CES paradigms can induce heterotopic mechanical perception amplification like the traditional 100 Hz CES paradigm but 10 Hz CES is associated with a less pain during the CES process (Study I).
2. Based on sample size calculation and reliability analysis for the outcome measures, in 10 Hz CES-induced pain LTP human model, superficial blood flow is reliable for neurogenic inflammation assessment and painful pinprick and light stroking stimuli are reliable for measuring heterotopic perception amplification (Study II).
3. CPM can depress heterotopic mechanical LTP-like perception amplification to non-painful mechanical pinprick and light stroking stimuli whereas not to painful pinprick stimuli. CPM may not modulate homotopic pain perception and peripheral neurogenic inflammation (Study III).

- **Limitations and Perspectives:**

1. The reposition of EPE during the measurement of the homotopic pain amplification in this project may cause variations and mask pain perception changes. Therefore, further research with fixed EPE placed on the skin would provide more comprehensive understanding for homotopic pain LTP.
2. The stimulation intensity of SES used in this project (10×Dth) might not be strong enough when using single electrical pulse to activate neurogenic nociceptors. Therefore, SES with stronger stimulation intensities (20×DTh or higher) may activate nociceptors more efficiently which may help to present homotopic pain LTP.
3. The pin electrodes used in this project might be not thin enough to efficiently activate superficial nociceptive nerve endings. Therefore, from a technical point of view, a thinner pin electrodes may be a potential further research to compare the efficiency to induce LTP-like cutaneous pain amplification in the future.
4. In study I and II, there was no non-CES control session/site. The absence of homotopic pain LTP compared to the baseline assessments might be due to habituation to SES. Therefore, it is possible that habituation may have covered the homotopic pain LTP. The participation of non-CES control session/site would increase the scientific impact of the study.

5. Event-related potentials (ERPs) can be used to investigate the responsiveness of the central nervous system to electrical stimuli when there are no changes of pain perception in the homotopic area.
6. Compared with high-frequency CES, 10 Hz CES is closer to discharging frequency of nociceptors during inflammatory/neuropathic pain conditions. Therefore, 10 Hz CES paradigm may have more biological significance and can be used in potential drug testing studies.
7. The electrical stimulation patterns can be further explored in future studies to be more like the irregular low frequency discharging, such as the number of impulses or bursts, burst time intervals, bursts of different frequencies, stimulation time duration, electrical stimulation intensity, etc.
8. CPM, as shown to be dysfunctional in chronic pain patients, showed its pain modulatory role in the development of cutaneous pain amplification after CES. This not only provides a better understanding of CPM endogenous pain inhibitory function but may also help to develop new and efficient analgesic treatments which could specially interact with CPM in chronic pain patients.

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