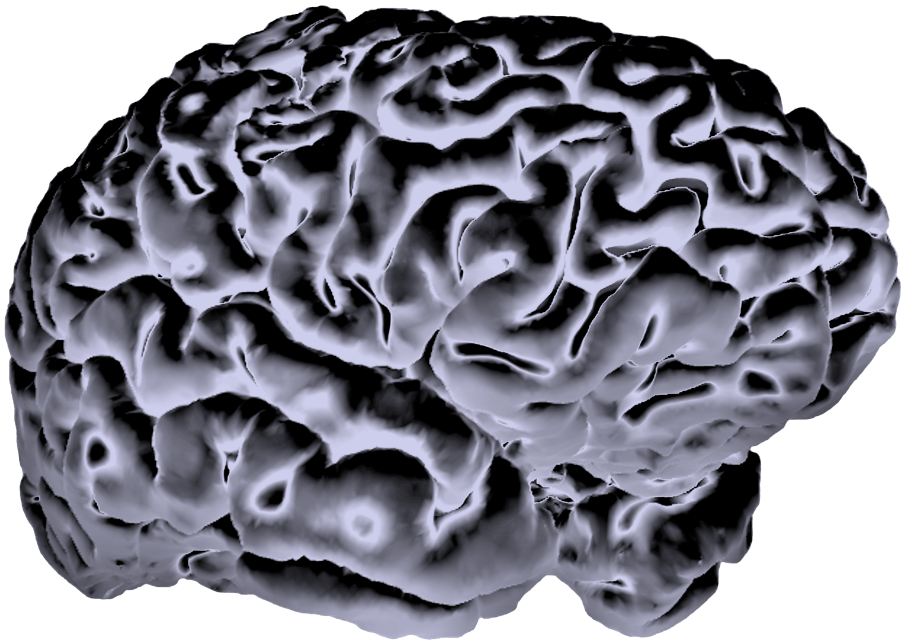
UNIVERSITY OF COPENHAGEN
FACULTY OF HEALTH AND MEDICAL SCIENCES

Secondary hyperalgesia

- Studies on phenotypic expression, brain anatomy, and brain connectivity in healthy men



PhD thesis

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Preface

I found my way into research during medical school where I attended a lecture with the distinguished professor in anaesthesiology, Jørgen Berg Dahl. Immediately after the lecture, I walked over to the professor and asked him how I could get to work with him. He told me to contact him again on a later time so he could set up a formal meeting. Three e-mails, two weeks, and one interview later, we arranged for a six-month stay at the department the upcoming semester. When the six months came to an end, I was fortunate enough that Jørgen (now on a first-name basis) offered me a six-month extension. During the next couple of years, I finished medical school and kept working together with Jørgen on various projects. One year after I completed medical school, I was offered a position as a PhD-student with Jørgen as the primary supervisor. I accepted the position without a doubt in my mind and began my three-year period as a PhD-student at the Department of Anaesthesiology at Rigshospitalet.

During my three years at Rigshospitalet, I have been fortunate to meet and work with extremely kind, helpful, and intelligent people. All have in their own way helped me during the last three years, and I would not be without a single one.

First and foremost, I want to thank my supervisors who have guided me through the past three years with dedication and a high degree of professionalism. To Jørgen, who saw potential in a blue-eyed medical student in his fourth year, and who has been a constant source of inspiration, a dedicated researcher, and always remembered to ask me how I felt when the long hours were taking its toll on me. To Sohail, who always pushed me to do my best and inspired me to do better, and not only became a mentor, but also a good friend. To Jørn, who taught me all I know about research methodology, and who always seems to amaze me with his keen eye for detail and his dry sense of humour. And lastly, to Johan, who expertly helped me with MRI calculations, guided me through computer programs I had never heard of, and laughed with me over expressions such as “Subcortical Bert”. Thank you.

My gratitude also goes out to my co-authors. Thanks to Rebecca Østervig for the hundreds of hours spent in the pain laboratory. Thanks to Christian Pipper for the extensive statistical computations and for always answering my questions with a high degree of patience. Thanks to Janus Nybing for the solid work in getting the MRI-sequence to work. Thanks to Inger Havsteen for providing expert consultation in neuroanatomy, and thanks to Anders Christensen and

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I also extend my gratitude to Chief of the anaesthesiology department, Jakob Trier Møller, for providing me with office space, solid advice, and a good laugh when I needed one. The same goes out to the current and previous staff at the department, Marianne, Linda, Charlotte, and Helle, and to Tina Calundann for always helping me with layout and images.

Also, thanks to Professor Lars Rasmussen for his expert guidance, his challenging questions, and of course, his jokes. A special thanks to my closest colleagues and PhD comrades in arms, Maria Louise, Kamilia, Marco, Camilla, Heidi, Julie, and Pia, who I have spent so much time with the last three years. Also, thanks to Manuel Pereira for professional sparring and good company.

Finally, a last thank you to my friends and family who have given me moral support (and warm food) when they knew I needed it, and to my girlfriend, Emilie who always supported me and with whom I could not fathom to be without.

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List of papers

This thesis is based on the following papers:

1. Hansen MS, Wetterslev J, Pipper CB, Østervig R, Asghar MS, Dahl JB. The Area of Secondary Hyperalgesia following Heat Stimulation in Healthy Male Volunteers: Inter- and Intra-Individual Variance and Reproducibility. PLoS One 2016;11(5):e0155284.

2. Hansen MS, Wetterslev J, Pipper CB, Asghar MS, Dahl JB et al. Heat pain detection threshold is associated with the area of secondary hyperalgesia following brief thermal sensitization: a study of healthy male volunteers. J Pain Res 2017;10:265-74.

3. Hansen MS, Asghar MS, Wetterslev J, Pipper CB, Mårtensson J, Becerra L, Christensen A, Nybing JD, Havsteen I, Boesen M, Dahl JB. The propensity to develop central sensitization is not correlated to pain relevant brain structures - a 3-tesla MRI study of healthy male volunteers. *Under review*.

4. Hansen MS, Becerra L, Dahl JB, Borsook D, Mårtensson J, Christensen A, Nybing JD, Havsteen I, Boesen M, Asghar MS. Brain resting state connectivity in the development of secondary hyperalgesia. *Under review*.

List of abbreviations

AMPA	Amino-3-hydroxy-5-methyl-4-isoxazole propionate
AUC	Area under the curve
BDNF	Brain-derived neurotrophic factor
BOLD	Blood oxygen level dependent
BTS	Brief thermal sensitization
CI	Confidence interval
CNS	Central nervous system
CV	Coefficient of variation
DTI	Diffusion tensor imaging
EBLUP	Estimated best linear unbiased predictor
fMRI	Functional magnetic resonance imaging
FMRIB	Oxford centre for functional magnetic resonance imaging of the brain
FSL	Functional magnetic resonance imaging brain software library
HADS	Hospital anxiety and depression scale
HADS-A	Hospital anxiety and depression scale – Anxiety
HADS-D	Hospital anxiety and depression scale – Depression
HPDT	Heat pain detection threshold
IC	Independent component
ICA	Independent component analysis
ICC	Intraclass correlations
IQR	Interquartile range
MNI	Montreal neurological institute
MRI	Magnetic resonance imaging
NMDA	N-methyl-D-aspartate
PCS	Pain catastrophizing scale
p-TS	Pain during 1 minute thermal stimulation
VAS	Visual analogue scale

List of figures and tables

Figures

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Dansk resume (Danish summary)

Smerter påvirker et stort antal mennesker i deres dagligdag, og ca. en femtedel af den europæiske befolkning, uanset køn, alder, uddannelse og etnicitet, lider af kroniske eller hyppigt tilbagevendende smerter. Dette har ofte svære, invaliderende psykiske og sociale følger for individet og udgør en massiv udfordring for sundhedsvæsenet.
Central sensibilisering; et fænomen der involverer talrige forandringer i det centrale nervesystem, har vist sig at bidrage til udviklingen af smerter samt spille en væsentlig rolle i udviklingen af kroniske smerter. Central sensibilisering kan manifestere sig på flere måder, bl.a. som *sekundær hyperalgesi*. Ved sekundær hyperalgesi ses øget følsomhed for tryk i rask væv der omgiver et skadet væv. Sekundær hyperalgesi kan fremkaldes hos raske mennesker ved brug af eksperimentelle smertemodeller, og forskning tyder på, at der er stor variation i hvordan sekundær hyperalgesi udtrykkes hos raske individer. Således udvikler nogle individer store sekundære hyperalgetiske arealer, mens andre udvikler små arealer. Ved at udløse sekundær hyperalgesi under standardiserede forhold kan central sensibilisering undersøges under kontrollerede betingelser. Vores viden om central sensibilisering er begrænset, og det er endnu uvist om nogle individer er disponeret til at udvikle central sensibilisering, og om dette indebærer en øget risiko for at udvikle svære smerter og kroniske smertetilstande.
Denne Ph.d.-afhandling udgøres af fire studier. Det overordnede formål med disse studier var at øge indsigten i fænomenet central sensibilisering ved at undersøge om den individuelle disposition til at udvikle central sensibilisering, vurderet ved sekundær hyperalgesi som surrogatmarkør, var associeret med kvantificerbare karakteristika hos raske, unge mænd.

I studie I fandt vi at den eksperimentelle smertemodel, kort termal sensibilisering, udløste et sekundært hyperalgetisk areal som var reproducerbart over tid, samt at den inter-individuelle variation var høj. Dette betyder at kort termal sensibilisering kan anvendes til at undersøge sekundær hyperalgesi hos raske mænd, og derfor kunne vi med fordel anvende metoden i vores efterfølgende studier.
I studie II fandt vi, at et stigende sekundært hyperalgetisk areal var associeret med en faldende varme-smerte tærskel. På trods af en høj-signifikant association kunne det enkelte individs varme-smerte tærskel kun forklare 19% af variationen i det sekundære hyperalgetiske areal.

Dette fund tyder på, at udviklingen af sekundær hyperalgesi og varme-smerte tærskler er to delvist uafhængige processer som repræsenterer forskellige aspekter af smertefysiologien.
I studie III MR-scannede vi forsøgsdeltageres hjerner og fandt, at størrelsen af det sekundære hyperalgetiske areal ikke var associeret med volumen af de hjernestrukturer som er relevante for smertebearbejdning. Dette resultat indikerer, at det enkelte individs evne til at udvikle central sensibilisering ikke afhænger af de smerterelevante hjernestrukturers volumina.
I studie IV anvendte vi funktionel MR-scanning til at undersøge forsøgsdeltagernes hjerneaktivitet i hvile. Vi fandt, at et stigende sekundært hyperalgetisk areal var associeret med ændret aktivitet i talrige hjernestrukturer i hvile, og at individer med forskellig størrelse sekundært hyperalgetisk areal udviste forskellig hjerneaktivitet i hvile.

Denne afhandling giver indsigt i flere aspekter af central sensibilisering. Specifikt finder vi at 1) kort termal sensibilisering kan bruges til at undersøge sekundær hyperalgesi hos raske, unge, mænd, og herved formentlig anvendes til at fænotype raske unge mænd på basis af deres sekundære hyperalgetiske arealer; 2) udviklingen af sekundært hyperalgetisk areal og varme-smerte tærskler repræsenterer to forskellige og delvist uafhængige aspekter af smertefysiologien; 3) dispositionen til at udvikle central sensibilisering, vurderet ved udviklingen af sekundær hyperalgesi, er ikke associeret med volumina af smerterelevante hjernestrukturer; og 4) individer med forskellig størrelse sekundært hyperalgetiske areal udviser muligvis forskellig hjerneaktivitet i hvile.
Samlet set tyder vores fund på, at måden hvorpå raske, unge mænd registrerer og bearbejder smerter er associeret med størrelsen af deres sekundære hyperalgetiske areal.

English summary

Pain constitutes a major health care problem and affects all populations regardless of age, sex, income, or ethnicity. Chronic pain is highly prevalent, affecting up to one-fifth of the European population, often with multiple concomitant sequelae including depression, inability to work, and social alienation. Evidence suggests that central sensitization; a phenomenon that encompasses multiple changes in the central nervous system (CNS) contributes to pain hypersensitivity and the maintenance of acute and chronic pain. Secondary hyperalgesia is a clinical manifestation of central sensitization where receptive field expansion facilitates signalling from mechanical stimulation in non-injured tissue adjacent to injured tissue to be perceived as painful. Secondary hyperalgesia can be elicited using experimental pain models, and evidence indicates that the size of the secondary hyperalgesia area following stimulation with an experimental pain model varies between different individuals, with some individuals developing large areas and others developing small areas. By eliciting secondary hyperalgesia under standardised conditions, researchers are able to study central sensitization in healthy volunteers in a controlled setting. It remains largely unknown if certain individuals have a higher innate propensity to develop central sensitization, and if these individuals are at higher risk of developing pain hypersensitivity and chronic pain conditions.

The overall aim with the four studies included in this thesis was to increase the insight on central sensitization, and investigate if individual propensities to develop central sensitization, assessed as secondary hyperalgesia areas, were associated with quantifiable individual characteristics in healthy men.

In study I we found that the pain model, brief thermal sensitization (BTS), elicited secondary hyperalgesia with a high level of reproducibility, and that it could be employed in investigations of secondary hyperalgesia in healthy men.

In study II we found that an increasing area of secondary hyperalgesia was associated with a decreasing heat pain detection threshold (HPDT). However, although highly significant, the association was weak (R^2 of 19%), indicating that HPDT only provided a modest explanation of the inter-individual variation in secondary hyperalgesia area, suggesting that HPDT and areas of secondary hyperalgesia may represent two independent pain entities.

In study III we applied anatomical magnetic resonance imaging (MRI) and found that the size of the secondary hyperalgesia area was not associated with the volume of the caudate nuclei or any other predefined brain structure relevant for pain processing. Our findings suggest that the propensity to develop central sensitization, assessed as secondary hyperalgesia, is not associated with pain relevant brain structure volume.
In study IV we applied resting state functional MRI and found that an increasing size of secondary hyperalgesia area was associated with both increased and decreased connectivity in multiple brain structures, and that individuals with different phenotypic expressions of secondary hyperalgesia exhibited significant differences in resting state connectivity.

In summary, the findings from the studies included in this thesis provide insight in some aspects of central sensitization. Specifically, the present findings indicate that 1) BTS can be employed in the investigation of secondary hyperalgesia in healthy young men and may have the potential to phenotypically assess healthy young men based on their area of secondary hyperalgesia; 2) areas of secondary hyperalgesia and HPDT may represent two relatively distinct independent pain entities; 3) the propensity to develop central sensitization, assessed as secondary hyperalgesia, is not correlated to the volume of pain relevant brain structures; and 4) that individuals with different phenotypic expression of secondary hyperalgesia may exhibit significantly different resting state connectivity.
Finally, the present findings suggest that healthy men with different phenotypic expressions of secondary hyperalgesia may potentially process pain differently.

1. Introduction

In the healthy individual, pain acts a protective mechanism keeping us from further injury by generating rapid withdrawal reflexes and creating unpleasant sensations inducing complex behavioural adaptions to avoid future contact with the noxious stimulus[1]. However, when pain becomes pathological, the beneficial effects wane, and the adaptive mechanisms of pain are no longer protective. Pain as a symptom and a disease is a major health care problem and affects all populations regardless of age, sex, income, or ethnicity[2]. Pain is a frequent cause of primary care consultations[3], a substantial problem in the postoperative period[4], and constitutes a huge socio-economic burden[5]. Persistent pain is highly prevalent affecting up to one-fifth of the European population[6], often with multiple concomitant sequelae including depression, inability to work, and social alienation[6]. Improvement of pain therapy, identification of novel therapeutic targets, and new insights in to basic pain physiology are therefore not only of high importance to the individual patient, but also vital for the society as a whole.

Considerable efforts have been invested into studying the reasons why some individuals develop higher pain responses than others, and if some individuals are more prone to develop chronic pain. Several predictive variables have been identified including sex[7-9], genetics[10-14], and psychological factors[15-17], but it remains unknown if some individuals have an innate higher propensity to develop high pain responses, and if potential causal factors exist.

Large cohort studies have revealed a high comorbidity of several diverse pain conditions without clear inflammatory or neuropathic pathology[18-20]. A co-occurrence of pain conditions such as fibromyalgia, low back pain, and chronic tension type headache in the same patients suggests that these conditions may have a common mechanistic basis[21]. Likewise, findings from several studies have suggested that a heightened sensitivity or reactivity of the central nervous system (CNS) may be a common feature of these syndromes, and a potential enhanced capacity to produce or maintain a heightened sensitivity of the CNS may serve as a primary pathological defect in some of these conditions[21]. The CNS's ability to modulate and amplify nociceptive signalling, and hereby increase the sensitivity of the CNS to peripheral noxious stimuli, is labelled central sensitization[1,21]. The overall aim with this thesis was to increase the insight on central sensitization, and investigate if individual propensities to develop central sensitization, assessed as secondary hyperalgesia areas, were associated with quantifiable individual characteristics.

2. Background

2.1 Central sensitization

Central sensitization represents an altering or amplification of neuronal signalling, inducing augmented pain responses and profound changes in the fundamental properties of pain sensation[21]. Central sensitization is a phenomenon that encompasses multiple changes in the CNS contributing to pain hypersensitivity and the maintenance of acute and chronic pain[1,21]. Multiple mechanisms are involved in central sensitization and comprises upregulation of amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) and N-methyl-D-aspartate (NMDA) receptors[1], upregulation and release of brain-derived neurotrophic factor (BDNF) in the dorsal horn neurons[22], down-regulation of inhibitory interneurons in the dorsal horn, and dorsal horn neural sprouting[23]. Moreover, changes in astrocytes, microglia, and gene transcription, as well as supraspinal brain activity may be involved in the maintenance of central sensitization[21]. Central sensitization disrupts the otherwise normal nociceptive stimulus-response relationship and represents a state in which pain can be elicited by supra- and subthreshold inputs of both noxious and innocuous character[1,21]. Clinically, central sensitization manifests as allodynia (painful perception of non-painful stimulation), secondary hyperalgesia (increased painful perception in non-injured tissue adjacent to injured tissue), aftersensations (perception of a stimulation after the stimulation has terminated), and enhanced temporal summation (augmented perception of pain due to repetitive painful stimulation)[1,21,24].

A repeated or sustained intense noxious stimulus induces acute activity-dependent central sensitization. This serves as an adaptive and protective mechanism by limiting the use of a potential injured body part, hereby facilitating healing. Central sensitization in the healthy individual may therefore be an adequate response to intense noxious stimulation. However, central sensitization becomes pathological when the noxious stimulation, inflammation, or pathological process persist, which results in a longer-lasting transcription depending central sensitization[1].

The pathological effects of central sensitization are seen in various chronic pain conditions, and central sensitization is believed to be important in the transition from acute to chronic pain, and in the maintenance of the chronic pain state. It remains largely unknown if certain

individuals have a higher innate propensity to develop central sensitization, and if these individuals are at higher risk of developing pain hypersensitivity and chronic pain conditions.

2.1.1 Secondary hyperalgesia

Secondary hyperalgesia is a clinical manifestation of central sensitization where receptive field expansion facilitates signalling from mechanical stimulation in non-injured tissue adjacent to injured tissue to be perceived as painful[1,21,24]. Secondary hyperalgesia can be elicited using standardised pain models, allowing researchers to study central sensitization in healthy volunteers under standardised conditions[25-29]. Cutaneous secondary hyperalgesia can be induced by different stimuli such as, heat[25-28,30-37], cold[38,39], electrical[40] , and chemical[41-44] stimulation. The most commonly used model for eliciting cutaneous secondary hyperalgesia involves heat stimulation, often in combination with capsaicin[26,30-35,37,45-47].

Evidence indicates that the magnitude of experimentally induced areas of secondary hyperalgesia varies between individuals, but remain fairly constant within each individual; suggesting that areas of secondary hyperalgesia may represent a phenotypic characteristic[25,29]. It has been suggested that individuals suffering from chronic pain conditions, including fibromyalgia and rheumatoid arthritis, display larger areas of secondary hyperalgesia when compared to healthy individuals[48,49]. Moreover, current evidence suggests a correlation between increasing area of secondary hyperalgesia surrounding surgical incisions and the development of postoperative persistent pain[50-53]. The correlation, independent of surgical characteristics such as, length of incision, volume of deep tissue trauma, and nerve lesion severity, indicates that the development of secondary hyperalgesia reflects individual predisposition rather than intraoperative trauma. Areas of secondary hyperalgesia following surgery or following standardised stimulation with pain models may thus be used as a model to evaluate the individual level of central sensitization.

2.2 Pain models

Pain models are necessary when investigating basic physiologic responses to pain under standardised conditions. Pain models applying standardised heat, chemical, or electrical stimulation can readily induce central sensitization in healthy volunteers. Moreover, other types of pain models applying stimuli such as pressure, heat, and cold can be applied to investigate tolerance and thresholds for various stimuli. In the studies presented in the current thesis, we applied the pain models *Brief thermal sensitization* (BTS), *Heat pain detection threshold* (HPDT), and Pain *during 1 minute thermal stimulation* (p-TS).

2.2.1 Brief thermal sensitization

Brief thermal sensitization (BTS) induces primary and secondary hyperalgesia by applying a three-minute long heat stimulation of the skin[26,32,33,36,54]. Primary hyperalgesia is induced at the site of the stimulation, and secondary hyperalgesia is located in normal tissue around the traumatized area. The area of secondary hyperalgesia has reduced thresholds for mechanical stimulation and can be estimated by pin-prick stimulation. Secondary hyperalgesia to pin-prick stimulation is mediated by myelinated heat- and mechano-sensitive type I and myelinated mechano-sensitive (heat-insensitive) A-fibre nociceptors[24,55,56]. However, recent evidence indicates that only mechano-sensitive (heat-insensitive) A-fibre nociceptors are involved in the mediation of secondary hyperalgesia to punctate mechanical stimulation[57].
BTS has been used in several studies[26,32,33,36,54], and a meta-analysis investigating three separate heat pain models (BTS, heat/capsaicin sensitization, and a 47°C burn injury) found that BTS had the highest level of reliability[29].

2.2.2 Heat pain detection threshold and pain during one minute thermal stimulation

Heat pain detection threshold (HPDT) and pain during one minute thermal stimulation (p-TS) have been applied in several studies[32,35,36,58] and are used as measures of the cutaneous heat pain sensitivity. The two tests supplement each other, each describing different aspects of cutaneous heat pain sensitivity. HPDT provides an estimate of the temperature at which heat stimulation is perceived as painful, whereas p-TS supplements with a measure of how painful the cutaneous heat stimulation is perceived at a specific temperature. According to animal and human studies, the rapid and continuous heating of the skin during HPDT and p-TS respectively

may be transmitted by myelinated A-fibre type II mechano- and heat-sensitive nociceptors (in hairy skin) and unmyelinated mechano- and heat-sensitive C fibres[55].

2.3 Magnetic resonance imaging

Magnetic resonance imaging (MRI) can be applied when studying the anatomical and functional properties of the CNS.

MRI relies on the magnetic properties of specific atomic nuclei. The hydrogen nucleus is present in water molecules and is abundant throughout the human body, making it ideal to support measurements at high spatial resolution. The MRI-scanner produces a strong magnetic field partially aligning the spin-state of all hydrogen nuclei. By applying a varying magnetic field, resonant radio frequency waves are emitted, and the spinning nuclei are flipped/rotated. When the varying magnetic field is turned off, the protons will oscillate and gradually return to their equilibrium (precession), simultaneously emitting a radio signal. The radio signals emitted from the oscillating hydrogen nuclei are detected using antennas (coils) and used to create images. The time it takes for the hydrogen nuclei to realign with the strong magnetic field, as well as the strength of the radio signals emitted by the nuclei, depend on the environment and positions of the nuclei. This means that hydrogen nuclei in different tissues return to their equilibrium at different rates, which allows the MRI-scanner to distinguish among different tissues and produce detailed images of soft tissue structures[59,60].

Using functional MRI (fMRI), we can indirectly estimate the neuronal activity of the brain. Augmented neuronal activity increases the demand for oxygen, inducing an increase in local blood flow to the respective brain region. The local increase in blood flow overcompensates for the temporary activity-induced decrease in oxygenated haemoglobin resulting in a relative decrease in deoxygenated haemoglobin[61]. Since oxygenated and deoxygenated haemoglobin have different magnetic properties[62,63], with deoxy-haemoglobin being paramagnetic, the relative decrease in deoxy-haemoglobin influences the MR-signal, introducing the use of Blood-Oxygen-Level-Dependent (BOLD) contrast to detect brain activity[59].

The BOLD-signal is commonly used in neuroscience as a non-invasive method to evaluate brain activity during task-management, external stimulation, or in the resting individual (resting state). With resting state fMRI, we can detect spontaneous, low-frequency (<0.1 Hz) synchronous fluctuations in the BOLD-signal. Brain structures demonstrating high temporal

correlated oscillations are believed to be connected in intrinsic connectivity networks, and activity in these networks is believed to reflect the functional communication between different brain structures[64]. Consequently, resting state fMRI allow us to evaluate resting state connectivity networks and the brain's functional organization in the resting individual. Resting state fMRI provides a unique opportunity to easily investigate network connectivity in individuals not able to perform tasks and offers a non-invasive approach to investigate pain processing in the CNS[65,66].

3. Objectives

The main objective of this thesis was to increase the insight on central sensitization in healthy individuals, and to investigate whether individual levels of central sensitization, assessed as secondary hyperalgesia areas, were associated with cutaneous heat pain sensitivity, structural brain anatomy, and functional brain connectivity. Accordingly, we conducted four studies with the following research questions.

1. Does the clinical pain model, *brief thermal sensitization (BTS)*, elicit a reproducible area of secondary hyperalgesia?

The objective of this study was to investigate the intra- and inter-individual variance in secondary hyperalgesia area elicited by the clinical pain model, BTS. Secondarily, we aimed to investigate how precise the Pain Catastrophizing Scale (PCS), Hospital Anxiety and Depression Scale (HADS), HPDT, and p-TS predicted the size of the area of secondary hyperalgesia.

2. Is the propensity to develop central sensitization, assessed as secondary hyperalgesia area, associated with the heat pain detection threshold (HPDT)?

The objective of this study was to investigate if the area of secondary hyperalgesia was associated with the HPDT. We hypothesised that the area of secondary hyperalgesia and HPDT represented two predominantly independent entities, and that the area of secondary hyperalgesia was poorly explained by the HPDT.

3. Is the propensity to develop central sensitization, assessed as secondary hyperalgesia area, associated with the volume of brain structures relevant for pain processing?

The objective of this study was to investigate the association between the size of the area of secondary hyperalgesia and the volumes of brain structures relevant for pain processing. The primary outcome measure focused on the volume of the caudate nuclei, and the secondary outcome measures focused on the volumes of the primary somatosensory cortex, anterior and mid cingulate cortex, putamen, nucleus accumbens, globus pallidus, insula, and the cerebellum.

4. Is the propensity to develop central sensitization, assessed as secondary hyperalgesia area, associated with the connectivity in known resting state networks?

The objective of this study was to investigate the association between the size of the area of secondary hyperalgesia and the connectivity in known resting state networks, and to investigate possible differences in resting state connectivity when comparing participants with small (lower quartile) vs. large (upper quartile) areas of secondary hyperalgesia.

4. Methods

Below follows a brief summary of the general methodology. Detailed information regarding the study designs, methods and analyses is available in the supplementary manuscripts. A short presentation of the design, methods, and results for each individual study will be presented in section 5.

4.1 General methodology

We conducted four prospective studies, all approved by the Danish Committee on Health Research Ethics and by the Danish Data Protection Agency. All four studies were registered at ClinicalTrials.gov (study I: NCT02166164, study II: NCT02527395, study III and IV: NCT02567318), and study protocols for study II, and study III and IV were published as separate publications[67,68].

In study I, we included 50 participants, and 121 participants were included in study II, III, and IV.

4.1.1 Study participants

Healthy male participants aged 18-35 years were included in all four studies. Oral and written informed consent was obtained prior to inclusion in all studies. In study I and II, the participants received EUR 20 per hour for their participation. In study III and IV, the participants received EUR 67 for their participation in the entire study. Participants who completed study II were subsequently included in study III and IV.

A summary of inclusion and exclusion criteria is listed in table 1.

Table 1. Inclusion and exclusion criteria in study I-IV

	Study I	Study II	Study III	Study IV
Participants (n)	50	121*	121*	121*
Inclusion criteria				
Sex	Men	Men	Men	Men
Age (years)	18-35	18-35	18-35	18-35
Other	NA	NA	Participation and completion of study II	
Exclusion criteria				
General exclusion criteria applied in study I-IV	Weekly intake of >21 units of alcohol, or intake of >3 units of alcohol within 24 hours before study day Substance abuse, assessed by the investigator Use of prescription medicine within 30 days before study day Chronic pain Psychiatric diagnoses Body Mass Index >30 kg/m^2 or <18 kg/m^2			
Study specific exclusion criteria	Analgesics within 2 days before study day	Analgesics within 3 days before study day Antihistamines within 2 days before study day Neurological illness Eczema, wound, or sunburn on the sites of stimulation	Analgesics within 3 days before study day Antihistamines within 2 days before study day Neurological illness Eczema, wound, or sunburn on the sites of stimulation Caffeine within 24 hours before study day Unwillingness to be informed regarding potential pathological findings in relation to MRI Trauma resulting in pain and administration of analgesics in the period between pain testing and MRI scan Head trauma in the period between pain testing and MRI Contraindications to MRI	

* Represents the same 121 participants
Abbreviations: MRI, magnetic resonance imaging; NA, not applicable

4.1.2 Pain models

In the four studies included in this thesis, we applied three different cutaneous heat pain models, and one method to evaluate secondary hyperalgesia. All heat stimulations were applied by the computer-controlled Somedic Senselab MSA Thermotester^{TM} using a 2.5 x 5 cm thermode.

Brief thermal sensitization (BTS)

BTS is a cutaneous heat pain model where the skin is heated to 45°C for three minutes. After the three minutes heating of the skin, the area of secondary hyperalgesia was quantified with the thermode remaining on the skin at a 45°C temperature. The assessment of secondary hyperalgesia took approximately 1-2 minutes, resulting in a total time of heat stimulation of maximum 5 minutes. The thermode was placed anterior on the upper right thigh in the midline between the anterior superior iliac spine and the base of patella (figure 1)[26,32,33,36,54].

Assessment of secondary hyperalgesia

In the studies included in this thesis, we evaluated the size of the secondary hyperalgesia area by pin-prick stimulation with a monofilament (Von Frey hair) with a nominal value of 18 (bending force 490 Millinewton) in four linear paths arranged with the thermode as centre. The pin-prick stimulation began on normal skin well outside the area of secondary hyperalgesia and advanced in steps of 5 mm/second. When the participant reported a clear change in sensation (increased tenderness, burning, pricking), the location was marked with a felt pen, and longitudinal and transverse axes were measured with a measuring tape for rectangular area calculation (figure 1)[26,45,47,69].

Heat pain detection threshold (HPDT)

HPDT represents the lowest temperature at which heat stimulation is perceived as painful. We evaluated the HPDT by placing the thermode with a starting temperature of 32°C on the volar side of the dominant lower arm and increasing the temperature with 1°C/second. When the participant perceived the heat stimulation as painful, he pressed a button, the temperature was recorded, and the thermode returned to the initial temperature of 32°C. If the thermode reached a temperature of 52°C, the thermode would automatically return to 32°C, and the HPDT would be recorded as 52°C. The HPDT was calculated as a mean of four stimulations with

an interval of 6-10 seconds (figure 1)[32,35,36,58]. During the HPDT assessment, the participant was not able to see the computer screen.

Pain during one minute thermal stimulation (p-TS)

p-TS was evaluated by placing the thermode on the volar side of the non-dominant lower arm. The skin was heated to 45°C for one minute, while the participant continuously evaluated the pain caused by the heating thermode[32,35,36,58]. The pain was evaluated on an electronic visual analogue scale (VAS) (Somedic USB-VAS) ranging from 0-100 mm, where 0 mm represented "no pain", and 100 mm represented "worst pain imaginable". A maximum VAS-score (p-TS VAS-max) and an area under the curve (p-TS VAS-AUC) for a one minute period were automatically calculated by the software provided with the electronic VAS (figure 1). During the p-TS assessment, the participant was not able to see the computer screen.

4.1.3 Psychological scales

In the studies presented in this thesis, we applied two different psychological tests.

Hospital Anxiety and Depression Scale

The Hospital Anxiety and Depression Scale (HADS) is a questionnaire containing 14 questions, with seven questions evaluating anxiety (HADS-A) and seven questions evaluating depression (HADS-D)[70]. Points are given on a four-point Likert scale ranging from 0-3. HADS evaluates symptoms of anxiety and depression, and the total test score is a measure of the individual's level of distress. The highest achievable score of HADS is 42, but to properly interpret HADS, both the total HADS-score and the subscales (HADS-A and HADS-D) should be evaluated. HADS-A and HADS-D are interpreted in the following way[71]:

- 0-7 points: Normal (no signs of depression/anxiety)
- 8-10 points: Mild level of depression/anxiety
- 11-15 points: Moderate level of depression/anxiety
- 16-21 points: Severe level of depression/anxiety

HADS has been demonstrated to be applicable in hospitalized patients as well as healthy individuals[72].

Pain Catastrophizing Scale

The Pain Catastrophizing scale (PCS) consists of 13 questions evaluated on a five-point Likert scale ranging from 0-4[73]. The PCS can be subdivided into three sub-scales, each evaluating a central element of catastrophizing; *rumination*, *magnification*, and *helplessness*. Individuals taking the test are asked to reflect on past painful experiences and evaluate how they felt during the painful experience by answering the 13 questions of the PCS. The highest achievable score is 52, but as different questions evaluate rumination, magnification, and helplessness, the PCS can be grouped into three separate sections:

- Rumination: Sum of questions 8, 9, 10, and 11 (max score of 16)
- Magnification: Sum of questions 6, 7, and 13 (max score of 12)
- Helplessness: Sum of questions 1, 2, 3, 4, 5, and 12 (max score of 24)

PCS can be applied in healthy individuals as well as patients[74].

Figure 1. Pain models

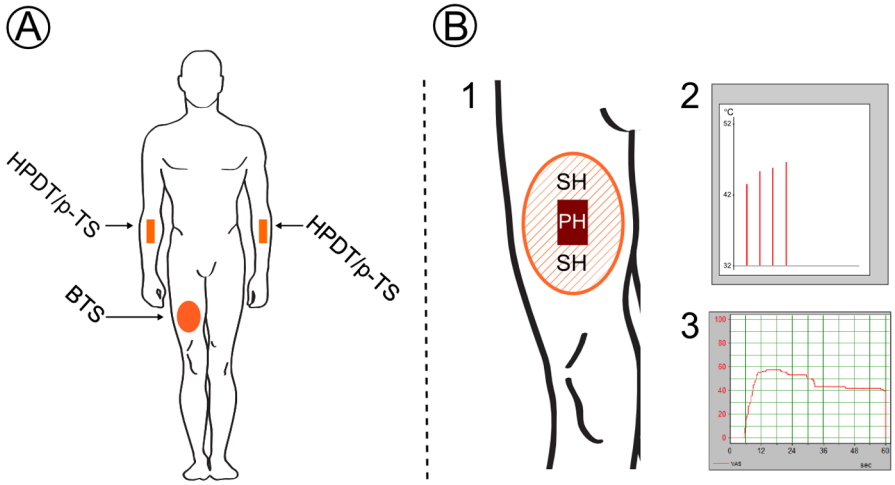

Figure 1 depicts the pain models applied in the studies.
Figure A provides an outline of the anatomical locations of the pain model testing. BTS was conducted anterior on the upper right thigh in the midline between the anterior superior iliac spine and the base of patella. HPDT was evaluated on the volar side of the dominant lower arm. p-TS was evaluated on the volar side of the non-dominant lower arm.
Figure B-1 provides an illustration of the development of primary and secondary hyperalgesia following BTS. The dark-red area corresponds to the location of the 45°C thermode with the corresponding development of primary hyperalgesia (PH). The orange-hatched area corresponds to the development of secondary hyperalgesia (SH).
Figure B-2 provides an illustration of the measurement of the HPDT. The HPDT was calculated as a mean of four stimulations with an interval of 6-10 seconds.
Figure B-3 provides an illustration of the measurement of p-TS. Pain evaluation was done with an electronic VAS-score (0-100 mm). A maximum VAS-score (p-TS VAS-max) during the 1 minute thermal stimulation and an area under the VAS-curve (p-TS VAS-AUC) were obtained.
Abbreviations: HPDT, heat pain detection threshold; p-TS, pain during one minute thermal stimulation; BTS, brief thermal sensitization; PH, primary hyperalgesia; SH, secondary hyperalgesia; VAS, visual analogue scale.

5. Summary of study I-IV

This section briefly presents the aim, design, methods, and results of study I-IV with focus on the primary results. A detailed account of study I-IV is given in the accompanying papers.

5.1 Study I

5.1.1 Aim

This prospective study was designed to evaluate the intra- and inter-individual variance in secondary hyperalgesia areas elicited by BTS with two independent investigators.

5.1.2 Design

The study involved one information day and four identical study days (figure 2).
On the information day, each participant was introduced to the pain models BTS, HPDT, and p-TS, and given the psychological tests PCS and HADS. The participants completed the questionnaires at home and handed them in on the first study day in concealed opaque envelopes to ensure blinding of the investigators.
On each study day, the participants were tested with BTS, HPDT, and p-TS in a predefined randomised sequence. Each study day began with BTS followed by a randomised sequence of HPDT and p-TS so that on two study days, the test sequence was 1) BTS, 2) HPDT, 3) p-TS, and on the other two study days, the test sequence was 1) BTS, 2) p-TS, and 3) HPDT (figure 2).
Two different investigators were employed to perform the pain testing on the four study days with the order of the days being randomised by a computer generated allocation sequence provided by Copenhagen Trial Unit. This ensured that each participant was tested by the same investigator twice, and that the investigator was not responsible for testing the same participant on two consecutive study days (figure 2). The results of the pain testing on the individual study days were placed in opaque sealed envelopes to ensure that the investigators were unable to see the previous test results. To prevent a potential carry-over effect of the pain testing, each study day as well as the information day were separated by a minimum of seven days.

Figure 2. Design of study I

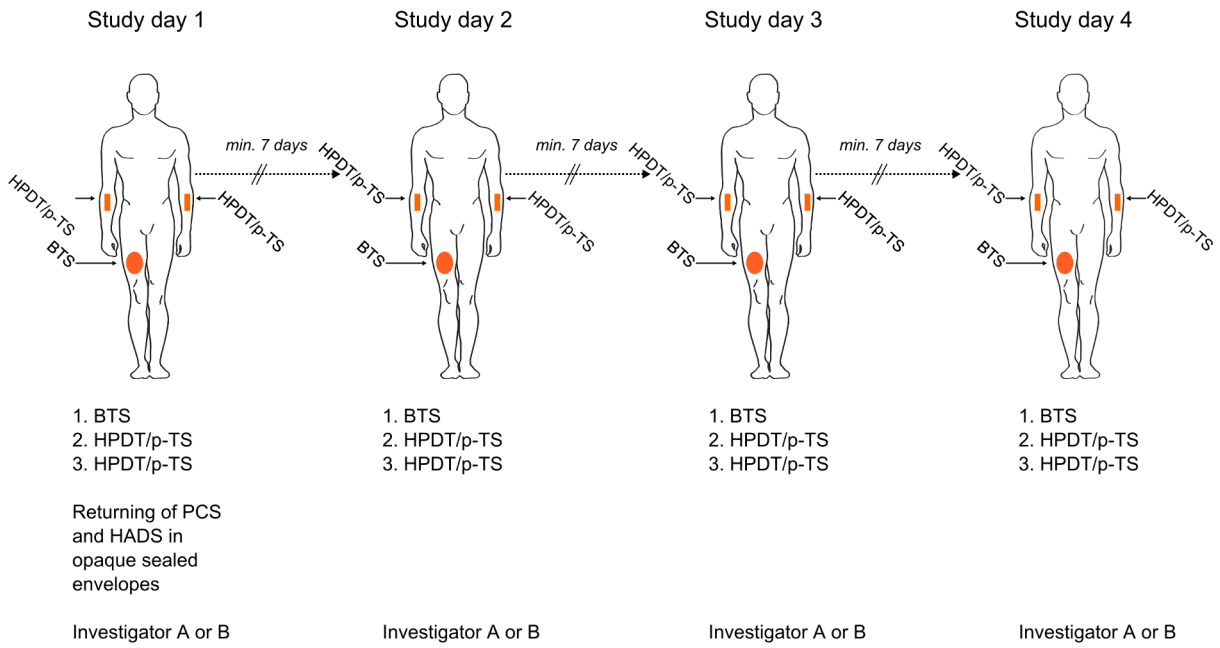

Figure 2 depicts the design of study I. The study consisted of one information day and four study days. On the information day, the participants were introduced to the pain tests and given the PCS and HADS to fill out at home and return on study day one. On each study day, the participants were tested with BTS, HPDT, and p-TS. Each study day began with BTS followed by either HPDT or p-TS depending on the randomised test sequence. Each investigator performed the tests on two separate study days with the order of the study day being randomised so that the same investigator was not responsible for testing the same participant on two consecutive study days.
Abbreviations: BTS, brief thermal sensitization; HPDT, heat pain detection threshold; p-TS, pain during one minute thermal stimulation; PCS, Pain Catastrophizing Scale; HADS, Hospital Anxiety and Depression Scale.

5.1.3 Outcome measures

Primary outcome measure

The primary outcome of our study was the intra- and inter-participant variance and the intra- and inter-investigator variance of the secondary hyperalgesia areas elicited by BTS on four separate study days with two different investigators.

Secondary outcome measures

In our secondary outcome measures, we investigated how precise the HPDT, p-TS VAS-AUC, p-TS VAS-max, HADS, PCS, and the HADS- and PCS sub-scores predicted the size of the secondary hyperalgesia area on the four study days.

5.1.4 Sample size estimation

To account for the complicated study design, we conducted statistical simulations using parameter values derived from a previous study[26]. The simulations included scenarios with varying number of study days and investigators with up to 10% missing data in a "missing at random" scenario. The variance of the secondary hyperalgesia areas in the obtained data was estimated using a variance component model (linear mixed model) including random effects of participant and investigator in 1000 simulated datasets with the number of participants ranging from 20 to 100. Estimates of the intraclass correlation coefficient (ICC) and the coefficient of variation (CV) were extracted. The simulations demonstrated that in a study with two investigators, four study days, fifty study participants, and 10% of the study participants with missing observations following the first study day, we would be able to determine an ICC of 0.78 with a SD of 0.066 (95% confidence interval (95% CI) 0.65 to 0.91) and a CV of 0.25 with a SD of 0.039 (95% CI 0.17 to 0.33). We accepted this study design to be sufficient in discerning the relevant variance components with acceptable precision.

5.1.5 Statistical analyses

Areas of secondary hyperalgesia, HPDT, p-TS VAS-max, and p-TS VAS-AUC were obtained as estimated best linear unbiased predictors (EBLUPs).

To evaluate the primary outcome measure, the variance of secondary hyperalgesia areas was estimated using a variance component model including random effects of participant and investigator. Intra- and inter-participant variance and intra- and inter-investigator variance of secondary hyperalgesia areas were reported as ICCs and CV.

To evaluate the secondary outcome measures, the ability of PCS, HADS, HPDT, p-TS VAS-max, and p-TS VAS-AUC to predict variation in the size of the secondary hyperalgesia area was evaluated using multiple linear regression. Model reduction was conducted with backwards elimination with a 5% cut-off level. Significance of individual predictors was assessed using analysis of variance methods, and p-values corresponded to F-tests and were evaluated at a 5% significance level.

5.1.6 Results

Fifty-four participants were assessed for eligibility; two did not meet inclusion criteria, and two declined to participate, resulting in inclusion of fifty participants. All fifty study participants completed the four study days and were analysed for the primary and secondary outcome measures. The median areas of secondary hyperalgesia elicited by BTS on study day one to four were 312.9 cm^2 (interquartile range (IQR) 256.6 to 457.0), 294 cm^2 (IQR 250.5 to 417.3), 339.3 cm^2 (IQR 231.7 to 389.9), and 310.1 cm^2 (IQR 244.1 to 413.3) respectively (figure 3).

Figure 3. Areas of secondary hyperalgesia elicited by brief thermal sensitization on the four study days

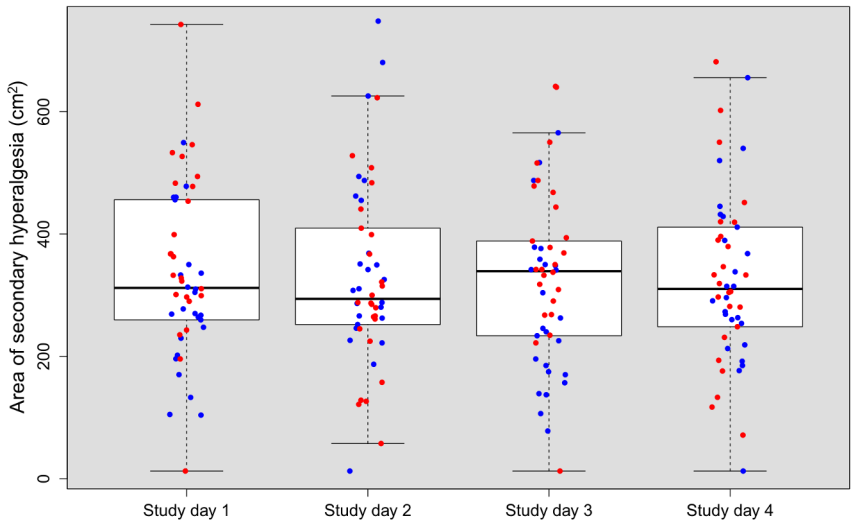

Figure 3 depicts areas of secondary hyperalgesia on the four study days.
Medians and interquartile ranges are displayed. Upper and lower whiskers indicate upper and lower quartile +/- 1.5 of the interquartile range respectively. Coloured points correspond to each participant's individual measurements of secondary hyperalgesia areas. Blue colour indicates that the participant has been tested by investigator "A" and red colour that the participant has been tested by investigator "B".

Primary outcome measures

Measurements of secondary hyperalgesia areas on the four study days revealed an intra-participant intra-investigator correlation of 0.85, (95% CI 0.78 to 0.90), an intra-participant inter-investigator correlation of 0.82 (95% CI 0.69 to 0.89), an inter-participant intra-investigator correlation of 0.03 (95% CI 0.0 to 0.16), and a CV of 0.17 (95% CI 0.14 to 0.21)

Secondary outcome measures

HPDT was identified as a significant predictor of the area of secondary hyperalgesia with an R^2 of 0.20 (p=0.0006). The prediction interval for the secondary hyperalgesia area given a HPDT of 46°C was estimated to 53.14 to 515.43 cm^2. PCS, HADS, p-TS VAS-max, and p-TS VAS-AUC did not significantly predict the area of secondary hyperalgesia.

5.1.7 Conclusion

Our findings demonstrated that BTS induced an area of secondary hyperalgesia with a low intra-participant variance and a high inter-participant variance compared to the inter-investigator variance. BTS induced secondary hyperalgesia with a high level of reproducibility and can be employed in investigations of secondary hyperalgesia in healthy male participants. Finally, our findings indicated that BTS can be used to phenotype healthy men based on their area of secondary hyperalgesia.

5.2 Study II

5.2.1 Aim

This prospective study was designed to investigate the association between HPDT and the size of the secondary hyperalgesia area induced by BTS. Based on the exploratory findings from study I, we hypothesised that HPDT and the area of secondary hyperalgesia elicited by BTS represented two predominantly independent entities, and that the area of secondary hyperalgesia was poorly explained by HPDT.

5.2.2 Design

The design of study II was very similar to the design in study I. However, study II entailed only one information day and two study days (figure 4). Similar to Study I, on the information day the participants were introduced to BTS, HPDT, and p-TS, and were given the PCS and HADS to complete at home. On the two study days, the participants were tested with BTS, HPDT, and p-TS in a predefined randomised computer generated allocation sequence provided by Copenhagen Trial Unit. Since our primary aim was to investigate the association between BTS and HPDT, the sequence was randomised so that on one study day the test sequence was 1) BTS, 2) HPDT, 3) p-TS, and on the other study day the sequence was 1) HPDT, 2) BTS, and 3) p-TS.

Similar to study I, the results of the pain testing on the individual study days were placed in opaque sealed envelopes to ensure that the investigator was unable to see previous test results. Furthermore, each of the two study days and the information day were separated by a minimum of seven days (figure 4).

Figure 4. Design of study II

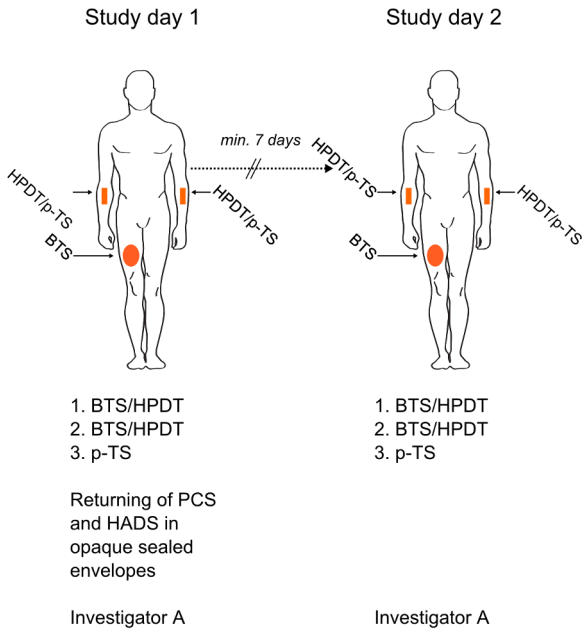

Figure 4 depicts the design of study II. The study consisted of one information day and two study days. On the information day, the participants were introduced to the tests and were given the PCS and HADS to fill out at home and return on study day one. On each study day, the participants were tested with BTS, HPDT, and p-TS. Each study day began with either BTS or HPDT, depending on the randomised test sequence, followed by p-TS. Only one investigator performed the tests on the two separate study days.

Abbreviations: BTS, brief thermal sensitization; HPDT, heat pain detection threshold; p-TS, pain during one minute thermal stimulation; PCS, Pain Catastrophizing Scale; HADS, Hospital Anxiety and Depression Scale.

5.2.3 Outcome measures

Primary analysis

The primary analysis investigated the association between HPDT and the size of the area of secondary hyperalgesia induced by BTS.

Secondary analyses

The secondary analyses investigated the association between the size of the area of secondary hyperalgesia and p-TS VAS-AUC, p-TS VAS-max, HADS, PCS, and the HADS and PCS sub-scores.

5.2.4 Sample size estimation

We conducted statistical simulations using data from the first two study days in study I.
To investigate a significant association between HPDT and the size of the secondary hyperalgesia area, we applied multiple linear regression in 1000 simulated datasets with the number of participants ranging from 50-250. The power was then calculated as the fraction of the number of times the calculated p-value was below 0.05. Since we hypothesised that the size of the secondary hyperalgesia area and HPDT were two independent entities, we aimed to achieve a very high power. The simulation based sample size analyses demonstrated that with an α of 0.05 and a β of 0.01, 120 participants was needed in order to achieve a power of 99.9%.

5.2.5 Statistical analyses

HPDT, p-TS VAS-AUC, p-TS VAS-max, and areas of secondary hyperalgesia were obtained as EBLUPs. In the primary analysis, the association between HPDT and the size of the secondary hyperalgesia area was investigated by linear regression.
In the secondary analyses, the association between the size of the secondary hyperalgesia area and p-TS VAS-AUC, p-TS VAS-max, PCS, and HADS was investigated by multiple linear regression. Model reduction was conducted with backwards elimination with a 5% cut-off level. P-values corresponded to F-tests and were evaluated at a 5% significance level

5.2.6 Results

One-hundred-thirty-one participants were assessed for eligibility, five did not meet the inclusion criteria, three declined to participate, and two were not included due to other reasons. Consequently, a total of 121 participants were included in the study. All 121 participants completed the study and were included in the final analyses. The median HPDT was 45.57°C (IQR 43.79 to 46.61), and the median size of the secondary hyperalgesia area was 447.78 cm^2 (IQR 346.2 to 528.99) (figure 5)

Primary analysis
Evaluation of secondary hyperalgesia areas and HPDT on two separate study days demonstrated a significant association between HPDT and the size of the secondary hyperalgesia area (p<0.0001). The expected change in secondary hyperalgesia area due 1°C increase in HPDT was estimated to -27.38 cm^2 (95% CI -37.77 to -16.98 cm^2). The R^2 was estimated to 0.19 with prediction limits at a HPDT of 46°C of 167.42 to 656.07 cm^2.

Secondary analyses
Our analyses showed a significant association between the HADS-D sub-score and secondary hyperalgesia area (p=0.046). The expected change due to a one-point increase in HADS-D sub-score was estimated to 11 cm^2 (95% CI 0.19 to 21.82). The R^2 was estimated to 0.03. No significant associations were identified in the other secondary outcome measures.

5.2.7 Conclusion

Our findings demonstrated a highly significant association between HPDT and the size of the secondary hyperalgesia area elicited by BTS. However, with a R^2 of only 0.19 and wide prediction limits, our results indicated that HPDT only offered a modest explanation of the inter-participant variation in the size of the secondary hyperalgesia area.

Figure 5. Areas of secondary hyperalgesia and heat pain detection thresholds

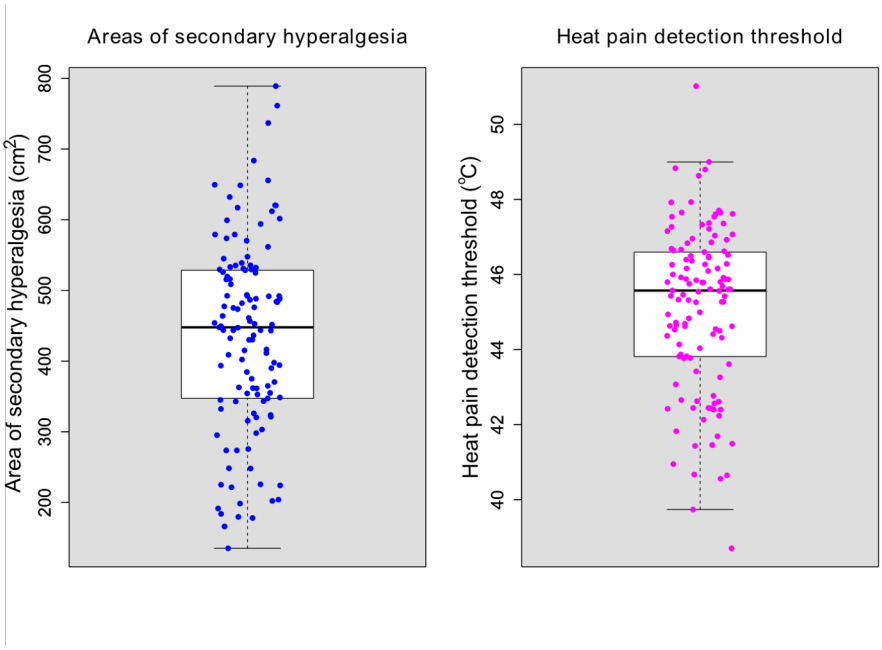

Figure 5 depicts areas of secondary hyperalgesia and HPDTs from the two study days.
Values of HPDT and secondary hyperalgesia areas were obtained as EBLUPs. Medians and interquartile ranges are displayed. Upper and lower whiskers indicate upper and lower quartile +/- 1.5 of the interquartile range respectively. Coloured points correspond to each participant's individual measurements of secondary hyperalgesia areas (blue) and HPDTs (purple).
Abbreviations: HPDT, heat pain detection threshold; EBLUP, best linear unbiased predictor.

5.3 Study III

5.3.1 Aim

This prospective study was designed to investigate the association between the size of the secondary hyperalgesia area and the volume of predefined brain structures relevant for pain processing, with the volume of the caudate nuclei as the primary outcome measure.

5.3.2 Design

All participants who completed study II were eligible for inclusion in study III; furthermore, results from the pain testing were extracted from study II and used for analyses in study III (figure 6). This included secondary hyperalgesia areas, HPDT, p-TS VAS-AUC, p-TS VAS-max, PCS, and HADS. The use of the pain testing results in study III is considered secondary use of data. Individual MRI-scans were conducted a minimum of 14 days and a maximum of 60 days after completion of study II to avoid any carry-over effects of the pain testing on the MRI scan.
On the day of the MRI, all participants underwent whole-brain MRI including anatomical T1-weighted, diffusion tensor imaging (DTI), and resting state fMRI (figure 6). All MRI scans were performed with a Siemens MAGNETOM Verio 3-tesla MRI-scanner with b17 software and a 32-channel head coil. No pain testing was conducted on the day of the MRI. Following completion of the MRI, all images were reviewed by an experienced radiology consultant. In the case of suspected pathological findings, the participant was excluded from the study. Per protocol[67], study III only reported data from the T1-weighted structural scans.

Figure 6. Design of study II, III, and IV

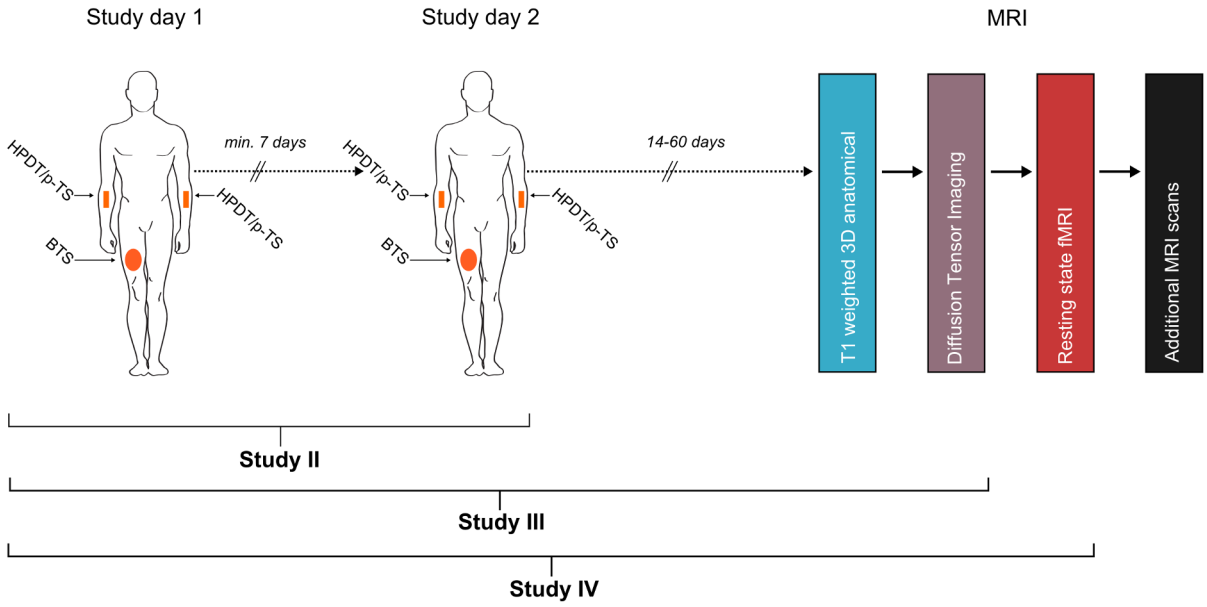

Figure 6 depicts the design of study II, III and IV.
All participants who completed study II were eligible for inclusion in study III and IV. Pain testing data from study II were extracted and applied in the analyses of study III and IV.
Individual MRI-scans were conducted at a minimum of 14 days, and a maximum of 60 days following completion of study II.
MRI scans were conducted in a fixed order starting with T1-weighted 3D anatomical scan (duration: 4 minutes) followed by DTI (duration: 12 minutes), resting state fMRI scan (duration: 8 minutes), and additional scans of technical or diagnostic character. Total duration of the MRI scan sequence was approximately 50 minutes.
Study III reported data from the T1-weighted structural scans, and study IV reported data from the resting state fMRI scans and used the structural data for co-registration purposes only. The additional MRI-scans were of technical character or done for diagnostics and were not included in the analysis.
Abbreviations: BTS, brief thermal sensitization; HPDT, heat pain detection threshold; p-TS, pain during one minute thermal stimulation; MRI, magnetic resonance imaging; fMRI, functional magnetic resonance imaging; DTI, diffusion tensor imaging.

5.3.3 Outcome measures

Primary analysis

The primary analysis investigated the association between the volume of the caudate nuclei and the size of the secondary hyperalgesia area.

Secondary analyses

The secondary analyses investigated the association between the size of the secondary hyperalgesia area and the volume of the following brain structures relevant for pain processing:

primary somatosensory cortex, anterior and mid cingulate cortex, putamen, nucleus accumbens, globus pallidus, insula, and the cerebellum.

Further exploratory analyses were conducted to investigate the association between the volume of the caudate nuclei and individual levels of HPDT, p-TS VAS-AUC, p-TS VAS-max, PCS, and HADS. Moreover, the included participants were divided into quartiles based on their individual area of secondary hyperalgesia. Subsequently, potential neuroanatomical differences in brain structures relevant for pain processing between participants with a small area of secondary hyperalgesia (lower quartile) and a large area (upper quartile) were investigated.
Finally, a post-hoc analysis investigating the association between the area of secondary hyperalgesia and the amygdala, hippocampus, and thalamus was also conducted.

5.3.4 Sample size estimation

We estimated the sample size based on a Z-test of the Fisher transformed Pearson correlation, using data from a previous study[25]. The analysis demonstrated that a sample size of 52 would allow us to detect a true correlation of R=-0.4 between the area of secondary hyperalgesia and the volume of the caudate nuclei with a statistical power of 0.80 (β=0.20) and a significance level of 0.025 to 0.05 according to the single step method. However, the sample size analysis was based on data from a study where only participants with small or large areas of secondary hyperalgesia were included, and since we included participants without any knowledge of their area of secondary hyperalgesia, we also expected participants with intermediate sizes of secondary hyperalgesia areas. To also secure a reasonable high sample size when conducting the exploratory analyses, we therefore planned to include 120 participants.

5.3.5 MRI preprocessing

All anatomical T1-weighted images were preprocessed and analysed using FreeSurfer imaging analysis suite version 5.3[75,76]. FreeSurfer is a software package for analysing human brain MRI images and allows semi-automatic volumetric segmentation of cortical and subcortical brain structures. Using FreeSurfer, cortical reconstruction was completed for all T1-weighted images. Cortical reconstruction creates computerised models of the brain using the T1-weighted MRI data from the individual study participants and includes a total of 31 data processing steps. Subsequently, volume estimates of subcortical structures were extracted using the

asegstats2table command, and cortical volume estimates were extracted according to the Desikan-Killiany cortical atlas[77]. Finally, all brain volumes were adjusted for inter-participant differences in intracranial volume using the method outlined by Raz et al.[78]. All brain volumes were extracted to a spread sheet for further statistical analyses.

5.3.6 Statistical analyses

In the primary analysis, the association between the area of secondary hyperalgesia and the volume of the caudate nuclei was investigated by multiple linear regression adjusted for age and body surface area.

In the secondary analyses, we applied multiple linear regression to investigate the association between the area of secondary hyperalgesia and the volume of the pain relevant brain structures described in 5.3.3 (secondary analyses and post-hoc analyses).

Likewise, the association between the volume of the caudate nuclei and the HPDT, p-TS VAS-AUC, p-TS VAS-max, PCS, and HADS was investigated by multiple linear regression. Finally, neuroanatomical differences (in predefined brain structures relevant for pain processing) between the groups of participants with large (upper quartile) vs. small (lower quartile) areas of secondary hyperalgesia were investigated using unpaired t-tests.

We also performed four separate post-hoc sensitivity analyses. In the first analysis, we adjusted for age, weight, body mass index, and mean arterial pressure. In the second analysis, we excluded all left-handed participants. In the third, we excluded all participants with non-Scandinavian ethnicity and in the fourth analysis we did not adjust for body surface area.

In all analyses P-values corresponded to Wald-tests and $P<0.05$ (controlled for family wise error rate) was considered statistically significant.

5.3.7 Results

One-hundred-and-twenty-one participants were included in the study and completed whole-brain MRI scan. Following review of the MRI scans, three participants were excluded due to suspected pathological findings, resulting in the inclusion of 118 participants in the final analyses. The median number of days between completion of study 2 and the MRI scan was 17 days (IQR 16 to 18).

Secondary hyperalgesia and caudate nuclei

The primary analysis revealed no significant associations between the volume of the caudate nuclei and the area of secondary hyperalgesia (right hemisphere p=0.13, left hemisphere p=0.12) (figure 7). Regression analyses demonstrated that a one-mm^3 increase in the volume of the right caudate nucleus resulted in an estimated increase in secondary hyperalgesia area of 0.103 cm^2 (95% CI -0.028 to 0.233). Likewise, a one-mm^3 increase in the volume of the left caudate nucleus resulted in an estimated decrease in secondary hyperalgesia area of -0.107 cm^2 (95% CI -0.239 to 0.025).

Figure 7. Associations between the size of the secondary hyperalgesia area and the volume of the caudate nuclei

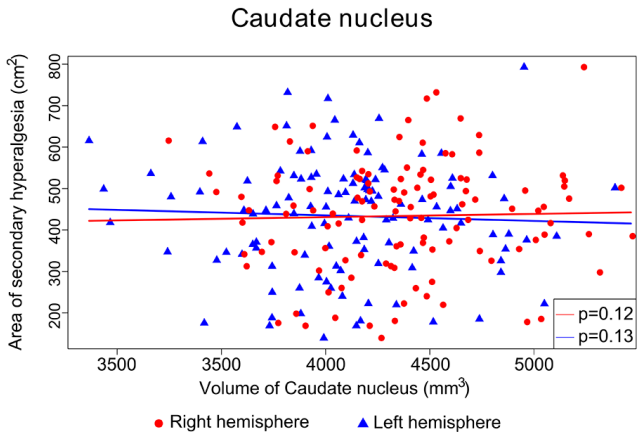

Figure 7 depicts a scatter plot illustrating the volume of the right (red dots) and left (blue triangles) caudate nucleus and the individual area of secondary hyperalgesia of each included participant. Individual volumes of the caudate nuclei were adjusted for intracranial volume, and individual areas of secondary hyperalgesia were adjusted for body surface area. We observed no significant association between the area of secondary hyperalgesia and the volume of the right and left caudate nucleus (right hemisphere p= 0.12, left hemisphere p=0.13).

Secondary hyperalgesia and brain structures relevant for pain processing

The secondary analyses revealed no significant association between the area of secondary hyperalgesia and the volume of the primary somatosensory cortex (right hemisphere *p*=0.11, left hemisphere *p*=0.76), anterior cingulate cortex (right *p*=0.33, left *p*=0.82) or mid cingulate cortex (right *p*=0.26, left *p*=0.91), putamen (right *p*=0.29, left *p*=0.05), nucleus accumbens (right *p*=0.27, left *p*=0.5), globus pallidus (right *p*= 0.35, left p=0.48), insula (right *p*=0.28, left *p*=0.08), or the cerebellum's white matter (right *p*= 0.44, left *p*=0.64) or cerebellum's cortex (right *p*= 0.62, left *p*=0.24) (figure 8). Moreover, the post-hoc analyses did not reveal any significant associations between the area of secondary hyperalgesia and the volume of the amygdala (right *p*=0.96, left *p*=1), hippocampus (right *p*=0.99, left *p*=1), or thalamus (right *p*=0.96, left *p*=0.96).

Likewise, no significant associations between the volume of the caudate nuclei and HPDT (right *p*=1, left *p*=1), p-TS VAS-AUC (right *p*=1, left *p*=1), p-TS VAS-MAX (right *p*=1, left *p*=1), PCS (right *p*=0.96, left *p*=0.94), or HADS (right *p*=0.26, left *p*=0.24) were found.

Furthermore, no significant differences in any of the identified brain structures relevant for pain processing were found when comparing participants with a small (lower quartile) vs. large (upper quartile) area of secondary hyperalgesia.

Lastly, the sensitivity analyses did not demonstrate different results in the primary and secondary analyses when compared to our original analyses.

5.3.8 Conclusion

Our study did not demonstrate any statistically significant associations between the area of secondary hyperalgesia and the volume of the caudate nuclei or any other predefined brain structures relevant for pain processing. Our findings indicated that the propensity to develop central sensitization was not correlated to pain relevant brain structure volume.

Figure 8. Associations between the size of the secondary hyperalgesia area and the volume of pain relevant cortical and subcortical brain structures

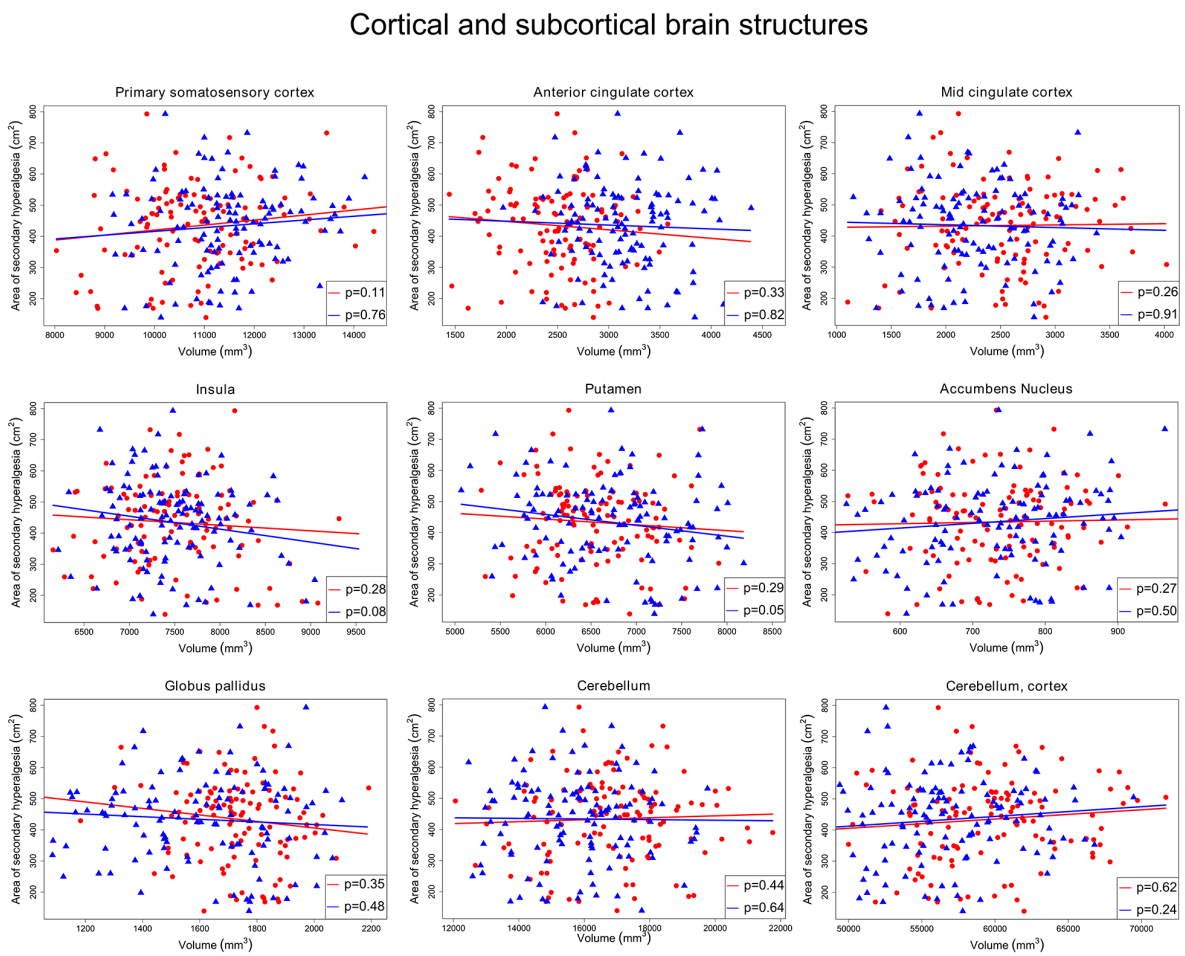

Figure 8 depicts scatter plots of individual volume measurements of brain structures relevant for pain processing belonging to the right hemisphere (red dots) and left hemisphere (blue triangles) and the individual areas of secondary hyperalgesia of each included participant. Volumes of individual brain structures were adjusted for intracranial volume, and individual areas of secondary hyperalgesia were adjusted for body surface area. We observed no significant association between the area of secondary hyperalgesia and the volume of brain structures relevant for pain processing.

5.4 Study IV

5.4.1 Aim

This study was designed to investigate the association between the size of the secondary hyperalgesia area and the connectivity in known resting state networks, and to investigate possible differences in resting state connectivity when comparing participants with a small vs. large area of secondary hyperalgesia.

5.4.2 Design

Resting state fMRI data collected in study III were per protocol[67] analysed in study IV. The study design in study IV was therefore identical to the design described in study III (figure 6).
During the resting state fMRI scan, the participants did not perform any tasks, nor did they receive pain stimulation. Every participant was instructed to stay awake and to keep the eyes open. If the participant fell asleep during the resting state fMRI scan he, was instructed to inform the investigator and the fMRI scan would then be discarded.
Heart rate, end-tidal pCO2, and respiration frequency were evaluated before, after and during the entire resting state fMRI scan.

5.4.3 Outcome measures

The outcome measures were per protocol specified as exploratory. We planned to investigate 1) the association between the size of the secondary hyperalgesia area and the connectivity in known resting state networks and 2) differences in resting state connectivity when comparing participants with a small (lower quartile) vs. large (upper quartile) area of secondary hyperalgesia.

5.4.4 Preprocessing of MRI data

T1-weighted anatomical images were preprocessed using the fMRI imaging brain software library's (FSL) brain extraction tool by applying robust brain centre estimation[79]. Individual T1-weighted images and the Montreal neurological institute (MNI)-152 brain atlas were used for co-registration only.
fMRI resting state data were preprocessed in three steps. Step 1: Individual participant data were preprocessed with the optimal number of independent components determined by the

FSL-melodic software[80-82] (http://fsl.fmrib.ox.ac.uk) and quality assessed for motion (≥3 degrees and/or ≥3 mm) and temporal scanner instability[83]. Step 2: Automatic denoising of individual resting state data was conducted by applying FMRIB's *ICA-based Xnoiseifier v1.064* (FIX)[84,85] (http://fsl.fmrib.ox.ac.uk/fsl/fslwiki/FIX). Step 3: Individual preprocessed data were concatenated and analysed in group analysis with FSL melodic applying a fixed number of 75 independent components[86].

Independent component analysis (ICA) is a data-driven approach that splits the 4D fMRI data into separate spatial maps with individual associated time courses. It is a model-free approach that analyses the BOLD-signal without an *a priori* knowledge of the regions of interest. Consequently, ICA results in identification of a number of independent components relating to brain activity or physiological noise[87]. By applying FIX, automatic removal of noise is conducted through multiple steps resulting in extraction of 180 spatial and temporal features for each independent component. This allows high-accuracy automatic removal of components deemed of no neurological origin[84,85]. In group analysis, manual selection with a fixed number of 75 independent components allows detailed and reliable evaluation of resting state networks.

In the analysis conducted in study IV, each of the 75 independent components were spatially cross-correlated with publicly available brain networks (templates) from healthy adults[88] (available at http://www.fmrib.ox.ac.uk/analysis/brainmap+rsns/). Additionally, two networks representing the salience network[89] and the basal ganglia network[90] were also included. A Pearson correlation of 0.25 was chosen as threshold and applied in determining spatial correspondence between the templates and the identified components. An independent component with a Pearson correlation above 0.25 was visually inspected and included for further analysis.

We planned to report results from networks relevant for pain processing only (Default mode network, Sensorimotor network, right Fronto-parietal network, Central executive network, Basal ganglia network, and the Salience network).

5.4.5 Statistical analyses

To investigate the association between the size of the secondary hyperalgesia area and the resting state connectivity, we conducted dual regression[91,92] including all participants. With dual regression, temporal dynamics and spatial maps of individual resting state fMRIs are regressed against the original data set, allowing participant and group comparisons. We applied

the effect of increasing secondary hyperalgesia area as a contrast, thus, individual areas of secondary hyperalgesia were demeaned and entered as an explanatory variable.
Prior to the next analysis, the included participants were divided into four quartiles based on their area of secondary hyperalgesia. We then investigated group differences when comparing participants with a small (lower quartile) vs. large (upper quartile) area of secondary hyperalgesia by conducting dual regression and applying the effect of a large area and the effect of a small area as separate contrasts.
Lastly, a post-hoc sensitivity analysis investigating the connectivity in the right Fronto-parietal network in right-handed participants only was performed to assess the robustness of the findings.
We applied Gaussian mixture modelling[93-95] to determine statistical thresholds for significance. Cluster peak values were identified, and a minimum cluster volume of 0.9 mm^3 was applied as a final threshold. Peak activity within each identified cluster was extracted, and the underlying brain structures were determined.

5.4.6 Results

One-hundred-twenty-one participants were included and underwent resting state fMRI scan. As described in study III, three participants were excluded due to pathological findings. Following quality assessment, two participants were excluded because of excessive head movement, and one participant was excluded because of scanner intensity instability. Consequently, data from 115 participants were included in the final analyses.

Effect of increasing area of secondary hyperalgesia
Significant positive correlations between increasing area of secondary hyperalgesia and connectivity in multiple brain structures were observed in the Sensorimotor network (total volume of positively correlated structures of 40.36 cm^3) and the Default mode network (total volume of 10.21 cm^3). Please see figures 9 and 10 in this thesis, and table 5 and figure 5 in paper IV.
Significant negative correlations between increasing area of secondary hyperalgesia and connectivity in multiple brain structures were observed in the Sensorimotor network (total volume of negatively correlated structures of 7.38 cm^3), the Default mode network (total

volume of 3.96 cm^3), and the right Fronto-parietal network (total volume of 5.14 cm^3). Please see figures 9 and 10 in this thesis and table 5 and figure 5 in paper IV.
No significant correlations were observed in the Central Executive network, the Salience network, or the Basal ganglia network.

Small versus large area of secondary hyperalgesia
In participants with a small area of secondary hyperalgesia, significantly increased connectivity in multiple brain structures was observed in the Sensorimotor network, the Default mode network, the Central executive network, and the Basal ganglia network. Please see figures 9 and 10 in this thesis and table 6 and figure 6 in paper IV.
In participants with a large area of secondary hyperalgesia, significantly increased connectivity in multiple brain structures was observed in the Sensorimotor network, the Default mode network, and the Central executive network. Please see figures 9 and 10 in this thesis and table 6 and figure 6 in paper IV.
No significant differences were observed in the Salience network or in the right Fronto-parietal network.

For specific details regarding the brain structures in terms of precise anatomical location and cluster size, please see tables 5 and 6 in the appending paper IV.

5.4.7 Conclusion

Our study revealed multiple significant positive and negative correlations between increasing size of secondary hyperalgesia area and resting state connectivity, as well as significant group differences when comparing participants with a small vs. large area of secondary hyperalgesia. The present findings indicated that individual propensity to develop central sensitization, assessed as phenotypic expression of secondary hyperalgesia, was associated with the resting state connectivity in multiple brain structures. Because of the exploratory nature of this study, the results should be interpreted with caution and require replication.

Figure 9. Resting state connectivity in the Sensorimotor, right Fronto-parietal, and Basal ganglia network

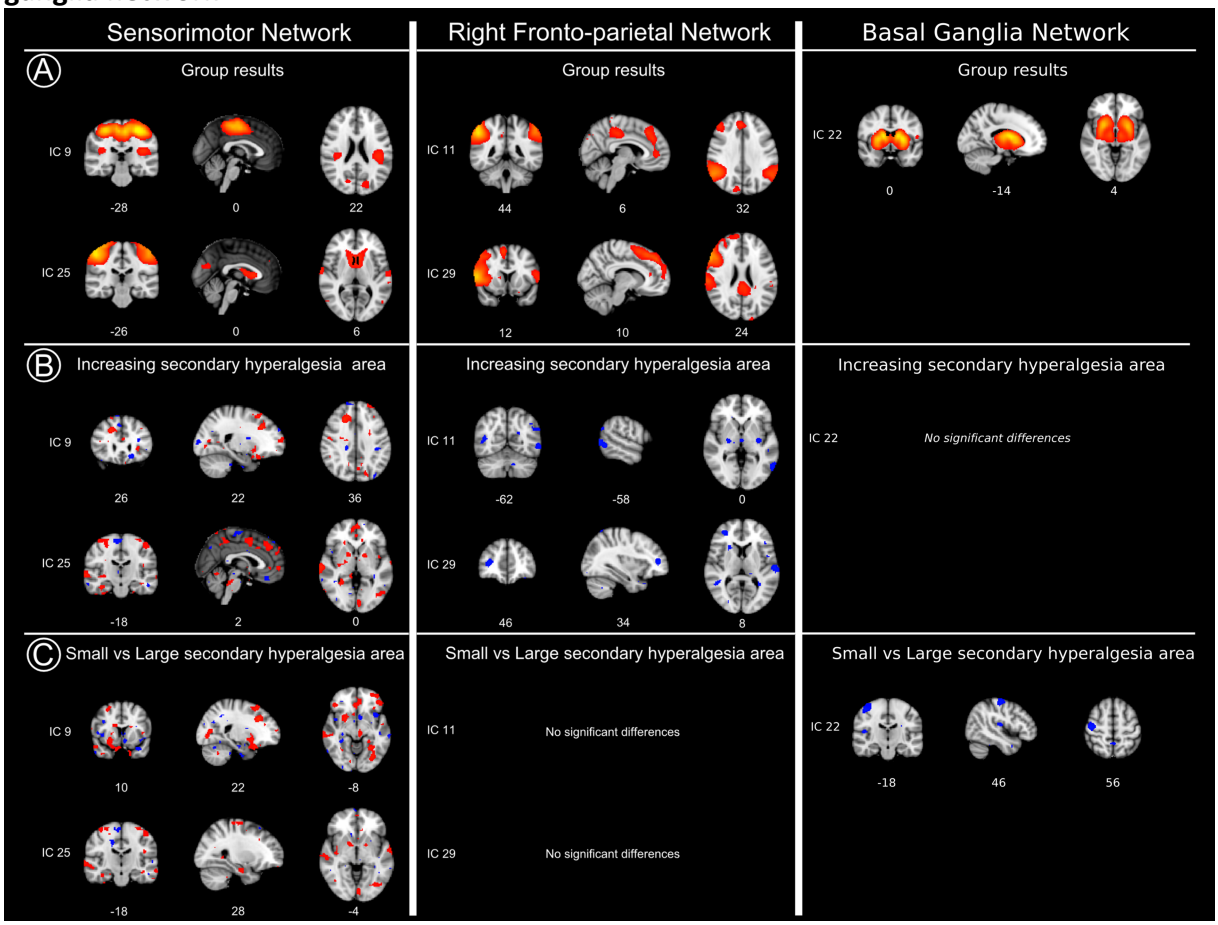

Figure 9 depicts resting state connectivity in the Sensorimotor, right Fronto-parietal, and Basal ganglia network.
A) Group results from all participants (n=115) showing the Sensorimotor network (comprised of IC 9 and IC 25), the right Fronto-parietal network (comprised of IC 11 and IC 29), and the Basal ganglia network (comprised of IC 22).
B) Resting state connectivity illustrating the effect of increasing secondary hyperalgesia area. Blue colours indicate brain structures where connectivity decreased with increasing area of secondary hyperalgesia, and red colours indicate brain structures where connectivity increased.
C) Resting state connectivity comparing participants with small (lower quartile) vs. large (upper quartile) areas of secondary hyperalgesia. Blue colours indicate brain structures with increased connectivity in participants with small areas of secondary hyperalgesia, and red colours indicate brain structures with increased connectivity in participants with large areas.
All statistically significant findings can be observed visually and cross referenced with the results displayed in table 5 and 6 in paper IV. Numbers refer to standard Montreal neurological institute atlas coordinates.
Abbreviations: IC, independent component

Figure 10. Resting state connectivity in the Default mode and Central executive network

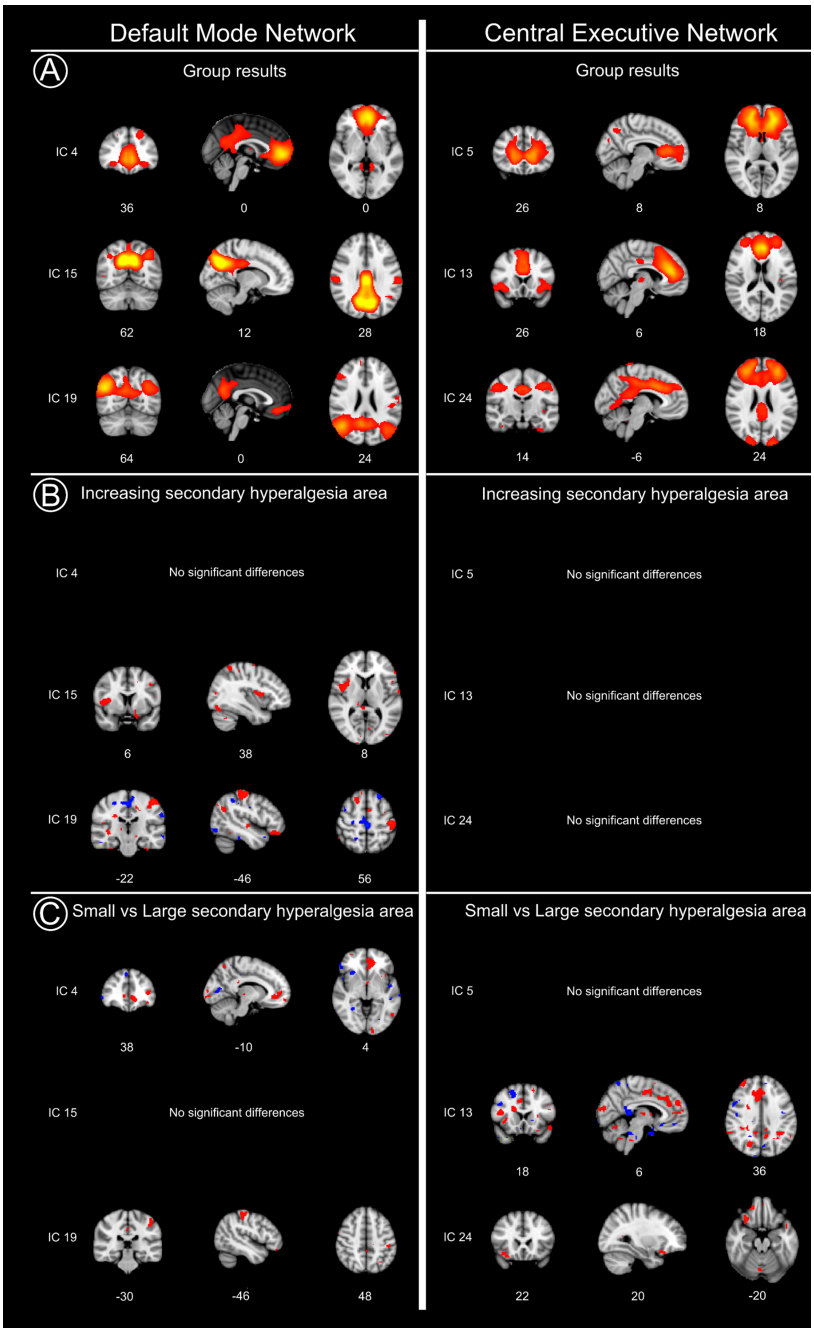

Figure 10 depicts resting state connectivity in the Default mode and the Central executive network.
A) Group results from all participants (n=115) showing the Default mode network (comprised of IC 4, IC 15, and IC 19) and the Central executive network (comprised of IC 5, IC 13, and IC 24).
B) Resting state connectivity illustrating the effect of increasing secondary hyperalgesia area. Blue colours indicate brain structures where connectivity decreased with increasing area of secondary hyperalgesia, and red colours indicate brain structures where connectivity increased.
C) Resting state connectivity comparing participants with small (lower quartile) vs. large (upper quartile) areas of secondary hyperalgesia. Blue colours indicate brain structures with increased connectivity in participants with small areas of secondary hyperalgesia, and red colours indicate brain structures with increased connectivity in participants with large areas.
All statistically significant findings can be observed visually and cross referenced with the results displayed in table 5 and 6 in paper IV. Numbers refer to standard Montreal neurological institute Atlas coordinates.
Abbreviations: IC, independent component.

6. Discussion

6.1 Principal findings

In study I, we found that the heat pain model BTS elicits secondary hyperalgesia with a high level of reproducibility, and that it can be employed in investigations of secondary hyperalgesia in healthy men, thus allowing phenotypic assessment of healthy men based on their area of secondary hyperalgesia.

In study II, we found that the HPDT is significantly associated with the size of the secondary hyperalgesia area elicited by BTS. However, with a R^2 of 19% and wide prediction limits, HPDT only provides a modest explanation of the inter-individual variation in secondary hyperalgesia, suggesting that HPDT and areas of secondary hyperalgesia may represent two independent pain entities.

In study III, we found that the size of the secondary hyperalgesia area is not associated with the volume of the caudate nuclei or any other predefined brain structures relevant for pain processing, suggesting that the propensity to develop central sensitization, assessed as secondary hyperalgesia area, is not correlated to the volume of pain relevant brain structures.

In study IV, we found that an increasing size of secondary hyperalgesia area is associated with both increased and decreased connectivity in multiple brain structures. Moreover, we found that participants with different phenotypic expressions of secondary hyperalgesia exhibited significantly different resting state connectivity. The results, however, are exploratory and should be interpreted with caution.

6.2 Strengths and limitations

6.2.3 Strengths

The studies included in this thesis have several strengths.
Firstly, we applied a rigorous methodology in all studies. All studies were registered at ClinicalTrials.gov, and the protocols of study II, III, and IV were published to increase transparency and to avoid post-hoc revisions and data dredging[67,68]. We conducted extensive statistical simulations to estimate the sample sizes in study I and II, and in all studies, we predefined all outcome measures of interest.

Secondly, the sequential design of our studies allowed us to evaluate the pain model, BTS, before applying it in the subsequent studies. The rigorous methodological design applied in study I enabled us to determine the reliability of the pain model in a standardised population with one or two investigators conducting the tests. The uniform general inclusion and exclusion criteria applied in the four studies ensured that the populations were similar with regard to several basic characteristics (table 1). The reliability of the BTS model was estimated in study I, and because of the homogeneity of the study populations, we were confident in applying the pain model in study II as well.

Thirdly, for inter-individual comparisons of participants, all areas of secondary hyperalgesia were adjusted for individual body surface area. Currently, there exists no gold standard for adjustment of secondary hyperalgesia areas when conducting inter-individual comparisons; however, we believe our approach to be a fair approximation.

Finally, in study II, III, and IV, we included a high number of participants. The high number of participants in study II resulted in an empirical power of 99.9%, substantially minimising the risk of type II errors and simultaneously accentuating the robustness of our findings.
In study III and IV, we present findings from the largest MRI study to date to investigate secondary hyperalgesia. We conducted all MRI scans on the same 3 tesla Siemens MRI-scanner over a short period of time, hereby reducing the risk of scanner drift. In study III, we attempted to minimise the risk of type I errors by predefining all anatomical brain structures of interest, and the primary and secondary outcome measures were thus based on known cortical and subcortical brain structures relevant for pain processing[25,96,97]. Moreover, in study III and IV, we

applied a hypothesis driven design, including participants regardless of their individual area of secondary hyperalgesia.

6.2.3 Limitations

The studies included in this thesis have some limitations.
Firstly, we applied strict inclusion and multiple exclusion criteria in all studies, resulting in inclusion of a very homogenous population. Consequently, the immediate results only apply to healthy, young men. Inclusion of a more heterogeneous population may have increased the variation in pain testing results and MRI findings. However, inclusion of both sexes could have introduced several factors of variations including possible sex and gender differences in pain responses[7,9,98], neuroanatomy, and fMRI findings[99-103], as well as a potential hormonal influence of the menstrual cycle on pain responses[104] and MRI findings[105-111]. Likewise, inclusion of chronic pain patients or individuals with various comorbidities would have introduced an unknown number of confounding variables complicating the interpretation of our findings. Additionally, the strict inclusion criteria as well as the nature of an experimental pain study may also have contributed to a sampling bias resulting in low inclusion of individuals with a tendency towards high pain catastrophizing and high anxiety. This type of sampling bias is difficult to avoid since healthy individuals with a high anxiety or fear towards pain may intentionally avoid voluntarily enrolment in these types of studies. Proper investigation of psychological variables and experimental pain responses should therefore involve consecutive inclusion of patients prior to surgery or specific inclusion of individuals with high psychological vulnerability.

Secondly, we did not evaluate genetics, stress, hormone levels, or dietary intake. Evidence indicates that high levels of serum cortisol and testosterone[112], diets high on tryptophan[113,114], and certain genetic markers[10-14] may influence the individual pain response. There is a lack of evidence investigating the influence of these variables on the development of secondary hyperalgesia. However, it cannot firmly be dismissed that the large inter-individual variation in secondary hyperalgesia areas, HPDT, and p-TS response partly is a result of inter-individual differences in genetics, diet, and stress levels.

Thirdly, we were not able to investigate all desired outcome measures in study III. Due to technical problems, images from the DTI sequence could not be analysed, and the outcome measures specified in the study protocol[68] investigating white matter microstructure and tractography could not be evaluated at present. Moreover, we were not able to sufficiently segment all the brain structures specified in the study protocol. Thus, presently we cannot conclude on possible associations between areas of secondary hyperalgesia and the volume of the secondary somatosensory cortex, the supplemental motor area, the substantia nigra, and the subthalamic nucleus.

Finally, the designs of Study II, III, and IV allow us only to conclude on associations. An inherent limitation of association studies is the inability to conclude on causality. Consequently, the findings in study II, III, and IV do not allow us to firmly conclude on causal factors. With the strict inclusion and exclusion criteria, we have attempted to minimise the influence of unknown variables; however, the exact causality of the inter-individual differences in secondary hyperalgesia areas cannot be derived from the present studies.

6.3 Current evidence and clinical implication

This thesis presents results from a series of studies aimed to investigate the development of secondary hyperalgesia in healthy men and its association with cutaneous heat pain sensitivity, structural brain anatomy, and resting state connectivity.

6.3.1 Secondary hyperalgesia

Cutaneous secondary hyperalgesia can be induced by applying standardised pain models. The development of experimentally induced cutaneous secondary hyperalgesia has been investigated in numerous studies[25-28,30-44,54]; however, only few studies have investigated the reliability or validity of the pain models[26,29,30,37]. Werner et al. conducted a well-performed meta-analysis investigating the development of secondary hyperalgesia using three pain models including the heat/capsaicin model (45°C cutaneous heat stimulation for 5 minutes followed by application of 0.075% capsaicin cream for 30 minutes), BTS (45°C for 3 minutes), and the burn injury model (47°C for 7 minutes).

A total of ten studies were included, resulting in an estimated pooled ICC of 0.69 (95% CI 0.61 to 0.76), 0.74 (95% CI 0.65 to 0.81), and 0.58 (95% CI 0.43 to 0.69) for the heat/capsaicin model, BTS,

and burn injury model respectively. In the analysis of BTS, four studies were included reporting ICCs ranging from 0.48 (0.07 to 0.76) to 0.84 (0.68 to 0.93) and CVs ranging from 14.2 (SD 18.3) to 29.9 (SD 19.5)[26,32,33,36].

Based on the study by Werner et al., we decided to apply the BTS model in our studies. However, in three of the four BTS-studies[26,32,33] included by Werner et al., BTS was applied following heat/capsaicin stimulation. Moreover, two of the four studies only had two study sessions. Currently, it remains unknown whether stimulation with the heat/capsaicin model prior to BTS affects the development of secondary hyperalgesia following BTS. Moreover, our statistical simulation analyses revealed that four study days were needed to discern the relevant variance components with an acceptable precision. Thus, in order to ensure that BTS could be used as a model for eliciting secondary hyperalgesia in healthy men, we conducted study I investigating inter- and intra-individual variance in the development of secondary hyperalgesia following BTS. The primary findings in study I with an intra-participant intra-investigator ICC of 0.85 (0.78 to 0.90) and a CV of 0.17 (0.14-0.21) confirmed to some extent the findings from previous studies[32,36] and enabled us to apply the BTS model in the subsequent studies.

The findings in study I also corroborate reports from previous studies, indicating that the size of experimentally induced areas of secondary hyperalgesia remains largely constant within each individual, suggesting that areas of secondary hyperalgesia may represent a phenotypic characteristic. Evidence also indicates that areas of secondary hyperalgesia may reflect individual predisposition to develop central sensitization. However, the impending question persists. Why do individuals from an otherwise homogenous population exhibit substantial inter-individual differences in the propensity to develop central sensitization assessed as secondary hyperalgesia areas? From study II we know that the cutaneous heat pain sensitivity only provided modest explanation for the inter-individual variance in secondary hyperalgesia areas. This indicates that although HPDT and secondary hyperalgesia may characterise some facets of pain sensitivity, the two measures may be dissociative and represent different partially independent pain entities. Similar conclusions were made in an additional study that reported no significant associations between temporal summation of pain, another manifestation of central sensitization, and heat pain threshold[115].

Findings from study II also indicated that the HADS-D sub-score only provided a very modest explanation for the inter-individual variation in secondary hyperalgesia areas. Though several clinical studies have reported significant associations between certain personality traits and

postoperative pain[15,16,116,117], the influence of psychological variables on experimental pain remains unclear[118]. Interestingly, studies of healthy volunteers found that pain-focused cognitive intervention reduced the area of secondary hyperalgesia[119], and that increasing pain-related catastrophizing was associated with increasing temporal summation of pain[120]. The findings indicate that central sensitization may indeed be influenced by pain-related cognition. In study II, we only found a weak association between secondary hyperalgesia area and HADS-D sub-score, and no significant associations with anxiety or pain catastrophizing. However, our study was not designed to detect these associations, and the inter-individual variation in PCS and HADS scores were very low. Further studies investigating the influence of psychological variables on central sensitization should attempt to include participants with high as well as low psychological vulnerability and apply fMRI to observe functional effects of possible psychological interventions.

6.3.2 Central sensitization and brain anatomy

MRI allows detailed estimation and detection of differences in brain structure, enabling researches to identify even minute structural changes with high accuracy[121-123]. In study III, we obtained anatomical images from all participants using high-resolution 3 tesla MRI in order to investigate if the area of secondary hyperalgesia was associated with the volume of pain relevant brain structures.

Numerous clinical studies investigating chronic pain patients have identified neuroanatomical correlates of chronic pain with reduced grey mater volume in several brain structures[124-128]. Central sensitization is believed to be an essential component of several chronic pain conditions. Nonetheless, in study III, we did not identify volume differences in pain relevant brain structures when comparing participants with small vs. large areas of secondary hyperalgesia, indicating that differences in the propensity to develop central sensitization were not related to pain relevant brain structure volume. However, neuroanatomical abnormalities found in chronic pain patients are not easily transferred into a setting involving healthy individuals only. Evidence indicates that grey matter abnormalities found in chronic pain patients are a consequence of experience-dependent neuronal plasticity due to persistent painful stimulation, and that some abnormalities are reversible if the pain is terminated[128-130]. Neuroanatomical comparisons between healthy individuals and chronic pain patients may therefore have limited applicability.

Studies of healthy participants have reported significant correlations between reduced grey matter volume of pain relevant brain structures and increased heat pain sensitivity[131,132] as well as

visceral sensitivity[133]. The findings of study III are not coherent with findings from previous studies. However, inter-study comparisons may prove difficult. In study III, our outcome measures primarily focused on associations between areas of secondary hyperalgesia rather than cutaneous heat pain sensitivity. Moreover, from study II, we know that HPDT only provides a moderate explanation of the inter-individual variance in secondary hyperalgesia areas.

In a recent MRI study of healthy participants, it was reported that the volume of the caudate nuclei was inversely correlated with the area of secondary hyperalgesia[25]. However, there exist important differences between the study by Asghar et al.[25] and study III. Asghar et al. included both male and female participants, whereas we solely included men. Moreover, Asghar et al. included participants based on their area of secondary hyperalgesia without adjusting for body surface area, whereas we included participants without prior knowledge of their individual area of secondary hyperalgesia and adjusted for body surface area. Finally, we predefined all anatomical brain structures of interest and included a comparatively higher number of participants.

The findings in study III are corroborated by reports indicating that individuals identified with high or low pain sensitivity do not demonstrate structural neuroanatomical differences[122]. Instead, high pain sensitizers are more disposed to develop grey matter reductions after repetitive noxious stimulation[122], suggesting that innate pain sensitivity is not related to structural anatomy but may indeed be caused by functional differences. Likewise, differences in innate propensities to develop central sensitization may be a result of differences in functional brain activity rather than neuroanatomy.

6.3.3 Central sensitization and fMRI

Functional imaging relies on brain haemodynamic and provides an indirect measure of brain activity. fMRI can be applied to investigate responses to external stimuli (e.g. pain) and tasks or to investigate the functional connectivity between brain areas in a stimulus- and task-free state (resting state)[96]. Investigations of the response to nociceptive stimulation using BOLD fMRI have led to the identification of several pain relevant brain structures. Combined with findings from psychological studies, results from fMRI studies have broadened the original concept of pain to involve not only the pain sensation itself but to also include emotional, affective, attentive, as well as motor responses[96]. Central sensitization is believed to be involved in the development of pain and in the transition from acute to chronic pain[21]. BOLD studies investigating brain activity during

central sensitization have identified multiple brain structures with increased activity in areas associated with somatosensory processing, cognition, affect, and pain modulation[25,134-138].

When comparing healthy individuals with large vs. small areas of secondary hyperalgesia, Asghar et al.[25] found that individuals with large areas had increased activity in brain structures related to the Default mode network (precuneus and posterior cingulate cortex) as well as the post central gyrus and the caudate nucleus during mechanical pin-prick stimulation within the secondary hyperalgesia area. In spite of the limitations discussed previously, these findings may indicate that individuals with different phenotypic expressions of secondary hyperalgesia differ in neuronal activation in response to painful stimulation.

Moreover, Seifert et al.[137] found an inverse correlation between the size of the secondary hyperalgesia area and activity within the medial prefrontal cortex, suggesting that individuals with a low propensity to develop central sensitization had a high capacity for descending endogenous analgesic modulation[137].

BOLD studies investigating brain activity in response to mechanical pin-prick stimulation in sensitized skin report of increased activation in multiple brain structures including the mesencephalic pontine reticular formation, somatosensory cortices, posterior cingulate cortices, and frontal middle gyri[41,136,138], indicating that central sensitization is a central phenomenon that is modulated by supraspinal activity in multiple brain structures.

The present findings in study IV indicate that increasing areas of secondary hyperalgesia are associated with the resting state connectivity in multiple brain structures including the primary and secondary somatosensory cortices, orbitofrontal cortices, dorsolateral prefrontal cortex, and temporal superior gyri. Our results suggest that individuals displaying large areas of secondary hyperalgesia may have an increased sensitivity to pain and an enhanced tendency towards pain appraisal and pain attention. Moreover, our results indicate that individuals with small areas of secondary hyperalgesia may possess a comparatively enhanced capacity for endogenous analgesic modulation.

The findings in study IV indicate that individuals with different phenotypic expressions of secondary hyperalgesia display significantly different resting state connectivity, suggesting that these individuals may indeed differ in their propensity to develop central sensitization.

Central sensitization induces a state of CNS hyper-excitability that disengages the classical stimulus-response relationship typical for pain perception. The individuals included in the four studies of this thesis were healthy and pain-free. This indicates that differences in phenotypic

expressions of secondary hyperalgesia, i.e. differences in central sensitization, are independent of current pain and comorbidities, suggesting that healthy, pain-free individuals may possess an innate predisposition towards developing central sensitization. Comprehending why (and if) some individuals are more prone to develop central sensitization, and if this conveys an increased risk of developing pain hypersensitivity or persistent pain, may introduce analgesic treatment strategies directed at normalising or reducing the inherent state of CNS hyper-excitability and identify possible biomarkers and targets for future analgesic therapy[21].

6.4 Future perspectives

The predictive value of secondary hyperalgesia areas in determining individual pain sensitivity and risk of developing persistent pain remains limited. Studies of healthy, young individuals cannot always be translated directly into a clinical day-to-day life, where the primary population suffering from pain often consists of patients with varying age, sex, and comorbidities. Future studies investigating the predictive value of secondary hyperalgesia areas should therefore involve patients undergoing surgery. Identification of pain sensitive individuals prior to surgery may be useful in planning individual postoperative analgesic therapy. Likewise, identification of high-pain sensitizers for inclusion in pharmaceutical research trials may improve future analgesic research considerably.

Studies investigating the basic properties of pain physiology, including central sensitization, are necessary in order to improve current analgesic therapy.

In study III, we provided evidence that the propensity to develop central sensitization, assessed as secondary hyperalgesia areas, is not associated with the volumes of pain relevant brain structures in healthy, young men. Imaging studies investigating healthy volunteers should therefore primarily focus on imaging modalities investigating brain function, e.g. fMRI. Also, in this line of research investigations of patients undergoing surgery, as well as individuals suffering from chronic pain are necessary, since not all findings in healthy individuals can be applied to “real-life” patients suffering from pain.

fMRI studies investigating the brainstem are also of interest, since evidence suggests that brainstem structures may be intricately involved in central sensitization. Likewise, studies investigating spinal cord function and the interaction between spinal- and supra spinal mechanisms may also yield promising results.

Finally, investigations on the endogenous opioid system may be of interest, since impairment of this protective mechanism may play a role in the development of persistent pain[139].

8. Conclusion

The studies included in this thesis collectively provide insight in the development of secondary hyperalgesia and its association with brain anatomy and connectivity. In study I, we demonstrated that BTS elicits secondary hyperalgesia in healthy, young men with a high level of reproducibility. BTS can therefore be utilized in future investigations of secondary hyperalgesia and employed to phenotype healthy men based on their area of secondary hyperalgesia. In study II, we found a significant association between HPDT and the size of the secondary hyperalgesia area; however, with a R^2 of 0.19 we concluded that HPDT only offered a moderate explanation of the inter-individual variation in secondary hyperalgesia area. In study III, we found that the size of the secondary hyperalgesia area was not associated with the volume of pain relevant brain structures in healthy men, suggesting that the propensity to develop central sensitization is not associated with the volume of pain relevant brain structures. Finally, in study IV, we found significant associations between the size of the secondary hyperalgesia area and the connectivity in multiple brain structures. Our findings indicate that individuals with different phenotypic expressions of secondary hyperalgesia indeed may display significantly different resting state connectivity. Accordingly, this suggests that healthy men with different phenotypic expressions of central sensitization may actually process pain differently. The exploratory nature of study IV must be kept in mind, and results should therefore be interpreted with caution. Consequently, further studies are needed in order to determine why some individuals have a higher propensity to develop central sensitization, and if these individuals are at a greater risk of developing pain hypersensitivity or chronic pain. Insight in these important physiological mechanisms may lead to novel findings and new strategies in the treatment of acute and chronic pain.

9. Conflicts of interest

None of the authors of the four studies included in this thesis have any conflicts of interest.

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PAPERS I - IV

PAPER I

PLOS ONE

RESEARCH ARTICLE

The Area of Secondary Hyperalgesia following Heat Stimulation in Healthy Male Volunteers: Inter- and Intra-Individual Variance and Reproducibility

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Abstract

Introduction

Clinical pain models can be applied when investigating basic physiologic pain responses in healthy volunteers. Several pain models exist; however, only few have been adequately validated. Our primary aim with this prospective study was to investigate the intra- and inter-individual variation in secondary hyperalgesia elicited by brief thermal sensitization (45°C for 3 min) in healthy volunteers.

Material and Methods

Fifty healthy volunteers were included. Areas of secondary hyperalgesia following brief thermal sensitization were investigated by 2 observers on 4 experimental days, with a minimum interval of 7 days. Additionally, heat pain detection threshold and pain during thermal stimulation (45°C for 1 min.), and the psychological tests Pain Catastrophizing Scale and Hospital Anxiety and Depression Score were applied.

Results

For areas of secondary hyperalgesia, an intra-observer intra-person correlation of 0.85, 95% CI [0.78, 0.90], an intra-observer inter-person correlation of 0.03, 95% CI [0.00, 0.16], and a coefficient of variation of 0.17, 95% CI [0.14, 0.21] was demonstrated. Four percent of the study population had areas of secondary hyperalgesia both below the 1st and above the 3rd quartile considering all included participants. Heat pain detection threshold predicted area of secondary hyperalgesia with an adjusted R^2 of 0.20 (P = 0.0006).

and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

Conclusions

We have demonstrated a low intra-individual, and a high inter-individual variation in thermally induced secondary hyperalgesia. We conclude that brief thermal sensitization produce secondary hyperalgesia with a high level of reproducibility, which can be applied to investigate different phenotypes related to secondary hyperalgesia in healthy volunteers.

Trial Registration

clinicaltrials.gov NCT02166164

Introduction

Clinical pain models are important in order to investigate basic physiologic pain responses in both healthy volunteers and patients. Such models play an important role in translational studies and are necessary to bridge the gap between animal and human pain research.

There are numerous pain models, employed to investigate different aspects of the human physiologic pain response [1, 2]. Models applying nociceptive stimulation to the skin, by heat [3–8], cold [9] or electrical stimuli [10] can be used in investigation of injury-induced sensitization of the central nervous system. Central sensitization is believed to be an important factor in the development and maintenance of pain, and represents an uncoupling of the nociceptive stimulus and the nociceptive response [11]. Likewise, central sensitization may have a prominent role in the inter-individual differences in pain sensitivity; the concept that different individuals experience different levels of pain when exposed to identical noxious or nociceptive stimuli.

A standardized heat injury of the skin results in primary hyperalgesia at the site of injury, and secondary hyperalgesia surrounding the traumatized area [1, 3–8]. Injury-induced secondary hyperalgesia is characterized by reduced thresholds for mechanical stimulation, and is supposed to result from an altered central processing of mechano- and nociceptive input in A-fibers from the periphery, so that activation of these fibers produce painful sensations [11–14].

Moreover, a significant inter-individual difference in the size of the area of secondary hyperalgesia may persist, implying that the development of secondary hyperalgesia may be a phenotypic expression [15]. The inter-individual differences in areas of secondary hyperalgesia may be due to genetic [16], physiologic, and psychological differences [17], as well as differences in brain activation during pain stimulation [18]; however, further studies are needed to confirm this hypothesis.

Several pain models investigating cutaneous sensitization exist—each investigating different aspects of cutaneous sensitization. Brief thermal sensitization (BTS) [4–8] induces short lasting cutaneous sensitization, ideal for multiple inductions throughout a study day. With the BTS-model the skin is heated to 45°C for 3 min., resulting in mild pain perception, and short lasting secondary hyperalgesia [4–8]. Thus, the BTS-model can be applied in investigation of central sensitization. To our knowledge there have been no prospective trials investigating intra-individual, inter-individual, and inter-investigator variances of areas of secondary hyperalgesia following BTS. Validation of the models is paramount, and methodological sound studies investigating the inter-and intra-individual reproducibility, as well as the inter- and intra-investigator reproducibility are needed in order to validate the use of the models in future scientific research [19, 20].

The primary aim of this prospective cohort study was to investigate the intra-individual and inter-individual variance in secondary hyperalgesia elicited by brief thermal sensitization in healthy male volunteers.

Material and Methods

The study was registered at clinicaltrials.gov (NCT02166164), and approved by the Danish Regional Committee on Health Research Ethics (Identifier: H-4-2014-027), and the Danish Data Protection Agency (Identifier: 30–1217). Informed written consent was obtained from all participants before inclusion in the study. The study was conducted at the Department of Anaesthesiology, 4231, Rigshospitalet, Copenhagen, Denmark, in the period from June 10, 2014 to September 17, 2014.

Study design

This prospective cohort study was designed to evaluate the method of BTS, and consisted of four identical experimental days and one information/inclusion day. To prevent carry-over effects, the information day and each of the four experimental days were separated by a minimum of seven days.

The participants were tested with three procedures, brief thermal sensitization (BTS), heat pain detection threshold (HPDT), and pain during 1 min. thermal stimulation (p-TS) (for definitions, see below) on the four separate experimental days in a predefined sequence (see Randomization and allocation concealment).

All the pain models were performed with the computer-controlled Somedic Senselab MSA Thermotester™; size 2.5x5 cm.

On the information day the participants were given the psychological tests Pain Catastrophizing Scale (PCS) and Hospital Anxiety and Depression Score (HADS). The participants completed the PCS and HADS questionnaires and handed them back on the first experimental day in a concealed opaque envelope to ensure blinding. Opening of the envelopes were postponed until all participants had completed all four experimental days.

In order to investigate the inter-investigator variance, two different investigators were responsible for the testing on the different experimental days. Each participant was tested on four different study days. Two different investigators performed the testing. Every participant was thus tested by each investigator independently on two separate days—the order of the days being randomized.

The investigators were trained in performing the assessments similarly, but conducted the tests independently of each other. Test results were placed in an opaque sealed envelope to ensure blinding between the two investigators.

Study participants

50 healthy male volunteers were included (Fig 1). Informed consent was obtained from all included participants. Participants were recruited by advertisement in the medical student magazine and online at www.forsøgspersoner.dk. Inclusion criteria were: Male sex, age $\geq$18 years and $\leq$35 years, speak and understand the Danish language, and signed informed consent. Exclusion criteria were: Failure to cooperate to the tests, alcohol and/or substance abuse, consummation of analgesics within 48 hours before experimental day, consummation of prescription medicine within the last 30 days before experimental day, history of chronic pain, psychiatric diagnoses, tattoos on the extremities, and a Body Mass Index (BMI) of >30 kg/m^2 and < 18 kg/m^2.

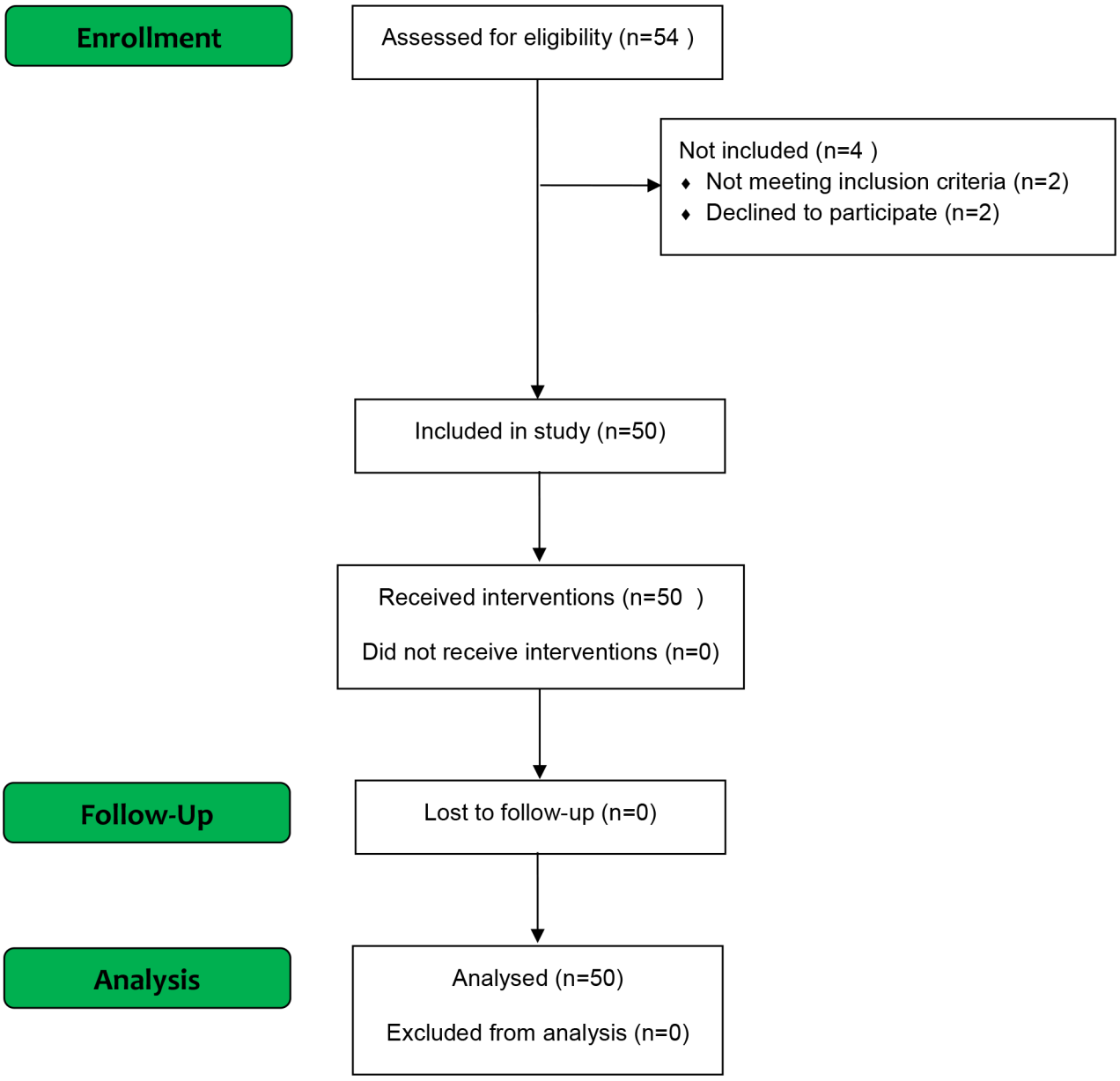

Fig 1. Flowchart of included study participants.

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Setting

The study was conducted in a quiet secluded room (temperature 22–25 degrees Celsius), where only the study participant and the responsible investigator were present. The participant was placed in a supine position during the assessment. The study was conducted during the time from 8.00 AM to 5.00 PM.

Pain models

Brief thermal sensitization (BTS). Induction of BTS was performed anterior on the right thigh, in the midline between the anterior superior iliac spine and the base of patella [4–8]. The

skin was heated to 45°C for 3 min. After 3 min., while the 45°C thermode was still placed on the skin, the assessment of secondary hyperalgesia was performed (see section "assessment of secondary hyperalgesia"). The assessment of secondary hyperalgesia took approximately 1–2 minutes, with a maximum duration of heat stimulation of 5 min.

Pain during 1 min. thermal stimulation (p-TS). Evaluation of p-TS was performed on the anterior aspect of the non-dominant volar side of the forearm [5, 21–24]. The participant's skin was heated to 45°C for 1 min., while the participant performed continuous evaluation of pain on an electronic visual analogue scale (VAS, 0 mm = no pain; 100 mm = worst pain imaginable). A maximum (Max.)-VAS and a VAS-Area under the curve (AUC) were registered. The participant was not able to see the computer-screen during the assessment.

Heat pain detection threshold (HPDT). The skin on the dominant anterior volar side of the forearm was heated with an increase in temperature of 1°C/sec (initial temperature 32°C) [5, 8, 21–24]. The study participant stated when the heat was perceived as painful by pressing a button, and the temperature was registered. The HPDT was calculated as an average of 4 stimulations. Each stimulation was performed with an interval of 6–10 seconds. The participant was not able to see the computer-screen during the assessment.

Assessment of secondary hyperalgesia. The area of secondary hyperalgesia was evaluated following BTS. The area was quantified by stimulation with a 19G monofilament (Von Frey hair) in 4 linear paths arranged 90° around the center of the heat-stimulation. The monofilament stimulation was initiated in normal skin, and advanced in steps of 5 mm with 1-second intervals towards the center of the heat-stimulation until the participant stated a clear change in the sensation (burning, intense pricking, tenderness). The borders were marked with a felt pen, and the transverse and longitudinal axes were measured with a pliable measuring tape for rectangular area calculation [4–8, 15, 21–27].

The area of primary hyperalgesia was defined as the area directly heated by the thermode (2.5x5 cm). The surrounding area with decreased mechanical thresholds was defined as the area of secondary hyperalgesia. For calculations, the area of the thermode (2.5x5 cm) was not subtracted from the total area of secondary hyperalgesia.

Psychological testing

Hospital Anxiety and Depression Scale (HADS). *HADS* is a questionnaire consisting of 14 questions [28]. HADS evaluates depression and anxiety, and can be subdivided in HADS-Anxiety and HADS-Depression. The highest achievable score is 42.

Pain Catastrophizing Score (PCS). *PCS* is a questionnaire consisting of 13 questions [29] and can be subdivided into 3 subtests that each evaluates the central elements in catastrophizing: Rumination, magnification, and helplessness. The highest achievable score is 52.

Outcomes

Primary outcome. To determine the intra- and inter-participant variance, and the intra- and inter-investigator variance of the secondary hyperalgesia areas following BTS on 4 separate experimental days with two different observers.

Secondary outcomes. To investigate:

1. How precise the scores of PCS and HADS predict the size of the area of secondary hyperalgesia.
2. How precise the subscales in PCS and HADS (PCS-rumination, PCS-magnification, PCS-helplessness, and HADS-Anxiety, HADS-Depression) predict the size of the area of secondary hyperalgesia.

3. How precise the HPDT evaluated on the 4 experimental days predicts the area of secondary hyperalgesia on the respective 4 experimental days.
4. How precise the Maximum VAS-score following p-TS evaluated on the 4 experimental days predicts the area of secondary hyperalgesia on the respective 4 experimental days.
5. How precise the VAS-AUC following p-TS evaluated on the 4 experimental days predicts the area of secondary hyperalgesia on the respective 4 experimental days

Sample size estimation

Estimation of the number of participants, investigators and experimental days were based on statistical simulations based on data from a previous study [4]. The simulations demonstrated that a scenario with 2 investigators, 4 experimental days and 50 study participants would enable us to discern the relevant variance components with acceptable precision.

For full documentation of statistical simulations, please see supporting information available online (S1 Appendix).

Randomization and allocation concealment

The reproducibility of the area of secondary hyperalgesia following BTS was the primary outcome. Thus, in order to avoid possible carry over effects of the HPDT and p-TS, all study days began with BTS. Testing with HPDT and p-TS were therefore subsequent to BTS on all study days; However, the sequence of testing (HPDT and p-TS) was randomized for each patient and each experimental day, so that on two of the four experimental days the sequence was: 1) BTS, 2) HPDT, 3) p-TS, and on the remaining two study days the sequence was: 1) BTS, 2) p-TS, 3) HPDT (Fig 2).

The investigator responsible for testing and registration of data on the respective experimental day was randomized, so the same investigator was not responsible for testing the same participant on two consecutive experimental days. The allocation sequence of participants to investigator, and the test allocation sequence (HPDT and p-TS) were randomly generated via a computer by the data manager at Copenhagen Trial Unit.

The randomization of the test allocation sequence was kept in opaque sealed envelopes prepared by Copenhagen Trial Unit to ensure allocation concealment. The envelopes remained sealed until immediately before the testing.

Statistical analysis

The variation in the areas of secondary hyperalgesia derived from the study participant, the experimental day, and the investigator was determined using a variance component model.

For each of the 5 secondary outcomes, the ability of PCS, HADS, HPDT, Max-VAS and VAS-AUC (following p-TS), to predict individual variations in areas of secondary hyperalgesia

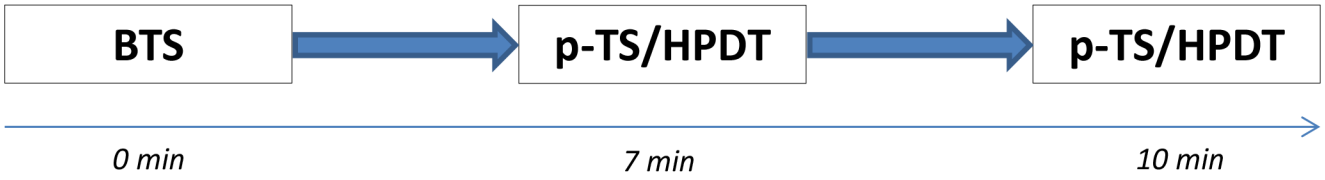

Fig 2. Sequence of clinical pain stimulation. Sequence of clinical pain stimulation. Sequence of p-TS and HPDT depends on randomization. Abbreviations: BTS, brief thermal sensitization; p-TS, pain during 1 min. thermal stimulation; HPDT, heat pain detection threshold; min, minutes.

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was investigated by linear regression on the estimated best linear unbiased predictors (EBLUPS) of individual secondary hyperalgesia extracted from the primary analysis. HPDT, Max-VAS, and VAS-AUC profiles were also summarized in terms of EBLUPS.

Significance of the predictors was assessed by Analysis of variance (ANOVA) methods and their predictive abilities were quantified with various summaries of prediction errors including 95% prediction intervals for the predictions. Model reduction was done by means of backwards elimination with a cut-off value of 5%.

The variation in the areas of secondary hyperalgesia described in the primary outcomes will be reported as Intraclass Correlations (ICC) and Coefficient of Variations (CV).

The predictive abilities of the variables described in the secondary outcomes are summarized by adjusted R^2 and illustrated by 95% predictive intervals for selected values of the remaining predictor.

Reproducibility of area of secondary hyperalgesia

We planned to categorize the participants in three groups according to the mean size of the area of secondary hyperalgesia: "Small-area" (1st quartile), "medium-area" (2nd and 3rd quartile), and "large-area" (4th quartile).

Based on the study performed by Werner et al. [15] we expected measures of reproducibility as detailed below:

1. Intraclass correlation coefficient (ICC) around 0.74, corresponding to an intra-participant variance of approximately 25% of the inter-individual variance.
2. A pooled mean intra-participant CV around 0.25
3. No more than 10% of the study participants change group from "small-area" to "large-area" or vice versa

Results

All study participants completed the study, and all study participants were analyzed for primary and secondary outcomes (Fig 1).

Data on participants' characteristics are presented in Table 1. The median interval between the information day, experimental day 1, 2, 3 and 4 was 12 (Range; 7–33), 9 (7–35), 15 (7–61) and 8.5 (7–37) days respectively.

No adverse or serious adverse events were reported.

The results from this study has previously been presented in abstract form [30].

Secondary hyperalgesia following BTS

The secondary hyperalgesia following BTS was evaluated on the 4 separate experimental days by 2 different investigators (1 investigator per experimental day). We found (i) an intra-

Table 1. Characteristics of included participants.

Variable	Mean (SD)	Range (min.-max.)
Age (years)	24 (3)	18–32
Height (m)	1,85 (0,1)	1.69–1.98
Weight (kg)	78 (9)	65–105
BMI (m^2/kg)	22,8 (2,1)	18.4–27.3

Abbreviations: BMI, Body Mass Index

doi:10.1371/journal.pone.0155284.t001

Table 2. Median size of the area of secondary hyperalgesia.

QST	Experimental day 1		Experimental day 2		Experimental day 3		Experimental day 4	
	Median (IQR)	Range	Median (IQR)	Range	Median (IQR)	Range	Median (IQR)	Range
BTS*	311.9 (256.6–457.0)	12.5–742.5	294.0 (250.5–417.3)	12.5–748.0	339.3 (231.7–389.9)	12.5–641.3	310.1 (244.1–413.3)	12.5–681.5

Median size and range of areas of secondary hyperalgesia following BTS on the four experimental days

* Medians, IQRs and ranges are given in Cm^2

Abbreviations: QST, Quantitative Sensory Testing; BTS, Brief thermal sensitization, IQR, Interquartile range

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investigator intra-participant correlation of 0.85, 95% CI (0.78, 0.90), (ii) an inter-investigator intra-participant correlation of 0.82 (0.69, 0.89), (iii) an intra-investigator inter-participant correlation of 0.03 (0.00, 0.16), and (iv) a coefficient of variation of 0.17 (0.14, 0.21).

Only 2 participants, 4% (1%, 13%) of the total population, had areas of secondary hyperalgesia in both below the 1st quartile and above the 3rd quartile, considering the total population. The sizes of the areas of secondary hyperalgesia as well as the results of the ICC are presented in Tables 2 and 3 and Fig 3, respectively.

Predictive factors of the area of secondary hyperalgesia

The ability of HPDT, Max-VAS (following p-TS), VAS-AUC (following p-TS), PCS and HADS to predict inter-individual variations in area of secondary hyperalgesia was investigated. After backwards elimination with a cut-off value of 5% only HPDT offered a statistically significant prediction of the area of secondary hyperalgesia with an adjusted R^2 of 0.20 (P = 0.0006). No other evaluated factors significantly predicted the area of secondary hyperalgesia. Results of HPDT, Max-VAS (following p-TS), VAS-AUC (following p-TS), PCS and HADS are presented in Tables 4 and 5 and Figs 4, 5 and 6.

Discussion

The aim of this study was to investigate the intra-individual, inter-individual, and inter-investigator variances of BTS-elicited areas of secondary hyperalgesia, in order to examine the reproducibility of the pain model. There are no gold standards for reproducibility; however, prior to our study we defined three criteria, hypothesized using data from previous studies. In order to confirm the reproducibility of the model, all three criteria had to be fulfilled (see methods). Firstly, we demonstrated an inter-investigator intra-participant correlation of 0.82 (0.69, 0.89), secondly we found a coefficient of variation of 0.17 (0.14, 0.21), and lastly we demonstrated that only two participants, 4% (1%, 13%) of the total population, had areas of secondary hyperalgesia in both below the 1st quartile and above the 3rd quartile, considering the total

Table 3. Main results.

Parameter	Result (95% CI)
$ICC_{Intra\text{-}investigator\ intra\text{-}participant}$	0.85 (0.78–0.90)
$ICC_{Inter\text{-}investigator\ intra\text{-}participant}$	0.82 (0.69–0.89)
$ICC_{Intra\text{-}investigator\ inter\text{-}participant}$	0.03 (0.0–0.16)
CV	0.17 (0.14–0.21)

Intraclass Correlations and Coefficient of Variation. Abbreviations: ICC, Intra Class Correlation; CV, Coefficient of Variation

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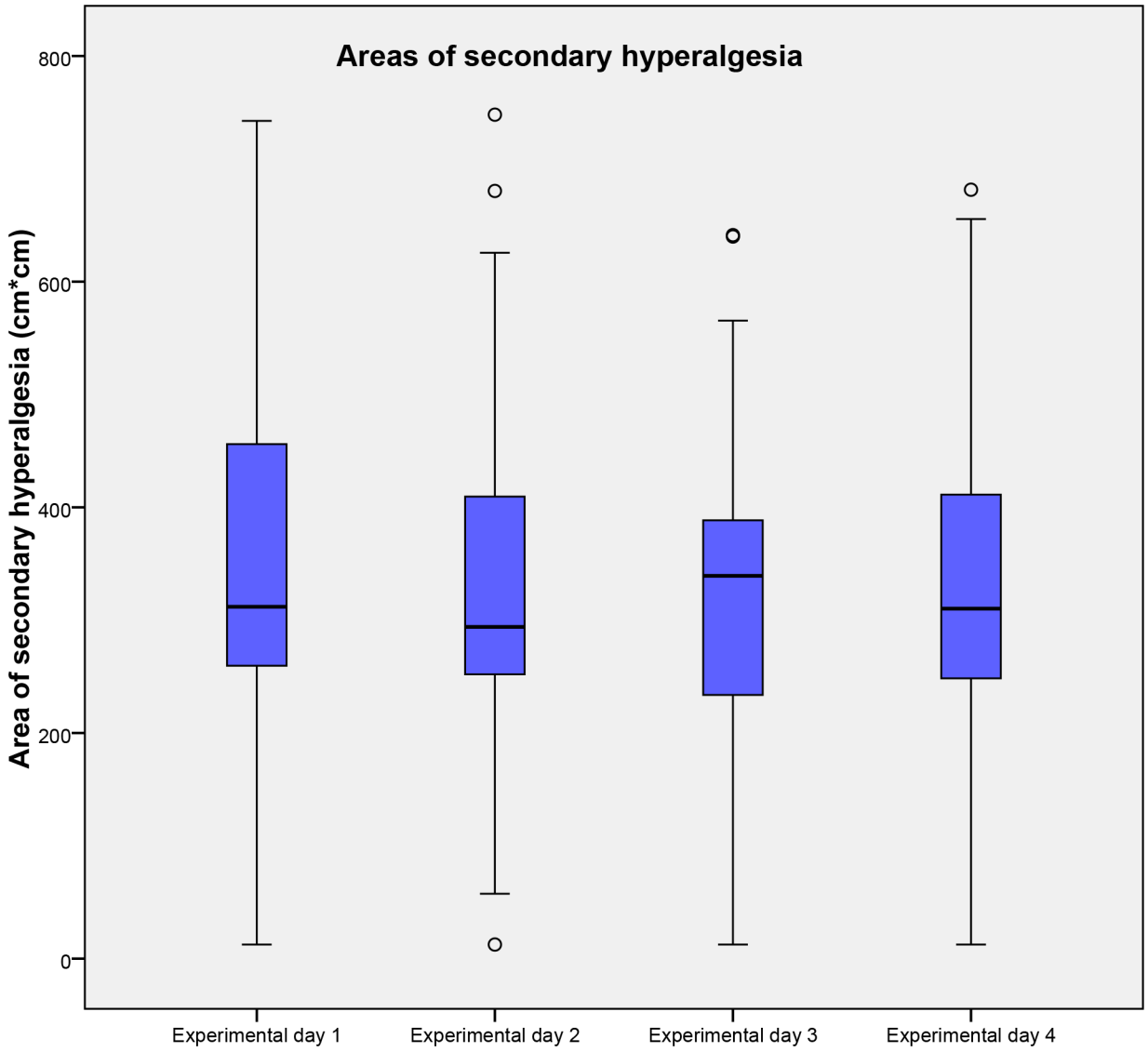

Fig 3. Areas of secondary hyperalgesia following BTS. Areas of secondary hyperalgesia on the 4 experimental days following brief thermal sensitization. Medians and interquartile ranges are displayed. Values higher than 1.5 times of upper quartile or lower quartile are designated as outliers and marked with °. Abbreviations: Cm, centimeter; BTS, Brief thermal sensitization.

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population. By evaluating the point estimates, all three criteria are fulfilled, and according to our pre-defined criteria, we have demonstrated that BTS is a reproducible model in regards to eliciting secondary hyperalgesia in healthy volunteers. The rather high ICCs and low CV demonstrate a high reliability and reproducibility respectively.

We also demonstrated that HPDT significantly predicted the area of secondary hyperalgesia. Our study was not designed to detect the correlation between HPDT and areas of secondary hyperalgesia; however, we find a highly significant result (P = 0.0006). An adjusted R^2 of 0.20 is nonetheless an indication that HPDT only offers a very modest explanation of the variation in BTS, which poses the question: How precise does HPDT predict areas of secondary hyperalgesia? In the current study, we estimated a prediction interval for BTS to (53.14–515.43) given a HPDT of 46°C, indicating wide prediction intervals. The possible variation of

Table 4. Heat pain detection threshold, and pain during 1 min. thermal stimulation.

QST	Experimental day 1		Experimental day 2		Experimental day 3		Experimental day 4	
	Median (IQR)	Range	Median (IQR)	Range	Median (IQR)	Range	Median (IQR)	Range
HPDT*	44.8 (42.4–46)	37.6–48.4	44.9 (43.1–46.3)	36.7–48.5	44.7 (42–46.4)	37.5–48.9	45.1 (43.6–46)	36.7–48.7
p-TS-max. VAS	23.0 (16–36)	0.0–72.0	27.0 (16–34)	0.0–71	25.0 (16.8–37.3)	3.0–64.0	27.0 (17.5–40)	2.0–71.0
p-TS-AUC VAS	853.8 (325.2–1198)	0.0–3663.0	851.8 (448.3–1275.3)	0.0–3620.50	936.2 (523.4–1216.2)	8.8–3400.7	901.7 (421.1–1416.6)	6.9–3294.5

Median, IQR and range of HPDT, p-TS-max VAS and p-TS-AUC VAS on the 4 experimental days.

* Medians, IQRs and ranges are given in °C

Abbreviations: QST, Quantitative Sensory Test; HPDT, Heat pain detection threshold; p-TS, pain during 1 min. thermal stimulation, Max., maximum; AUC, Area Under the Curve; VAS, Visual Analog Scale, IQR, Interquartile range

doi:10.1371/journal.pone.0155284.t004

BTS for a given HPDT value is huge and HPDT and secondary hyperalgesia following BTS may thus represent two different pain entities. HPDT has been demonstrated to be highly reproducible [31], and both HPDT and brief thermal sensitization activates peripheral A-delta and C-fibers [32]. However, the secondary hyperalgesia to punctate mechanical stimuli, that occurs as a result of central neuronal plasticity of the nociceptive system is mediated by A-fiber nociceptors, not C-fibers [11–14]. Thus, secondary hyperalgesia as a result of central sensitization elicited by BTS may be significantly distinct from HPDT. To our knowledge, no studies have investigated this issue, and further studies are needed to confirm this hypothesis.

Individual characteristics, such as sex, and obesity, may influence pain thresholds and tolerance [24, 33–37]. Likewise it remains unclear whether the menstrual cycle influences the pain sensitivity in healthy women [38]. In addition we have no knowledge of what effect tattoos have on peripheral cutaneous sensitivity. To account for these variables, we applied strict inclusion and exclusion criteria, in order to minimize the unknown factors of variation. This enabled us to focus on the ability of BTS to produce an area of secondary hyperalgesia, rather than the influence of the individual characteristics of the participant.

Several clinical studies have demonstrated an association between psychological factors and pain [39, 40]. In the present study the two psychological tests, HADS and PCS, did not significantly predict the area of secondary hyperalgesia. Our study was not designed to detect the correlation between psychological test scores and area of secondary hyperalgesia; however, post hoc analyses demonstrated that in order to investigate such a correlation with sufficiently high

Table 5. Scores of Pain Catastrophizing Scale and Hospital Anxiety and Depression Scale.

Psychological test	Median (IQR)	Range
$PCS_{Rumination}$	6 (3.8–8)	0–15
$PCS_{Magnification}$	2 (1–4)	0–7
$PCS_{Helplessness}$	3 (2–7)	0–19
PCS_{Total}	12 (7.8–18)	0–38
$HADS_{Anxiety}$	4 (1–6)	0–12
$HADS_{Depression}$	1 (1–3)	0–9
$HADS_{Total}$	5 (3–10)	0–18

Median, IQRs and range of the PCS and HADS. Total scores and scores of individual subtests are displayed. Abbreviations: PCS, Pain Catastrophizing Score; HADS, Hospital Anxiety and Depression Scale, IQR, Interquartile range

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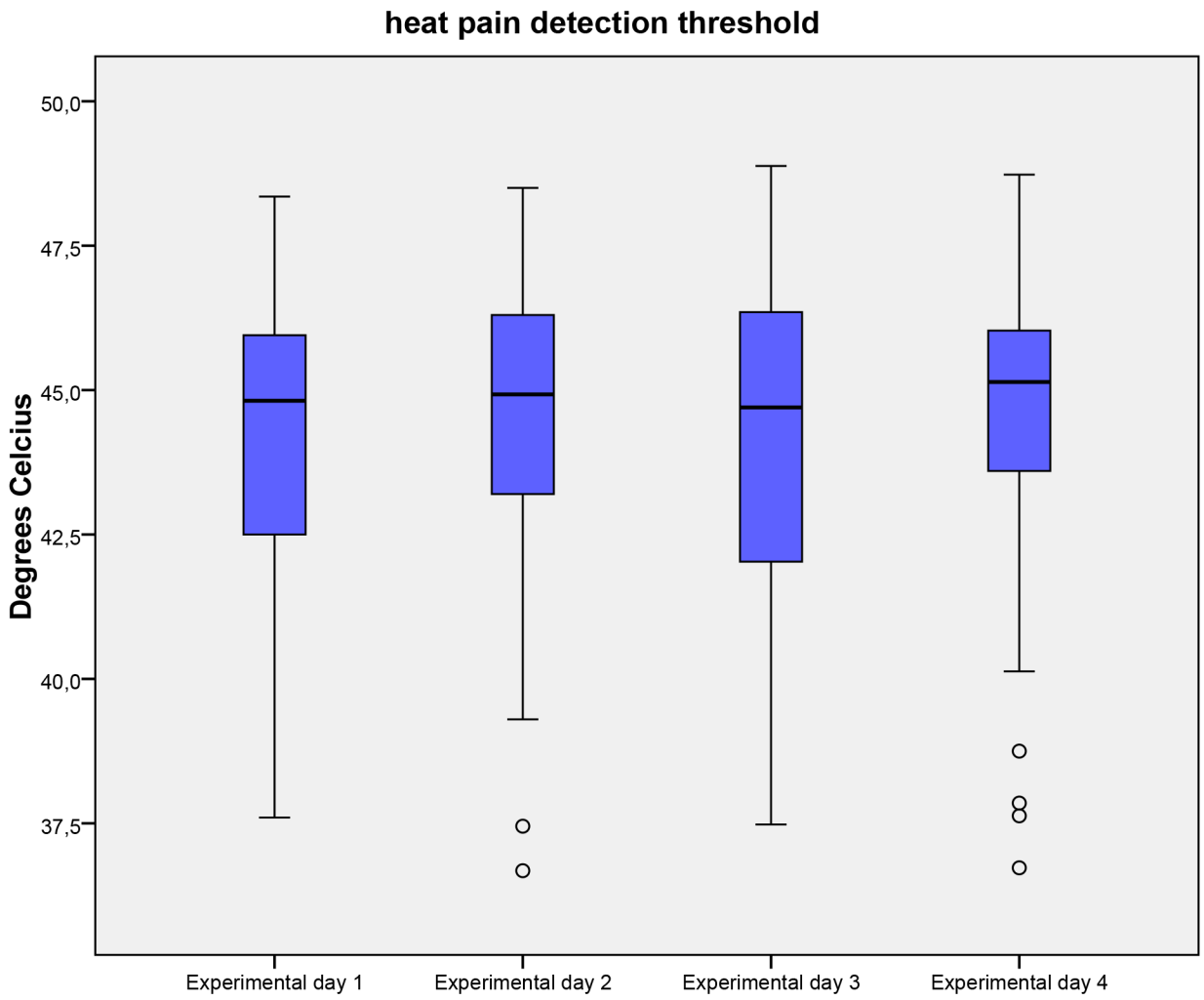

Fig 4. Heat pain detection threshold. Heat pain detection threshold on the 4 experimental days. Medians and interquartile ranges are displayed. Values higher than 1.5 times of upper quartile or lower quartile are designated as outliers and marked with °.

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power, 300 healthy participants should have been included in the study. A plausible reason for the weak correlation between psychological test scores and secondary hyperalgesia may be that we included a very homogenous population of healthy volunteers, that all had relatively low scores on HADS and PCS. By including healthy volunteers in an experimental pain study, it can be speculated that only few persons with a high anxiety-index or high-catastrophizing scores are enrolled, because they rarely consider volunteering in experimental pain studies. Thus, avoidance of sampling bias may be difficult. Therefore, in order to minimize sampling bias when investigating a possible association of high psychological vulnerability and experimental pain entities, consecutive inclusion of patients awaiting surgery, or specific inclusion of healthy volunteers with high psychological vulnerability seems necessary.

When evaluating the area of secondary hyperalgesia there exist different methods [4–8, 15, 18, 21–27, 41]. We chose a pragmatic approach that can be easily applied in a clinical setting by non-specialists, and evaluated the area of secondary hyperalgesia with the same polyamide filament (19g), in all the participants. Likewise, we chose a simple approach in calculating the area of secondary hyperalgesia, by using a 4 vector rectangular area calculation that has been

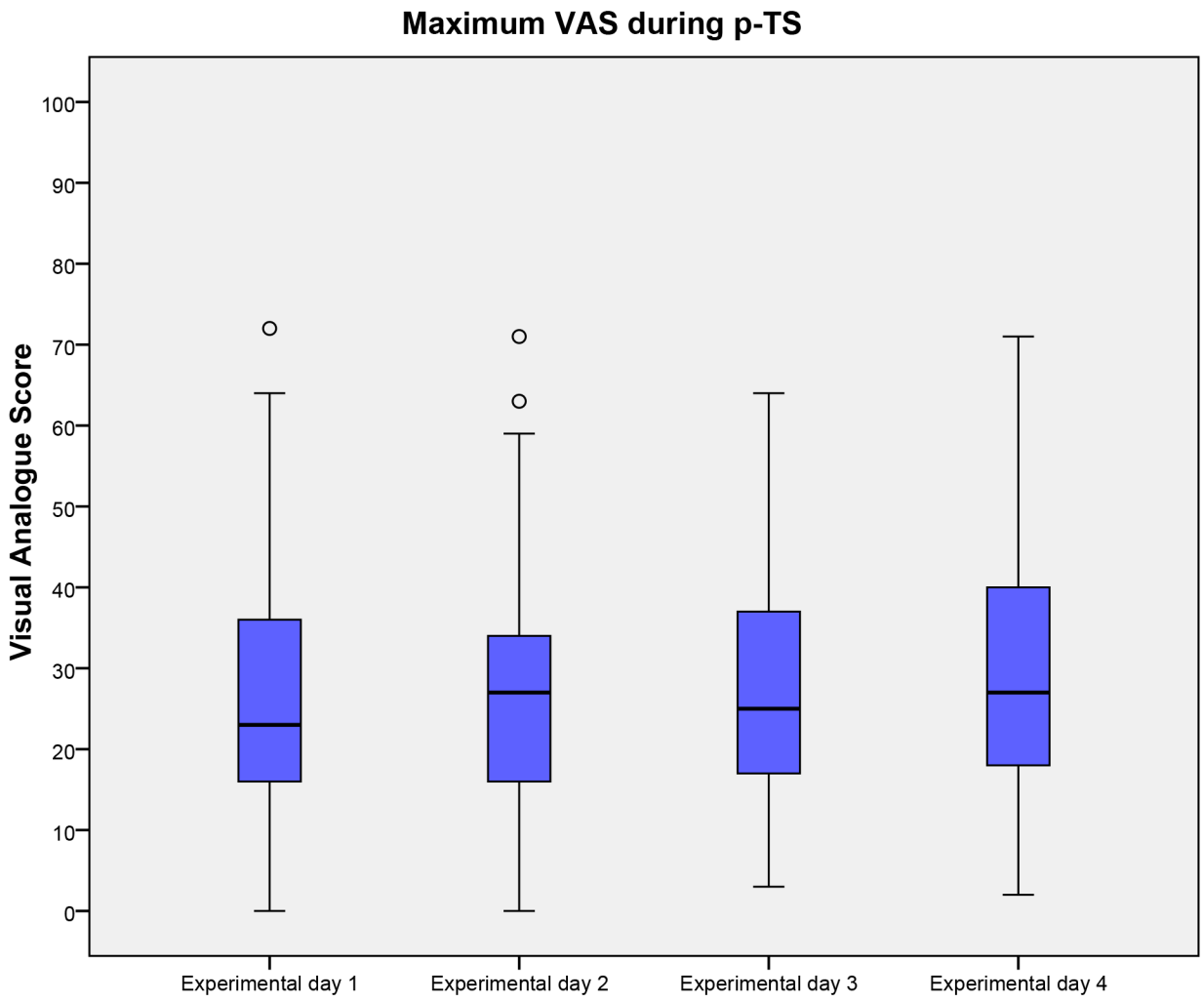

Fig 5. maximum VAS during p-TS. Maximum visual analogue score during 1 min. thermal stimulation on the 4 experimental days. Medians and interquartile ranges are displayed. Values higher than 1.5 times of upper quartile or lower quartile are designated as outliers and marked with °. Abbreviations: VAS, Visual analogue score; p-TS, Pain during 1 min. thermal stimulation.

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applied in several previous studies [4–8, 15, 21–27]. More favorable results may have been achieved by using the up-down method when applying punctate mechanical stimuli with the polyamide monofilaments [42]; however, our results demonstrate that the methods we applied were sufficient to demonstrate that BTS produce a reproducible area of secondary hyperalgesia.

The area of secondary hyperalgesia elicited by BTS may represent the level of central sensitization in the individual participant. Woolf describes that different pain hypersensitivity syndromes may share a common contribution of central sensitization, and hypothesizes that the comorbidity of different clinical pain syndromes may be explained by a "central sensitization syndrome" [11]. Thus, individuals with high pain sensitivity may share common factors that may be identified prior to the development of chronic pain. Moreover, it unlocks the possibility that individuals can be "phenotyped" in regards to their pain hypersensitivity. The assessment of secondary hyperalgesia may be a tool for investigation of central sensitization, and thus, be applied as a predictive factor of e.g. postoperative pain. To our knowledge, only few studies have investigated the assessment of secondary hyperalgesia as a possible predictive factor of

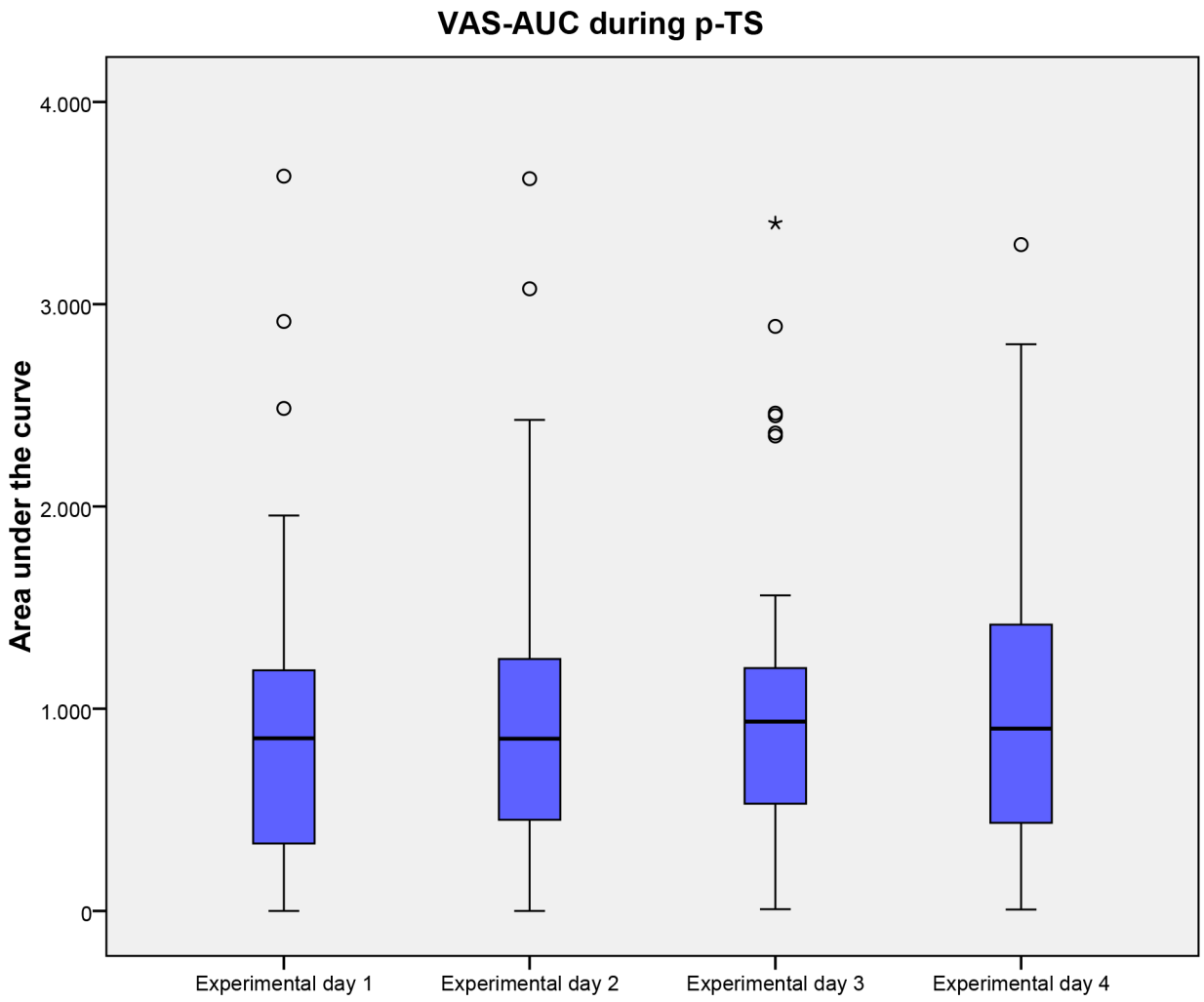

Fig 6. VAS-AUC during p-TS. Visual analogue score during 1 min. thermal stimulation on the 4 experimental days. Medians and interquartile ranges are displayed. Values higher than 1.5 times of upper quartile or lower quartile are designated as outliers and marked with °. Abbreviations: VAS, Visual analogue score; p-TS, Pain during 1 min. thermal stimulation; AUC, Area under the curve.

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postoperative pain, with one study demonstrating no correlations between area of secondary hyperalgesia following burn injury [43], and another demonstrating that postoperative secondary hyperalgesia around the surgical incision following an iliac crest bone harvest was predictive for the development of chronic postsurgical neuropathic pain [44]. Our results demonstrate a high inter-participant variance in the area of secondary hyperalgesia. The primary aim with this study was not to identify possible causal factors that could explain the high inter-participant variance; however our results are interesting and several factors could provide explanation for the remarkable inter-participant variance in an otherwise homogenous population. Factors such as stress [45], diet, including tryptophan intake [46, 47], hormone levels [45], skin receptor density, anatomical and functional brain differences [18], as well as genetics [16] could have influenced our results. The area of secondary hyperalgesia as a phenotypic indicator of pain hypersensitivity, and as a predictor for the development of acute and chronic pain is yet unexplored and further research is needed in order to clarify this.

The applicability of quantitative sensory testing (QST) and experimental pain models in translational and clinical studies has been widely debated. Should QST be implemented in the daily clinical practice, and should trials investigating analgesics implement the use of experimental pain models? When evaluating the increasingly body of research performed on QST and pain models, the main problem appears to be the heterogeneity in study methodology and statistical approaches [1, 48]. When using QST it is recommend that intra-participant reliability is determined [19]. Thus, before QST and experimental pain models can be fully implemented in clinical studies, reliability of the individual models is necessary. This means that several prospective methodological studies of the individual models should be performed [49]; evaluating intra- and inter-participant variance, as well as intra- and inter-investigator variance. Moreover, general accepted measures of reproducibility are needed. So far, no general recommendations have been proposed on how reproducible or reliable the various pain models or QSTs should be before they are implemented in translational or clinical research [50]. In the present study, we attempted to pre-define measures of reproducibility based on an earlier retrospective study [15]. This is in our opinion an important strength, and hypotheses regarding reproducibility/reliability should be implemented in study design as well as sample size analysis in future studies. If a test is not reliable and/or reproducible, then it cannot be used as tool in a diverse scientific community.

Our study has some limitations. Firstly, the two investigators were trained to perform BTS in precisely the same manner. That includes the assessment of secondary hyperalgesia, as well as the information given to the participants. This means, that if two entirely independent investigators were to perform the assessment without rigorous simultaneous training, the inter-investigator variance might increase. Moreover, even though our simulation study (see supporting information: S1 Appendix, simulation study 2, Figs 4 and 5) demonstrated that a scenario with 2 investigators and 50 study participants would enable us to discern the relevant variance components with acceptable precision, a higher number of independent observers is required to obtain final conclusions on the inter-observer variance and inter-study comparisons.

Secondly, we applied BTS on a highly homogenous population of healthy, male volunteers. Inclusion of women, elderly patients and chronic pain patients could potentially have increased the inter-participant variance with a clustering of effects in chronic pain patients. Thus, studies investigating a heterogeneous clinical population are needed in order to clarify the potential of BTS as a tool for evaluating both male and female patients, as well as the young and elderly population. Thirdly, we did not evaluate the participants' dietary intake or hormone levels. Studies have demonstrated that tryptophan may increase the pain sensitivity [46, 47], and cortisol and testosterone levels may influence the pain sensitivity [45]. Consequently, control of the dietary intake may have decreased intra-participant variance, and evaluation of hormone levels may have been an explanatory factor in the high inter-participant variance in the area of secondary hyperalgesia.

Lastly, even though our HPDT was within the range reported in previous studies [51], it may be that the three participants with a mean HPDT below 40°C possibly misunderstood the procedure.

In conclusion, our rigorous prospective study confirms earlier retrospective indications, that BTS produce a reproducible area of secondary hyperalgesia [15]. Furthermore, we have demonstrated a low intra-participant variance and a high inter-participant variance compared to inter-observer variance. BTS can therefore be applied in investigations of secondary hyperalgesia in healthy volunteers. However, to thoroughly determine that BTS is a reliable tool for pain research, other independent research groups should continue investigation of BTS in healthy volunteers and in more heterogeneous clinical populations.

Supporting Information

S1 Appendix. Statistical simulation study.
(PDF)

S2 Appendix. All relevant study data.
(XLSX)

S3 Appendix. Trend Checklist.
(PDF)

S4 Appendix. Study protocol, original language, Danish.
(DOCX)

S5 Appendix. Study protocol, translated, English.
(DOCX)

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Author Contributions

Conceived and designed the experiments: MSH JBD MSA JW CBP RØ. Performed the experiments: MSH RØ. Analyzed the data: MSH CBP JW. Wrote the paper: MSH JBD MSA JW CBP RØ.

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PAPER II

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CLINICAL TRIAL REPORT

Heat pain detection threshold is associated with the area of secondary hyperalgesia following brief thermal sensitization: a study of healthy male volunteers

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Introduction: The area of secondary hyperalgesia following brief thermal sensitization (BTS) of the skin and heat pain detection thresholds (HPDT) may both have predictive abilities in regards to pain sensitivity and clinical pain states. The association between HPDT and secondary hyperalgesia, however, remains unsettled, and the dissimilarities in physiologic properties suggest that they may represent 2 distinctively different pain entities. The aim of this study was to investigate the association between HPDT and BTS-induced secondary hyperalgesia.

Methods: A sample of 121 healthy male participants was included and tested on 2 separate study days with BTS (45°C, 3 minutes), HPDT, and pain during thermal stimulation (45°C, 1 minute). Areas of secondary hyperalgesia were quantified after monofilament pinprick stimulation. The pain catastrophizing scale (PCS) and hospital anxiety and depression scale (HADS) were also applied.

Results: A significant association between HPDT and the size of the area of secondary hyperalgesia ($p<0.0001$) was found. The expected change in area of secondary hyperalgesia due to a 1-degree increase in HPDT was estimated to be −27.38 cm^2, 95% confidence interval (CI) of −37.77 to −16.98 cm^2, with an R^2 of 0.19. Likewise, a significant association between HADS-depression subscore and area of secondary hyperalgesia ($p=0.046$) was found, with an estimated expected change in secondary hyperalgesia to a 1-point increase in HADS-depression subscore of 11 cm^2, 95% CI (0.19–21.82), and with R^2 of 0.03. We found no significant associations between secondary hyperalgesia area and PCS score or pain during thermal stimulation.

Conclusion: HPDT and the area of secondary hyperalgesia after BTS are significantly associated; however, with an R^2 of only 19%, HPDT only offers a modest explanation of the inter-participant variation in the size of the secondary hyperalgesia area elicited by BTS.

Keywords: pain, central nervous system sensitization, hyperalgesia, pain threshold, healthy volunteers, catastrophization, secondary hyperalgesia, central sensitization

Introduction

Clinical pain models may bridge the gap between animal and human research and may be applied in the investigation of pain sensitivity. Sufficient prediction of pain sensitivity, for example prior to surgery, may improve our ability to prevent severe acute and chronic pain following surgery,[1] as well improve the inclusion procedure in pharmaceutical drug trials by allowing initial grouping of participants in high- and low-pain responders.[2,3]

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Current evidence suggest that the development of secondary hyperalgesia to punctate mechanical stimuli after a cutaneous heat injury in healthy volunteers is mediated by heat- and mechanosensitive type-I and/or mechanosensitive (heat-insensitive) A-fiber nociceptors, and is due to changes in the central nervous system, that is, central sensitization.[4–9] Central sensitization encompasses a functional change in neuron properties and nociceptive pathways, with increased membrane excitability and synaptic efficacy, and decreased synaptic inhibition resulting in increased and sometimes pathological responses to mechanical and noxious stimulation.[4,9] The transcription-dependent long-lasting phase of central sensitization is assumed to play a key role in several pathological pain conditions, for example, osteoarthritis and fibromyalgia,[4,9–11] and investigation of secondary hyperalgesia following a standardized burn injury may therefore provide insight into central sensitization.

Studies in healthy volunteers have indicated that the size of the area of secondary hyperalgesia following standardized cutaneous sensitization procedures has a large inter-individual to intra-individual variance,[12,13] is modifiable by certain analgesics,[14–18] and may be predictive of individual pain responses.[4,9,19,20] The area of secondary hyperalgesia following the cutaneous heat pain model of brief thermal sensitization (BTS)[13,14,16,18,21,22] quantified by monofilament stimulation[12–14,16–18,21–28] has been demonstrated to be a reproducible phenomenon that may be used in phenotype characterization of healthy volunteers.[12,13]

Heat pain detection threshold (HPDT) has been applied in several studies,[17,18,22,25,29–33] and the acute first pain elicited by the rapid heating of the skin is believed to be transmitted in A-fiber type-II mechano- and heat-sensitive nociceptors (in hairy skin), and mechano- and heat-sensitive C fibers.[5] HPDT has been proven to be reproducible,[34] and evidence suggests that HPDT may have a predictive value when investigating postoperative pain.[35,36]

However, the dissimilarities in physiologic properties between secondary hyperalgesia to mechanical pinprick stimulation and HPDT suggest that they may represent 2 distinctively different pain entities.

As a first step to explore secondary hyperalgesia following BTS and its potential predictive abilities, we aim to investigate the association between HPDT and secondary hyperalgesia induced by BTS. We hypothesized that HPDT and areas of secondary hyperalgesia were two predominantly independent entities, and that the area of secondary hyperalgesia was poorly explained by HPDT.

Methods

The study was approved by the local Danish Committee on Health Research Ethics for the Capital Region (Identifier: H-8-2014-012) and the Danish Data Protection Agency (Identifier: 30–1436); the study is also reported on the international database clinicaltrials.gov (Identifier: NCT02527395).

The design and methods of this prospective study is based upon a previous study done by Hansen et al;[13] moreover, an extensive description of the design and methods of this study has been published in a preceding methods paper, which is publicly available for review.[37]

Study participants

Healthy male participants aged >18 and <35 years who could understand and speak the Danish language were included in the study. Written informed consent was obtained from all participants prior to inclusion, and all participants received EUR 20 (USD 27) per hour for their participation in the study. Participants were recruited by advertisement in the medical student magazine at Copenhagen University and online at www.forsøgspersoner.dk. Exclusion criteria were failure to cooperate with the tests, a weekly intake of >21 units of alcohol, consummation of >3 units of alcohol 24 hours before study day, substance abuse, intake of analgesics within 3 days before study day, intake of antihistamines 48 hours before study day, intake of prescription medicine and/or antidepressant medicine within 30 days before study day, neurological illnesses, chronic pain conditions, psychiatric diagnoses, tattoos on the extremities, eczema, wounds or sunburns at the sites of testing, and a body mass index (BMI) of >30 kg/m^2 and <18 kg/m^2.

Setting

The study was conducted in a quiet secluded room (temperature of 22°C–25°C), where only the investigator and the participant were present. The participants were placed in a supine position, on their back, throughout the assessments. The study was conducted during the time from 8 AM to 6 PM at the Department of Anesthesiology, 4231, Rigshospitalet, Copenhagen, Denmark in the period from October 1, 2015 to December 2, 2015.

Design

The study consisted of 1 screening/information day and 2 separate study days. To avoid a possible carry-over effect of the applied tests, the screening day and the 2 study days were separated with a minimum of 7 days.[13] Height, weight, arterial blood pressure, and pulse frequency of all participants were measured; moreover, data on age, right/left-handedness, and parental ethnicity were collected. On the 2 separate

study days, the study participants were tested with 3 types of pain models: BTS, HPDT, and pain during 1-minute thermal stimulation (p-TS) in a predefined sequence (see pain models and randomization and allocation concealment). On the information day, the participants were provided with the psychological tests, pain catastrophizing scale (PCS)[38–40] and hospital anxiety and depression scale (HADS)[41–43] (see psychological testing), which they completed at home and returned on the first study day in sealed opaque envelopes to ensure blinding. Opening of the envelopes was deferred until all participants had completed the study. All other assessments and tests were performed by the same investigator throughout the study (MSH).

Pain models

All pain testing was conducted with a computer-controlled thermode (MSA Thermotester™), size 2.5×5 cm

Brief thermal sensitization (BTS)

BTS was induced by placing the computer-controlled thermode on the skin of the participant, centrally on the anterior part of the right thigh in the midline between the anterior superior iliac spine and the base of patella (Figure 1). The starting temperature of the thermode was 32°C, and with an increase of 1°C/second, the thermode was heated to 45°C. After 3 minutes, the assessment of secondary hyperalgesia (see below) was conducted while the thermode at 45°C was still positioned on the skin of the participant.[14,16,18,21,22] The assessment of secondary hyperalgesia took ~1–2 minutes, resulting in a maximum duration of heat stimulation of 5 minutes.

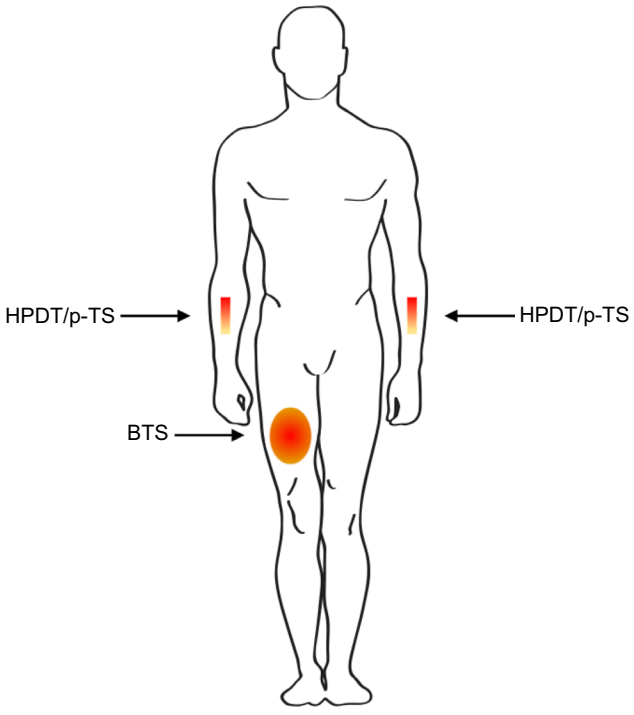

Figure 1 Anatomical location of pain model testing.
Notes: HPDT was performed on the anterior part of the dominant lower arm, p-TS was performed on the anterior part of the non-dominant lower arm, and BTS was performed centrally on the anterior part of the right thigh in the midline between the anterior superior iliac spine and the base of patella.
Abbreviations: BTS, brief thermal sensitization; HPDT, heat pain detection threshold; p-TS, pain during thermal stimulation.

Assessment of secondary hyperalgesia

The area of secondary hyperalgesia was quantified after pinprick stimulation with a 19G monofilament (von Frey hair) in 4 linear paths arranged in 90° around the center of the thermode. Stimulation began well outside the area of secondary hyperalgesia, minimum 15 cm from the edge of the thermode, and advanced in steps of 5 mm/second toward the thermode. When the participant stated a clear change in sensation (intense burning, pricking, and tenderness), the spot was marked with a felt pen, and the longitudinal and transverse axes were measured with a pliable measuring tape for rectangular area calculation.[12–14,16–18,21–28]

Heat pain detection threshold (HPDT)

HPDT was evaluated by placing the thermode on the skin of the participant on the anterior part of the dominant lower arm (Figure 1). The start temperature of the thermode was 32°C and the temperature was then increased by 1°C/second. When the participant perceived the heat as painful he pressed a button, the temperature was registered, and the thermode returned to a temperature of 32°C. If a temperature of 52°C was reached, the thermode would automatically return to 32°C and 52°C would be registered as the threshold. The HPDT was estimated as an average of 4 separate stimulations with an interval of 6–10 seconds.[13,14,16–18,22–25]

Pain during thermal stimulation (p-TS)

The thermode was placed on the participant's skin centrally on the anterior lower non-dominant arm (Figure 1). The start temperature of the thermode was 32°C, and with an increase of 1°C/second, the thermode was heated to 45°C and remained 45°C for 1 minute. During the 1 minute heating of the skin the participant evaluated the pain using the electronic visual analog scale (VAS; Somedic USB-VAS), with an index of 0–100 mm, where 0 mm represented "no pain", and 100 mm represented "worst pain imaginable". The software provided with the electronic VAS automatically calculated an area under the curve (AUC) and a maximum VAS score for the time period. The participant was not able to see the computer screen during the assessment.[17,22–25]

Psychological testing

Pain Catastrophizing Scale (PCS)

PCS is 13-point questionnaire on a 5-point Likert scale with values from 0 to 4. The highest achievable score is 52, and the PCS can be subdivided into 3 sections that evaluate 1) rumination, 2) magnification, and 3) helplessness.[38–40]

Hospital Anxiety and Depression Scale (HADS)

HADS is a 14-point questionnaire on a 4-point Likert scale with values ranging from 0 to 3. The highest achievable score is 53, and the HADS can be subdivided into 2 sections that evaluate 1) anxiety and 2) depression.[41–43]

Randomization and allocation concealment

The sequence of BTS and HPDT was randomized so that on 1 study day the sequence was: 1) BTS, 2) HPDT, and 3) p-TS, and on the other study day the sequence was: 1) HPDT, 2) BTS, and 3) p-TS. The randomization was performed with a computer-generated random allocation sequence, conducted by the Copenhagen Trial Unit, and stored in sealed opaque envelopes to secure adequate allocation concealment.

Test results and assessments for each study day were entered in a standardized case report form and placed in an opaque sealed envelope to ensure that the investigator was unable to see previous test results. Completed psychological tests were kept in sealed opaque envelopes and the blinding was first broken after all study participants had completed the study.

Outcome measures

Primary analysis

The association between HPDT and area of secondary hyperalgesia induced by BTS.

Secondary analyses

The association between area of secondary hyperalgesia induced by BTS and

1. VAS-AUC following p-TS
2. Max VAS-score following p-TS
3. PCS-score
4. HADS-score
5. PCS and HADS subscales (PCS-rumination, PCS-magnification, PCS-helplessness, HADS-anxiety, and HADS-depression)

Sample size

A simulation-based sample size calculation was performed with data from our previous study;[13] and with an α of 0.05 and β of 0.01, we estimated that a number of 120 participants were needed in order to provide an empirical power of 99.9% (for further description see the published protocol[37]). All simulation-based calculations were made using the open-source statistical programming environment R.[44]

Statistical analysis

Individual levels of areas of secondary hyperalgesia, HPDT, VAS-AUC, and VAS-max were obtained as estimated best linear unbiased predictors (EBLUPS). The association between area of secondary hyperalgesia and HPDT was evaluated by multiple linear regression adjusting for individual body surface area. Models were validated graphically by means of residuals and QQ plots. Normality of residuals was assessed by the Kolmogorov–Smirnov test. The ability of HPDT to predict the size of the area of secondary hyperalgesia was quantified by R^2 and illustrated with prediction limits.

In a secondary analysis, we additionally included VAS-AUC, Max VAS-score following p-TS, PCS-score, and HADS-score as predictors in a multiple linear regression on area of secondary hyperalgesia. The importance of these predictors was assessed by backward elimination with a 5% cut-off level.

p-Values corresponded to *F* tests and were evaluated at a 5% significance level.

Additionally, 3 post hoc sensitivity analyses were performed to assess the robustness of the findings. In the first sensitivity analysis, further adjustment by age, weight, BMI, and mean arterial pressure (MAP) was performed. In the second sensitivity analysis, only right-handed participants were included; and finally, in the third sensitivity analysis, only ethnic Scandinavians were included.

Body surface area was calculated using the Mosteller formula.[45] Distributions of variables are summarized by medians and interquartile ranges. All analyses were made using the open-source statistical programming environment R.[44]

Results

A sample of 131 healthy male volunteers was assessed for eligibility, and a total of 121 were included in the study (Figure 2). All 121 study participants completed the study, and data from all the participants were analyzed for the primary and secondary outcome measures. Of the 121 participants, 12 had one or more parents with non-Scandinavian

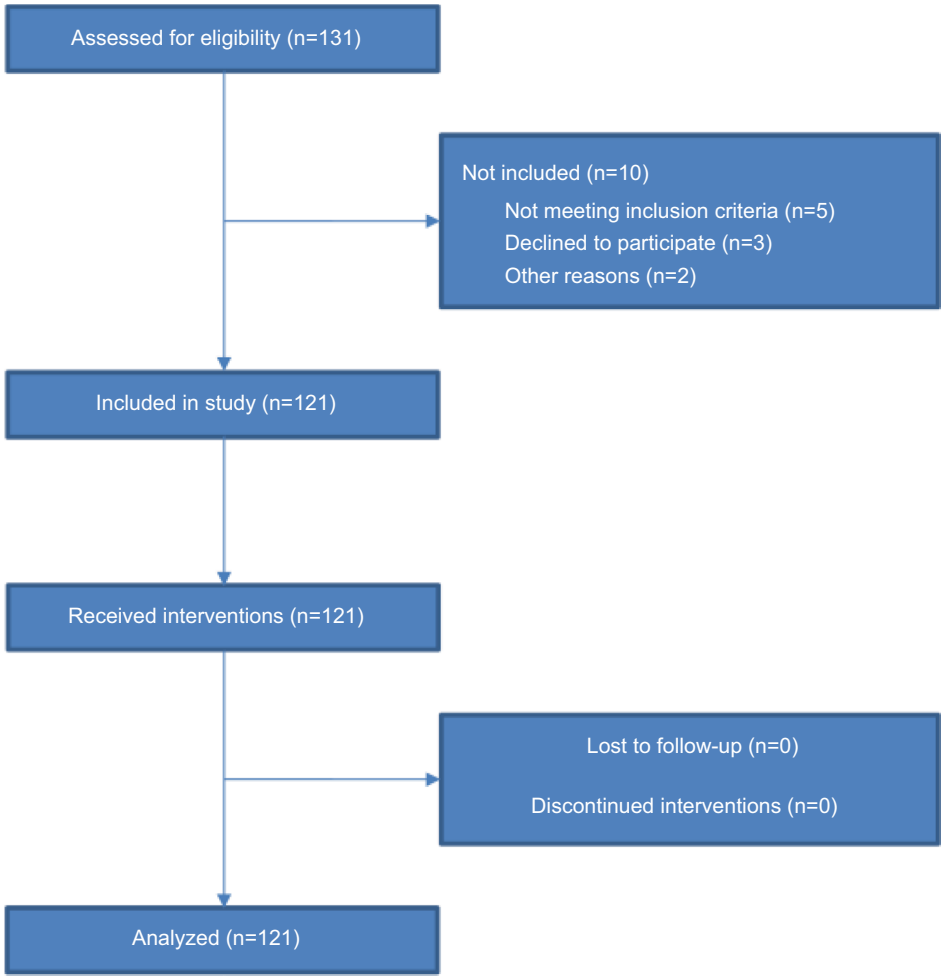

Figure 2 Flowchart of included study participants.

Table 1 Characteristics of included participants

Characteristic	Study participants (n=121)
Age (years)*	22 (20–24.5)
Height (m)*	1.84 (1.79–1.88)
Weight (kg)*	76.5 (70–85)
BMI (m^2/kg)*	22.79 (20.9–24.5)
MAP (mmHg)*	89.6 (84.3–96)
Pulse (beats/min)*	64 (58–70)
Non-Scandinavian ethnicity (n)	12
Left-handed participants (n)	16

Note: *Data are reported as median and interquartile range.
Abbreviations: BMI, body mass index; MAP, mean arterial pressure.

Table 2 Results from pain model testing

Variable	Median (IQR)	Range (min–max)
Area of secondary hyperalgesia (cm^2)	447.78 (346.19–528.99)	135.21–788.90
HPDT (°C)	45.57 (43.79–46.61)	38.70–51.01
p-TS VAS-max (mm)	32.82 (18.79–52.71)	2.41–95.99
p-TS VAS-AUC (mm^2)	1123.92 (649.34–1844.45)	82.65–4456.32

Notes: Median and range were estimated by calculating the estimated best linear unbiased predictors (EBLUPS).
Abbreviations: HPDT, heat pain detection threshold; IQR, interquartile range; max, maximum; min, minimum; p-TS, pain during thermal stimulation; VAS-AUC, visual analog scale area under the curve; VAS-max, maximum visual analog scale.

ethnicity and 16 were left-handed. The median interval between the 2 study days was 7 days (interquartile range [IQR], 7–8). Relevant data on the included participants' characteristics are presented in Table 1. The median size of the area of secondary hyperalgesia was 447.78 cm^2 (IQR, 346.19–528.99) and the median HPDT was 45.57°C (IQR, 43.79–46.61). Results from the p-TS, PCS, and HADS are presented in Tables 2 and 3. No adverse or serious adverse events were reported.

Primary analysis

We found a significant association between HPDT and the size of the area of secondary hyperalgesia ($p<0.0001$). We estimated the expected change in area of secondary hyperalgesia due to a 1-degree increase in HPDT to −27.38 cm^2 with a 95% confidence interval (CI) of −37.77 to −16.98 cm^2. The R^2 was calculated to 0.19, and the prediction limits at a given HPDT of 46°C and body surface of 1.99 m^2 were estimated to 167.42–656.07 cm^2 (Figure 3).

Table 3 Psychological test scores, total, and subscores

Variable	Median (IQR)	Range (min–max)
PCS-helplessness	4 (2–6.5)	0–17
PCS-rumination	5 (3–8)	0–12
PCS-magnification	3 (1–4)	0–10
PCS-total	12 (7–17)	1–31
HADS-anxiety	4 (2–6)	0–16
HADS-depression	1 (1–3)	0–13
HADS-total	6 (3–8.5)	0–21

Abbreviations: HADS, hospital anxiety and depression scale; IQR, interquartile range; PCS, pain catastrophizing scale.

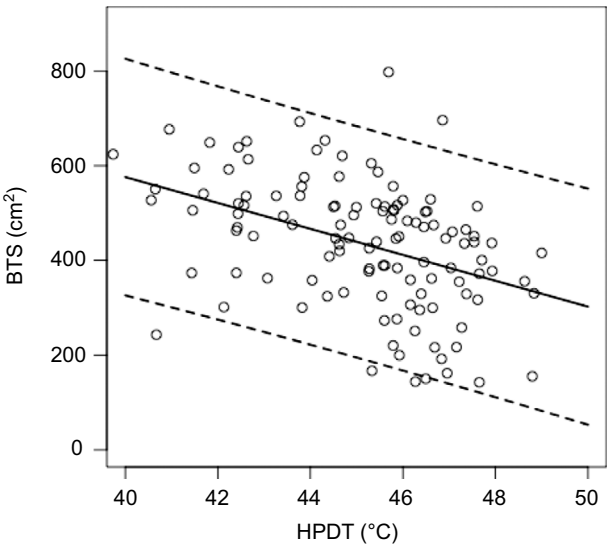

Figure 3 Predictions of areas of secondary hyperalgesia (following BTS) by HPDT.
Notes: Points correspond to individual participant measurement of secondary hyperalgesia areas. The solid line corresponds to the predictions of secondary hyperalgesia areas and HPDT, and the dashed line corresponds to 95% prediction limits.
Abbreviations: BTS, brief thermal sensitization; HPDT, heat pain detection threshold.

Secondary analyses

We found a significant association between HADS-depression score and area of secondary hyperalgesia (p=0.046). The estimated expected change in secondary hyperalgesia area to a 1-point increase in HADS-depression was 11 cm^2 (95% CI, 0.19–21.82; R^2, 0.03). No significant associations were found in any of the other secondary outcome measures.

Post hoc analyses

The 3 post hoc sensitivity analyses did not demonstrate noticeably different results when compared to our primary analysis.

Discussion

In the present study, we aimed to investigate the association between HPDT and secondary hyperalgesia elicited by BTS. We demonstrated a significant association between HPDT and secondary hyperalgesia, where increasing levels of HDPT were associated with decreasing sizes of secondary hyperalgesia areas. In addition to the highly significant association, we found an R^2 of 19%, illustrating that HPDT only offers a modest explanation of the inter-participant variation in secondary hyperalgesia following BTS. The estimated prediction interval for areas of secondary hyperalgesia at an HPDT of 46°C and a body surface of 1.99 m^2 were estimated to 167.42–656.07 cm^2 (Figure 3), indicating that although we find a highly significant result, HPDT and areas of secondary hyperalgesia are only modestly associated. Likewise, we also found a significant association between increasing HADS-depression subscore and increasing size of secondary hyperalgesia area; however, R^2 was estimated to 3%, and in this study, HADS-depression subscore only offered a very modest explanation for the variation in the area of secondary hyperalgesia.

We have applied an experimental pain model (BTS) with a high reliability (intraobserver intra-participant correlation of 0.85).[13] Moreover, our post hoc sensitivity analyses did not demonstrate noticeable differences compared to our primary analysis, illustrating the robustness of our results. In our study, BMI, age, MAP, left-handedness, and ethnicity did not have any influence on the association between HPDT and secondary hyperalgesia to mechanical punctate stimuli. The high number of included participants provides an empirical power of 99.9%, which practically eliminates the risk of type-II errors and once again illustrates the robustness of our results.

The results in this study confirm the results from our previous study where a significant association was demonstrated with an R^2 of 20%.[13] Likewise, in our current study we find a high inter-participant difference in areas of secondary hyperalgesia ranging from 135 to 788 cm^2 (Table 2), as well as high inter-participant differences in HPDT ranging from 38.7°C to 51.02°C (Table 2).

The weak association between HPDT and secondary hyperalgesia area is noteworthy because it has been suggested that both parameters may to some extent be important in categorizing pain sensitivity; however, evidence on the predictive value of these parameters is contradicting with diverse results both for[20,46–48] and against[31,36] HPDT and secondary hyperalgesia areas as predictors of pain sensitivity. The physiologic properties in the neural mediation of HPDT and secondary hyperalgesia may, in part, account for the weak association. HPDT is primarily mediated by A-fiber type-II mechano- and heat-sensitive nociceptors,

and mechano- and heat-sensitive C fibers, and secondary hyperalgesia to punctate mechanical stimuli is mediated by heat- and mechano-sensitive type-I and mechanosensitive (heat-insensitive) A-fiber nociceptors.[5–7] In a recent study, results even suggested that secondary hyperalgesia was mediated only by heat-insensitive mechanosensitive A-fiber nociceptors.[8] Finally, it is believed that the development of secondary hyperalgesia is caused by central sensitization due to changes in the central nervous system,[4,7,9] which leads to the suggestion that HPDT and secondary hyperalgesia to mechanical punctate stimuli may be 2 distinctively different pain entities. A biological explanation, although speculative, may be that HPDT represent an acute warning system against nociceptive stimuli, whereas secondary hyperalgesia represents a somewhat later occurrence of sensitization of central neurons, which may serve other purposes in the nociceptive process.

Studies have demonstrated that patients suffering from persistent pain due to rheumatoid arthritis or fibromyalgia display larger areas of secondary hyperalgesia when compared with healthy individuals.[49,50] Likewise, clinical studies have indicated that increasing sizes of secondary hyperalgesia areas surrounding surgical wounds are associated with an increased risk of developing chronic pain following surgery.[20,51] These findings indicate that a large area of secondary hyperalgesia is found in persons with high levels of central sensitization. Thus, the investigation of secondary hyperalgesia areas may provide insight in individual levels of central sensitization. With Woolf's description of a central sensitization syndrome,[4] where pain hypersensitivity syndromes may share common contributions of central sensitization, the investigation of secondary hyperalgesia may provide insight into already known pain hypersensitivity syndromes, and may also contribute to the phenotyping of pain sensitivity in healthy persons. A recent brain magnetic resonance imaging study of healthy volunteers indicated that participants with differences in areas of secondary hyperalgesia exhibited structural and functional differences when comparing healthy participants with a large vs small area of secondary hyperalgesia,[52] suggesting differences in sensory discrimination, pain suppression, and avoidance behavior. However, the practical applicability of secondary hyperalgesia areas is not yet fully understood or described, and thorough investigations of central sensitization, as well as factors influencing individual propensity to develop central sensitization may have a role in the future of analgesic therapy and pain research.

Contrary to our previous study,[13] we found a significant association between increasing HADS-depression subscore and increasing size of secondary hyperalgesia area. However, R^2 was estimated to 3%, and in this study, HADS-depression subscore only offered a very modest explanation for the variation in secondary hyperalgesia areas. Several clinical studies have demonstrated significant associations between postoperative pain and personality traits, such as depression, anxiety, and pain catastrophizing.[53–56] Moreover, in a study by Salomons et al,[57] it was demonstrated that pain-focused cognitive training reduced the area of secondary hyperalgesia in healthy volunteers. However, in a recently published review it was concluded that the influence of psychological variables on experimental pain responses is still largely unclear.[58] Our very strict inclusion criteria, that specifically excluded women, chronic pain patients, and persons with prior psychological history, may have resulted in a sampling bias that reduced the inter-individual variance of secondary hyperalgesia areas, PCS (IQR, 7–17), and HADS score (IQR, 3–8.5), and could be responsible for the weak association between HADS, HPDT, and secondary hyperalgesia. A sufficient investigation of psychological variables and pain should attempt to conduct consecutive inclusion of patients prior to, for example, surgery or restricted inclusion of volunteers with high psychiatric vulnerability.

Our study has some limitations. 1) As emphasized before, we applied very strict inclusion criteria, and consequently, included a very homogenous population; inclusion of, for example, females and chronic pain patients could potentially have increased the inter-individual variance and resulted in a higher R^2. However, individual characteristics, such as sex,[25,59–62] obesity,[63] and menstrual hormone cycle,[64] may potentially influence pain thresholds and sensitivity and to accommodate for all these variables, hereby minimizing the unknown factors of variation, and to focus only on the association between HPDT and secondary hyperalgesia areas, we chose to apply very strict inclusion criteria. Additionally, BTS has only been validated in healthy male volunteers,[13] and consequently, the results of this study only apply to young and healthy, male volunteers.

2) We did not evaluate dietary intake, stress and hormone levels, genetics, brain anatomy, or skin receptor density of the included participants. Studies have suggested that diets high on tryptophan,[65,66] high stress levels of serum cortisol and testosterone,[67] and even certain genetic markers[68–72] may influence the pain sensitivity. An inter-participant differentiable diet and hormone level, as well as differences in stress levels, genetics, and brain anatomy could be explanatory factors

of the high inter-individual variance of HPDT and areas of secondary hyperalgesia.

Finally, 3 patients reported HPDTs well outside the interquartile ranges, 2 patients <40°C and 1 patient >50°C, which may indicate that they misunderstood the procedure.

In our study, HPDT only offered a modest explanation of the inter-individual size of the area of secondary hyperalgesia; and the inter-individual differences in secondary hyperalgesia observed in numerous studies remain largely unexplained. Studies investigating postoperative pain and secondary hyperalgesia before and after surgery could provide insight on the predictive value of secondary hyperalgesia areas, and finally, as secondary hyperalgesia is believed to occur as a result of central neuronal plasticity,[4,9] future research should attempt to investigate variables in the central nervous system in both patients and healthy participants, with modalities such as structural and functional magnetic resonance imaging, electroencephalography, and magnetoencephalography.

In conclusion, our study demonstrated a statistically significant association between HPDT and the size of the area of secondary hyperalgesia. However, with an R^2 of only 19%, HPDT offers only a modest explanation of the inter-participant variation in the size of the secondary hyperalgesia area elicited by BTS.

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Disclosure

The authors report no conflicts of interest in this work.

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PAPER III

Title: The propensity to develop central sensitization is not correlated to pain relevant brain structures - a 3-tesla MRI study of healthy male volunteers

Running head: Central sensitization and brain anatomy

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Abstract

Central sensitization plays a pivotal role in maintenance of pain, and is believed to be intricately involved in several chronic pain conditions. One clinical manifestation of central sensitization is secondary hyperalgesia. The degree of secondary hyperalgesia presumably reflects individual levels of central sensitization. The objective of this study was to investigate the association between areas of secondary hyperalgesia and volumes of the caudate nuclei and other brain structures involved in pain processing.

We recruited 121 healthy male participants; 118 were included in the final analysis. All participants underwent whole brain magnetic resonance imaging (MRI). Prior to MRI, all participants underwent pain testing. Secondary hyperalgesia was induced by brief thermal sensitization. Additionally, we recorded heat pain detection thresholds (HPDT), pain during one minute thermal stimulation (p-TS) and results of the Pain Catastrophizing Scale (PCS) and Hospital Anxiety and Depression score (HADS).

We found no significant associations between the size of the area of secondary hyperalgesia and the volume of the caudate nuclei or any of the following structures: primary somatosensory cortex, anterior and mid cingulate cortex, putamen, nucleus accumbens, globus pallidus, insula and the cerebellum. Likewise, we found no significant associations between the volume of the caudate nuclei and HPDTs, p-TS, PCS and HADS.

Our findings indicate that the size of the secondary hyperalgesia area is not associated with the volume of brain structures relevant for pain processing. This suggests that the propensity to develop central sensitization, assessed as secondary hyperalgesia, is not correlated to brain structure volume.

Introduction

Nociceptive stimuli can elicit sensitization of neurons in the central pain pathways.
This phenomenon of central sensitization is a manifestation of the plasticity in the central nervous system (CNS) and represents the CNS's ability to alter and produce augmented pain responses by amplification of synaptic inputs and recruitment of subthreshold neurons. Central sensitization is believed to be a contributing factor for individual pain sensitivity, and may play a pivotal role in the maintenance and chronification of pain[1,2].

Central sensitization can readily be investigated in humans with pain models utilizing either heat[3,4], cold[5], chemical[6] or electrical[7] stimulation. Noxious heat stimulation to the skin produces primary hyperalgesia at the site of injury and secondary hyperalgesia with reduced thresholds for mechanical stimulation in the non-injured skin surrounding the injury[8-10]. Current evidence indicates that secondary hyperalgesia following a standardized heat injury is a result of central sensitization[1,2,11] and is expressed differently among individuals, where some individuals develop small while others develop large secondary hyperalgesia areas[9,12]. In addition, individuals will continue to develop secondary hyperalgesia of similar magnitude when exposed to the same noxious stimuli[9,12]. Secondary hyperalgesia is thus a robust phenomenon that can be used to phenotypically characterize individuals[9,12].

The occurrence of central sensitization in different chronic pain conditions suggests that certain individuals may be predisposed towards developing central sensitization[1,2,9]. An important question is why, and if, some individuals have a higher propensity for developing central sensitization, and if such individuals have a subsequent higher risk of developing pain hypersensitivity and chronic pain[1]. Currently, no sufficient explanation of the high inter-individual variance in secondary hyperalgesia areas has been provided. Understanding these variations may lead to crucial insights into central mechanisms of pain and possibly to identification of biomarkers for central sensitization.

A recent exploratory brain MRI study found structural and functional differences when comparing healthy volunteers with a small vs. large area of secondary hyperalgesia[13], demonstrating an inverse correlation of the volume of the caudate nuclei and the area of secondary hyperalgesia. The caudate nuclei are essential for the integration and control of motor, sensory, and

motivational information[14]; however, studies suggest that they are also activated during pain expectancy[15], are involved in the modulation and suppression of pain[16], and are important sites for the sensory processing and spatial location of noxious stimuli[17]. Moreover, clinical studies have indicated that reduced grey matter volume of the caudate nuclei is seen in patients with various chronic pain conditions[18-20]. Several other brain structures, including the primary somatosensory cortex, anterior and mid cingulate cortex, basal ganglia, insula and the cerebellum have been demonstrated to be intricately involved in pain processing[21,22], illustrating the comprehensiveness of pain perception.

The aim of the current study was to determine whether differences in brain anatomy were associated with the propensity to develop central sensitization, assessed as areas of secondary hyperalgesia. Specifically, we investigated if the size of the secondary hyperalgesia area was associated with the volume of the caudate nuclei and other brain structures relevant for pain processing.

Materials and Methods

The study was approved by the Danish Committee on Health Research Ethics for the Capital Region (H-15010473) and the Danish Data Protection Agency (RH-2015-149). In addition, the study was registered on Clinicaltrials.gov (NCT02567318).
A detailed description of the study design and methods has been published previously[23].

Design

Briefly, the study consisted of two separate parts: Part 1: Pain testing and Part 2: MRI scans.

Pain testing

The isolated results from the pain testing have been presented in a separate publication[24]. In addition, these data have been used for the analyses of the present MRI data.
The pain testing was conducted at a minimum of 14 days and at a maximum of 60 days prior to the MRI scans to avoid any carry-over effects. For details regarding the pain testing please see the published protocol[23]. Briefly, all included participants were tested with the following pain models (fig. 1):

Brief thermal sensitization (BTS)

A computer-controlled thermode (Somedic MSA Thermotester; size 2.5 x 5 cm.) was placed on the upper right thigh. The thermode was then heated to 45°C for three minutes. Afterwards the assessment of secondary hyperalgesia (see below) was conducted while the 45°C heated thermode remained on the skin of the participant[4,9,12,25]. The assessment took approximately 1-2 minutes, resulting in a maximum duration of the heat stimulation of 5 min.

Assessment of secondary hyperalgesia

The area of secondary hyperalgesia was quantified after stimulation with a monofilament (Von Frey hair) with a nominal value of 18 (bending force 490 mN) in 4 linear paths arranged in 90° around the centre of the thermode. Stimulation began well outside the area of secondary hyperalgesia, and advanced in 5 mm/sec intervals towards the centre of the thermode. When clear change in sensation occurred (intense burning, pricking, tenderness) the location was

marked, and the longitudinal and transverse axes were measured for rectangular area calculation[4,8,9,12].

Heat pain detection threshold

The individual heat pain detection threshold (HPDT) was evaluated by placing the thermode on the anterior part of the dominant lower arm. The temperature of the thermode was then increased by 1°C/second from a baseline of 32°C, until the participant perceived the heat as painful and pressed a button. The HPDT was estimated as an average of 4 separate stimulations with an interval of 6-10 seconds[8,26].

Pain during one minute thermal stimulation (p-TS)

The thermode was placed on the lower non-dominant arm, and was heated to 45°C for one min. During the one minute heating the participant evaluated the pain using the electronic Visual Analog Scale (VAS) (Somedic USB-VAS), with an index of 0-100 mm, where 0 mm represented "no pain", and 100 mm represented "worst pain imaginable". A VAS area under the curve (VAS-AUC) and a maximum VAS-score was calculated by the computer software[8,26].

MRI scans

On the day of the MRI scans each included participant underwent multimodal whole brain MRI scans (fig. 1); no other tests or assessments were conducted on this study day. The total duration of the MRI scans was approximately 50 min. Following completion of MRI-scans, all images were reviewed by an experienced radiology consultant. In the case of suspected pathological findings, the participant was informed hereof and was referred to a specialist in neurology for further examination.

MRI data acquisition and imaging protocols

All MRI scans were performed with a Siemens MAGNETOM Verio 3-tesla MRI scanner, with b17 software, and a 32-channel head coil.

Anatomical images

Anatomical images were obtained using a T1-weighted 3D FLASH (160 sagittal slices, matrix 256x256 mm, Field of view 256 mm, echo time (TE) 2.98 ms, repetition time (TR) 2300 ms, Slice 1 mm, in plane resolution 1x1 mm, flip angle 9°).

Additional MRI sequences not analysed:

We also performed the following MRI sequences: Diffusion tensor imaging, resting state epi single shot functional MRI, arterial spin labelling, b0 field maps, T2-FLAIR, T2-weigthed TSE sequence, and GRE hemo sequences. Due to technical problems, we were not able to use the DTI-scans for analysis in this study, and consequently the secondary and exploratory outcome measures described in the published study protocol could therefore at present not be evaluated. As reported in the published study protocol[23], results from the resting state functional MRI will be reported in a separate paper.

The remaining MRI scans were either of technical character or for diagnostics, and will per protocol not be reported in this paper.

Physiological measurements

Pulse frequency, respiration frequency, and end-tidal PCO_2 were measured during the entire scan session, including before and after the resting state scan.

Psychological testing

The participants were tested with two separate psychological tests.

Pain Catastrophizing Scale (PCS) is a 13-point questionnaire on a five-point Likert scale with values from 0-4. The highest achievable score is 52, and the PCS can be subdivided in 3 sections that evaluate Rumination, Magnification, and Helplessness[27].

Hospital Anxiety and Depression Scale (HADS) is a 14-point questionnaire on a four-point Likert scale with values ranging from 0-3. The highest achievable score is 53, and the HADS can be subdivided in two sections that evaluate anxiety and depression[28].

Setting

All MRI scans were conducted at the Department of Radiology, Bispebjerg and Frederiksberg Hospitals, Copenhagen, Denmark. The pain testing[24] was conducted at the department of Anaesthesiology, Rigshospitalet, Copenhagen, Denmark. Data were collected in the period from October 2015 to December 2015. All analyses were conducted at the department of Anaesthesiology, Rigshospitalet, and at the Section of Biostatistics, Faculty of health, Copenhagen University, Denmark.

Study participants

Healthy male volunteers aged 18-35 years, who had participated in preceding pain testing[24] were included in the study. Oral and written informed consent was obtained from all participants prior to inclusion in the study. The participants received EUR 67 (USD 74) for their participation in the study. Inclusion and exclusion criteria are listed in table 1.

Outcome measures

Primary analysis

To investigate the association between the volume of the left and right caudate nuclei and the magnitude of the area of secondary hyperalgesia induced by BTS.

Secondary analyses

To investigate the association between the magnitude of the area of secondary hyperalgesia and cortical as well as subcortical brain structures relevant for pain processing (primary somatosensory cortex, anterior and mid cingulate cortex, putamen, nucleus accumbens, globus pallidus, insula and the cerebellum).

Exploratory analyses

To investigate the association between the volume of the left and right caudate nuclei and the following five parameters: 1. HPDT; 2. p-TS max. VAS-score; 3. p-TS VAS-AUC; 4. PCS; and 5. HADS scores.

To investigate possible neuroanatomical differences between participants displaying a small area of secondary hyperalgesia (lower quartile) compared to participants displaying a large area of secondary hyperalgesia (upper quartile). The same cortical and subcortical brain structures as specified in the primary and secondary analyses were included in the analysis.

Sample size analysis

Sample size estimation was based on a Z-test of the Fisher transformed Pearson correlation with results from a previous study[13]. With a true correlation of R=- 0.4 between the area of secondary hyperalgesia and the volume of the caudate nuclei, and with a significance level of 2.5-5% according to the single step method, a sample size of 52 was needed to obtain a power of 0.80 (β=0.20). Our sample size estimation was based on results from a study were only participants that produced small or large area of secondary hyperalgesia were included. In the present study, we aimed to include participants without prior knowledge of their areas of secondary hyperalgesia and thus also expected inclusion of participants with intermediary size areas of secondary hyperalgesia. To secure a reasonable high sample size when comparing the upper and lower quartiles based on area of secondary hyperalgesia we aimed to include 120 participants.

Structural MRI preprocessing

Anatomical T1W-weighted images were preprocessed and analysed using the FreeSurfer imaging analysis suite version 5.3, which is freely available for download online (http://surfer.nmr.mgh.harvard.edu/) and is a semi-automatic software that performs volumetric segmentation of cortical and subcortical structures[29-31]. Cortical volumes were extracted according to the Desikan-Killiany cortical atlas[32]. To avoid possible confounding due to inter-participant head size differences, all volumes were adjusted for intracranial volume using a method based on the analysis of covariance approach outlined by Raz et al.[33]. All volumes were extracted to a spread sheet for separate data analysis.

Statistical analyses

Individual levels of secondary hyperalgesia, HPDT, p-TS VAS-max, and p-TS VAS-AUC was obtained as estimated best linear unbiased predictors (EBLUPs)[24].

Primary analysis

The area of secondary hyperalgesia was adjusted for body surface area. Individual body surface areas were calculated using the Mosteller formula[34]. The association between the volume of the left and right caudate nucleus and the magnitude of the secondary hyperalgesia area was estimated by multiple linear regression. The ability to predict the size of the secondary hyperalgesia area by measurement of the caudate nuclei volume was quantified by R^2. P-values were adjusted for multiple testing using the single step method[35].

Secondary analyses

The association between the magnitude of the secondary hyperalgesia area and the volume of the cortical and subcortical brain structures relevant for pain processing (primary somatosensory cortex, anterior and mid cingulate cortex, putamen, accumbens nucleus, globus pallidus, insula and the cerebellum) was estimated by multiple linear regression. Model reduction was performed by backwards elimination with a 5% cut-off level.

Exploratory analyses

The association between the volume of the left and right caudate nucleus and HPDT, p-TS VAS-max, p-TS VAS-AUC, PCS and HADS respectively was evaluated per exploratory outcome by multiple linear regression. The findings were adjusted for multiple testing using the single step method[35].

Possible neuroanatomical differences between participants displaying a small area of secondary hyperalgesia (lower quartile) and participants displaying a large area of secondary hyperalgesia (upper quartile) were estimated using unpaired t-test. The findings were adjusted for multiple testing using the single step method[35].

Sensitivity analyses

Four sensitivity analyses were performed to assess the robustness of the findings.

In the first sensitivity analysis, further adjustments by age, weight, BMI, and MAP were performed. To adjust for hand-dominance a second sensitivity analysis were performed where only right-handed participants were included. To adjust for difference in ethnicity a third sensitivity analysis where performed where only ethnic Scandinavians were included. To evaluate the impact of individual body surface, we conducted a fourth sensitivity analysis where we did not adjust for body surface area.

Post-hoc analyses

A post-hoc analysis was conducted to investigate the association between the area of secondary hyperalgesia and brain structures not included in the per-protocol analyses, but with a possible relevance for pain processing. Thus, the association between the magnitude of the secondary hyperalgesia area and the volume of the left and right amygdala, hippocampus, and thalamus were estimated by multiple linear regression. Moreover, when comparing participants with a small and large area of secondary hyperalgesia possible differences in the volume of these three structures were estimated using unpaired t-test. P-values were adjusted for multiple testing using the single step method; however, P-values in the per-protocol planned analyses were not adjusted further by inclusion of the additional brain structures in the post-hoc analysis.

P-values corresponded to Wald-tests and $p<0.05$ were evaluated as significant.

All statistical analyses that were not computed by the MRI software were calculated using the open-source statistical programming environment R (R Core Team (2014). (R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/).

Results

121 healthy participants were included in the study. All participants completed the MRI-scans, but following clinical review, 3 participants were excluded due to suspected pathological findings. Thus, 118 participants were included in the final analysis (fig. 2). Of the 118 included participants, 10 were left-handed, and 15 had one or more parents with non-Scandinavian ethnicity. The median interval between the completion of the preceding study session with pain testing and the MRI scan was 17 days (interquartile range (IQR) 16 to 18).

The median size of the secondary hyperalgesia area was 448 cm^2 (IQR 346 to 528) with a range of 135 to 789 cm^2 (table 2).

Basic characteristics for the 118 participants and evoked pain results extracted from the preceding study session are displayed in table 2. No adverse or serious adverse events were reported.

Secondary hyperalgesia and caudate nuclei

We found no significant associations between the volume of the right and left caudate nucleus and the size of the area of secondary hyperalgesia (right hemisphere, single-step adjusted p=0.13, left hemisphere, single-step adjusted p=0.12). The adjusted R^2 was estimated to 0.007, and our regression analyses demonstrated that a one-mm^3 increase in the volume of the right caudate nucleus resulted in an estimated increase of 0.10 cm^2 in secondary hyperalgesia area (with a family-wise adjusted 95% confidence interval (95% CI) of (-0.03 to 0.23)). Likewise, a one-mm^3 increase in the volume of the left caudate nucleus resulted in an estimated decrease of -0.11 cm^2 in secondary hyperalgesia area (95% CI (-0.24 to 0.03)) (fig. 3).

Secondary hyperalgesia and cortical and subcortical areas

We found no significant association between the size of the area of secondary hyperalgesia, and the volume of the primary somatosensory cortex (right hemisphere *p*=0.11, left hemisphere *p*=0.76), anterior cingulate cortex (right *p*=0.33, left *p*=0.82) and mid cingulate cortex (right *p*=0.26, left *p*=0.91), putamen (right *p*=0.29, left *p*=0.05), nucleus accumbens (right *p*=0.27, left *p*=0.5), globus pallidus (right *p*= 0.35, left p=0.48), insula (right *p*=0.28, left *p*=0.08) or the cerebellum's white matter (right *p*= 0.44, left *p*=0.64) and cortex (right *p*= 0.62, left *p*=0.24) (fig. 4).

In the post-hoc analyses we found no significant associations between the size of the secondary hyperalgesia area and the volume of the amygdala (right *p*=0.96, left *p*=1), hippocampus (right *p*=0.99, left *p*=1), and thalamus (right *p*=0.96, left *p*=0.96).

Caudate nuclei and pain testing results

We found no significant associations between the volume of the right and left caudate nuclei and HPDT (right caudate nucleus *p*= 1, left caudate nucleus *p*=1), p-TS VAS-max (right *p*=1, left *p*=1) or p-TS VAS-AUC (right *p*=1, left *p*=1).

Caudate nuclei and anxiety, depression and pain catastrophizing

We found no significant associations between PCS and the volume of the caudate nuclei (right caudate nuclei *p*=0.96, left caudate nuclei *p*=0.94) or HADS and the caudate nuclei (right *p*=0.26, left *p*=0.24) score.

Small vs. Large area of secondary hyperalgesia

Following stratification based on areas of secondary hyperalgesia, the median area size in the groups including the lower (N=29) and upper quartile (N=29) was 261 cm^2 (IQR 203 to 319) and 579 cm^2 (IQR 516 to 629) respectively (table 3).

When comparing participants with a small area of secondary hyperalgesia (lower quartile) vs. participants with a large area (upper quartile) we found no significant differences in the volumes of the caudate nucleus (right *p*=1, left *p*=1) (fig. 5), the primary somatosensory cortex (right *p*= 0.91, left *p*=1), anterior cingulate cortex (right *p*=0.991, left *p*=1) and mid cingulate cortex (right *p*=1, left *p*=1), putamen (right *p*=1, left *p*=0.75), nucleus accumbens (right *p*=1, left *p*=0.98), globus

pallidus (right p=1, left p=1), insula (right p=1, left p=0.93),or the cerebellum's white matter (right p=1, left p=1) and cortex (right p=0.98, left p=1).

Likewise, in the post-hoc analyses we did not detect any significant differences in volumes of the amygdala (right p=1, left p=0.99), hippocampus (right p=0.98, left p=1) and thalamus (right p=0.89, left p=0.99) when comparing participants with a small vs. large area of secondary hyperalgesia.

Sensitivity analyses

Adjustment of age, weight, BMI and MAP did not demonstrate different results when comparing to our primary analysis. Secondly, we found that exclusion of left-handed participants or participants with non-Scandinavian ethnicity did not change the results markedly. Lastly, applying secondary hyperalgesia areas without adjusting for body surface area did not change the results markedly.

Discussion

The major question addressed by this study is whether differences in the propensity to develop secondary hyperalgesia and thus central sensitization are related to differences in the volume of brain structures in healthy volunteers.

We found that phenotypic expression of secondary hyperalgesia was not associated with differences in the volume of the caudate nuclei, nor was it associated with differences in the volumes of the primary somatosensory cortex, anterior and mid cingulate cortex, putamen, nucleus accumbens, globus pallidus, insula, cerebellum, amygdala, hippocampus and thalamus.

The occurrence of pain is dependent on both peripheral mechanisms and the excitability of the central nervous system. Sensitization of the central nervous system is characterized by enhanced responsiveness of nociceptive neurons to normal or subthreshold afferent inputs, and is believed to play an important role in various pain conditions such as osteoarthritis, fibromyalgia, headache and neuropathic pain[1,2].

Even though central sensitization was characterized more than three decades ago, its pathophysiology still remains elusive. It is believed that alterations in synaptic efficacy, membrane excitability, transmission inhibition, as well as changes in microglia, astrocytes, and gene transcription leads to the changes in functional properties that are characteristic for central sensitization[1,2]. Clinically, the effects of central sensitization can be observed as enhanced temporal summation, allodynia, hyperalgesia, and after-sensations (perception of a stimulus after the stimulus has been terminated)[2]. One essential feature of central sensitization is secondary hyperalgesia, i.e. expansion of receptive fields enabling input from non-injured tissue to be perceived as painful[1].

Previous studies have indicated a link between the magnitude of secondary hyperalgesia area and persistent pain. Patients suffering from fibromyalgia or rheumatoid arthritis display larger areas of secondary hyperalgesia compared to healthy individuals[36,37]. Moreover, following iliac crest bone harvest[38] and after abdominal surgery[39,40] and thoracotomy[41], a correlation was demonstrated between increasing size of secondary hyperalgesia area and the development of persistent pain. Interestingly, no correlation was found between the magnitude of secondary hyperalgesia and surgical characteristics (length of incision, volume of deep tissue trauma and nerve lesion

severity), which suggests that secondary hyperalgesia reflects individual predispositions to develop central sensitization[38]. One study found no correlation between pre-surgical areas of secondary hyperalgesia and postoperative pain following arthroscopy[42]. However, in this study secondary hyperalgesia was assessed to predict pain 1-10 days postoperatively and not to predict persistent or chronic pain.

In the present study, we used high-resolution MRI at 3-Tesla to investigate if volume estimates of brain structures involved in pain processing would correlate to the area of secondary hyperalgesia induced by a thermal injury. MRI permits precision measurement and detection of minute differences in brain structure[43,44]. We found no significant association between areas of secondary hyperalgesia and the volume of the caudate nuclei. This is emphasized by the estimated R^2, indicating that only 0.0068% of the variation of in secondary hyperalgesia area is explained by the volume of the caudate nuclei. Moreover, we found no significant associations between heat pain detection thresholds or pain during one minute thermal stimulation and the volume of the caudate nuclei, indicating that cutaneous heat pain sensitivity is also not related to the volume of the caudate nuclei. Finally, we found no significant associations between the area of secondary hyperalgesia and the volume of any other pain relevant brain structures.
Our findings indicate that the predisposition for central sensitization, assessed as secondary hyperalgesia area, is not related to brain structure volume, and that individual levels of central sensitization are not determined by cortical or subcortical structural volume differences.
In contrast, a previous exploratory MRI study reported a correlation between the volume of the caudate nuclei and the area of secondary hyperalgesia[13]. In both the present and the former study, the neuroanatomy of healthy participants was examined by 3-Tesla MRI. However, there are important differences between the two studies: Firstly, as opposed to the former study, we examined predefined anatomical areas of interest (brain structures related to pain processing[21,22]) and corrected our data for total body surface in the analysis of the present data. Secondly, in the present study we included a high number of healthy male participants (N = 121), without prior knowledge of their individual areas of secondary hyperalgesia, as compared to inclusion of fewer participants of both gender (N= 40) based on the magnitude of the secondary hyperalgesia area (and with a disproportionally higher number of females in one group) in the study by Asghar et al.[13]. The difference in method of inclusion is especially important since it may have contributed significantly to the differences in results. Inclusion based on area of secondary hyperalgesia

increases the inter-participant differences in development of secondary hyperalgesia, and may produce more visible results; however, we believe that our approach in the current study is more robust since it is strictly driven by the hypothesis, and not data driven. Nonetheless, this may have resulted in a lower inclusion of participants with small or large areas of secondary hyperalgesia, and increased the risk of type 2 errors.

We performed the MRI scans on average 17 days after the pain testing to avoid carry-over effects. It has been demonstrated that areas of secondary hyperalgesia following BTS remain stable over a period of minimum 4 weeks[12]. This allowed for investigation without the risk of recording neuroanatomical changes due to recent or repetitive pain stimulations. We included a large number of participants (n = 121) making this the largest MRI study of secondary hyperalgesia reported so far. There were no missing data and no protocol violations. Moreover, we based our primary and secondary outcome measures on known cortical and subcortical brain structures relevant for pain and central sensitization[13,21,22]. We conducted separate sensitivity analyses to test the robustness of our findings, and did not find different results compared to our primary analysis. Finally, based on the high number of included participants combined with a stringent methodological approach we believe our findings to be robust and of high quality.

Studies of healthy participants have indicated that reduced grey matter volume of pain relevant structures is correlated with increased visceral sensitivity[45] as well as increased heat pain sensitivity[46]. Results from the present study are not coherent with those findings. However, HPDTs have been demonstrated only to offer moderate explanation of the inter-individual variations in secondary hyperalgesia[24]; suggesting that cutaneous heat pain sensitivity and areas of secondary hyperalgesia represent two distinctively different pain entities. Moreover, to the authors' knowledge, no studies have investigated the association between visceral sensitivity and secondary hyperalgesia areas.

Several studies of chronic pain patients have identified neuroanatomical correlates of chronic pain[18,20,47] and reported reduced grey matter volumes in multiple pain relevant brain structures. However, comparisons between structural abnormalities found in pain free healthy individuals and chronic pain patients have limited value, since evidence suggest that structural grey matter abnormalities observed in chronic pain patients are a result of experience-dependent neuronal plasticity, and that these abnormalities are reversible when the pain stimulus is terminated[47-50]. In

support of this, a study of healthy individuals reported that initial MRI-scans did not show structural differences between individuals characterized with high and low pain sensitivity, but following repetitive noxious stimulation the high pain sensitizers were more prone to develop grey matter density reductions[43]. This suggests that healthy individuals with high innate pain sensitivity are more prone to develop structural abnormalities comparable to chronic pain patients, but also that pain sensitivity is not influenced by the structural anatomy of the brain.

The present study has some limitations. Firstly, we applied strict inclusion criteria resulting in a homogenous population of healthy male participants. Inclusion of both sexes would have introduced several variations such as possible neuroanatomical differences related to sex[51,52], hormonal influence of the menstrual cycle on MRI findings[53,54], and possible interaction between the menstrual cycle and pain responses[55]. Moreover, the BTS method has only been validated in healthy male volunteers[12]. Secondly, due to limitations in the software and the ancillary anatomical atlas we were not able to segment the secondary somatosensory cortex, the supplementary motor area, the substantia nigra, and the subthalamic nucleus as we had specified in our published study protocol[23]. Finally, we included participants regardless of hand-dominance and ethnicity. MRI-studies often include right-handed participants only, additionally, ethnicity may influence pain thresholds[56]. Our sensitivity analyses; however, did not show any differences in findings when excluding left-handed or non-Scandinavian participants illustrating the robustness of our results.

In conclusion, we did not find significant associations between the area of secondary hyperalgesia induced by a BTS and the volume of the caudate nuclei or any other predefined brain structures involved in pain processing, indicating that the propensity to develop central sensitization is not correlated to the volume of pain related brain structures.
We suggest that future studies of contributing factors to central sensitization should include investigations of the functional connectivity of the CNS, the endogenous opioid system, relevant molecular mechanisms and psychological factors. Potential findings in future studies may shed light on the aetiology of central sensitization and ultimately provide us with novel pharmaceutical targets in the treatment of acute and chronic pain.

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Author contributions

All authors contributed in the conception and design of the study, critically revised the manuscript, and approved the final manuscript. MSH performed the data collection, MRI data processing and contributed to the primary writing of the manuscript. CBP conducted the statistical calculations. JDN contributed to the data collection. MSA and JM contributed to the MRI data processing and the design of the MRI-sequence. LB, AC, MB and IH contributed to design of the MRI sequence, and JBD and JW contributed to the design and methodology of the pain testing.

Conflicts of interest

The authors declare that they have no conflicts of interests

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Tables and figures

Inclusion criteria	Exclusion criteria
Age ≥18 years and ≤35 years Speak and understand the Danish language Male sex Signed informed consent Participation and completion of the study: "Heat pain detection threshold is associated with the area of secondary hyperalgesia following brief thermal sensitization: a study of healthy male volunteers"[24]	Inability to cooperate to the test Weekly intake of >21 units of alcohol, or intake of >3 units of alcohol within 24 hours before study day Substance abuse, assessed by the investigator Consummation of analgesics within 3 days before study day Consummation of antihistamines within 2 days before study day Consummation of antidepressant medication within 30 days before the study day Consummation of prescription medicine within 30 days before the study day Consummation of caffeine within 24 hours before study day. Neurological illnesses Chronic pain Psychiatric diagnoses Eczema, wounds or sunburns on the sites of stimulation Body Mass Index >30 kg/m^2 or <18 kg/m^2. Unwilling to receive information regarding potential pathological findings in relation to the MRI. Trauma resulting in pain and administration of analgesics in the period between pain testing and MRI scan. Head trauma in the period between the pain testing and the MRI. Contraindications to MRI (claustrophobia, pacemaker implant, artificial heart valve, cochlear/stapes prosthetics, irremovable insulin pump, neuro-stimulator, metal from previous surgery, metallic foreign objects, catheters, shunts, draining tubes, and surgical procedures within the last 6 weeks (subjected to individual evaluation)).

Table 1. Inclusion and exclusion criteria
Abbreviations: MRI, Magnetic resonance imaging.

Variable	Median (IQR)	Range (min-max)
Age (years)	22 (20-24)	18-33
Height (m)	1.84 (1.79-1.88)	1.68-2.03
Weight (kg)	76.8 (70.0-84.8)	57-110
BMI (m^2/kg)	22.82 (21.02-24.51)	18.12-28.63
MAP (mm Hg)	90 (84-96)	73-117
Heart rate (bpm)	64 (58-70)	46-97
Pain testing results		
Area of secondary hyperalgesia (cm^2)	448 (346-526)	135-789
HPDT (°C)	45.57 (43.78-46.60)	38.70-51.01
p-TS VAS-max (mm)	33.5 (18.79-53.41)	2.41-95.99
p-TS VAS-AUC	1151 (648-1850)	83-4456
Psychological test results		
PCS-helplessness	4 (2-6.25)	0-17
PCS-rumination	5 (3-8)	0-12
PCS-magnification	3 (1-4)	0-10
PCS-total	12 (7-17)	1-31
HADS-anxiety	4 (2-6)	0-13
HADS-depression	1 (1-3)	0-16
HADS-total	6 (3-8.25)	0-21

Table 2. Basic characteristics, pain testing results, and psychological test results of the 118 participants included in the analysis

All medians and ranges of the area of secondary hyperalgesia, heat pain detection thresholds and pain during one minute thermal stimulation have been estimated by calculating the estimated best linear unbiased predictors (EBLUPs). Test results of PCS and HADS were extracted following completion of all MRI-scans.

Abbreviations: IQR, Interquartile range; BMI, body mass index; bpm, beats per minute; MAP, mean arterial pressure; HPDT, heat pain detection threshold; p-TS, pain during one minute thermal stimulation; VAS-max, maximum visual analogue scale; VAS-AUC, visual analogue scale area under the curve; PCS, Pain Catastrophizing Scale; HADS, Hospital Anxiety and Depression Scale.

Characteristic	Small area (lower quartile)	Large area (upper quartile)
Number of participants (n)	29	29
Area of secondary hyperalgesia (cm^2)	261 (203-319)	579 (516-629)
HPDT (°C)	46.32 (45.56-47.11)	43.34 (41.93-44.59)
p-TS VAS-max (mm)	28.15 (17.03-41.56)	49.44 (26.74-70.61)
p-TS VAS-AUC (mm^2)	1002 (566-1408)	1720 (991-2801)
PCS-helplessness	3 (1.25-6.5)	4 (2.25-6.75)
PCS-rumination	5 (3-7)	5 (4-7)
PCS-magnification	2.5 (1-4)	2.5 (1-4)
PCS-total	11 (6.25-16.75)	12.5 (9-16.75)
HADS-anxiety	3 (2-6)	4.5 (3-6.75)
HADS-depression	1 (0-2)	2 (1-4)
HADS-total	4 (2.25-7.5)	7 (4.25-9)

Table 3. Results from pain testing and psychological testing of the upper and lower quartile based on magnitude of secondary hyperalgesia area adjusted for body surface.

Numbers are reported in median and interquartile ranges.

All medians and ranges of area of secondary hyperalgesia, heat pain detection thresholds and pain during one minute thermal stimulation have been estimated by calculating the estimated best linear unbiased predictors (EBLUPs).

Abbreviations: IQR, interquartile range; HPDT, heat pain detection threshold; p-TS, pain during one minute thermal stimulation; VAS-max, maximum visual analogue scale; VAS-AUC, visual analogue scale area under the curve; min, minimum; max, maximum; PCS, Pain Catastrophizing Scale; HADS, Hospital Anxiety and Depression Scale.

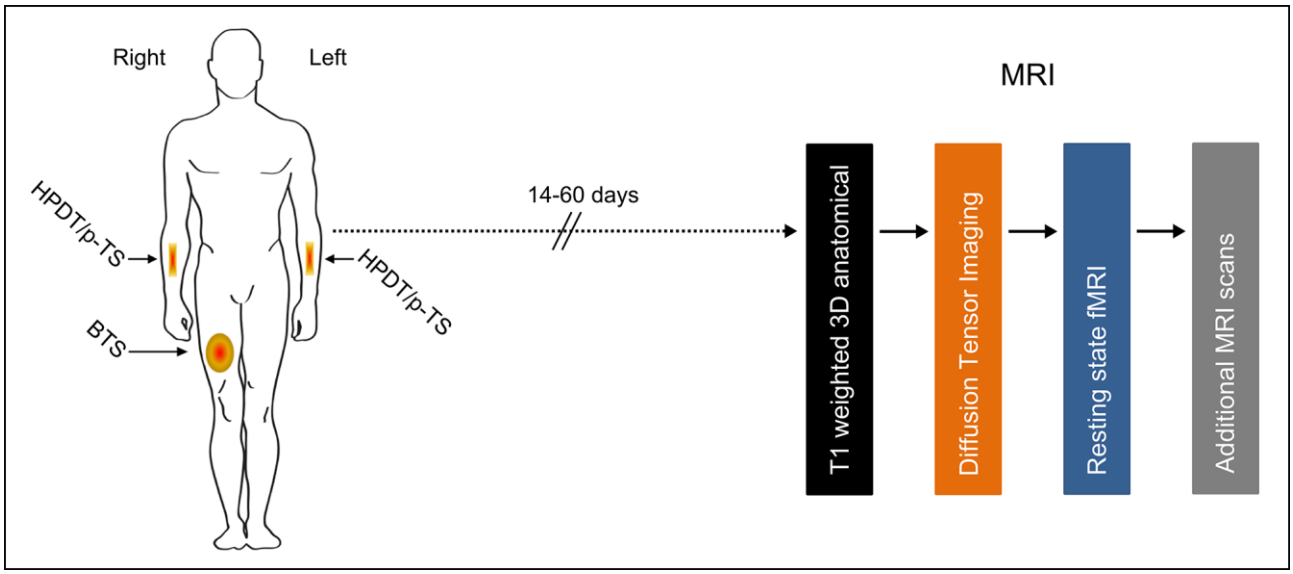

Figure 1. Anatomical presentation of the location of the pain testing and MRI scan sequence

Brief thermal sensitization was conducted centrally on the anterior part of the right thigh in the midline between the anterior superior iliac spine and the base of patella. Heat pain detection threshold was evaluated on the anterior part of the dominant lower arm; pain during one minute thermal stimulation was evaluated on the anterior part of the non-dominant lower arm. The MRI scans were performed a minimum period of 14 days and maximum 60 days after the pain testing.

The MRI scans were conducted in fixed order, starting with T1-weighted 3D anatomical scan (duration: 4 minutes) followed by diffusion tensor imaging (duration: 12 minutes), resting state fMRI scan (duration: 8 minutes) and additional scans of technical or diagnostic character. Total duration of MRI scan sequence was approximately 50 minutes.

Abbreviations: BTS, brief thermal sensitization; HPDT, heat pain detection threshold; p-TS, pain during one minute thermal stimulation; MRI, magnetic resonance imaging; fMRI, functional magnetic resonance imaging.

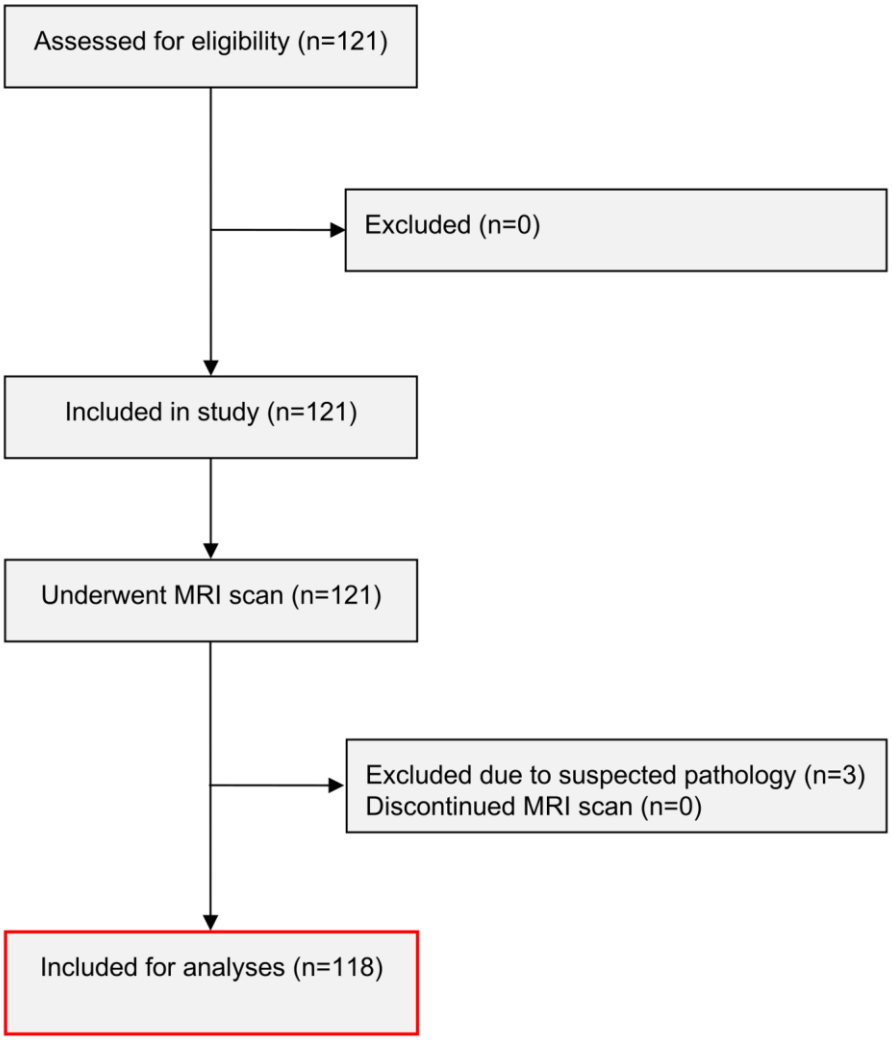

Figure 2. Flowchart of included study participants
121 participants were assessed for eligibility and included in the study. Three participants were excluded due to pathological findings following magnetic resonance imaging, and consequently 118 participants were included in the final analysis.
Abbreviations: MRI, magnetic resonance imaging.

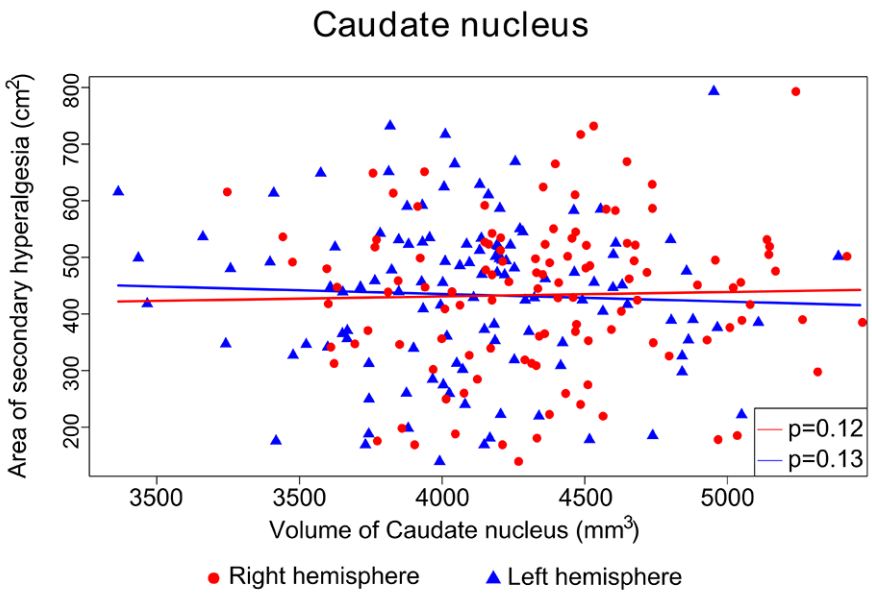

Figure 3. Associations between the area of secondary hyperalgesia and the volume of the caudate nuclei
Scatter plot of the volume of the right (red dots) and left (blue triangles) caudate nucleus and the corresponding area of secondary hyperalgesia of each included participant. Individual volumes of the caudate nuclei were adjusted for intracranial volume, and individual areas of secondary hyperalgesia were adjusted for body surface area. Regression lines demonstrate no significant association between area of secondary hyperalgesia and the volume of the right and left caudate nucleus (right hemisphere, p= 0.12, left hemisphere, p=0.13).

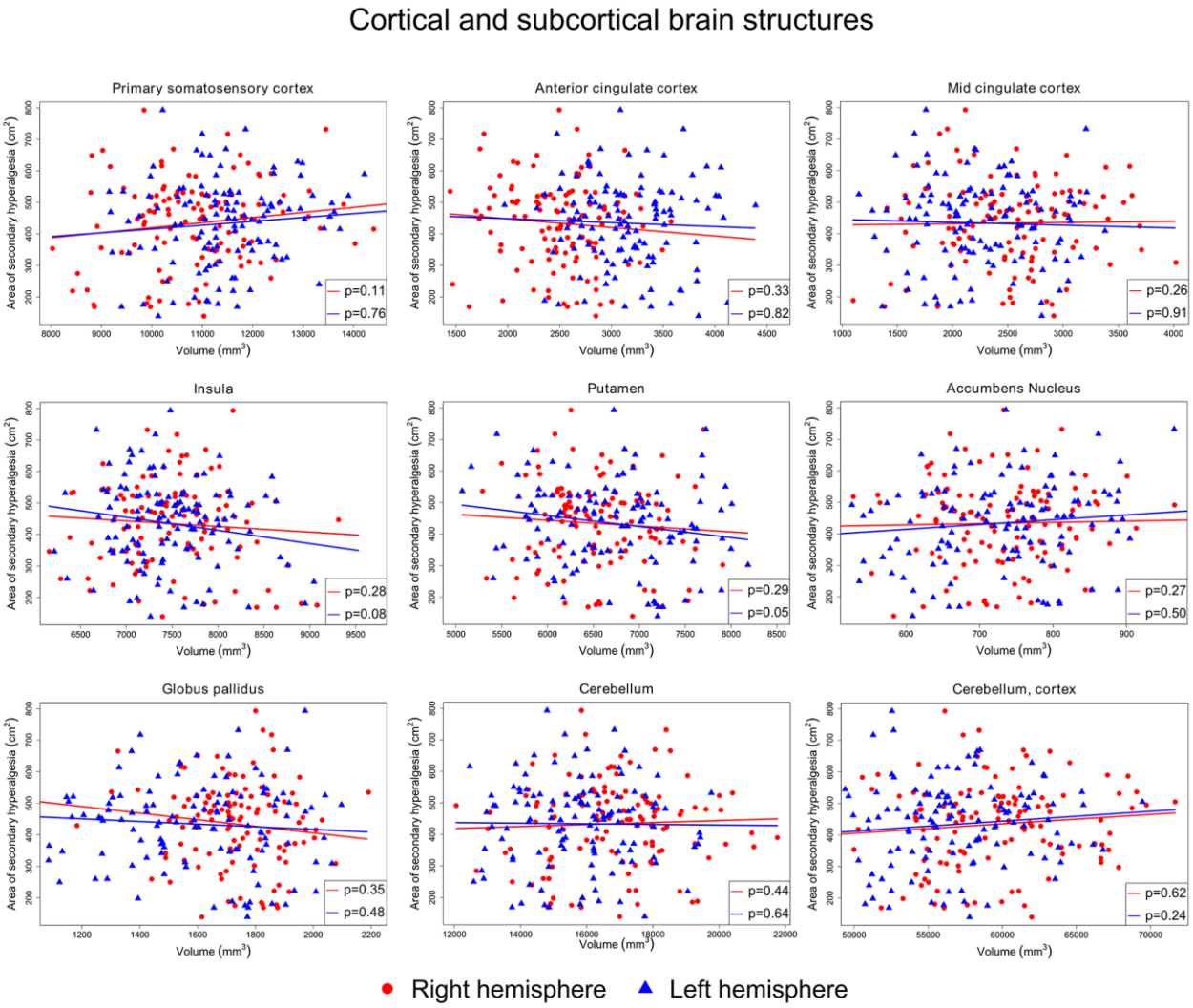

Figure 4. Associations between the size of the secondary hyperalgesia area and the volume of relevant cortical and subcortical brain structures

Scatter plots of individual volume measurements of brain structures relevant for pain processing belonging to the right hemisphere (red dots) and left hemisphere (blue triangles). Volumes of individual brain structures were adjusted for intracranial volume, and individual areas of secondary hyperalgesia were adjusted for body surface area. Red and blue regression lines and p-values ≥ 0.05 demonstrate no significant association between the size of the secondary hyperalgesia area and the volume of brain structures relevant for pain processing.

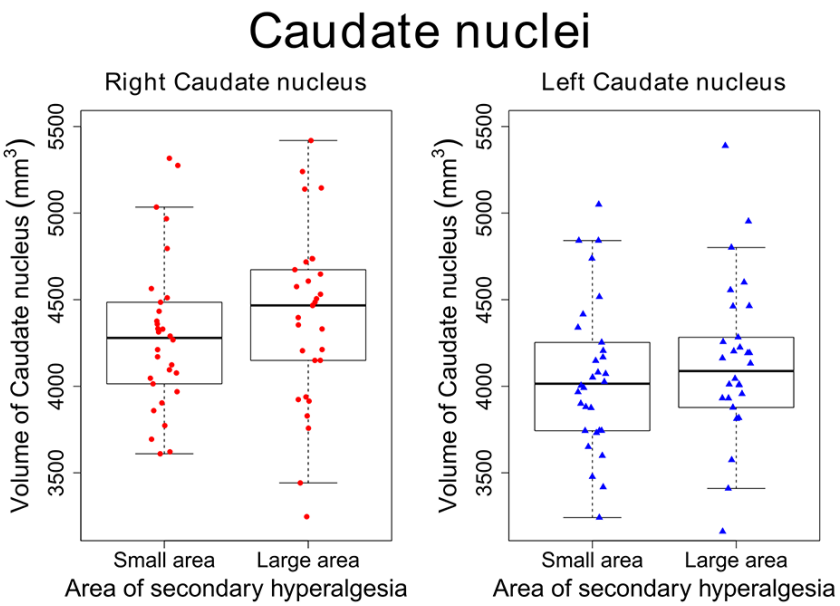

Figure 5. Volumes of caudate nuclei when comparing participants with a small versus large area of secondary hyperalgesia

Boxplot of the right (dots) and the left (triangles) volumes of the caudate nuclei corresponding to the participants with small (lower quartile) and large (upper quartile) areas of secondary hyperalgesia respectively. Points correspond to individual volume measurements, the thick horizontal line corresponds to median volume of the caudate nuclei, and whiskers indicate borders of 1.5 times the upper or lower quartile. There was no significant difference between the groups (small vs. large areas of secondary hyperalgesia) regarding the volume of the caudate nucleus in either the left (p=1) or the right (p=1) hemisphere.

PAPER IV

Title: Brain Resting State Connectivity in the Development of Secondary Hyperalgesia

Running head: Resting state connectivity and secondary hyperalgesia

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Abstract

Introduction

Central sensitization is a condition in which there is an abnormal responsiveness to nociceptive stimuli. As such the process may contribute to the development and maintenance of pain. Factors influencing the propensity for development of central sensitization have been a subject of intense debate and remain elusive. Injury-induced secondary hyperalgesia can be elicited by experimental pain models in humans, and is believed to be a result of central sensitization. Secondary hyperalgesia may thus reflect the individual level of central sensitization. The objective of this study was to investigate possible associations between increasing size of secondary hyperalgesia area and brain connectivity in known resting state networks.

Methods

We recruited 121 healthy participants (male, age 22 (SD 3.35)) who underwent T1-weighted and resting state functional magnetic resonance imaging. Prior to the scan session, areas of secondary hyperalgesia following brief thermal sensitization (3 min. 45°C heat stimulation) were evaluated in all participants.

Results

115 participants were included in the final analysis. We found that increasing area of secondary hyperalgesia was positively correlated with increasing connectivity in the Sensorimotor- and Default mode networks. We also observed a negative correlation (decreasing connectivity) with increasing secondary hyperalgesia area in the Sensorimotor-, Fronto-parietal-, and Default mode networks.

Conclusion

Our findings indicate that increasing area of secondary hyperalgesia is associated with increasing and decreasing connectivity in multiple networks, suggesting that differences in the propensity for central sensitization, assessed as secondary hyperalgesia areas, may be expressed as differences in the resting state central neuronal activity.

Keywords

Pain; Secondary hyperalgesia; Central sensitization; Resting state fMRI; MRI

Introduction

Central sensitization plays a pivotal role in development and maintenance of pain hypersensitivity and is believed to play an equally crucial role in the transition from acute pain to chronic pain conditions[1,2]. Central sensitization characterizes the central nervous system's (CNS) capacity to alter neuronal signalling by enhancing responsiveness of central nociceptive neurons to normal or subthreshold afferent inputs[1,2]. The clinical manifestations of central sensitization can be observed as allodynia, enhanced temporal summation, hyperalgesia, and after-sensations[1,2]. Secondary hyperalgesia is an essential manifestation of central sensitization where expansion of receptive fields enables neuronal signalling from non-injured tissue adjacent to injured tissue to be perceived as painful[1]. By applying experimental secondary hyperalgesia pain models, central sensitization can be induced in healthy participants[3-7]. Previous studies have demonstrated that areas of secondary hyperalgesia following a thermal injury vary among individuals, where some individuals develop a large area, while others develop a small area of secondary hyperalgesia. The areas of secondary hyperalgesia remains largely constant within each individual[3,7,8], suggesting that it is a phenotypic characteristic[7]. Clinical studies indicate that an increasing size of secondary hyperalgesia area surrounding a surgical incision is correlated with an increased risk of developing persistent pain after surgery[9-11]. The correlation was independent of surgical characteristics (deep tissue trauma, nerve lesion severity, and length of incision), suggesting that the development of secondary hyperalgesia may rely on individual predispositions to develop central sensitization[12]. In addition, chronic pain patients suffering from fibromyalgia or rheumatoid arthritis exhibit larger areas of secondary hyperalgesia compared to pain free healthy individuals[13,14]. Causal factors responsible for the differences in the propensity to develop central sensitization remain largely unexplored, and identification of relevant mechanisms and potential biomarkers may be instrumental in classifying novel targets for analgesic intervention.

As a first step to explore the pathophysiology of secondary hyperalgesia and central sensitization we found that an increasing area of secondary hyperalgesia was associated with a decreasing heat pain detection threshold (HPDT). However, our findings also revealed that HPDT only offered a modest explanation of the inter-individual variation in secondary hyperalgesia areas[15]. As a second step, we investigated if the size of the secondary hyperalgesia area was associated with the volume of pain related brain structures and found no significant associations, indicating that the

propensity to develop central sensitization is not associated with brain structure volume (Hansen MS, Asghar MS, Wetterslev J, et al. The propensity to develop central sensitization is not correlated to pain relevant brain structures - a 3-tesla MRI study of healthy male volunteers. Unpublished data pending review).

Current evidence suggests that supraspinal activity in the brainstem, subcortical and cortical structures may be essential in the development and maintenance of secondary hyperalgesia[16,17]. Presently, we therefore aim to investigate if the propensity to develop central sensitization, assessed as secondary hyperalgesia areas, is associated with the functional connectivity of known resting state networks.

Material and methods

The study was approved by the Danish Committee on Health Research Ethics for the Capital Region (H-15010473) and the Danish Data Protection Agency (RH-2015-149). The study is also registered on Clinicaltrials.gov (NCT02567318). The study protocol with a detailed description of study design has been published previously[18]. Below follows a brief description of the design and methods of the study.

Design

The study consisted of two separate parts conducted on separate days: (1) Pain testing and (2) MRI scans.

Pain testing

Pain testing was performed in a previous study session[19], and the isolated pain testing results have been presented in a separate publication[15]. In the present study we used these data for analysis of the MRI data.

The pain testing was conducted a minimum of 14 days and a maximum of 60 days before the MRI-scan to avoid potential carry-over effects. Briefly, the participants were tested with the cutaneous heat pain model, *brief thermal sensitization* (BTS), on two separate study days. Here the skin of the anterior right thigh was heated to 45°C for 3 minutes using a computer controlled thermode (Somedic MSA Thermotester; size 2.5 x 5 cm.), eliciting primary hyperalgesia at the site of the injury, and secondary hyperalgesia surrounding the heat injury. The assessment of secondary hyperalgesia was conducted while the 45°C thermode was still placed on the skin of the participant[4,20-23]. The assessment took approximately 1-2 minutes, resulting in a maximum duration of heat stimulation of 5 min.

The area of secondary hyperalgesia was quantified following stimulation with a monofilament (Von Frey hair) with a nominal value of 18 (bending force 490 mN) in 4 linear paths arranged in 90° with the thermode as centre. Stimulation was initiated well outside the area of secondary hyperalgesia and progressed in 5 mm/s intervals towards the centre of the thermode. When the participant stated a clear change in sensation (intense burning, pricking, tenderness) the place was

marked, and the longitudinal and transverse axes were measured for rectangular area calculation[4,7,8,20-30]. For details regarding the pain testing please see the published protocol[18].

MRI scans

On the MRI day, the participants did not perform any tasks or pain stimulation and thus no additional assessments, tests or stimulations were conducted besides the MRI scans. The duration of the study day was approximately 50 min in which each participant underwent multimodal whole brain MRI scans. After completion of the MRI scans, all anatomical images were reviewed by an experienced radiology consultant. In case of suspected pathological findings, the participant was informed hereof and was referred to a specialist in neurology for further examination.

MRI acquisition and image protocol

The MRI scans were performed with a Siemens MAGNETOM Verio 3 tesla, software version b17, and a 32-channel head coil. Anatomical images were obtained using T1-weighted 3D FLASH (160 sagittal slices, matrix 256x256 mm, Field of view 256 mm, echo time (TE) 2.98 ms, repetition time (TR) 2300 ms, Slice 1 mm, in plane resolution 1x1 mm, flip angle 9°).

Resting-state images were acquired as an epi single shot (42 axial 3 mm slices, gap 0 mm, field of view 192 mm, TR=2250 ms, TE =26 ms, acceleration factor R=2 matrix size 64x64 mm, flip angle 82°, 200 volumes). During the resting state fMRI scans the participants were instructed to stay awake and keep their eyes open. If the participant fell asleep they were instructed to inform the investigator, and the fMRI scan would be discarded.

Additional MRI sequences not analysed

We also performed the following MRI sequences: Diffusion tensor imaging, Arterial spin labelling, b0 field maps, T2-Flair, T2-weighted TSE sequence and GRE hemo sequence. These scans were either of technical character or for diagnostic purposes, and will per protocol not be reported in this paper.

Physiological measurements

Heart rate, respiration frequency, and end-tidal PCO_2 were measured during the entire scan session including before and after the resting state scan. Functional MRI measurements can be

influenced by changes in physiology. These data were captured in order to ensure that physiological parameters remained stable.

Setting

MRI scans were conducted at the Department of Radiology, Bispebjerg and Frederiksberg hospitals, Copenhagen, Denmark. Pain testing[19] was conducted at the department of Anesthesiology, Copenhagen University Hospital, Rigshospitalet, Copenhagen, Denmark. Analyses of MRI data were carried out at the department of Anesthesiology, Rigshospitalet, Copenhagen, Denmark, and at the Center for Pain and the Brain, Childrens hospital, Boston, USA. Data was collected in the period from October 2015 to December 2015.

Participants

Healthy male volunteers aged 18-35 years, who had completed the preceding pain testing session[19] were included in the study. Prior to inclusion, oral and written informed consent was obtained from all participants. The participants received EUR 67 (USD 74) for their participation in the study. Inclusion and exclusion criteria are listed in table 1.

Outcome measures

The outcome measures have per protocol been specified as exploratory[18]. The specific outcome measures were: (1). To determine the association between the size of the area of secondary hyperalgesia and the connectivity in networks measured by resting state fMRI (rsfMRI). (2) To define differences in connectivity networks measured by rsfMRI when comparing participants with a small (lower quartile) versus large (upper quartile) area of secondary hyperalgesia.

Preprocessing of MRI data

Preprocessing of anatomical data

T1W anatomical images for each participant were preprocessed using FSL Brain Extraction Tool (BET) applying robust brain centre estimation[31].

Presently, the anatomical images we used for co-registration purposes of the functional images only. Per protocol, results from the structural MRI data and correlation to the areas of secondary hyperalgesia will result in a separate publication.

Preprocessing of resting state data

Initially, data was preprocessed for each individual participant (motion correction, temporal high pass filtering with a 100 s time constant, single session ICA), with the optimal number of independent components determined by the FSL-Melodic software (http://fsl.fmrib.ox.ac.uk), and subsequently quality assessed for motion (≥3 degrees and/or 3mm) and temporal scanner stability using the method described by Friedman and Glover[32]. Images were inspected to detect large (>20%) changes in signal intensity. Secondly, automatic de-noising of resting state data was conducted by applying FIX, with a threshold of 1[33,34] (http://fsl.fmrib.ox.ac.uk/fsl/fslwiki/FIX), which removes temporal components of the data that are deemed of no neurological origin. Lastly, the preprocessed data was concatenated and analysed in group analysis with FSL Melodic to estimate common independent components with a fixed number of 75 independent components[35] (spatial smoothing with 5 mm kernel, multi-session temporal concatenation). Individual preprocessed T1W images and the MNI-152 brain atlas were used for co-registration.

Intrinsic Network Identification

Subsequent to the Independent component analysis (ICA), each of the identified components were spatially cross-correlated (Pearson correlation) with healthy adults' networks as available at: http://www.fmrib.ox.ac.uk/analysis/brainmap+rsns/. The dataset including 20 separate networks were selected[36], and in addition, two networks representing the salience network[37] and the basal ganglia network were included[38]. A Pearson correlation of 0.25 was set as threshold and applied in determining significant spatial correspondence between the templates and the components identified in the present data set. If one component was correlated with more than one template network, the template with the highest correlation was chosen as appropriate network. Each of

the identified components with a Pearson correlation above 0.25 were visually inspected and included for further analysis.

Since our aim with this study was related with pain processing we planned to report results from the Default mode network (DMN), right Fronto-parietal network (FPN), Central executive (CEN), Basal ganglia network (BGN), Salience and Sensorimotor network (SMN) only.

Statistical analyses

Areas of secondary hyperalgesia were adjusted for individual body surface area in all analyses[39].

Statistical analyses of resting state data

Dual regression was carried out as described by Beckmann et al.[40] and Filippini et al.[41], where temporal information from the fMRI data are integrated across multiple distributed networks identified in the group ICA analysis. By applying dual regression, temporal dynamics and associated spatial maps of the individual participant can be estimated by regression against the original data set, which allows inter-participant and inter-group comparisons.

Two separate dual regression analyses were performed:

1. Investigating the effect of an increasing size of secondary hyperalgesia area

We included all participants and applied area of secondary hyperalgesia as an explanatory variable. In this analysis, we used the effect of the increasing size of the secondary hyperalgesia area as a contrast: evaluating the association between connectivity and increasing size of secondary hyperalgesia area. The mean secondary hyperalgesia area was subtracted from each participant's individual area of secondary hyperalgesia (demeaned), and the result was entered in the analysis as an exploratory variable. To limit a potential confounding effect of age, we included age as a covariate in the design matrix. A positive correlation indicated brain structures where connectivity increased with increasing sizes of secondary hyperalgesia areas. A negative correlation indicated brain structures where connectivity decreased with increasing sizes of secondary hyperalgesia areas.

2. Comparison of participants with a small and large area of secondary hyperalgesia

Based on the size of the secondary hyperalgesia area the population was divided into four quartiles. Differences in resting state connectivity were estimated by comparison of the

participants with large areas of secondary hyperalgesia (upper-quartile) with participants with small areas of secondary hyperalgesia (lower-quartile). To assess group differences between the lower and upper quartile we created the following contrasts: I. Effect of small area of secondary hyperalgesia (small vs large area of secondary hyperalgesia), II. Effect of large area of secondary hyperalgesia (large vs small area of secondary hyperalgesia).

Post-hoc analysis

We had no exclusion criteria based on hand-dominance. Thus to test the robustness of our results we conducted a post-hoc analysis investigating the connectivity of the right FPN in right-handed participants only. We conducted the post-hoc analysis for the right FPN, because the FPN was the only lateralised network that was included in the analysis.

Inference

We aimed to apply a false discovery rate (FDR) to determine statistical thresholds for significance. To conduct FDR, data must follow a standard normal distribution. Thus, we conducted a one-sample Kolmogorov-Smirnov test[42,43], to test if our statistical maps had a standard normal distribution. However, the null-hypothesis was rejected, and consequently, FDR could not be performed[44]. As an alternative method for inference we applied Gaussian mixture modelling[45-47]. With mixture modelling the Z-statistic images for each contrast in the dual regression analyses were subjected to alternative hypothesis testing. By applying a Markov random-field, we were able to regularize the labelling of voxels into “null”, “hyper-connected” or “hypo-connected”, and promote spatially neighbouring voxels to have similar labels, allowing us to estimate the number of truly independent samples in the present data set[48]. The parameters for the Markov random field and the mixture modelling were estimated from data by applying the iterated conditional modes algorithm[49], and posterior probability maps estimating the probability of activation of a given voxel according to the labels of nearby voxels were created with a threshold of 0.5 or above. Statistical connectivity maps were spatially clustered using in-house software, and cluster peak values were identified. A minimum cluster volume of 0.9 mm^3 (7 smoothed native space voxels) were applied as final threshold, and peak activity within each cluster was extracted.
The brain structures underlying the identified clusters were determined using a publicly available atlas (Fmri.wfubmc.edu/software/pickatlas).

Results

Participant Data

121 participants were included in the study and underwent whole brain MRI. Prior to data analysis, three participants were excluded due to pathological findings. Following quality assessment two participants were excluded due to head movement (> 3 degrees and/or > 3 mm), while data from one participant was excluded due to scanner intensity instability. Thus, a total of 115 participants were included in the final analysis (fig. 1). Of the 115 participants, 10 had one or more parents with non-Scandinavian ethnicity, and 15 were left-handed. The median number of days between the completion of the preceding study session and the MRI-scan was 17 days, interquartile range (IQR) (16-18). Demographic characteristics for the 115 participants are presented in table 2. We did not detect any changes in heart rate, respiratory frequency and end-tidal CO_2 during the MRI-scan session (for physiological measurements see table 3); and no participants fell asleep during the MRI-scan session. No adverse or serious adverse events were reported.

Areas of secondary hyperalgesia

The median size of the area of secondary hyperalgesia was 447 cm^2 (IQR 348 to 525). The median sizes of secondary hyperalgesia areas in the lower (small area) and upper quartile (large area) were 273 cm^2 (IQR 201 to 320) and 579 cm^2 (IQR 515 to 640) respectively. For basic characteristics of the participants with large and small areas of secondary hyperalgesia only, see table 4.

Network identification

We identified the Medial, Lateral and Occipital visual networks, DMN, Cerebellum, SMN, Auditory, CEN, right and left FPN, Salience, and BGN.

3.4. Network connectivity

Effect of increasing area of secondary hyperalgesia on specific brain networks

Sensorimotor network

A significant positive correlation was observed in the *Frontal* rectus, superior orbital, inferior orbital, middle orbital, superior medial, superior, inferior triangular, supplemental motor area,

precentral, *occipital* calcarine, inferior, cuneus, *temporal* superior, middle, fusiform, lingual, *cingulum* posterior, *parietal* postcentral, angular, and *insula* posterior. Total volume of positively correlated structures was 40.36 cm^3. A significant negative correlation was observed in the *Frontal* middle orbital, middle, rectus, paracentral lobule, *occipital* cuneus, middle. Total volume of negatively correlated structures was 7.384 cm^3 (fig. 2 and 5, table 6).

Right Fronto-parietal network

No significant positive correlation was observed. A significant negative correlation was observed in the *temporal* middle, superior, *frontal* middle orbital, and *occipital* superior. Total volume of negatively correlated structures was 5.136 cm^3 (fig. 2 and 5, table 6).

Default mode network

A significant positive correlation was observed in the *frontal* rectus, superior orbital, *parietal* inferior, postcentral, angular, and the *insula* anterior. The total volume for positive correlated structures was 10.208 cm^3. A negative correlation was observed in the *frontal* middle, paracentral, and the *occipital* inferior. The total volume for negative correlated structures was 3.96 cm^3 (fig. 3 and 5, table 6).

Central executive network

No significant correlations were observed (fig. 3, table 6).

Salience network

No significant correlations were observed (table 6).

Basal ganglia network

No significant correlations were observed (fig. 4, table 6).

Small versus large areas of secondary hyperalgesia on resting state network measures.

Differences in resting state connectivity when comparing participants with a small (lower quartile) vs. large (upper quartile) area of secondary hyperalgesia are reported below.

Sensorimotor network

In participants with a small area of secondary hyperalgesia a significantly increased connectivity was observed in the *occipital* middle, cuneus, *insula* anterior, and the *frontal* supplemental motor area. In participants with a large area of secondary hyperalgesia a significantly increased connectivity was observed the *frontal* middle orbital, superior, superior medial, inferior orbital, inferior triangular, middle orbital, superior orbital, rectus, *occipital* rolandic operculum, inferior, *temporal* middle, lingual, superior *cingulum* posterior, and subcortically in Putamen and Hippocampus (fig. 2 and 6, table 7).

Right Fronto-parietal network

No significant differences in connectivity were observed when comparing participants with a small or large area of secondary hyperalgesia.

Default mode network

In participants with a small area of secondary hyperalgesia a significantly increased connectivity was observed in the left *frontal* inferior triangular, *parietal* postcentral, and the *occipital* calcarine gyrus. In participants with a large area of secondary hyperalgesia a significantly increased connectivity was observed in the *parietal* precuneus and postcentral, and *cingulum* anterior and posterior (fig.3 and 6, table 7).

Central executive network

In participants with a small area of secondary hyperalgesia a significantly increased connectivity was observed in the *frontal* middle, inferior operculum, *parietal* superior, *occipital* calcarine, *temporal* inferior, *cingulum* posterior, *insula* anterior, and subcortically in the Putamen.
In participants with a large area of secondary hyperalgesia a significantly increased connectivity was observed in the *frontal* superior medial, superior, middle orbital, inferior orbital, supplemental motor area, *parietal* postcentral, inferior, *occipital* rolandic operculum, middle, *cingulum* middle, and subcortically in the Caudate (fig. 3 and 6, table 7).

Salience network

No significant differences in connectivity were observed when comparing participants with a small or large area of secondary hyperalgesia (table 7).

Basal ganglia network

In participants with a small area of secondary hyperalgesia a significantly increased connectivity was observed in the *frontal* precentral and *parietal* inferior. No significant increased connectivity was observed in participants with large areas of secondary hyperalgesia (fig. 4 and 6, table 7).

Post-hoc analysis

Post-hoc analysis was conducted with inclusion of the right handed participants only, investigating the connectivity of the right FPN. We found no significant correlations for increasing area of secondary hyperalgesia. However, when comparing participants with a small and large area of secondary hyperalgesia, we found increased connectivity in the *frontal* middle*, parietal* precuneus, *occipital* cuneus, and the *Cingulum* middle in participants with small areas of secondary hyperalgesia. In participants with large areas of secondary hyperalgesia we found increased connectivity in the *parietal* supramarginal (supplemental table 1).

Discussion

In the present study we investigated possible correlations between connectivity in known resting state networks and increasing size of secondary hyperalgesia area in healthy men, to determine if the propensity to develop central sensitization is associated with differences in resting state connectivity. In addition, we investigated possible differences in resting state connectivity between participants with small and large areas of secondary hyperalgesia. We report significant associations between increasing size of secondary hyperalgesia area and connectivity in the SMN, DMN, and the right FPN. Likewise, significant differences in connectivity were found between groups with small and large areas of secondary hyperalgesia in the SMN, DMN, CEN, and BGN. The data supports the notion that the basal brain state, as reflected by resting state connectivity, may be an indicator of individual responsiveness in regards to nociceptive drive and in limiting the degree of experimentally induced secondary hyperalgesia. As such the model presented may be a marker for resilience to central sensitization.

Networks and secondary hyperalgesia

Sensorimotor network (SMN)

We found significant positive correlations between increasing size of secondary hyperalgesia areas and connectivity as well as group differences in the SMN and multiple brain structures. The SMNs include the primary (SI) and secondary somatosensory cortices (SII) and the motor system including the supplemental motor area[36,50]. The SI records pain intensity and location, and is involved in discrimination of pain, while the SII plays an important role in integration of pain stimuli and in pain memory, learning and attention[51-53]. In addition the SMN is also involved in perception of peripheral sensory inputs, generation of motor responses in general, and in response to pain[54]. The positive correlation in SI (postcentral gyrus) and SII (rolandic operculum) may indicate a heightened sensitivity to nociceptive stimuli, as well as an augmented pain processing and pain memory system in participants with large areas of secondary hyperalgesia. Our findings are supported by a previous rsfMRI study that reported a positive correlation between temporal summation of pain (perception of increasing pain due to repetitive nociceptive stimulation) and increased connectivity between the Thalamus and SI in healthy individuals[55].
In addition, we report positive correlations in the superior temporal gyrus and the orbitofrontal cortex (OFC); involved in pain expectancy, as well as decision making and pain appraisal

respectively[56-58]. The OFC is a part of the endogenous analgesic system and has projections to the anterior cingulate cortex (ACC) and the dorsolateral prefrontal cortex (DLPFC). However, interestingly we neither found increasing connectivity in the ACC nor in the DLPFC, which indicates that participants with large areas of secondary hyperalgesia do not express increased endogenous analgesic capacity. Individuals with a large area of secondary hyperalgesia may thus express a heightened sensitivity to pain, influenced by a potential increased focus on pain through an enhanced pain expectancy and pain appraisal.

Fronto-parietal network (FPN)

We found a significant although discrete correlation between decreasing connectivity in four clusters and increasing size of secondary hyperalgesia area. The FPNs consist of the lateral prefrontal cortices and the posterior parietal cortices[59]. The FPNs are lateralized networks where the FPN in the dominant hemisphere is involved in self-awareness, perception, somesthesis and pain[36]; specifically in endogenous pain modulation i.e. expectancy-induced modulation of pain[60]. The FPN in the non-dominant hemisphere is involved in cognition, language and memory[36]. When comparing participants with small and large areas of secondary hyperalgesia, we found no differences in connectivity. Surprisingly, two clusters with a negative correlation between connectivity and increasing area of secondary hyperalgesia were observed in the OFC and in the superior temporal gyrus. This is remarkable since a positive correlation between connectivity in these structures and increasing area of secondary hyperalgesia was observed in the SMN.

Default mode network (DMN)

In the present study the positive correlations in the inferior parietal lobules as well the increased connectivity in the posterior cingulate cortex in participants with large areas of secondary hyperalgesia suggest subtle but significantly increased connectivity of the DMN. The DMN includes the posterior cingulate cortex, precuneus, ventral and dorsal medial prefrontal cortex, lateral and inferior parietal cortex, parts of the lateral temporal cortex, the entorhinal cortex and the hippocampus[36,61-66]. The DMN is deactivated during external task-related performance and activated when a person is not focusing on the external environment[61]. Increased connectivity of the DMN, and in particular, posterior cingulate cortex, has also been reported in chronic pain patients[67-69]. BOLD studies investigating the DMN have reported diverse findings ranging from increased activity in the precuneus and posterior cingulate cortex in participants with large areas

of secondary hyperalgesia[3], to an inverse correlation between size of secondary hyperalgesia area and activity in the medial prefrontal cortex[70]. The present findings may indicate similarities between participants with large areas of secondary hyperalgesia and chronic pain patients; however the precise mechanism and influence on pain processing as well as the spatial extent remain elusive.

Central executive network (CEN)

The CEN includes the DLPFC, the posterior parietal cortex, and the ACC and the paracingulate cortex[36,71]. The CEN is involved in attention control, working memory, and other high-level cognitive functions[71]. In the current study we only found differences in connectivity when comparing groups with small vs. large areas of secondary hyperalgesia, indicating a non-linear relationship between CEN connectivity and the area of secondary hyperalgesia. Particularly, we found increased connectivity in a cluster located in the DLPFC in participants with small areas of secondary hyperalgesia. The DLPFC is involved in several cognitive processes, including working memory, as well as descending pain inhibition and placebo analgesia[17,57,72-76].

Current evidence indicates that the DLPFC is involved in downward anti-nociceptive regulation, inhibiting ascending nociceptive signalling, by recruiting the rostral ACC and subsequently subcortical brain structures including the PAG[74,76]. In relation to the present study our findings suggest that participants with small areas of secondary hyperalgesia may exhibit a comparatively increased connectivity in the DLPFC suggesting an improved endogenous analgesic capacity. Finally, increased connectivity was observed in three separate clusters in the Mid cingulate cortices in participants with large areas of secondary hyperalgesia, suggesting that individuals with large areas of secondary hyperalgesia may possess an enhanced tendency towards attention to pain and pain avoidance[77].

Basal ganglia network (BGN)

No significant correlations between the size of the area of secondary hyperalgesia and connectivity in the BGN were observed. However, increased connectivity was observed in two clusters (precentral gyrus and parietal inferior lobule) in participants with small areas of secondary hyperalgesia, suggesting a non-linear relationship between connectivity and secondary hyperalgesia area. The BGN includes the putamen, globus pallidus, substantia nigra, the caudate -, the accumbens- and the subthalamic nuclei[78,79]. The BGN receives input from several cortical and

subcortical structures and is involved in integration of motor-, sensory, and motivational information. In addition, the basal ganglia are intricately involved in pain processing, exerting influence on the pain modulation, as well as affective and cognitive aspects of pain[78].
The exact role of the precentral gyrus in pain processing remains elusive; however, motor cortex stimulation in treatment of various pain syndromes has been applied for several years[80-82]. It has been suggested that motor cortex activity influences a network of pain relevant brain structures, and may therefore be involved indirectly in the endogenous analgesic pain modulation via top-down inhibitory regulation[80]. Presently, this suggests that participants with small areas of secondary hyperalgesia may have comparatively higher endogenous analgesic capacities.

Model for evaluating brain risk for pain chronification

Our findings indicate that healthy pain free individuals with a large area of secondary hyperalgesia may exhibit a comparatively increased sensitivity to pain, with an enhanced tendency towards pain attention and pain appraisal. In contrast, individuals with a small area of secondary hyperalgesia may possess a comparatively increased endogenous analgesic capacity.

Central sensitization of pain defines the CNS's capacity to augment or modify pain so it no longer clearly reflects the peripheral stimulus[1,2]. It leaves the CNS in a state of hyper-excitability, and uncouples the classic stimulus-response pathway usual for pain perception[1,2]. Secondary hyperalgesia is a clinical manifestation of central sensitization that may represent a phenotypic characteristic of the individual propensity to develop central sensitization[1,2,7,8]. Our findings indicate that variations in secondary hyperalgesia areas are correlated with resting state connectivity in healthy pain free men, indicating that some individuals may indeed have an enhanced propensity to develop central sensitization. This enhanced propensity is independent of previous injury and comorbidities, suggesting that healthy pain free individuals may possess an innate predisposition towards developing central sensitization, with a potentially increased risk of developing pain hypersensitivity and chronic pain.
Understanding why and if some individuals are more predisposed to develop central sensitization, and whether this entails an increased risk of developing pain hypersensitivity and chronic pain, may allow for novel analgesic treatment strategies targeted at normalizing or reducing the state of increased CNS hyper-excitability [1]. Finally, evaluation of secondary hyperalgesia areas may be a

method for identifying individuals with high propensities to develop central sensitization for inclusion in medical research trials, and improvement of acute analgesic therapies.

Limitations

Participant characteristics

We included a homogenous population of healthy young men, limiting the generalizability of our findings, as well as the inter-individual variability in secondary hyperalgesia areas and cerebral anatomy. However, inclusion of both sexes would have introduced a number of unknown variables that could have influenced our findings, such as potential neuroanatomical differences related to sex[83-86], as well menstrual and hormonal influences on MRI findings[84,87-91] and pain responses[89,92]. Moreover, the BTS method has only been validated in young healthy men[8].

Potential basis for differences in individual resting state connectivity

Several factors may influence individual resting state connectivity including age[93], genetics[94], previous injury, and prior childhood trauma[95]. Connectivity changes in the healthy aging brain have been reported in multiple studies with reduced connectivity in the DMN and Salience network, persisting even after controlling for brain atrophy and age related structural changes[93]. To accommodate this, we included age as a covariate in our analyses to adjust for a potential influence. Although possible childhood trauma was not an exclusion criterium *per see*, we only included healthy volunteers rendering the influence of major childhood traumas negligible.

It was not possible to take genetic factors in to account in the present study. In addition we had no exclusion criteria based on the participants' ethnicity. However, the study population was largely homogeneous with regard to ethnicity. Only ten of the 115 participants included in the final analysis had one or more parents with non-Scandinavian ethnicity. Although this does not rule out the influence of the genetic factors directly, it does make the study population more homogenous. Ethnicity may influence pain thresholds[96-99], and ethnicity and cultural differences may play a role in network functionality[100]; however, this issue is largely unexplored, and to the authors' knowledge, there exists no evidence of differentiated distributions of pain relevant networks according to different ethnic groups.

Hand dominance

We had no exclusion criteria based on hand-dominance, and the inclusion of left-handed participants is not always preferred, but primarily relevant when investigating fMRI during tasks or the lateralized networks during rest. To test the robustness of our results we therefore conducted a post-hoc analysis investigating the connectivity of the only lateralised network included in this study, the right FPN, in right-handed participants only. The post-hoc analysis demonstrated only discrete discrepancies when comparing to our primary analysis (supplemental table 1).

Technical Caveats

Due to a field of view of 192 mm we were not able to include the cerebellum or the brain stem in our resting state analyses.

Conclusions

In this study of 115 healthy participants we found significant correlations between increasing size of secondary hyperalgesia area and connectivity in multiple networks, as well as significant differences in several networks when comparing participants with small and large areas of secondary hyperalgesia. Our findings indicate that individuals with large areas of secondary hyperalgesia display significantly different resting state connectivity when compared to participants with small areas. Presently, this suggests that differences in the propensity for developing central sensitization are expressed as differences in the resting state central neuronal activity, and that, healthy individuals may in fact process pain differently depending on their individual expression of central sensitization.

The most prominent findings were observed in the Sensorimotor network; however, due to the exploratory nature of the study the results must be interpreted with caution. Future studies are needed to elucidate the relevance of supraspinal connectivity in central sensitization.

Competing interests

The authors declare that they have no competing interests

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Author contributions

All authors contributed in the conception and design of the study, and critically revised the manuscript. All authors read and approved the final manuscript.

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Tables and figures

Inclusion criteria	Exclusion criteria
Age ≥18 years and ≤35 years Speak and understand the Danish language Male sex Signed informed consent Participation and completion of the study: "Heat pain detection threshold is associated with the area of secondary hyperalgesia following brief thermal sensitization: a study of healthy male volunteers"[15]	Inability to cooperate to the test Weekly intake of >21 units of alcohol, or intake of >3 units of alcohol within 24 hours before study day Substance abuse, assessed by the investigator Consummation of analgesics within 3 days before study day Consummation of antihistamines within 2 days before study day Consummation of antidepressant medication within 30 days before the study day Consummation of prescription medicine within 30 days before the study day Consummation caffeine within 24 hours before study day. Neurological illnesses Chronic pain Psychiatric diagnoses Eczema, wounds or sunburns on the sites of stimulation Body Mass Index >30 kg/m^2 or <18 kg/m^2. Unwilling to receive information regarding potential pathological findings in relation to the MRI. Any kind of trauma resulting in pain and administration of analgesics in the period between pain testing and MRI scan. Head trauma in the period between the pain testing and the MRI. Contraindications to MRI (claustrophobia, pacemaker implant, artificial heart valve, cochlear/stapes prosthetics, irremovable insulin pump, neuro-stimulator, metal from previous surgery, metallic foreign objects, catheters, shunts, draining tubes, and surgical procedures within the last 6 weeks (subjected to individual evaluation)).

Table 1. Inclusion and exclusion criteria
Abbreviations: MRI, Magnetic resonance imaging

Characteristics	
Age (years)	22 (3.35)
Height (m)	1.84 (0.07)
Weight (kg)	77.8 (11.19)
BMI (m^2/kg)	22.9 (2.56)
MAP (mm Hg)	96 (6.47)
Heart rate (beats/min)	69 (9.02)
Non-Scandinavian ethnicity (n)	10
Left-handed participants (n)	15

Table 2. Basic characteristics of 115 included participants
Abbreviations: SD, Standard deviation; BMI, body mass index; MAP, mean arterial pressure.

Variable	Before T1-Weighted MRI	After T1-Weigthed MRI	Before resting state scan	After resting state scan
Heart rate (beats/min)	66 (59-78)	63 (56-72)	63 (56-69)	62 (54-70)
Respiratory frequency*	14 (12-17)	15 (12-17)	14 (12-17)	14 (12-17)
End-tidal CO_2 (mm Hg)	3.5 (3.1-3.9)	3.5 (3.2-3.8)	3.5 (3.1-3.9)	3.4 (3.1-3.8)

Table 3. Measurements during MRI-scan session
Data are presented as median, (Interquartile range).
*Respiratoy frequency are evaluated as breaths/min

Characteristic	Lower Quartile (n=29)	Upper Quartile (n=29)
Age (years)	23 (3.50)	21 (2.53)
Height (m)	1.84 (0.06)	1.83 (0.07)
Weight (kg)	80.3 (10.32)	74.5 (13.57)
BMI (m^2/kg)	23.7 (2.45)	22 (2.69)
Surface area (m^2)	2.02 (0.15)	1.94 (0.21)
MAP (mm Hg)	96 (7.20)	94 (6.67)
Heart rate (beats/min)	70 (10.03)	69 (10.49)
Area of secondary hyperalgesia (cm^2) (median, IQR), unadjusted	273 (201.2-320.8)	579 (515.8-640.4)

Table 4. Comparisons of quartiles based on area of secondary hyperalgesia adjusted for body surface
Numbers reported are means with standard deviations in parentheses or as medians with interquartile ranges.
Abbreviations: BMI, body mass index; MAP, mean arterial pressure; IQR, Interquartile range

Table 5. Effect of increasing area of secondary hyperalgesia on resting state connectivity in specific brain networks.
The table depicts brain regions with significantly increased or decreased connectivity according to increasing area of secondary hyperalgesia in specific resting state networks. Coordinates and maximum statistical values (Z-stat) are given for peak activity, and volume of cluster of activity is given in mm^3.
Abbreviations: Lat, lateralisation; Vol, volume; R, right; L, left

	Brain Region	Lat.	z-stat	X(mm)	Y(mm)	Z(mm)	Vol(cm)
Sensorimotor Network							
Positive correlation (Large area of secondary hyperalgesia --> high connectivity)	***Cortical***						
	Frontal						
	Superior	R	2,816	6	38	36	0,968
	Superior Orbital	R	3,665	18	62	12	1,088
	Inferior Orbital	L	3,4626	-50	38	-8	1,4
	Middle Orbital	R	4,5778	22	26	36	2,208
	Superior Orbital	R	3,6183	22	18	56	1,352
	Superior Medial	L	2,9637	-2	58	0	1,44
	Rectus	R	4,5408	10	38	-24	1,232
	Inferior Triangular	R	3,3318	50	30	28	1,224
	Rectus	R	3,5675	14	18	-16	1,048
	Supplemental Motor Area	L	2,675	2	6	48	1,152
	Precentral	R	2,541	34	-10	64	1,176
	Occipital						
	Inferior	L	3,1415	-30	-78	-8	0,912
	Inferior	L	2,8885	-34	-70	-8	0,968
	Inferior	L	2,688	-46	-70	-4	1,208
	Calcarine	L	2,6433	-2	-86	-4	0,936
	Calcarine	R	2,6249	26	-54	8	1,504
	Cuneus	L	2,7674	-6	-98	16	1,232
	Temporal						
	Superior	L	4,0087	-54	-6	-12	0,92
	Superior	R	3,5732	66	-18	0	1,992
	Superior	L	2,3467	-38	-34	12	1,696
	Superior	L	2,6171	-54	-38	12	1,312
	Middle	R	2,7023	46	-74	20	1,256
	Middle	R	3,5479	66	-30	-4	1,496
	Fusiform	R	3,1319	38	-18	-24	1,728
	Fusiform	L	3,8004	-30	-54	-8	1,6
	Lingual	R	4,1158	18	-82	0	0,976
	Cingulum						
	Post	L	3,6757	-2	-42	20	1,248
	Parietal						
	Postcentral	R	3,3077	34	-30	68	1,848
	Angular	L	2,4498	-54	-58	24	1,256
	Angular	L	3,4128	-42	-70	40	0,984
	Insula						
	Insula Posterior	R	3,7823	38	-14	-4	1
Negative correlation (Large area of secondary hyperalgesia --> low connectivity)	**Cortical**						
	Frontal						
	Middle	L	3,9614	-30	58	12	1,008
	Middle Orbital	R	3,8621	30	58	24	1,064
	Rectus	L	3,8267	-2	38	-20	0,928
	Paracentral Lobule	L	4,2752	-6	-30	60	1,416
	Occipital						
	Cuneus	L	4,167	-10	-78	24	1,576
	Middle	L	3,501	-38	-86	24	1,392
Right Fronto-parietal network							
Positive correlation (Large area of secondary hyperalgesia --> high connectivity)	No statistically significant correlations						
Negative correlation (Large area of secondary hyperalgesia --> low connectivity)	**Cortical**						
	Frontal						
	Middle Orbital	R	3,2585	34	46	8	0,992
	Temporal						
	Middle	L	3,3153	-58	-62	0	1,464
	Superior	L	3,4894	-58	-22	12	1,6
	Occipital						
	Superior	R	2,9845	22	-86	36	1,08

Table 5 continued

	Brain Region	Lat.	z-stat	X(mm)	Y(mm)	Z(mm)	Vol(cm)
Deafult Mode Network							
Positive correlation (Large area of secondary hyperalgesia --> high connectivity)	**Cortical**						
	Frontal						
	Rectus	L	3,6253	-6	26	-24	1,056
	Superior_Orbital	R	3,2528	18	22	56	0,944
	Parietal						
	Inferior	R	3,5095	34	-50	52	1,312
	Inferior	L	3,6034	-30	-58	44	1,248
	Postcentral	L	3,7749	-46	-22	56	2,144
	Angular	L	3,4934	-50	-62	28	1,072
	Insula						
	Anterior	R	4,2791	38	6	8	2,432
Negative correlation (Large area of secondary hyperalgesia --> low connectivity)	***Cortical***						
	Frontal						
	Middle	L	3,215	-26	34	40	1,336
	Paracentral Lobule	L	4,2877	-2	-26	56	1,448
	Occipital						
	Inferior	L	3,4524	-42	-78	-8	1,176
Central Executive Network							
Positive correlation (Large area of secondary hyperalgesia --> high connectivity)	No statistically significant correlations						
Negative correlation (Large area of secondary hyperalgesia --> low connectivity)	No statistically significant correlations						
Salience Network							
Positive correlation (Large area of secondary hyperalgesia --> high connectivity)	No statistically significant correlations						
Negative correlation (Large area of secondary hyperalgesia --> low connectivity)	No statistically significant correlations						
Basal ganglia Network							
Positive correlation (Large area of secondary hyperalgesia --> high connectivity)	No statistically significant correlations						
Negative correlation (Large area of secondary hyperalgesia --> low connectivity)	No statistically significant correlations						

Table 6. Effect of small versus large areas of secondary hyperalgesia on resting state connectivity in specific brain networks.
The table depicts brain regions with significantly increased or decreased connectivity when comparing participants with small versus large areas of secondary hyperalgesia. Coordinates and maximum statistical values (Z-stat) are given for peak activity, and volume of cluster of activity is given in mm^3.
Abbreviations: Lat, lateralisation; Vol, volume; R, right; L, left

	Brain Region	Lat.	z-stat	X(mm)	Y(mm)	Z(mm)	Vol(cm)
Sensorimotor Network	**Cortical**						
Small area > Large area	*Frontal*						
	Supplemental Motor Area	R	3,2497	10	-14	64	0,944
	Occipital						
	Middle	L	2,643	-38	-78	36	0,912
	Cuneus	L	3,3931	-10	-78	24	1,048
	Insula						
	Anterior	L	2,9518	-38	14	-12	1,04
Large area > Small area	**Cortical**						
	Frontal						
	Superior	L	3,0254	-22	38	32	1,032
	Superior Medial	R	3,5281	14	38	-24	0,904
	Superior Medial	L	2,5646	-2	58	32	1,408
	Superior Orbital	R	3,386	22	22	56	1,72
	Middle Orbital	R	3,0588	38	50	4	1,336
	Middle Orbital	R	4,7691	22	26	36	3,68
	Inferior Orbital	L	3,0971	-46	38	-8	1,44
	Inferior Triangular	L	2,9985	-46	34	12	1,112
	Rectus	R	2,4161	18	14	-16	2,184
	Occipital						
	Rolandic Operculum	L	2,8012	-46	-22	12	1,536
	Inferior	L	3,1986	-38	-70	-8	1,296
	Temporal						
	Superior	R	2,5913	62	-18	-4	1,168
	Middle	R	3,5143	66	-30	-4	1,704
	Lingual	L	3,5369	-26	-54	-8	1,232
	Middle	L	2,7001	-54	-70	12	1,728
	Lingual	R	4,0684	18	-82	0	1,504
	Cingulum						
	Post	L	3,0233	-2	-42	20	1,328
	Sub-Cortical						
	Putamen	R	3,1277	26	6	-8	0,92
	Hippocampus	R	3,2224	30	-10	-20	1,264
Right Fronto-parietal network							
Small area > Large area	No statistically significant differences						
Large area > Small area	No statistically significant differences						
Deafult Mode Network	**Cortical**						
Small area > Large area	*Frontal*						
	Inferior Triangular	L	3,7878	-50	18	20	0,984
	Parietal						
	Postcentral	L	4,5932	-22	-38	64	2
	Occipital						
	Calcarine	L	4,1641	-18	-70	12	1,64
Large area > Small area	**Cortical**						
	Parietal						
	Precuneus	L	4,442	-14	-62	56	0,984
	Postcentral	L	3,687	-46	-30	48	1,08
	Cingulum						
	Anterior	L	3,4459	-10	38	-4	1,832
	Post	L	3,6498	-2	-46	24	1,056

Table 6 continued

	Brain Region	Lat.	z-stat	X(mm)	Y(mm)	Z(mm)	Vol(cm)
Central Executive Network							
Small area > Large area	**Cortical**						
	Frontal						
	Middle	L	2,8386	-38	54	8	1,152
	Inferior Operculum	L	3,1268	-50	10	4	1,304
	Parietal						
	Superior	R	3,4231	18	-82	48	1,144
	Occipital						
	Calcarine	L	2,8133	-10	-90	-8	1,304
	Temporal						
	Inferior	R	2,7688	58	-38	-20	1,016
	Cingulum						
	Post	R	3,7448	6	-38	8	1,576
	Insula						
	Anterior	L	3,4018	-46	6	-4	0,984
	Anterior	R	3,4154	30	22	-20	0,936
	Sub-Cortical						
	Putamen	R	3,0313	26	-10	8	1,184
Large area > Small area	**Cortical**						
	Frontal						
	Superior Medial	L	3,116	-14	62	4	1,68
	Superior	R	2,9096	6	50	28	2,32
	Middle Orbital	R	3,0187	34	46	36	3,28
	Inferior Orbital	R	3,4338	42	26	-16	1,168
	Supplemental Motor Area	L	2,7311	-2	2	60	1,128
	Parietal						
	Postcentral	L	3,3245	-50	-10	24	1,568
	Inferior	R	2,7716	38	-46	44	1,544
	Occipital						
	Rolandic Operculum	R	3,5488	58	6	16	1,496
	Middle	L	2,3917	-34	-86	4	0,928
	Cingulum						
	Middle	R	2,5862	6	18	36	2,104
	Middle	L	3,0919	-2	-2	44	1,296
	Middle	R	3,2893	18	-34	48	1,928
	Sub-Cortical						
	Caudate	R	3,2421	14	6	16	1,976
Salience Network							
Small area > Large area	No statistically significant differences						
Large area > Small area	No statistically significant differences						
Basal ganglia Network							
Small area > Large area	**Cortical**						
	Frontal						
	Precentral	R	3,788	46	-18	56	2,312
	Parietal						
	Inferior	L	3,3275	-26	-70	44	1,256
Large area > Small area	No statistically significant differences						

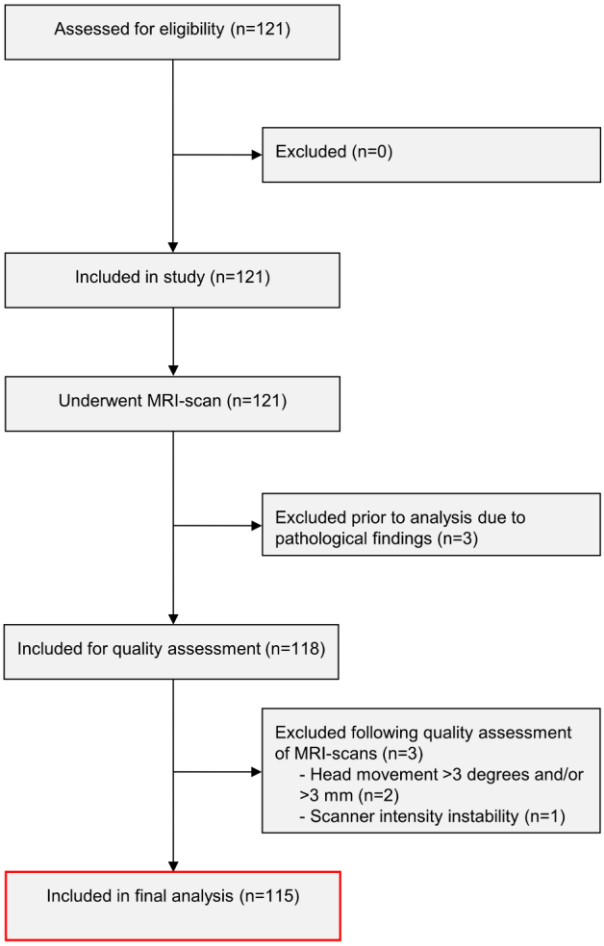

Figure 1. Flowchart of included participants
121 participants were assessed for eligibility and included in the study. 3 participants were excluded due to pathological findings following magnetic resonance imaging (MRI), and 3 participants were excluded due to head movement (n=2) and scanner intensity instability (n=1). Consequently, 115 participants were included in the final analysis.

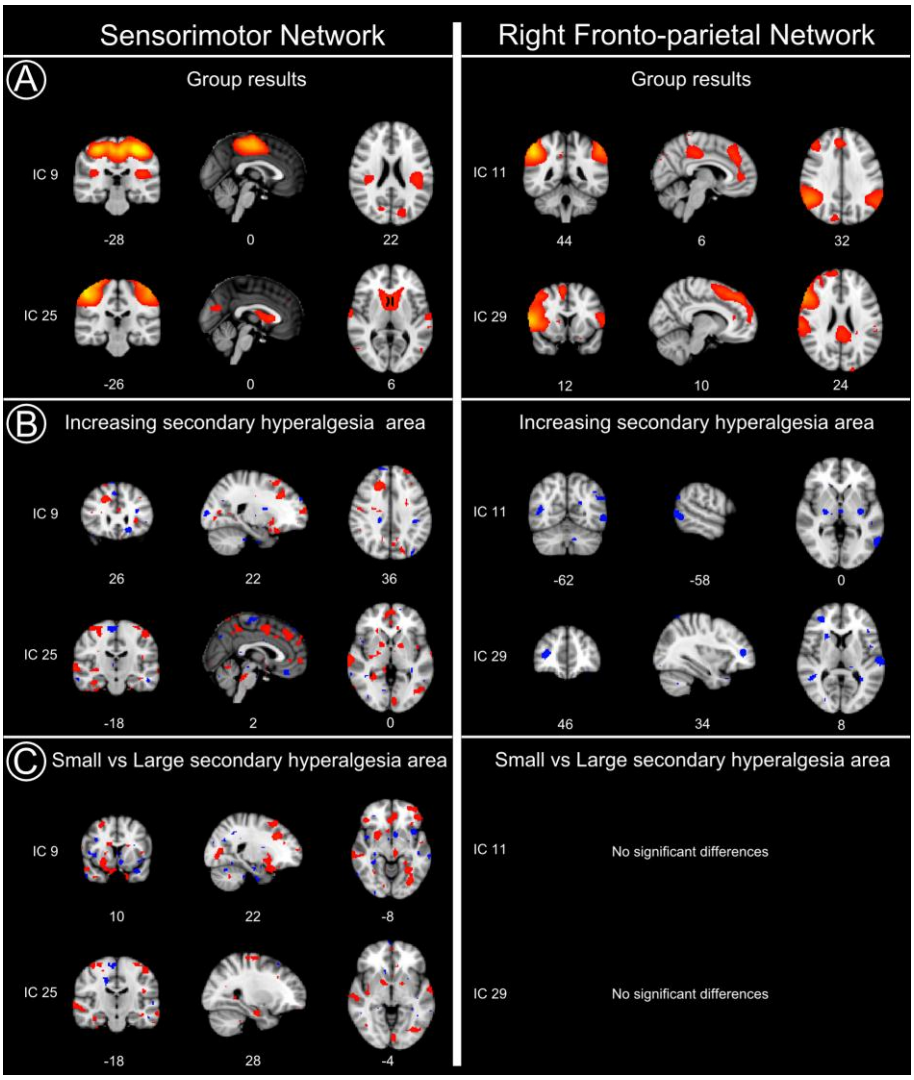

Figure 2. Resting state connectivity in the Sensorimotor and the right Fronto-parietal network

A) Group results from all participants (n=115) showing the Sensorimotor network (comprised of IC 9 and IC 25), and the right Fronto-parietal network (comprised of IC 11 and IC 29).

B) Resting state connectivity illustrating the effect of increasing secondary hyperalgesia area. Blue colour indicates brain structures where connectivity decreased with increasing area of secondary hyperalgesia, and red colours indicate brain structures where connectivity increased. In the Sensorimotor network, we found multiple brain structures displaying increased connectivity including the orbitofrontal gyri, postcentral gyrus, and temporal superior gyrus, and only few structures displaying decreased connectivity including the frontal middle gyrus. In the right Fronto-parietal network, we found no brain structures displaying increased connectivity, and only few brain structures displaying decreased connectivity including the temporal middle and superior gyrus.

C) Resting state connectivity comparing participants with small (lower quartile) and large (upper quartile) areas of secondary hyperalgesia. Blue colours indicate brain structures with increased connectivity in participants with small areas of secondary hyperalgesia, and red colours indicate brain structures with increased connectivity in participants with large areas. In the Sensorimotor network, we found few brain structures displaying increased connectivity in participants with small areas of secondary hyperalgesia including the supplemental motor area and the insula, and multiple brain structures displaying increased connectivity including the rolandic operculum and orbitofrontal gyri in participants with large areas. No statistically significant differences were observed in the right Fronto-parietal network.

All statistically significant findings can be observed visually and cross referenced with the results displayed in table 5 and 6. Numbers refers to standard MNI Atlas coordinates.

Abbreviations: IC, Independent component

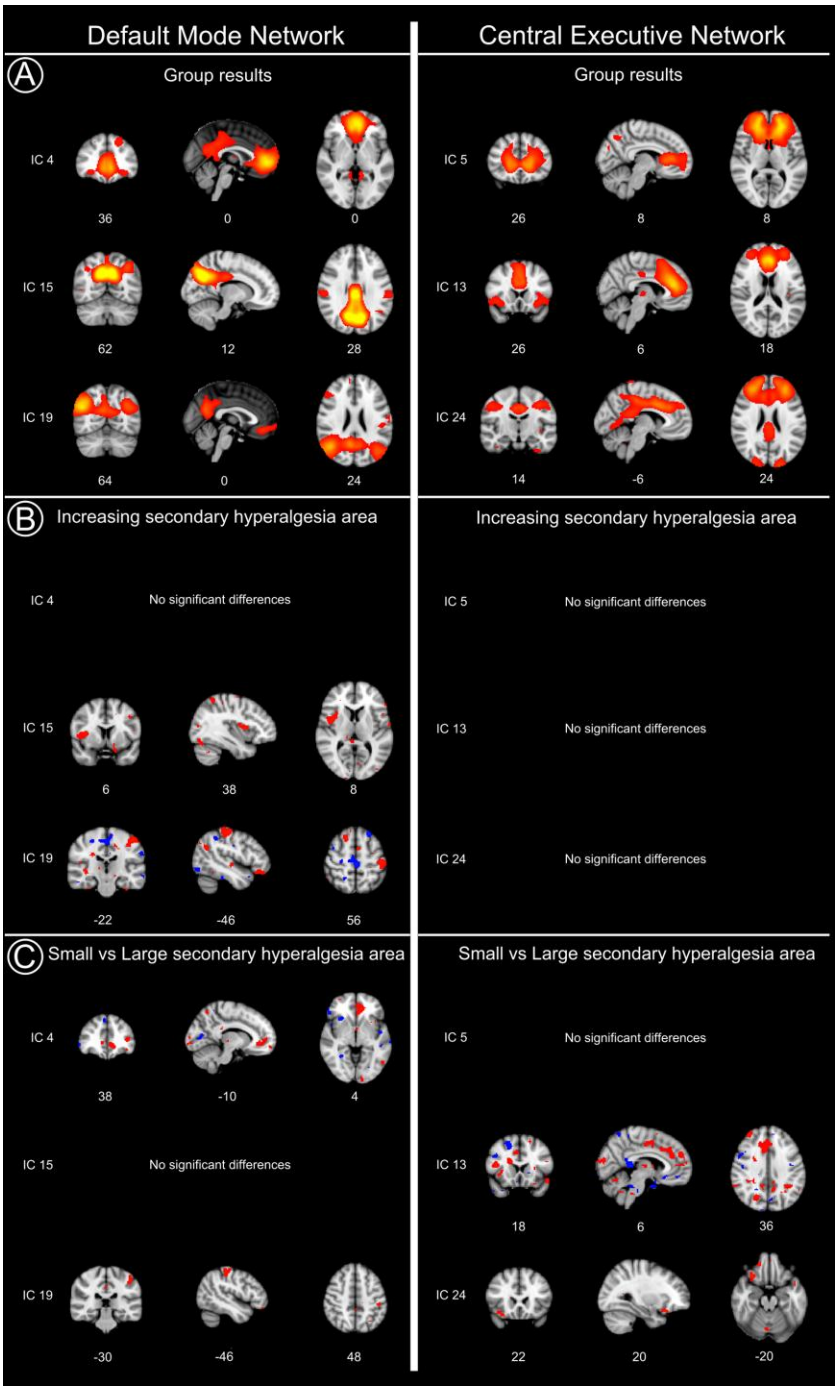

Figure 3. Resting state connectivity in the Default mode and the Central executive network

A) Group results from all participants (n=115) showing the Default mode network (comprised of IC 4, IC 15 and IC 19), and the Central executive network (comprised of IC 5, IC 13, and IC 24).

B) Resting state connectivity illustrating the effect of increasing secondary hyperalgesia area. Blue colour indicates brain structures where connectivity decreased with increasing area of secondary hyperalgesia, and red colours indicate brain structures where connectivity increased. In the Default mode network, we found few brain structures displaying increased connectivity including the postcentral gyrus and the anterior insula, and few structures displaying decreased connectivity including the frontal middle gyrus and the paracentral lobule. In the Central executive network, no statistically significant correlations in connectivity were observed.

C) Resting state connectivity comparing participants with small (lower quartile) and large (upper quartile) areas of secondary hyperalgesia. Blue colours indicate brain structures with increased connectivity in participants with small areas of secondary hyperalgesia, and red colours indicate brain structures with increased connectivity in participants with large areas. In the Default mode network, we found few brain structures displaying increased connectivity in participants with small areas of secondary hyperalgesia including the inferior triangular and the postcentral gyrus, and few structures displaying increased connectivity in participants with large areas including the postcentral gyrus and the anterior and posterior cingulum. In the Central executive network, we found multiple brain structures displaying increased connectivity in participants with small areas of secondary hyperalgesia including the frontal middle gyrus and the inferior operculum, and multiple structures displaying decreased connectivity in participants with large areas including the middle and inferior orbital gyri, and the middle cingulum.

All statistically significant findings can be observed visually and cross referenced with the results displayed in table 5 and 6. Numbers refers to standard MNI Atlas coordinates.
Abbreviations: IC, Independent component

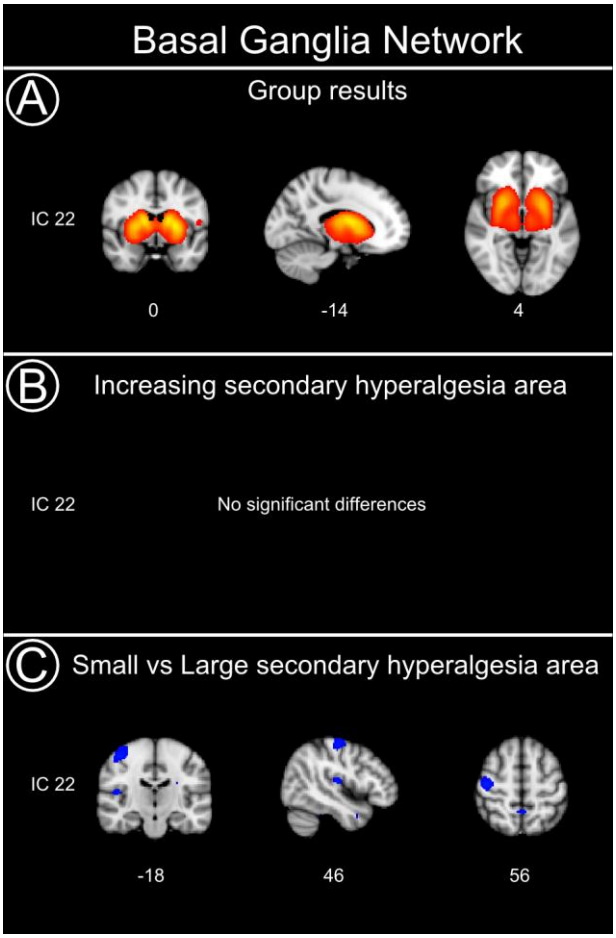

Figure 4. Resting state connectivity in the Basal ganglia network
A) Group results from all participants (n=115) showing the Basal ganglia network (comprised of IC 22).

B) Resting state connectivity illustrating the effect of increasing area of secondary hyperalgesia. No statistically significant correlations were observed in the network.

C) Resting state connectivity comparing participants with small (lower quartile) and large (upper quartile) areas of secondary hyperalgesia. Blue colours indicate brain structures with increased connectivity in participants with small areas of secondary hyperalgesia. In the Basal ganglia network, we found few structures displaying increased connectivity in participants with small areas of secondary hyperalgesia including the precentral gyrus and the parietal inferior gyrus. No significant increased connectivity was observed in participants with large areas of secondary hyperalgesia.

All statistically significant findings can be observed visually and cross referenced with the results displayed in table 5 and 6. Numbers refers to standard MNI Atlas coordinates.
Abbreviations: IC, Independent component

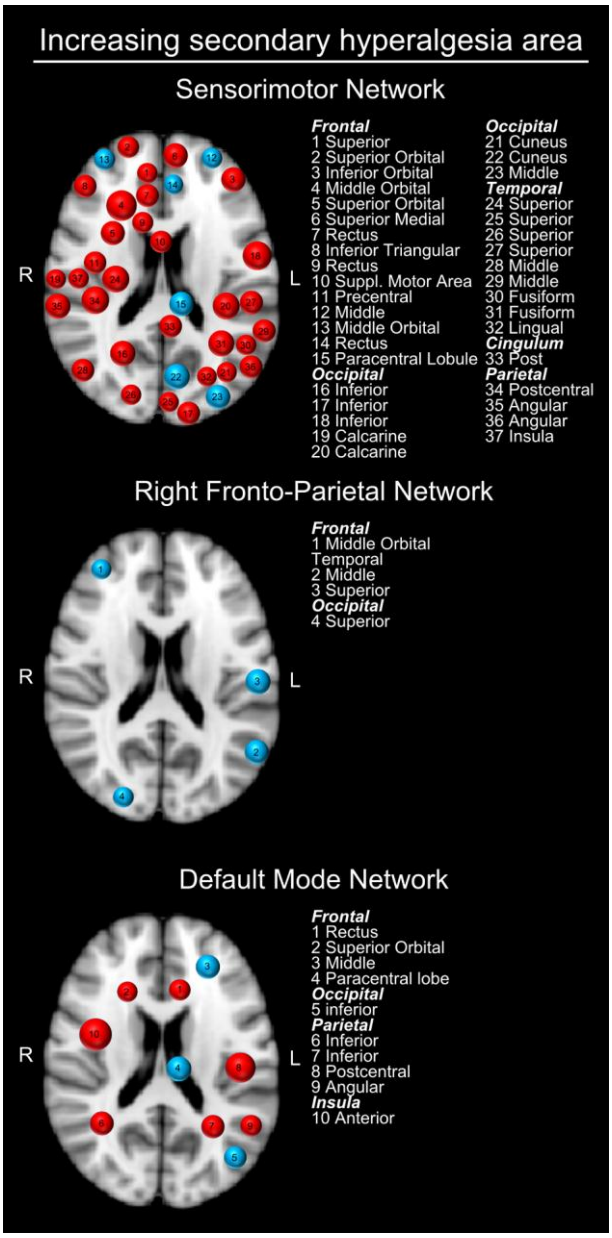

Figure 5. Summary of the significant resting state connectivity in the Sensorimotor network, right Fronto-parietal network and the Default mode network.
Overview illustrating the statistically significant effect of increasing secondary hyperalgesia area on resting state connectivity. Blue points indicate brain structures where connectivity decreased with increasing area of secondary hyperalgesia, and red points indicate brain structures where connectivity increased. The area of the points corresponds to volume estimates of the individual brain structures. Placement of points is based on exact X and Y MNI coordinates cross referenced to, and displayed as in table 5 and 6, with a fixed Z-coordinate of 18, on the MNI-152.

In the Sensorimotor network, we found multiple brain structures displaying increased connectivity including orbitofrontal gyri, postcentral gyrus, and temporal superior gyrus, and few structures displaying decreased connectivity including the frontal middle gyrus.

In the right Fronto-parietal network we found no brain structures displaying increased connectivity, and 4 structures displaying decreased connectivity including the temporal middle and superior gyrus.

In the Default mode network we found few brain structures displaying increased connectivity, including the postcentral gyrus and the anterior insula and few structures displaying decreased connectivity including the frontal middle gyrus and the paracentral lobule.
Abbreviations: R, right; L, left

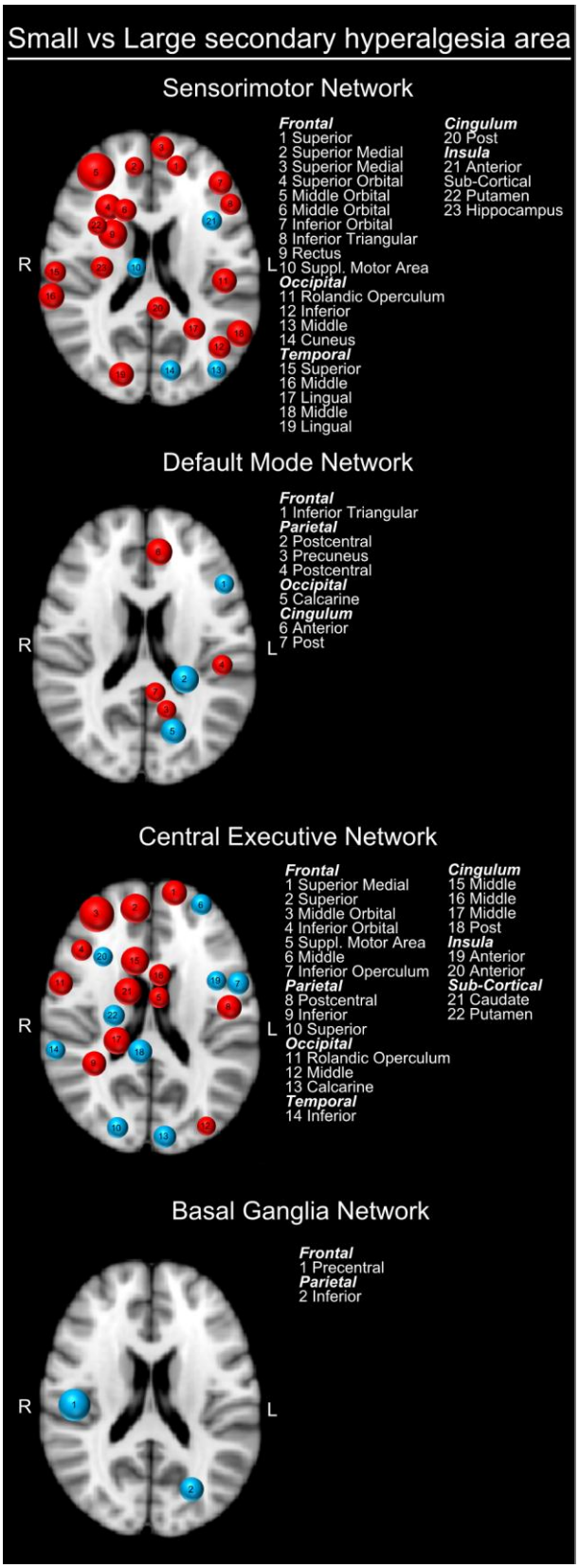

Figure 6. Summary of the significant resting state connectivity in the Sensorimotor network, Default mode network, Central executive network, and the Basal ganglia network.
Summary illustrating the statistically significant connectivity when comparing participants with small (lower quartile) and large (upper quartile) areas of secondary hyperalgesia. Blue points indicate brain structures with increased connectivity in participants with small areas of secondary hyperalgesia, and red points indicate brain structures with increased connectivity in participants with large areas. The area of the points corresponds to volume estimates of the individual brain structures. Placement of points is based on exact X and Y MNI coordinates cross referenced to, and displayed as in table 5 and 6, with a fixed Z-coordinate of 18, on the MNI-152.

In the Sensorimotor network, we found few brain structures displaying increased connectivity in participants with small areas of secondary hyperalgesia including supplemental motor area and the insula, and multiple structures displaying increased connectivity in participants with large areas including the rolandic operculum and orbitofrontal gyri.

In the Default mode network we found few structures displaying increased connectivity in participants with small areas including the inferior triangular and the postcentral gyrus, and few structures displaying increased connectivity in participants with large areas including the postcentral gyrus and the anterior and posterior cingulum.

In the Central executive network we found several structures displaying increased connectivity in participants with small areas including the frontal middle gyrus and the inferior operculum, and several structures displaying increased connectivity in participants with large areas including the middle and inferior orbital gyri, and the middle cingulum.

In the Basal ganglia network we found only two structures displaying increased connectivity in participants with small areas of secondary hyperalgesia, the precentral gyrus and parietal inferior gyrus, and no structures displaying increased connectivity in participants with large areas.
Abbreviations: R, right; L, left.

Supplemental table 1. Post-hoc analysis

The table depicts brain regions with significantly increased or decreased connectivity according to increasing area of secondary hyperalgesia in specific resting state networks in right-handed participants only. Coordinates and maximum statistical values (Z-stat) are given for peak activity, and volume of cluster of activity is given in mm^3.

Abbreviations: Lat, lateralisation; Vol, volume; R, right; L, left

	Brain Region	Lat.	z-stat	X(mm)	Y(mm)	Z(mm)	Vol(cm)
Right Fronto-parietal network							
Positive correlation (Large area of secondary hyperalgesia --> high connectivity)	No statistically significant correlations						
Negative correlation (Large area of secondary hyperalgesia --> low connectivity)	No statistically significant correlations						
Small area > Large area	**Cortical**						
	Frontal						
	Middle	L	3,527	-34	18	36	1,008
	Parietal						
	Precuneus	R	3,3616	2	-42	56	0,944
	Occipital						
	Cuneus	R	3,4214	6	-78	40	1,04
	Cingulum						
	Middle	R	3,7969	2	-26	52	1,488
Large area > Small area	**Cortical**						
	Parietal						
	SupraMarginal	R	3,8403	54	-38	32	2,344