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Inflammation and pain in skin and deep tissues

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INFLAMMATION AND PAIN
IN SKIN AND DEEP TISSUES

BY
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INFLAMMATION AND PAIN IN SKIN AND DEEP TISSUES

PhD Thesis by

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Preface

The present thesis is submitted to Aalborg University, in order to fulfill the requirements for the degree of Doctor of Philosophy in Clinical Science and Biomedicine.

The thesis is based on Silvia Lo Vecchio's work (2010-2013) as a PhD student employed at the Department of Health Science and Technology, Aalborg University, Denmark, which was performed under the supervision of Professor Lars Jelstrup Petersen and Professor Thomas Graven-Nielsen.

Acknowledgments

I wish to express my profound gratitude to Professor Lars Jelstrup Petersen and Professor Thomas Graven-Nielsen for all their supervision work and their great support to this PhD project. I would also like to thank the other co-authors for formulating the scientific objectives of this dissertation: Sara Finocchietti, Parisa Gazerani and Lars Arendt-Nielsen, for their help, advice, and great contribution to all my studies. I would also like thank the secretaries Susanne Nielsen Lundis and Lone Schødt Andersen and the technical staff, Knud Larsen in particular, for the help they offered me and for their patience.

I would like to express my warm thanks to all my colleagues and friends, for their moral support and for being as a family to me during these years.

At last, I would like to thank my family for their unconditional support, Simone for standing everyday by my side and giving me encouragement and endless love.

Table of Contents

1   Aim of the PhD Project

The aim of this PhD thesis was to investigate the time course and the sensory changes induced by UVB inflammation and clarify the existence of possible convergence between skin and deep tissues. In particular this thesis is focused on the following objectives:

1st objective: to evaluate the change in sensitivity after inflammation inside and outside the irradiated skin and to investigate whether UVB-induced cutaneous inflammation would enhance the pain responses from the underlying deep somatic areas;

2nd objective: to evaluate any possible convergence or cumulative effects between cutaneous and deep-tissue hyperalgesia by combining UVB cutaneous model of hyperalgesia with intramuscular sensitization evoked by delayed-onset muscle soreness (DOMS);

3rd objective: to further investigate the pattern of mechanical hyperalgesia and allodynia induced by UVB irradiation by application of repeated heat stimuli and whether sensitization of the deep tissues underlying UVB-irradiated skin might alter the response of the UVB-model to heat rekindling.

2 PhD studies

Based on the objectives of the thesis, three studies were designed and performed. The study 1 dealt with the first objective, whereas study 2 and study 3 of the thesis dealt with the second and third objectives, respectively.

Study 1: Hyperalgesia and allodynia to superficial and deep-tissue mechanical stimulation within and outside a UVB-irradiated skin area.

In the Study 1, the ultraviolet-B (UVB) inflammatory model was applied on the skin of healthy subjects in order to investigate 1) changes in cutaneous vaso-responses induced by UVB-induced inflammation, 2) changes in mechanical pain sensitivity within and outside the UVB-induced inflammatory area, and 3) the effect of topical anesthesia on the UVB-induced primary and secondary hyperalgesia.

Study 2: Combined inflammatory pain models in skin and deep tissues.

In the Study 2, in order to investigate the existence of convergent facilitation between cutaneous and deep tissues hyperalgesia, the UVB cutaneous inflammatory model was combined with delayed-onset muscle soreness (DOMS) produced by eccentric activity in the muscle tissue below the UVB-irradiated skin.

Study 3: Heat-rekindling in UVB-irradiated skin and NGF-sensitized muscle: experimental models of prolonged mechanical hypersensitivity.

The Study 3 was designed to test the efficacy of repeated heat stimuli on mechanical sensitivity on the UVB model alone and in combination with nerve growth factor (NGF) sensitized muscle. In this third study, to allow the development of a full inflammatory response after UVB irradiation, the muscle soreness was induced 24 h after irradiation. To avoid mechanical stretching of the injured skin caused by the eccentric contractions, a different method was selected for the induction of deep tissues hyperalgesia. Since one of the most supported theories about DOMS indicates the NGF as one of the fundamental substance implicated in the development of muscle soreness induced by

DOMS (Murase et al., 2010), the cutaneous UVB-model was applied in combination with NGF-induced muscle soreness. This study was designed in order to investigate: 1) whether heat application would either maintain or enlarge the areas of allodynia and hyperalgesia induced by the UVB model, 2) whether heat rekindling could have a different effect on sensitivity when tested on the area of allodynia or hyperalgesia, 3) whether the combination between UVB-model and NGF-induced muscle sensitization may change the responsiveness of UVB-irradiated skin to heat rekindling.

3   The PhD study structure
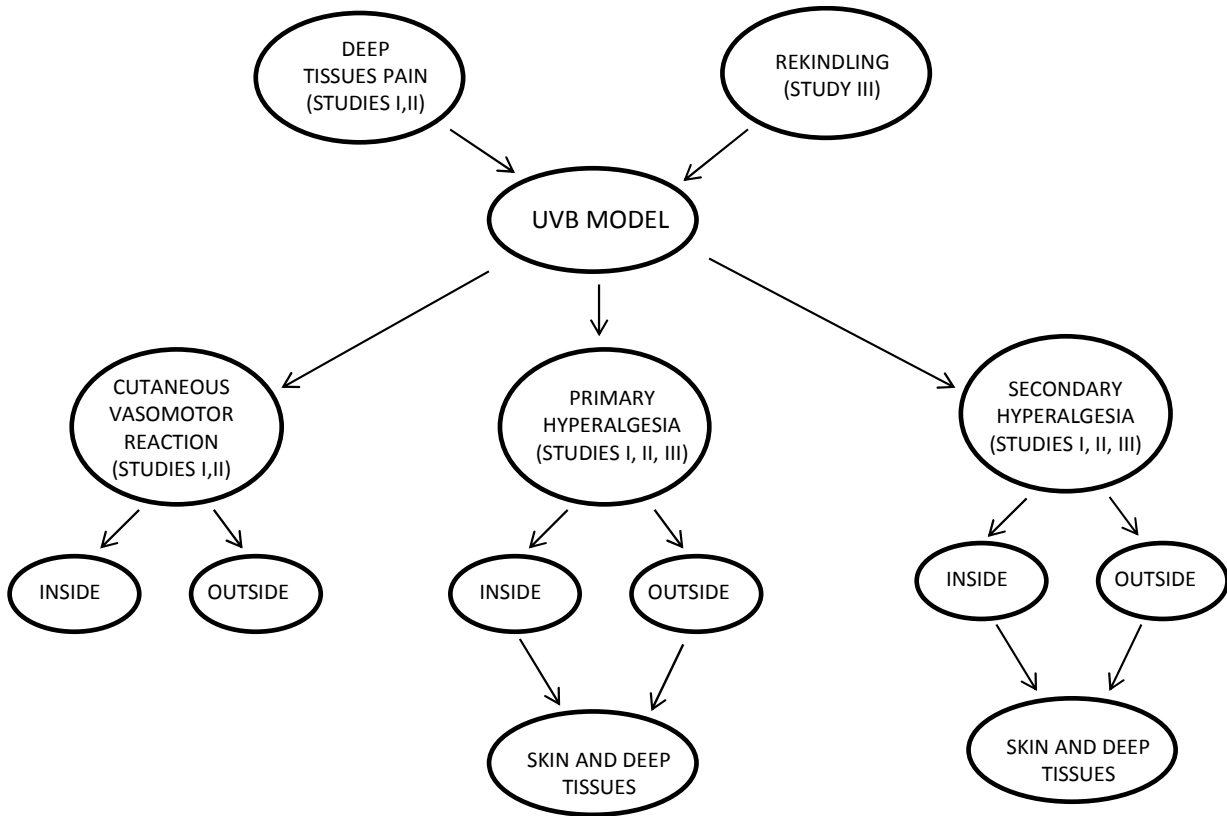
4   Introduction

4.1   General Aspects of Pain

According to the International Association for the Study of Pain (IASP), pain is defined as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (IASP, 1994). Pain comprises a complex experience involving not only the transduction of noxious stimuli but also cognitive and emotional aspects of the stimuli when it is processed in higher cortical areas in the brain (Basbaum et al., 2009). It is considered a personal experience and can only be studied in human beings, the only specie capable of communicating emotional and sensory experience (Pedersen 2000). Pain is commonly divided in acute and chronic pain. The acute pain, considered as a protective phenomenon, appears quickly after tissues injury and disappears gradually by a healing process (Kuner 2010). If the nociceptive input persists after healing, a state of chronic pain can develop (Jensen et al., 2003).

The term nociceptor indicates a sensory receptor that responds to relatively high magnitude or potentially noxious stimuli. The nociceptors, thinly myelinated Aδ and unmyelinated C afferents (polymodal nociceptors), are peripheral nerve endings located in different tissues of the body including skin, connective tissues, blood vessels, viscera and deep somatic tissues like muscles and joints. The process of nociception refers to the recognition of a noxious stimulus from a nociceptor and its transmission to the brain through peripheral and central neurons (Holdcroft and Jaggar 2005; Kidd and Urban 2001; Woolf 1983). Alteration of the pain path induces hyper responsiveness or hypersensitivity (Basbaum et al., 2009). Pain hypersensitivity appears in two forms:

• Hyperalgesia, defined as an "increased response to a stimulus which is normally painful" (IASP, 1994).

• Allodynia, defined as "pain due to a stimulus which does not normally provoke pain" (IASP, 1994).

After a stimulus, when hyperalgesia and allodynia are both present, the only term that can be used is "hyperalgesia" (Lindblom et al., 1986; Pedersen 2000). The term primary hyperalgesia indicates an increase in nociceptor responsiveness in the site of injury, while secondary hyperalgesia indicates an increase in responsiveness to normal painful stimuli in the undamaged area surrounding the injury (**Fig 1**), (Ali et al., 1996; Meyer et al., 2006). There are two processes contributing to hyperalgesia defined as the peripheral sensitization (increased responses of nociceptors located in

the peripheral tissues) and sensitization of central mechanisms (increased responses from dorsal horn neurons, involving the central nervous system) (Graven-Nielsen and Arendt-Nielsen 2010). In sensitization of central mechanisms, the central integrative mechanisms are up-regulated, leading to facilitated temporal summation of pain, defined as the progressive intensification in pain perception in response to a stimulus sequence having identical intensity (Graven-Nielsen and Arendt-Nielsen 2010).
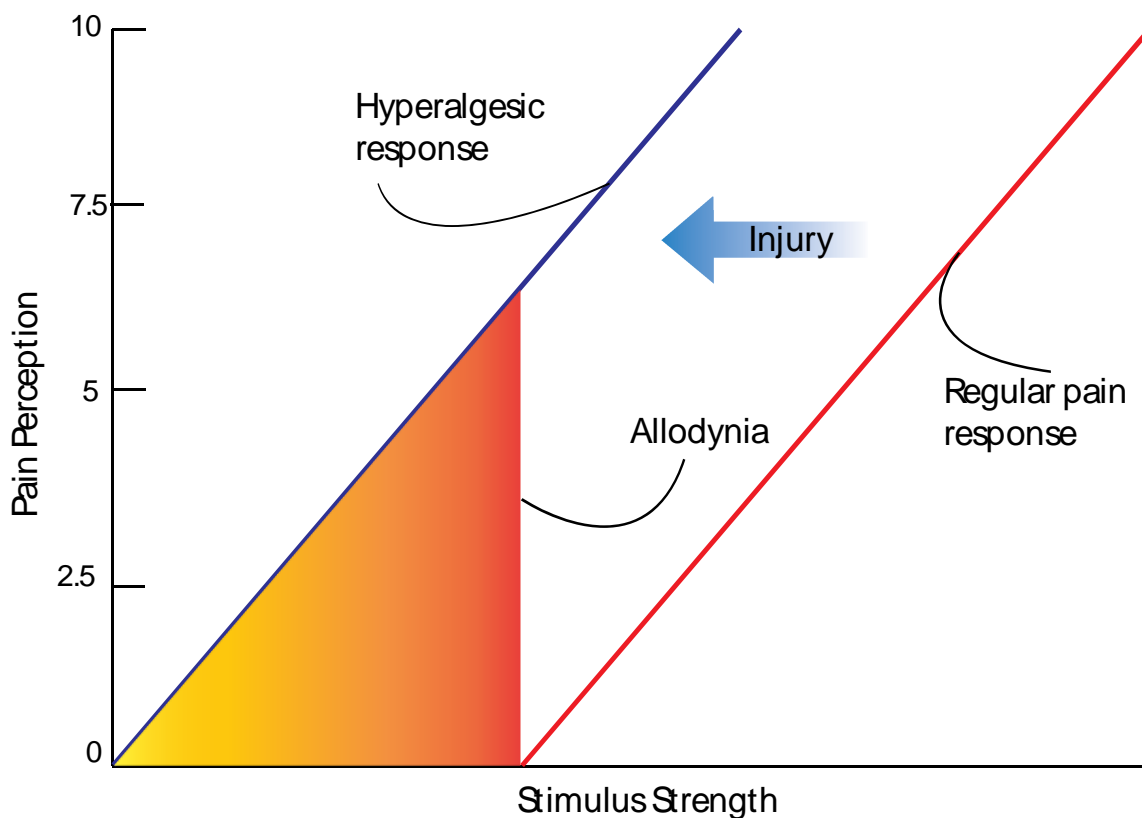
Figure 1: Schematic representation of allodynia and hyperalgesia. The two curves represent the relation between the stimulus intensity and the pain response.

4.2 Inflammatory Pain

The term inflammatory pain indicates the pain originating from a tissue injury, irritation or infection, which usually lead to inflammation (Holdcroft and Jaggar 2005). The inflammatory process is part of the biological response of the body to a wide range of insults, like pathogens,

damaged cells, or irritants (Ferrero-Miliani et al., 2007). This natural "defense" process determines hyperalgesia and an increased blood flow in the area, accompanying with an accumulation of fluid (edema). The symptoms of inflammation consist of warmth and redness of the skin, along with swelling and pain (Holdcroft and Jaggar 2005). Those reactions contribute to prevention of further injury and to the resolution of the damage. Inflammatory pain and inflammation are mediated by release of inflammatory mediators, called also "inflammatory soup", from damaged cells that increase the sensitivity of nociceptors to noxious thermal or mechanical stimuli (Basbaum et al., 2009; Holdcroft and Jaggar 2005; Huang et al., 2006). These inflammatory mediators include extracellular proteases and protons, arachidonic acid, neurotransmitters, bradykinin, NGF and ATP. Among these inflammatory mediators, some substances like protons, ATP and serotonin can act directly on the nociceptors by interacting with ion channels or specific receptors located on the surface of the sensory nerve endings innervating the area (Basbaum et al., 2009; Julius and Basbaum 2001). Other mediators, such as bradykinin and NGF, act by binding to metabotropic receptors and membrane receptors that work through a secondary messenger (Julius and Basbaum 2001). All primary sensory nociceptors make synaptic connections at the level of the spinal cord with dorsal horn neurons. These neurons then, transmit pain messages to specific parts of the brain, such us thalamus, reticular formation, and cerebral cortex (Julius and Basbaum 2001). Several clinical conditions are characterized by the presence of inflammatory reaction, including rheumatoid arthritis, a common chronic inflammatory disorder involving the synovial membrane and the joints (Choy and Panayi 2001) and fibromyalgia, a disorder of generalized musculoskeletal pain (Wolfe 1991).

4.3 Experimental pain models of cutaneous inflammation

Human experimental pain models that mimic human clinical pain conditions are valuable tools in the study of pain mechanisms and analgesic effectiveness of drugs in early stages of clinical trials (Arendt-Nielsen et al., 2007; Ren and Dubner 1999). A pain model to be considered ideal must provide a reproducible and standardized condition and a high accuracy of the pain assessments (Gustorff et al., 2004a; Sycha et al., 2005). It also has to be simple to perform, without any sign of spontaneous pain, and it must cause stable and reproducible primary and secondary hyperalgesia without inducing tissue or psychological damages (Petersen and Rowbotham 1999). The most important challenge when dealing with human experimental pain models is to evaluate if mechanisms seen using experimental pain models can actually reflect the mechanisms seen in clinical conditions of pain as inflammatory and musculoskeletal disorders (Schmelz M. et al., 2010).

Cutaneous experimental pain models are the most used ones, mostly because of the easy access to the skin (Staahl and Drewes 2004), and a widespread range of inflammatory substances and irritants can be used to produce temporary and reversible tissues injury and hyperalgesia in the skin (Zhang and Ren 2011). The most important pain models used to induce pain hypersensitivity in cutaneous and subcutaneous tissues are burn and freeze injury models, ultraviolet irradiation model (UVB model), and capsaicin model. Capsaicin, the component of chili peppers, is one of the most used chemically-induced pain models and it causes a type of inflammation called *neurogenic inflammation* (Staahl and Drewes 2004). Several studies have confirmed that capsaicin induces flare reaction, hyperalgesia and allodynia in the area of application and in the adjacent area (Bishop et al., 2009; Zhang and Ren 2011). Burn and freeze injury models are frequently used in humans to induce either primary or secondary hyperalgesia by application of warm (52°C/30-45 s) or cold (-28°C) stimuli, respectively (Zhang and Ren 2011).

4.3.1 Ultraviolet-B Pain Model

Ultraviolet-B (UV-B) irradiation model is a well-known translational model since it has been used to induce cutaneous inflammatory pain in both animals (Bishop et al., 2007; Davies et al., 2011) and humans (Bishop et al., 2010; Gustorff et al., 2013; Harrison et al., 2004). This model causes an erythema called "sunburn" along with thermal and mechanical hyperalgesia (Bishop et al., 2009; Gustorff et al., 2013). In this PhD project, the UVB inflammatory pain model was used to induce inflammation, allodynia and hyperalgesia in human skin. These reactions are present in a variety of chronic inflammatory states, such as rheumatoid arthritis, and can be used in healthy volunteers to study the pain system under well controlled settings (Handwerker and Arendt-Nielsen 2013; Staahl and Drewes 2004). The UVB model has been chosen since it is a stable and reproducible model that has been extensively investigated both in animals and humans and does not cause spontaneous pain or tissues damage (Bishop et al., 2009; Davies et al., 2011; Gustorff et al., 2004b; Gustorff et al., 2013). Moreover, the UVB model is able to induce a long-lasting hyperalgesic reaction in comparison to other models such as capsaicin-induced inflammation (Pitcher et al., 2008; Sycha et al., 2003).

Humans are normally exposed to UVB radiation through natural sunlight but there are also artificial sources of UVB radiation such as sun-beds, phototherapy lamps or tungsten-halogen lamps (Honigsmann 2002). In this PhD project, UVB irradiation was applied using a calibrated UVB source (wavelength 290-320nm; Saalmann Multitester, Saalmann, SBC LT 400 Herford, Germany) on different skin sites. The skin was irradiated by using UVB dose of 3 times the individual MED (calculated for each test person). The individual minimal erythematic dose (MED) is known as the minimum amount of UVB energy (J/cm2) that 24 h after exposure produces an erythematic area with distinct borders.

Ultraviolet-B (UV-B) ranging from 290 to 320 nm is a component of sunlight and can be considered the most effective in inducing the cutaneous sunburn reaction (Benrath et al., 2001). This reaction is generally classified as a superficial or first-degree burn (McStay ; Soter 1990), and is considered an acute inflammatory reaction caused by an excessive exposure of the epidermis to UVB radiations (Honigsmann 2002). The UVB-irradiation is mostly absorbed by the chromophores present in the epidermis and leads to destruction of epidermal cells, like keratinocytes and to release of numerous cytokines and inflammatory mediators into the skin (Honigsmann 2002). A large number of these mediators such as nitric oxide, histamine, bradykinin, prostaglandins, cytokines,

and NGF are involved in the sensitization of the peripheral nociceptors located on the sensory neurons innervating the region of the irradiation (Dray 1995; Kidd and Urban 2001). Skin inflammation produced by UVB induces an erythema called "sunburn" accompanied by thermal and mechanical hyperalgesia and changes in tissues perfusion in the area of irradiation (area of primary hyperalgesia) and in the adjacent areas (area of secondary hyperalgesia) (Gustorff et al., 2004a; Gustorff et al., 2004b; Sycha et al., 2005) (**Fig 2**). These characteristics of the UVB model peak 24 h after irradiation and have been demonstrated in men and rodents (Bishop et al., 2009; Davies et al., 2011; Gustorff et al., 2013). UVB irradiation also induces increased pigmentation and thickening of the epidermis (Honigsmann 2002). Several studies have concluded that the decrease in thermal pain threshold induced by the UVB-model is transmitted through unmyelinated C-fibres, whereas the decrease in mechanical pain thresholds is transmitted through myelinated Aβ and Aδ fibres (Bishop et al., 2009; Gustorff et al., 2004a; Sycha et al., 2005). An overview of the human studies conducted on the UVB-pain model is shown in **Table 1**. In general, all the studies presented in the table concluded that UVB-irradiation induced both thermal and mechanical hyperalgesia in the area of irradiation but presented controversial results regarding the existence of secondary hyperalgesia.

In addition to studying pain mechanisms and pathways, the UVB model can also be considered a valid tool for testing or screening of analgesic drugs. Recently, this model has been used to test the efficacy of opioids, morphine and remifentanil (Gustorff et al., 2004b; Koppert et al., 2004; Staahl et al., 2009) and non-steroidal anti-inflammatory drugs (NSAIDs), ibuprofen and rofecoxib (Bickel et al., 1998; Sycha et al., 2005; Sycha et al., 2003). **Table 2** shows an overview of all the human studies investigating the effect of drugs via the UVB model. All these studies confirmed that the UVB pain model can be used as a valid tool for screening different analgesic drugs. The papers presented in the tables have mostly been selected through the databases PubMed and Medline using the following search strings: UVB and hyperalgesia; time course of UVB-induced inflammation, UVB and pain.

Even though the UVB model has been well characterized both in animals and humans (Bishop et al., 2009; Bishop et al., 2007; Davies et al., 2011; Gustorff et al., 2013; Harrison et al., 2004), the presence of secondary hyperalgesia in this model is still controversial. For this reason, in this PhD project one of the aims was to study the alteration in cutaneous vaso-response, mechanical and pressure pain sensitivity within and outside the UVB-irradiated area and a possible convergence between inputs coming from the irradiated skin and the underling deep somatic area.

What is also still unknown is the possibility of an interaction between superficial and deep inflammatory models, when those are applied in combination, even though the existence of convergence between afferent nociceptive fibres from superficial and deep structures has been demonstrated (Hoheisel and Mense 1990; Yu and Mense 1990). Based on this evidence, this PhD study also evaluated whether intramuscular sensitization may affects the UVB-induced cutaneous hyperalgesia when combining it with the UVB inflammatory model.

Figure 2: Image of upper arm irradiated with 3xMED after removal of the EMLA cream. Data taken from Study III.

Author and Year	Title	Aim	Tests performed	Results regarding primary hyperalgesia	Results regarding secondary hyperalgesia	Conclusion
Hoffman and Schmelz (1999)	Time course of UVA- and UVB-induced inflammation and hyperalgesia in human skin	To evaluate dose–response and time course of inflammation and hyperalgesia following UVA and UVB irradiation.	HPPT, mechanical hyperalgesia (assessed using impact stimulation).	Only UVB Irradiation induced HPPT decrease and mechanical hyperalgesia	Not reported	UVB- but not UVA-irradiation can be used as experimental model to induce both thermal and mechanical hyperalgesia.
Gustorff and colleagues (2004)	The sunburn pain model: the stability of primary and secondary hyperalgesia over 10 hours in a crossover setting	To investigate repeatability and stability of PH and SH over 10 h.	HPTT, area of SH (assessed using 150 g von Frey), Electrical Pain Tolerance Thresholds (5 Hz & 250 Hz).	Decrease of HPTT. Decrease of Electrical PTT at 250 Hz.	Pin-prick SH present. No SH to HPTT. No SH to Electrical PTT	The UVB model presents both high within-day stability and between-day repeatability for both PH and SH. UVB-induced SH has been demonstrated for the first time in this study.
Bishop and colleagues (2009)	Ultraviolet-B induced inflammation of human skin: Characterisation and comparison with traditional models of hyperalgesia	To investigate the dose dependence and time course of UVB inflammation.	Mechanical hyperalgesia (assessed by 10 g von Frey), HPPT, allodynia (assessed using brush).	The mechanical hyperalgesia was present and HPPT decreased.	No allodynia or hyperalgesia were present.	The UVB model produces changes in sensory processing within the area of PH with only minimal changes in the area of SH.
Gustorff and colleagues (2013)	The pattern and temporal profile of somatosensory changes in the human UVB sunburn model reveal the presence of peripheral and central sensitization	To characterize time course and magnitude of sensory changes evoked by UVB irradiation in both primary and secondary hyperalgetic areas.	HPPT, CPT, PPT, MPT, DMA, mechanical pain sensitivity	CPT increased, HPPT, MPT and PPT decreased.	DMA, MPT decreased	The UVB model induced primary hyperalgesia accompanied by a moderate degree of secondary hyperalgesia.
Weinkauf and colleagues (2013)	Modality-specific nociceptor sensitization following UV-B irradiation of human skin	To study if UVB irradiation may increase the axonal excitability of nociceptors.	Mechanical impact stimuli, MPT, PPT, electrical pain thresholds, HPPT	HPPT, MPT, PPT, mechanical impact stimuli and electrical pain thresholds decreased.	Not reported	UVB irradiation induced increased axonal excitability.
Mørch and colleagues (2013)	The UVB cutaneous inflammatory pain model: a reproducibility study in healthy volunteers	To study the test-retest reliability of the UVB inflammatory model.	DMA (brush), tactile perception threshold (assessed using von Frey), PPT, HPPT, SH	DMA, PPT and HPPT decreased	SH was present	UVB model induced neurogenic inflammation and hyperalgesia with highly reproducibility within and between sessions.

Table 1. Overview of human studies using the UVB model.

Abbreviations used: HPR = heat pain response, HPPT = heat pain perception threshold, HPTT = heat pain tolerance threshold, CPT= cold pain threshold, PPT= pressure pain threshold, MPT= mechanical pain threshold, DMA= dynamic mechanical allodynia, WPT= warmth perception threshold, PH = primary hyperalgesia, SH = secondary hyperalgesia.

Author and Year	Title	Aim	Drug tested	Tests performed	Results regarding primary hyperalgesia	Results regarding secondary hyperalgesia	Conclusion
Bickel and colleagues (1998)	Effects of anti-hyperalgesic drugs on experimentally induced hyperalgesia in man	To compare the anti-hyperalgesic effects of Ibuprofen on PH.	Ibuprofen	HPPT, mechanical hyperalgesia (assessed using impact of 5, 9, and 13 m/s), repetitive pinching.	3xMED: UVB induced both heat hyperalgesia and mechanical hyperalgesia for 9 and 13 m/s.	Not reported	Ibuprofen had an analgesic effect on both thermal and mechanical PH. The k-agonist did not showed any effect on UVB irradiation.
Koppert and colleagues (1999)	Peripheral antihyper-algesic effect of morphine to heat, but not mechanical, stimulation in healthy volunteers after ultraviolet-B irradiation	To study the peripheral effects of Morphine.	Morphine	Mechanical hyperalgesia (assessed using impact stimuli) and HPPT.	Morphine reduce heat hyperalgesia but showed no effect on mechanical hyperalgesia.	Not reported	The antihyperalgesic effect of morphine was present in the irradiated area.
Sycha and colleagues (2003)	A simple pain model for the evaluation of analgesic effects of NSAIDs in healthy subjects	To evaluate the effectiveness of UVB model in testing NSAIDs	Ibuprofen	HPPT, HPTT	Ibuprofen increased both HPPT and HPTT.	Not reported	The effect of ibuprofen was significant and the UVB model is a valid tool for testing NSAIDs
Gustorff and colleagues (2004)	The Effects of Remifentanil and Gabapentin on Hyperalgesia in a new extended inflammatory skin pain model in healthy volunteers	To test the effect of two different drugs alone and in combination.	Remifentanil and Gabapentin	HPTT, HPPT, area of SH (assessed using 150 g von Frey),	Decreased of both HPTT and HPPT. Remifentanil increased both HPPT and HPTT in the area of PH and in the normal skin.	Remifentanil reduced SH. Gapapentin; no reduction.	Opioid analgesia was demonstrated in the UVB model of PH and SH.
Sycha and Colleagues (2005)	Rofecoxib attenuates both primary and secondary inflammatory hyperalgesia: a randomized, double blinded, placebo controlled crossover trial in the UVB pain model	To study the effects of rofecoxib on PH and SH induced by UVB irradiation.	Rofecoxib	HPTT, HPPT and area of SH (assessed using 150 g von Frey),	Rofecoxib increased both HPTT and HPPT.	Rofecoxib reduced SH.	Rofecoxib induced peripheral effects and reduced SH in inflammatory pain.
Eisenach and colleagues (2010)	Effects of Intrathecal Ketorolac on Human Experimental Pain	To assess the role of spinal Cyclo-oxygenase on hyper-sensitivity.	Ketorolac	Allodynia (to stroking) and mechanical hyperalgesia (assessed using 225 mN von Frey)	Small areas of Hyperalgesia and allodynia unaffected by ketorolac.	Areas of SH unaffected by ketorolac. Allodynia not present.	Intrathecal ketorolac reduced the area of allodynia after UVB but showed no effect on pain raising from acute heat stimuli or capsaicin.
Ortner and	Dose response of	To determine	Tramadol	HPPT, CPT,	Tramadol but	Both	In inflammatory

colleagues (2011)	tramadol and its combination with paracetamol in UVB induced hyperalgesia	tramadol and tramaldol + paracetamol effect on primary and secondary hyperalgesia.	and Paracetamol	mechanical hyperalgesia and allodynia (assessed using 256 mN pin-prick), DMA	not paracetamol decreased CPT and mechanical hyperalgesia	Tramadol and Paracetamol reduced SH.	hyperalgesia tramadol exerts a weak anti-hyperalgesia. Adding a small dose of paracetamol enhances thermal anti-hyperalgesia.
Rössler and colleagues (2013)	Central origin of pinprick hyperalgesia adjacent to an UV-B induced inflammatory skin pain model in healthy volunteers	To evaluate if SH can be reduced by superficial local anaesthetic	Lidocaine	Mechanical hyperalgesia (assessed using 256 mN pin-prick) and allodynia (to stroking), HPPT, CPT, PPT, WPT.	Presence of area of hyperalgesia and allodynia. All the measurements were not affected by the lidocaine perfusion.	Hyperalgesia and allodynia were not affected by the anaesthetic block.	Mechanical hyperalgesia was unchanged after application of anaesthetic 8 h after irradiation confirming the presence of central mechanism mediating secondary hyperalgesia.

Table 2. Overview of human studies testing drugs in the UVB model

Abbreviations used: HPR = heat pain response, HPPT = heat pain perception threshold, HPTT = heat pain tolerance threshold, CPT= cold pain threshold, PPT= pressure pain threshold, DMA= dynamic mechanical allodynia, WPT= warmth perception threshold, PH = primary hyperalgesia, SH = secondary hyperalgesia.

4.4 Deep-Tissues Pain Models

Several different methods can be used to induce experimental pain for the quantitative assessment of the pain sensitivity in deep tissue. These methods can be divided in two major categories: the exogenous models are methods involving external stimuli (such as pressure stimulation or injection of algesic substances like NGF or saline injections), whereas the endogenous methods are methods inducing pain in deep-tissue by physiological stimuli such as eccentric exercise (Graven-Nielsen and Arendt-Nielsen 2010). The muscle nociceptors are free nerve ending nociceptor and are divided in group III, corresponding to the cutaneous Aδ-fibres, and group IV, corresponding to the cutaneous C-fibres.

 In this PhD project, in order to investigate the effect of a possible convergence between cutaneous (the UVB cutaneous pain model) and deep-tissue hyperalgesia, the delayed onset muscle soreness (DOMS) pain model was applied. In addition, the NGF model was used in association with the UVB model in order to investigate whether the combination between cutaneous and muscle hyperalgesia may influence the responsiveness of the UVB model. Both DOMS and NGF pain

models are well established models to induce deep-tissue hyperalgesia (Andersen et al., 2008; Gibson 2007).

4.4.1 Exercises-induced Delayed Onset Muscle Soreness Model

Eccentric exercise is an endogenous method to evoke experimental deep tissue pain commonly used to induce muscle hyperalgesia (Gibson 2007). The eccentric activity is characterized by elongation of muscle during simultaneous contraction. When the external load outdoes the ability of the muscle to resist the load, the muscle is forced to lengthen, producing an active tension (Nie et al., 2007; Stauber 1989). Eccentric exercise induces delayed onset muscle soreness (DOMS) that occurs as a result of changes in the muscle fibres within 24 and 48 hours after eccentric exercise (Armstrong 1984) and manifests as aching pain accompanied by tenderness and stiffness (Dannecker et al., 2003). Those sensations usually diminish within 5–7 days after the exercise (Nie et al., 2005). The exercises-induced DOMS is a well-established and long-lasting model and resembles some characteristics seen in several clinical conditions in musculoskeletal pain or ischemic muscle pain (Handwerker and Arendt-Nielsen 2013; Nie et al., 2005). DOMS has been investigated in both humans and animals (Cheung et al., 2003; Murase et al., 2010; Taguchi et al., 2005), and the research results reflect events seen in acute inflammation (Smith 1991). To explain the development of DOMS, different mechanisms have been proposed based on biochemical, ultrastuctural and histological studies conducted both in animals and humans (Murase et al., 2010). One of the most supported mechanisms is based on the existence of minor muscle traumas causing an increased release of inflammatory substances (bradykinin, histamine, prostaglandins, glutamate and neutrophils) as a consequence of the inflammatory response. This has been confirmed by MacIntyre and co-workers who found a great presence of neutrophils in the eccentric-exercised muscle tissue compared to the non-exercised muscle for up to 4 h post-exercise as a consequence of the increased blood circulation in the muscle after eccentric activity (MacIntyre et al., 2000; Nosaka and Clarkson 1996). Due to the inflammatory process, DOMS has been attributed to peripheral sensitization but central mechanisms may also be involved since facilitated temporal summation to repetitive pressure stimulations during DOMS has been demonstrated (Nie et al., 2006). Another important theory considers NGF as the key substance in maintaining DOMS induced by eccentric contraction (Murase et al., 2010). In experiments conducted in rats, Murase and co-workers demonstrated that intramuscular administration of anti-NGF antibody 6 h after eccentric contractions could block completely the induction of muscle mechanical hyperalgesia (Murase et al., 2010). Murase's experiment also confirmed a 12 h delay of the NGF mRNA up-regulation after eccentric activity

that may explain the delayed development of DOMS following eccentric contraction, which is in line with similar reports in humans (Murase et al., 2010).

DOMS is usually induced in the muscles of arm and leg (Nie et al., 2005) but is not commonly induced in the lumbar muscles, even though low back is often involved in clinical pain conditions. In this PhD project, DOMS in the lumbar extensors was induced by asking subjects to perform 50 back exercises (eccentric contraction of the lumbar muscles, **Fig 3**).
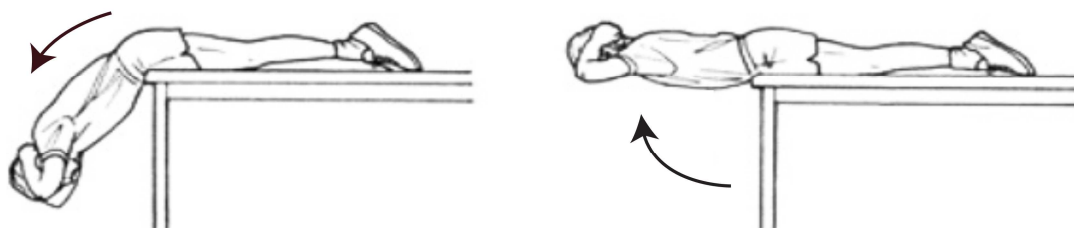
Figure 3: Schematic representation of the eccentric contraction of the lumbar muscles performed by the subjects.

The exercises-induced DOMS model presents several limitations. Based on the Likert scale results (Study II) it can be assumed that this model does not induce an equal level of muscle soreness and hyperalgesia in all individuals undergoing this procedure. This is probably due to gender difference (MacIntyre et al., 2000) or differences in muscle training among the subjects. Furthermore, DOMS does not induce pain at rest, typically present in musculoskeletal conditions such as fibromyalgia (Gracely et al., 2003), and has also the disadvantage of being difficult to control since this method involves numerous muscle groups within the investigated region (Staahl and Drewes 2004).

This study is the first to assess the possibility of an interaction between superficial and deep sensitization utilizing established models of skin and muscle hyperalgesia. Moreover, the DOMS model has been chosen since it peaks around 24 h post-exercises similar to peak of the effects in response to UVB model in the skin. This will allow a better evaluation of the pain changes induced by both methodologies applied in combination.

4.4.2　Nerve Growth Factor Pain Model

Several studies have shown that muscular hyperalgesia can be experimentally induced by application of various substances into the muscles. This sensitization mimics the inflammatory condition and the decrease in pain thresholds present in some disorders involving the musculoskeletal system, making the models very useful from a clinical perspective (Mørk et al., 2003; Staahl and Drewes 2004). The nerve growth factor (NGF) is a molecule member of a family of peptides called neurotrophins and plays a key role in the development of the peripheral nervous system and in the inhibition of programmed cell death in neurons in the peripheral nervous system (Frade and Barde 1998; Rueff and L.M. 1996). NGF is normally secreted from various tissues, including muscles and can bind to neurons expressing the trkA receptor (Andersen et al., 2008; Rueff and L.M. 1996). The role of NGF in the inflammation has been investigated by several groups (Donnerer et al., 1992; Rueff and L.M. 1996; Woolf et al., 1994). As a direct consequence of the inflammatory process, the NGF production increases in the periphery and, in case of persistent inflammation, it can be transported backward from the periphery toward the dorsal root ganglion (Goedert et al., 1981; Lewin and Mendell 1993). The increased concentration of NGF in the skin during inflammation is due to its release from keratinocytes that can be induced by tumor necrosis factor alpha (TNFα) and interleukins (IL-1β), which are released from mast cell and macrophages during inflammation (Lindholm et al., 1987; Rueff and L.M. 1996). It has also been demonstrated that the exposure of nociceptors to NGF increases the release of the neuropeptides such as substance P and calcitonin gene-related peptide (CGRP), both related to the development of pain and hyperalgesia (Greco et al., 2008; Rueff and L.M. 1996; Woolf et al., 1994). A confirmation of the direct role of NGF in hyperalgesia emerged from studies applying NGF injections in both animals and humans. Systematic injections of NGF into rats cause hypersensitivity to noxious heat and mechanical stimuli that persists for days (Frade and Barde 1998). Moreover, intramuscular injection of NGF in the tibialis anterior muscle in healthy volunteers induced mechanical hyperalgesia one day after the injection, lasting up to 7 days after the administration (Andersen et al., 2008). A large number of studies have also investigated the possibility of reversing inflammatory pain by blocking the action of NGF using anti-NGF antibodies or similar agents able to bind free molecules of NGF (Frade and Barde 1998; Rueff and L.M. 1996; Woolf et al., 1994). All studies confirm that inflammatory hyperalgesia was reduced by blocking NGF, confirming that the release of NGF is necessary to produce a hyperalgesic reaction. Several disorders, including arthritis and cystitis, have also been shown to include the inflammatory process associated with

increased NGF release (Hudspith et al., 2006). The direct relationship among inflammation, NGF, and the long-lasting muscle hyperalgesia induced by this model are the principal reasons explaining the selection of this model in this PhD project to investigate if NGF-muscle sensitization of the muscle below the UVB-irradiated area can modify the responsiveness of UVB-irradiated skin to heat rekindling of the UVB area. Moreover, this is a completely different model from the DOMS model since it is a chemical model.

5   General methodological approaches

5.1   Assessment of Vasomotor Response

Inflammatory process and its associated changes are usually reflected in the skin, where it is also possible to assess cutaneous blood flow changes. These have been investigated in several areas of medicine, such as dermatology, plastic surgery, and vascular medicine (Clauw et al., 2011; Essex and Byrne 1991). The cutaneous microcirculation includes two different horizontal plexuses. The first is located 1-1.5 mm under the surface of the skin, whereas the second one is located at the intersection between the dermis and the subcutaneous tissue (Braverman 2000; Clough and Church 2002). A wide range of techniques can be used to measure the cutaneous blood flow, such thermography, a technique allowing a continuous monitoring of the skin temperature (Jasemian et al., 2012), ultrasound, laser-Doppler flowmetry, allowing measurement of microvascular tissue perfusion based on the Doppler effect (Wardell et al., 1993), and laser-Doppler imaging (Clough and Church 2002). Among these different methodologies, the laser-Doppler imaging technique (LDI) is considered one of the best validated techniques for use in a clinical experiments (Clough and Church 2002) and was employed in this PhD project as described below.

5.1.1   Laser Doppler Imaging

The LDI scanner is a device that measures the cutaneous blood flow by using a mirror deflecting laser beam onto the skin surface. A 2 mW, 633-nm, red helium-neon laser scans the surface of the skin, and light, back-scattered from moving red blood cells, is shifted in frequency according to the Doppler principle and the flux is expressed in arbitrary unit (AU) (Newton et al., 2001). The erythrocytes velocity is then calculated and represents a relative measure of cutaneous blood flow (Gustorff et al., 2013). At the end of the measurement, the instrument produces a color two-dimensional image representing the erythrocytes flux up to a depth of ~0.6-1 mm (Clough and Church 2002). In this PhD project, the alteration in blood perfusion following an inflammatory process in the skin and deep-tissues was quantified by the laser Doppler imaging technique (Moor Laser Doppler Imager V3.08, Moor Instruments Ltd, Devon, UK). The laser head was always positioned at a fixed distance of 30 cm above the irradiated skin, with the laser beam perpendicular to the skin and the scan was performed with a 256 x 256 pixel resolution. The measurement was

performed in a temperature-controlled room, with the subject resting in a bed. Prior to and during the study sessions, the use of any substance (caffeine, nicotine, alcohol) that could influence the blood flow measurements was prohibited. Several studies have shown that nicotine and alcohol intake can induce changes in cutaneous blood flow (Waeber et al., 1984; Wilkin and Fortner 1985). Among these different methodologies, the laser-Doppler imaging technique (LDI) was employed in the PhD project since it allows remote skin blood flow measurements without any skin contact (Clough and Church 2002), avoiding changes of the results due to skin manipulation in the inflamed area. Another reason for choosing this methodology is that the LDI permits measurement of a large area of the skin allowing seeing the changes in blood flow inside and outside the irradiated areas in a single measurement.

5.2   Assessment of Mechanical Pain Sensitivity

The mechanical pain thresholds (MPT) in the skin were determined using weight-calibrated pin-prick stimulators (Aalborg University, Denmark). This is a set of weight-calibrated metal probes that are used to determine the area of secondary hyperalgesia (Staahl and Drewes 2004). Pinprick stimulation seems to activate mostly Aδ-fibres and the feeling is reported as pricking or ''first pain''(Staahl and Drewes 2004). The pin-prick stimulators have a metal probe with a diameter tip of 0.6 mm and different weights and the MPT is defined as the lightest pin-prick stimulator that can induce a sharp sensation. The pin-prick stimulator represents a rapid sensory assessment for the estimation of MPT and is simple to perform. In order to produce a constant evaluation of MPT, the test should always be performed by the same investigator. Moreover, the pin-prick stimulator should be pressed gently against the skin so that the pin-prick sensation is only caused by the applied force and based on the calibrated weight of the stimulator (Chan et al., 1992). Since the stimulation induced by the pin-prick stimulator may last for a few seconds, it is also very important to apply the successive stimulation with a reasonable gap time when it is applied repeatedly (Chan et al., 1992).

The von Frey hair stimulator is a tool used to determine touch perception thresholds (Aesthesiometer, Somedic AB, Hörby, Sweden) and consists of a set of 19 filaments having different diameter mounted on plexiglas handles. In this project only the 26 g stimulator has been used to map the allodynic area after UVB irradiation or after heat rekindling since it corresponds to a light touch and is not painful in normal skin. When the applied stimulation reaches a certain

pressure, the filament bends, allowing the activation of Aβ-fiber mediating touch sensation and the measurement of the allodynic area (Staahl and Drewes 2004).

In this project two different approaches were used to map the areas of hyperalgesia and allodynia. For mapping the hyperalgesic area, the pin prick corresponding to the MPT of the subjects was used, whereas a single von Frey hair stimulator (26 g) has been used for mapping the allodynic area.

5.3   Heat Rekindling Protocol

The heat rekindling is a technique based on the application of mild heat stimuli to enhance afferent input from an already established sensitization. The concept behind the heat rekindling is that the heat stimulation sustains and enhances the initial response of the applied hyperalgesic model (Eisenach et al., 2010). Heat rekindling has been initially applied in combination with the capsaicin pain model. A mild and non-painful heat stimulus of 40°C for 5 minutes was applied resulting in a reinforcement of the secondary hyperalgesic area for up to 4 h (Petersen and Rowbotham 1999). Recently, the heat rekindling has been used in combination with the UVB-inflammatory pain model in order to enhance the effect of the UVB irradiation on the area of secondary allodynia and hyperalgesia (Cookson et al., 2005; Davies et al., 2011; Eisenach et al., 2010). In their recent study, Eisenach and collaborators, demostrated that heat rekindling performed after cutaneous UVB irradiation induced a larger area of secondary hyperalgesia compared to UVB alone (Eisenach et al., 2010). Several studies have also used the heat/UVB model to test the efficacy of a wide range of drugs. In a recent study conducted by Wang and co-workers, the UVB/heat pain model has been used to test the efficacy of two different drugs: rofecoxib, a cyclooxigenase-2 (Cox-2) inhibitor, and ketamine, a N-Methyl-D-aspartate (NMDA) receptor antagonist (Wang et al., 2005). The results of this study confirm that the UVB/heat model can be used to test the effects of analgesic drugs targeting different receptors. Table 3 shows an overview of the human studies that have investigated the UVB/heat model as a pain model and for drug studies.

In this PhD project, heat rekindling was performed on the skin by a thermod stimulator attached precisely on the irradiated area. Two consecutive cycles of rekindling have been performed using the same paradigm indicated in the heat/capsaicin model. In order to investigate whether heat rekindling could prolong or enhance the secondary area of hyperalgesia and allodynia induced by UVB-irradiation, the rekindling was performed 72 h after irradiation, when the hyperalgesic effect of UVB started to decrease.

Author and Year	Title	Aim	Tests performed	Results	Drug tested	Conclusion
Wang and Colleagues (2005)	Antihyperalgesic profiles of rofecoxib and ketamine in a new healthy volunteer model of inflammatory pain	To explore peripheral and central aspects of the UVB/heat model.	Laser Doppler imaging, PH, SH and allodynia.	Rofecoxib reduces PH and SH whereas Ketamine only reduces SH.	Rofecoxib and Ketamine	The UVB/heat model can be used to test drugs with different mechanism of action.
Cookson and Colleagues (2005)	A new human model of inflammatory pain and sensitization evoked by ultraviolet (UV)-irradiation combined with heat rekindling	To enhance the central mechanism involved in the UVB model by combining UVB and heat.	Flare, HPPT, MPT	UVB-irradiation induces flare and a decrease of both HPPT and MPT. Increase of SH after heat rekindling	None	UVB irradiation induced primary and secondary hyperalgesia that can be enhanced by heat rekindling.
Eisenach and Colleagues (2010)	Effects of intrathecal ketorolac on human experimental pain	To investigate the role of spinal cyclooxygenase in human by injection of cyclooxygenase inhibitor ketorolac.	Areas of hyperalgesia and allodynia, peripheral oxyhemoglobin saturation, blood pressure and heart rate	Heat rekindling increased the UVB hyperalgesic area	Ketorolac	Ketorolac reduced hypersensitivity induced by UVB alone and UVB plus heat.

Table 3. Overview of human studies using the Heat/UVB model.

Abbreviations used: HPR = heat pain response, HPPT = heat pain perception threshold, HPTT = heat pain tolerance threshold, MPT= mechanical pain threshold, PH = primary hyperalgesia, SH = secondary hyperalgesia.

5.4 Assessment of Pressure Pain Sensitivity

Pressure algometry is one of the most frequently used techniques for the quantification of mechanical sensitivity and pain. This method, involving pressure stimulation to induce pain from deep structures, can be considered an experimental equivalent to palpation in the clinical practice (Graven-Nielsen and Sergerdahl 2001) and has been used extensively (Jensen et al., 1986). It is believed that both Aδ- and C-fibres are involved in the perception of the pain arising from pressure stimulation (Staahl and Drewes 2004).

Manual pressure algometry has been used for evaluation of sensitivity to pain and the assessment of pressure perception but this methodology has a certain amount of variability, even though it is considered a very reliable method for measuring pressure pain thresholds (Fischer 1987; Potter et al., 2006; Reeves et al., 1986). Recently, in order to ensure delivering of consistent pressure during the assessments, the computer-controlled algometer (**Fig 4A**) has been used to evaluate pressure

pain sensitivity in different structures (Finocchietti et al., 2011). This methodology, by reducing the variability of the measurements, allows an accurate construction of the stimulus-response function, a curve relating graded pressure stimulations and pain intensity (Graven-Nielsen et al., 2004). Pressure stimulation is applied perpendicularly to the skin surface where the pressure pain threshold (PPT) and the pressure pain tolerance (PPTO) can be easily measured. PPT is defined as the point at which a feeling of pressure changes into a feeling of pain. PPTO is defined as the greatest pain which the subject can tolerate (Finocchietti et al., 2011). The subject presses a push button twice: on the first push, the PPT is recorded, and on the second push the PPTO is registered and the stimulation is stopped. Additionally, the subject rates the pain intensity continuously during the pressure stimulation on an electronic visual analogue scale (VAS 0-10 cm) where 0 indicated "no pain", and 10 indicated "maximal pain" (Graven-Nielsen et al., 2004). The pressure vs. VAS function can be extracted to allow establishing how this curve can be modulated in response to pain and inflammation (**Fig 1**). Several studies have investigated the effects of cutaneous sensitization on pressure pain sensitivity. Among these studies, Gustorff and co-workers and Weinkauf and collaborators have concluded that UVB irradiation induced a decrease of PPT assessed using a handheld algometer (Gustorff et al., 2013; Weinkauf et al., 2013). Numerous studies, have also investigated the effect of deep-tissues sensitization on pressure pain sensitivity. Some of these studies using different pain models, reported that deep-tissues sensitization also induced a decrease of PPT (Binderup et al., 2010; Jensen and Norup 1992; Nie et al., 2009b).

The major limitation related to this technique is that the pressure algometry is non-specific since both receptors in the skin and in deeper structure can be activated at the same time by the pressure stimulation itself (Staahl and Drewes 2004). In order to minimize the effect of the shear strains on the skin, the probes are usually covered with a rubber disc so that the cutaneous nociceptor activation can be reduced.
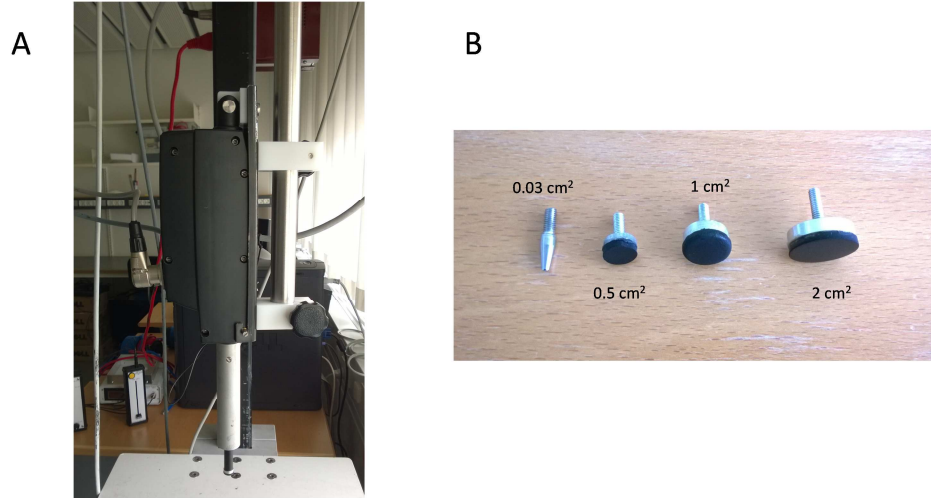
Figure 4. (A) The computer-controlled pressure algometer. (B) The four different probes used in the experiments.

6 Main findings

6.1 Cutaneous Blood Flow (BF)

In this project, the development of the inflammatory response in the skin following the application of the two different pain models was validated by laser Doppler imaging. In the first experiment, the change in cutaneous blood flow was validated after UVB irradiation of the skin, in the area of primary and secondary hyperalgesia (**Fig 5**).
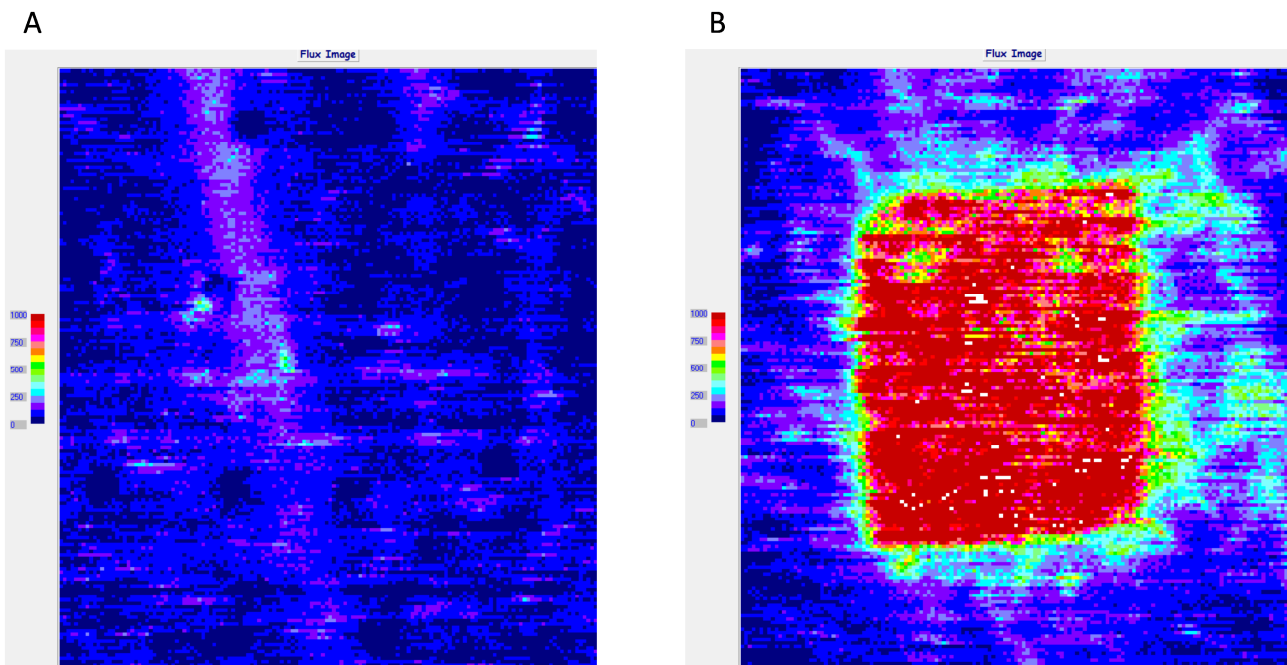
Figure 5. Images of cutaneous blood flow before (panel A) and after (panel B) UVD irradiation in the forearm of one subject. Data are taken from Study I.

Several studies have evaluated the change in blow flow following UVB irradiation. In previous studies, it was found that UVB irradiation induces an increase in cutaneous blood flow in the area of primary hyperalgesia, but conflicting results were found regarding the increase of blood flow in the area of secondary hyperalgesia (Benrath et al., 2001; Bishop et al., 2009). Bishop and collaborators (2009) reported increased BF in the area of irradiation but no observable vascular reactions in the area surrounding the UVB-irradiated site, whereas Benrath et al. (2001) found, in the forearm of

healthy volunteers a 10-fold increase in BF in the inflamed skin and a significant increase up to 1cm outside the irradiated area. In line with the findings from Benrath et al, in the first study of this PhD project an increase of 8-fold from baseline skin blood flow was found both in the arm and in the back and also a small but significant increase in skin blood flow up to 1.5 cm outside the irradiated area (**Fig 6**). It is believed that UVB inflammation releases a number of vasoactive inflammatory mediators acting directly on the vasculature (Angst et al., 2008). Several studies have also demonstrated that this response also involves a neurogenic component as repeated capsaicin administration prior to UVB irradiation can reduce or even block this reaction (Benrath et al., 2001; Benrath et al., 1995; Rukwied et al., 2008). Recent studies have found an increase in production of nitric oxide (NO) by keratinocytes following UVB irradiation, showing that NO may also have an important role in inducing vasodilatation and erythema after irradiation (Clydesdale et al., 2001). In fact, Rhodes and collaborators used microdialysis technique in the UVB irradiated skin and confirmed that NO is one of the most important inflammatory mediators released that can directly be involved in the skin response to UVB irradiation (Clough and Church 2002; Rhodes et al., 2001).

In Study II of the PhD project, the change in skin perfusion was evaluated in the skin after UVB irradiation and eccentric activity. The results of that study confirmed that UVB induced a significant increase in vasodilatation in the irradiated area and up to 2 cm outside the UVB irradiated area. The same study (II), also reported a small increase in blood flow in the DOMS area up to 2 cm away from the irradiated site, probably caused by the muscular inflammatory process induced by DOMS. As already mentioned, the release of inflammatory markers and neutrophils as a consequence of the muscle traumas induced by eccentric activity might be the cause of this increase in cutaneous blood flow.

Data collected when combining the two pain model (UVB and DOMS), showed an increase in blood flow which was higher than values found in the DOMS site alone. This result might be a direct consequence of a potentiation of the responses when the two models are applied in combination. Moreover, when combining the two models, the increase in blood flow was smaller than the increase found in the UVB area, where only the irradiation was applied. It is possible to speculate that this result may be due to difference in cutaneous perfusion between lower and upper part of the back.
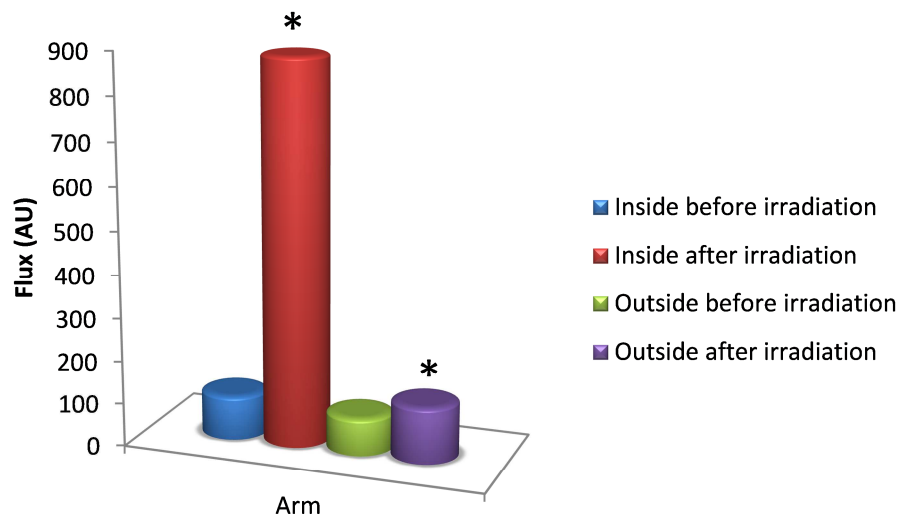
Figure 6. Mean (± SEM, N = 16) skin blood flow in arbitrary units (AU) before and 24 h after UVB irradiation inside and outside the area irradiated on the arm. Data taken from Study I.

6.2   Skin hyperalgesia to mechanical stimulation

In addition to the primary hyperalgesia, several studies have investigated the development of secondary hyperalgesia following cutaneous UVB irradiation using a wide range of different methodologies, for example, the 10 g von Frey filament used by Bishop and coworkers, the electronic   von Frey system used by Harrison and the 150 g rigid von Frey filament used by Gustorff and collaborators (Bishop et al., 2009; Gustorff et al., 2004b; Gustorff et al., 2013; Harrison et al., 2004). All these studies found contradictory results leading to the conclusion that the type of filament used in the assessment of skin hyperalgesia, might influence the outcome.

In this PhD project a pronounced cutaneous mechanical hyperalgesic area was present within the irradiated area 24 h after irradiation probably caused by the activation of Aδ and C fibres as a direct consequence of the release of pro-inflammatory mediators after tissues injury. This finding related to the existence of an area of primary hyperalgesia after UVB irradiation, is in agreement with the studies carried out by Bishop and Harrison (Bishop et al., 2009; Harrison et al., 2004). Moreover, in agreement with the finding of Gustorff and collaborators, an area of secondary hyperalgesia was demonstrated outside the irradiated area using weight-calibrated pin-pricks (Gustorff et al., 2004b; Gustorff et al., 2013). These findings are shown in **Fig 7**.
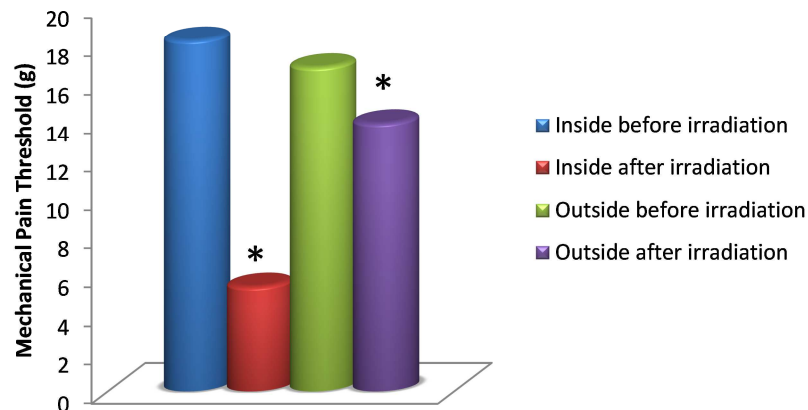
Figure 7. Mean (± SEM, N = 16) pin-prick pain thresholds (weight calibrated pin-prick instrument) before and 24 h after UVB irradiation inside and outside the area irradiated in the back. Data taken from Study II.

Based on the finding of this study and in the light of the results provided by other authors it is possible to conclude that in the assessment of skin hyperalgesia, the assessment modality used is highly important. This can explain why Bishop et al. (2009) could not map any area of secondary hyperalgesia after UVB irradiation. The light 10 g von Frey filament used in their study was not the optimal choice for demonstration of secondary hyperalgesia since this filament is not painful in normal skin. Moreover, in the assessment of mechanical hyperalgesia, the pin-prick represents a better choice with respect to the Von Frey hairs since the pressure exerted by the pin-prick stimulator is only related to the calibrated weight of the stimulator with no bias caused by the force applied by the investigator.

In a recent study by Rössler and collaborators, it was demonstrated that after UVB irradiation, the area of secondary hyperalgesia to pin-prick was not reduced by application of local anesthetics, suggesting the influence of a centrally-mediated mechanisms (Rössler et al., 2013).

When combining the UVB model with eccentric activity in the underlying muscle, the magnitude of decrease in pin-prick thresholds found in the location where the two models were combined presented no difference compared with the decrease found in the UVB location suggesting that the decrease in mechanical pain threshold could only be due to the effect of cutaneous UVB inflammation without any convergent facilitation or potentiating effect between skin and deep tissues hyperalgesia.

6.3   Pressure pain sensitivity by pressure algometry

Pressure algometry has been used for decades for the assessment of sensitivity to pain. In the first experiment the pressure pain sensitivity was evaluated after UVB irradiation of the skin. As showed for the mechanical pain threshold, the UVB irradiation induces an area of primary and secondary hyperalgesia to pressure stimuli (**Fig 8**). In this PhD project it was also hypothesized that the reduction in pressure pain thresholds both inside and outside the irradiated area may be influenced by sensitized responses of deep-tissue nociceptors. Anyway, no difference was found between the decrease in PPT in the UVB area and the decrease found in the area where the UVB and DOMS models were applied in combination. These findings suggest that the decrease in PPT might be only due to the cutaneous UVB inflammation and that DOMS does not have any effect on the UVB-related cutaneous hyperalgesia. A possible explanation for the lack of DOMS effect on PPT can be found in the model itself as already mentioned - it does not induce an equal level of muscle soreness in all subjects or the exercises protocol applied in this study did not induce an adequate level of muscle hyperalgesia.
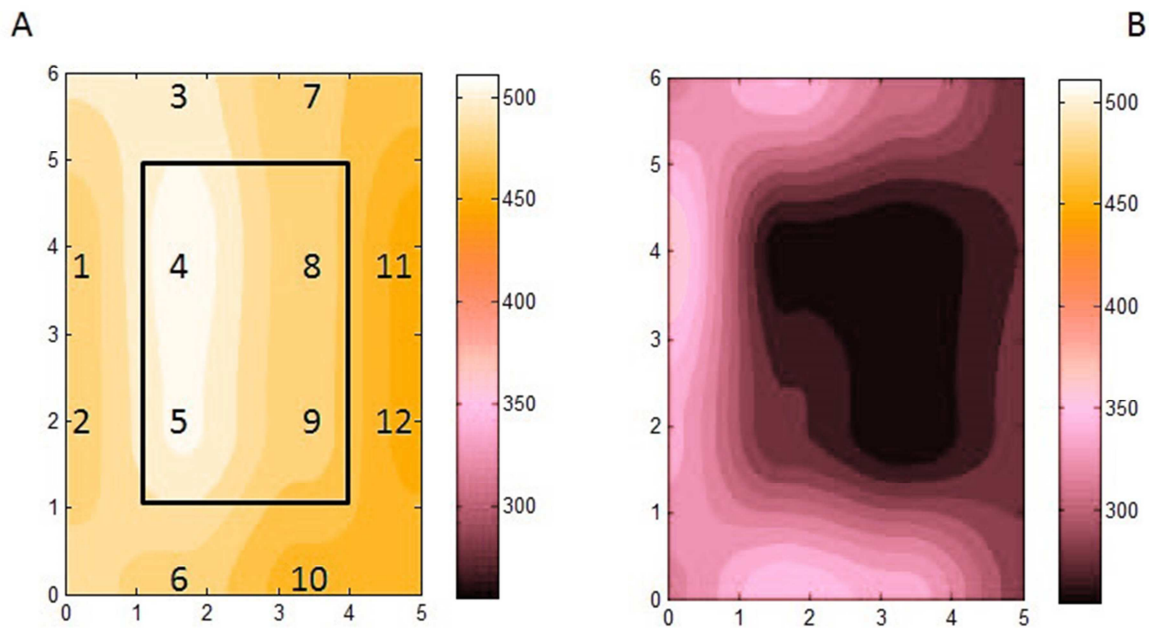
Figure 8. Pain pressure threshold maps before (A) and after (B) irradiation in the arm. The numbers indicate the points of stimulation, with points 3 and 7 are oriented towards the wrist, and points 6 and 10 towards the elbow. The maps are based on interpolated mean values (N = 16) from assessments with the 0.5 cm^2 flat probe. Data taken from Study I.

6.3.1 The effect of probe design on skin and deep-tissues

It is well known that probe shape and diameter are important factors in the assessment of pressure pain. Using computer models, previous studies demonstrated that probes with surface area larger than 1cm^2 can give a better evaluation of pain threshold in deep tissues, as the activation of muscle nociceptors is related to the deformation of the muscle tissue (Finocchietti et al., 2011; Takahashi and Mizumura 2004; Takahashi et al., 2005). In this PhD project four probes, different in shape and diameter, have been used: a flat tip probe with a diameter of 2.0 cm^2, a flat tip probe with a diameter of 1.0 cm^2, a flat tip probe with a diameter of 0.5 cm^2, and a V-shaped probe with a flat contact surface of 0.03 cm^2, the last one specially designed to elicit cutaneous pressure pain (**Fig 4B**). In order to minimize the painful skin stimuli coming from the sharp contact edges of the 2.0 cm^2, 1.0 cm^2, 0.5 cm^2 probes, these probes are covered with a rubber disc (Finocchietti et al., 2011). In Studies I and II it was found that the PPTs detected with the smallest probe (0.03 cm^2) were smaller than the thresholds detected with the other three larger probes (2.0 cm^2, 1.0 cm^2, 0.5 cm^2)

when the data are not adjusted for the probe area. Those findings suggest that with small probes less absolute force is required to induce pain than using probes with a large diameter ($> 1 \text{cm}^2$). This is in line with the findings by other authors reporting increasing threshold with increasing probe size (Greenspan and McGillis 1991; Jensen et al., 1986). Moreover, the decrease after UVB irradiation in PPT and PPTO using 2 cm^2 and 1 cm^2 probes suggests that the use of larger probes may lead to activation of both skin and deeper tissues nociceptors after induction of hyperalgesia or that these probes also stimulate the skin. This is in line with the findings reported by several studies where the authors tried to eliminate the contribution of the skin to pressure stimulation by inducing cutaneous anaesthesia (Graven-Nielsen et al., 2004; Kosek et al., 1999; Laursen et al., 1997). In all these studies, the authors concluded that also the inputs coming from the skin are involved in the assessment of pressure pain sensitivity in deep tissues.

6.3.2 Stimulus-Response Curve (SR curve)

The stimulus-response functions is a curve where graded pressure stimulations is related to pain intensity recorded trough a VAS. Most pressure algometry studies have not assessed the complete stimulus-response function. This PhD study is the first to assess how the pressure-intensity versus pain-intensity function is modulated in response to skin irradiation alone or in combination with DOMS-induced deep tissue soreness (Studies I and II). The data reported in Study I illustrated that, with all probes, 24 h after UVB irradiation, the stimulus response curve showed a left-shift and an increased slope both within and outside the UVB-irradiated area compared with baseline recordings with the most pronounced left-shift within the irradiated area (**Fig 9**). The left-shift of the stimulus-response curve results in lowering of the pain thresholds (allodynia) and an increase in pain to suprathreshold stimuli (hyperalgesia, **Fig 1**). This confirms the presence of areas of primary and secondary hyperalgesia to mechanical pressure stimuli induced by UVB irradiation. The Study I also revealed that the probe shape has an influence on the SR slope. In fact the analysis of the slope for the V-shaped probe suggests that the subjects raised the VAS scale faster with the smallest probe than with the other three probes both inside and outside the irradiated area. A possible explanation is that the cutaneous sensitization is more affected by the V-shaped probe than others probes. The data collected in the second experiment indicated that DOMS alone induced a left-shift of the stimulus-response curve and an increased slope, 24 h after eccentric exercises, but no additive effects were reported when combining the UVB and DOMS models together. The stimulus-

response function is based on the pain intensity recorded by VAS scale. The VAS scale is also one-dimensional without any information about pain quality. For this reason, in future studies it could be useful to use McGill pain questionnaire or other descriptors of pain to assess also the quality of pain besides its intensity.
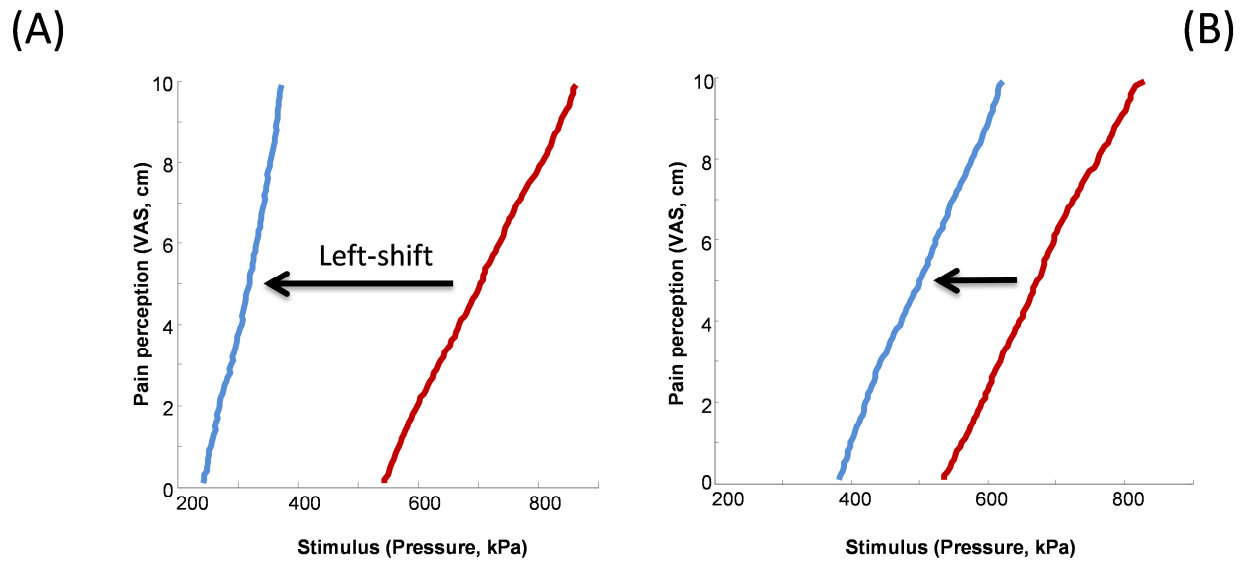
Figure 9. Pressure intensity versus VAS scores inside (A) and outside (B) the irradiated area on the back. The data are taken from Study I and the stimulus-response curves are based on the average of 16 subjects from assessments with the 0.5 cm^2 probe. Before irradiation (red curve), the VAS score increases moderately fast with increasing pressure stimulus intensity. After irradiation (blue curve), the stimulus-response curve is shifted to the left; indicating that a lower pressure is necessary to induce pain and an increased response to suprathreshold stimuli.

6.3.3 Temporal summation of pain

Temporal summation of pain (TSP) is the progressively increase of VAS score in response to repetitive pressure stimulations of the same strength and is thought to mimic the wind-up process occurring in dorsal horn neurons. This is a mechanism where the sensitivity of the neurons in the dorsal horn to C-fibre input can be intensified by repetitive stimulation (Hayashi et al., 2013; Mendell and Wall 1965). TSP to repetitive pressure stimulations has been used in skin and deep

tissues with electrical, mechanical and thermal stimuli (Arendt-Nielsen et al., 1994; Nie et al., 2009a; Staud et al., 2003) and in patients with chronic musculoskeletal pain it has been demonstrated that TSP is facilitated compared with healthy controls (Arendt-Nielsen et al., 2010; Staud et al., 2003). The TSP indicates that the increasing pain perception after repetitive stimulation having the same intensity is due to a central mechanism (Arendt-Nielsen et al., 1994; Nie et al., 2009b; Staud et al., 2003). In this PhD project, the TSP was evaluated using a sequential stimulation of 10 pressure stimuli, all equal to the subjective pain threshold. After the recording, the VAS score corresponding to each stimulus was extracted. In order to show the pure effect of TSP following the 10 stimuli, the values were normalized by subtracting the first VAS score from all the 10 values recorded. In the second study, it was found that UVB irradiation induced facilitated temporal summation in the irradiated area (**Fig 10**). It was also found that TSP was not affected by DOMS. This result might be explained, as already mentioned, by the lack of effect of the exercises protocol applied and is in contrast with several studies reporting facilitated temporal summation after eccentric activity (Bajaj et al., 2000; Nie et al., 2006). Moreover, no additional increase in TSP was found in the UVB+DOMS location compared to the UVB location suggesting that the phenomenon of TSP might be only due to the central effect induced by UVB-model (**Fig 10**). The presence of temporal summation induced by UVB irradiation can be considered of highly importance when testing analgesics having central effects.
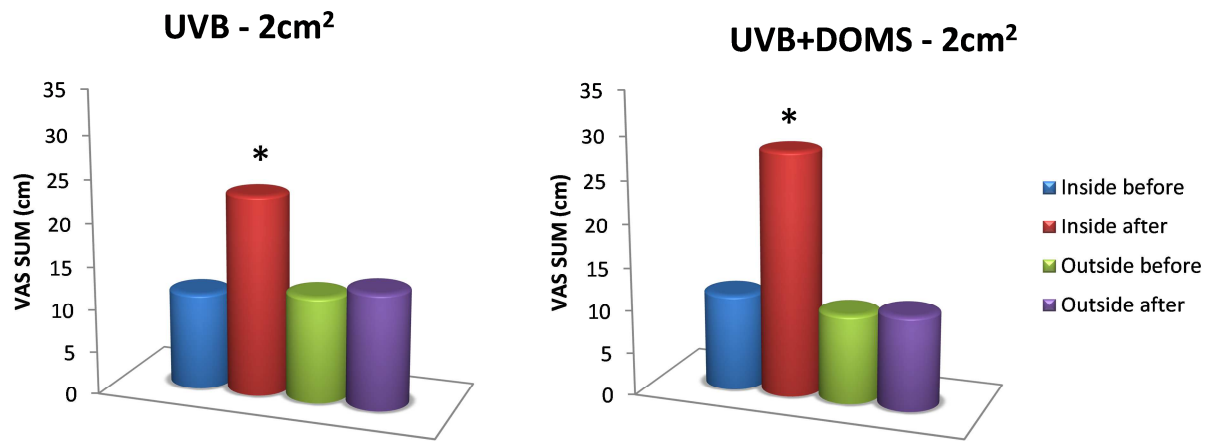
Figure 10. Mean (± SEM, N = 16) of the sum of the VAS scores (VAS sum) recorded before and after irradiation/exercise assessed with the 2 cm^2 flat probe. The VAS scores have been normalized by subtracting the first VAS score from all the 10 values and then the VAS sum was calculated. Data taken from Study II.

6.4   Effect of local cutaneous anesthesia on the UVB- inflammatory model

In order to understand whether the effect of UVB irradiation on both areas of primary and secondary hyperalgesia is of peripheral or central origin, the effect of topical anesthetic application on the UVB-induced primary and secondary hyperalgesia was evaluated. EMLA cream was applied 24 h after UVB irradiation. The EMLA anesthetic cream is a mixture of equal parts of lidocaine and prilocaine (1 g of cream contains 25 mg of lidocaine and 25 mg of prilocaine) and is usually used in clinical practice during surgery to decrease pain from cutaneous procedure in both adults and children (Taddio et al., 1998). It has also been proved that EMLA cream induces analgesia on normal skin and also in skin affected by dermatological conditions such as hyperhidrosis or postherpetic neuralgia (Lycka 1992). A study conducted by Arendt-Nielsen and collaborators, reported that the depth of analgesia induced by topical application of EMLA cream is about 5 mm (Arendt-Neilsen et al., 1989). It has been also proved that several factors may influence the anesthetic effect of the EMLA, such as the anatomical region where the cream is applied and the thickness of the skin (Arendt-Neilsen et al., 1989; Bjerring and Arendt-Nielsen 1990; Nielsen et al., 1992). In order to be effective, after its application the EMLA cream has to be covered in an occlusive dressing. Several studies have reported erythema and skin pallor after application of EMLA under occlusive dressing probably as a consequence of vasoactive properties of both lidocaine and prilocaine (Tahir et al., 2006). The results of Study I showed an increase in cutaneous pin-prick threshold following application of EMLA cream in uninjured skin, demonstrating that EMLA cream is able to induce hypoalgesia in intact skin. Results (Study I) also demonstrate that EMLA cream does not have anesthetic effect on the PPT or on the pin prick hyperalgesia on the UVB+EMLA area. A possible explanation of those findings can be related with the timing of application of the anesthetics. In this experiment the EMLA cream was applied on top of the irradiated area only 24 h after irradiation, when the UVB-induced hyperalgesia was already well-established. This may indicate that EMLA cream is only able to prevent the development of hyperalgesia if applied prior intervention, but it is not able to reverse the hyperalgesia after its full establishment. The most likely explanation is that the EMLA cream does not block the skin receptor once they have been sensitized. This is in line with the results of the study conducted by Rössler and collaborators in healthy volunteers (Rössler et al., 2013). In their study they concluded that the presence of mechanical hyperalgesia in the irradiated area was not altered by peripheral afferent blockade with local lidocaine anesthetic agent at 4 h after UVB irradiation (Rössler et al., 2013). It

is also possible that the skin works as a barrier to the cream diffusion making difficult to understand the amount of EMLA cream that actually reaches the nociceptors.

Study I also revealed unchanged pressure pain sensitivity after anesthetic application. This is in line with different studies reporting unchanged or even decreased PPT after EMLA administration (Graven-Nielsen et al., 1998; Graven-Nielsen et al., 2004; Kosek et al., 1995; Laursen et al., 1997). This result may indicate that also the deeper-structures beneath the UVB-irradiated skin could be sensitized possibly through a convergence of mechanisms involving both skin and deep tissues somatic nociceptors.

6.5   Interaction between Skin and Deep Tissues

The phenomenon of interaction between skin and deep tissues can be explained with the convergence-projection model, where afferent fibres coming from different areas as muscle and skin may make synaptic contact with the same dorsal horn neuron (Graven-Nielsen 2006; Hoheisel and Mense 1990). The existence of a possible convergence between afferent nociceptive fibres from superficial and deep structures has been already demonstrated in several studies (Hoheisel and Mense 1990; Yu and Mense 1990).

What is still unknown and what was evaluated in the second and in the third studies of this PhD project was the manifestation of a possible convergence between deep and superficial tissues when those inflammatory models of skin and muscle were applied in combination. It was hypothesized that when both models were applied, the increase in sensitivity will be superior with respect to the individual reactions.

The data reported after combination of cutaneous and deep tissue pain models in Study II indicates possible additive effect between the two models mediating the neurogenic inflammation, since the increase in blood flow in the area where the two models were applied in combination was higher than the increase present in the DOMS site (**Fig 11AB**), whereas no additive effect between the two models was present on data regarding mechanical hyperalgesia (**Fig 11CD**). These findings suggest that different mechanisms of neuronal convergence between deep and superficial somatic tissues might mediate processes of neurogenic inflammation and hyperalgesia.
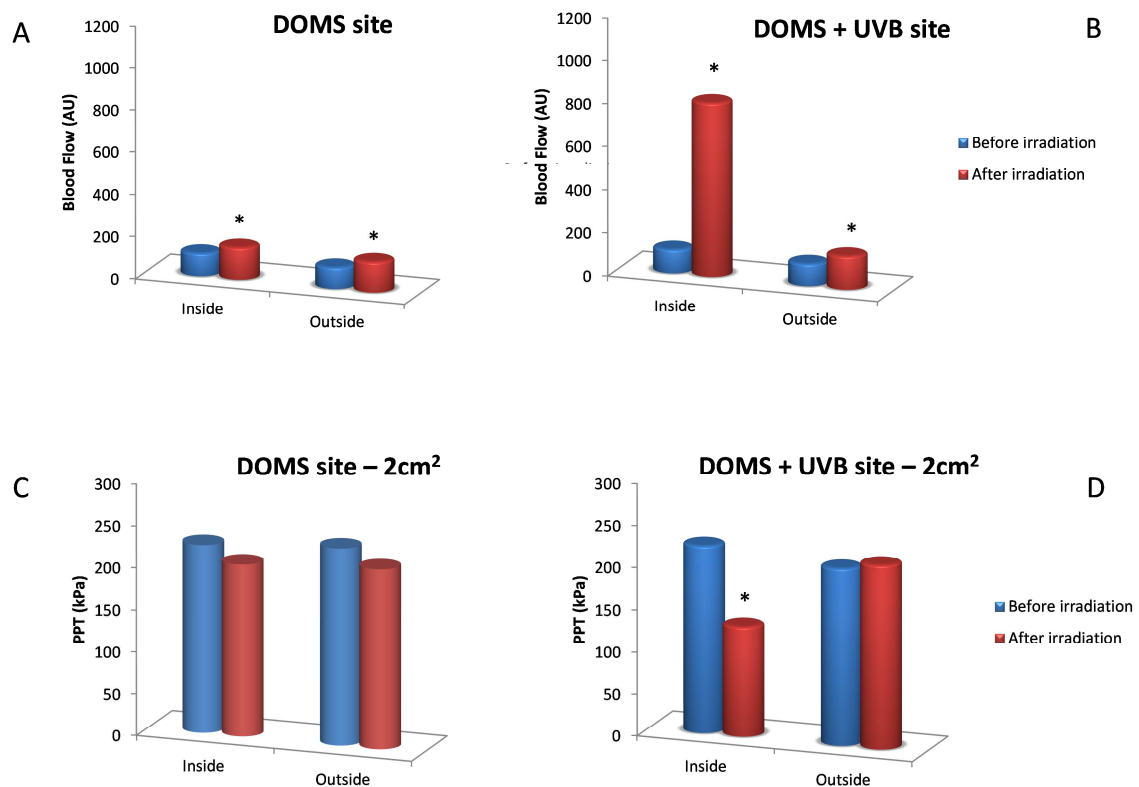
Figure 11. Mean (± SEM, N = 16) of blood flow (AB) and pressure pain threshold (CD) recorded before and after exercise and irradiation/exercise. The PPT has been assessed with the 2 cm^2 flat probe. Data taken from Study II.

6.6 UVB-induced areas of allodynia and hyperalgesia

The UVB model is one of the best characterized models of peripheral sensitization, since it induces primary hyperalgesia peaking between 24 and 48 h both in animals and humans (Weerasinghe et al., 2014). However, the existence of secondary hyperalgesia as a consequence of UVB irradiation has always been controversial and has been confirmed only by some groups (Davies et al., 2011; Gustorff et al., 2013; Weerasinghe et al., 2014). In different studies, conducted on both rats and humans, the authors agreed that the UVB-model is able to induce both areas of secondary allodynia and hyperalgesia through induction of central sensitization (Davies et al., 2011; Gustorff et al., 2013; Weerasinghe et al., 2014). The findings of Study III are in line with the results of these groups and demonstrated that UVB irradiation of the skin induces primary areas of allodynia and

hyperalgesia lasting up to 3 days, caused by the sensitization of peripheral nociceptors in the skin. Moreover, the UVB model also induced secondary areas of allodynia and hyperalgesia probably due to sensitization of dorsal horn neurons (**Fig 12**). The presence of secondary hyperalgesia after UVB irradiation is important to clarify the neural mechanism underling inflammatory pain and to better understand all the aspects of chronic pain.
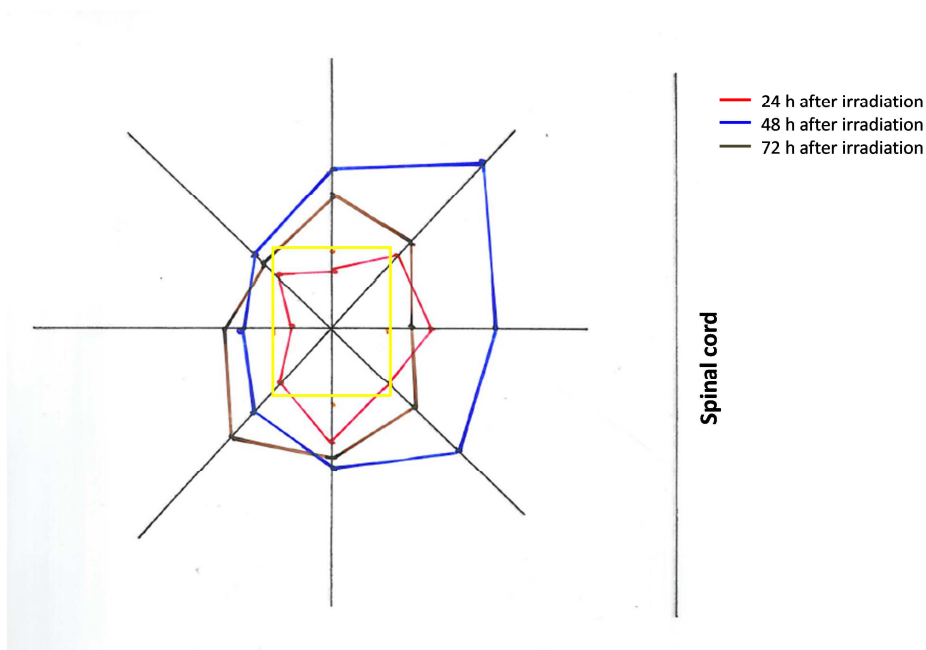
Figure 12. Example of mapping of the area of secondary allodynia 24, 48 and 72 h after irradiation in the back. The yellow square indicates the irradiated area, and the areas of allodynia have been mapped using a single 23 g von Frey filament. The data refers to a single subject and has been taken from Study III.

6.7   Effect of heat rekindling in UVB-irradiated skin and NGF-sensitized muscle

In the third study, the effect of heat rekindling on the areas of secondary allodynia and hyperalgesia induced by UVB irradiation was also investigated. It was found that application of mild heat to an already UVB-sensitized skin can maintain or facilitate allodynia and hyperalgesia for a longer period as showed on both panels of **Fig 13**. These results are in line with the finding of studies investigating the effect of the UVB and heat rekindling (UVB/HR) model in animals. The study

conducted by Davis and collaborators in rats provides evidence that the secondary hyperalgesic area induce by UVB-irradiation in the plantar hind paw was enhanced and prolonged by heat rekindling (Davies et al., 2011; Weerasinghe et al., 2014). In the most recent animal study, conducted by Weerasinghe and collaborators, the authors concluded that the increase of the area of UVB-induced secondary hyperalgesia after rekindling may be due to a more prolonged C-fiber input to the spinal cord induced by heat (Weerasinghe et al., 2014). The same conclusions have been shown by similar studies conducted in humans. Eisenach and co-workers investigated the effect of the cyclooxygenase inhibitor ketorolac on the UVB/HR model (Eisenach et al., 2010). In their study they concluded that heat rekindling increased the area oh hyperalgesia induced by UVB-irradiation and this process can be reversed by intravenous ketorolac administration (Eisenach et al., 2010). In conclusion, the UVB/HR model can be considered a valid tool for screening compounds having long lasting action.

The third study also investigated whether the addition of muscle sensitization to a previous UVB-sensitized skin could enhance or modified the rekindling effect. In order to avoid mechanical stretching of the irradiated skin caused by the eccentric contractions, the NGF model was used to induce deep tissues hyperalgesia. In this study it was hypothesized that the responsiveness to mechanical stimulation could be enhanced by a possible convergence mechanism between superficial and deep tissue sensory input (**Fig 14**). However, the data collected did not support this hypothesis since the muscle soreness induced by NGF did not have any effect on the UVB-induced areas of allodynia or to heat rekindling. These results can be due to the strong UVB effect on the skin masking the effect of the NGF (Study III). In the third study, the NGF was injected 24 h after UVB irradiation, when the UVB inflammation was full established. Modifying the time line of the study and performing both models the same day may have led to different results.
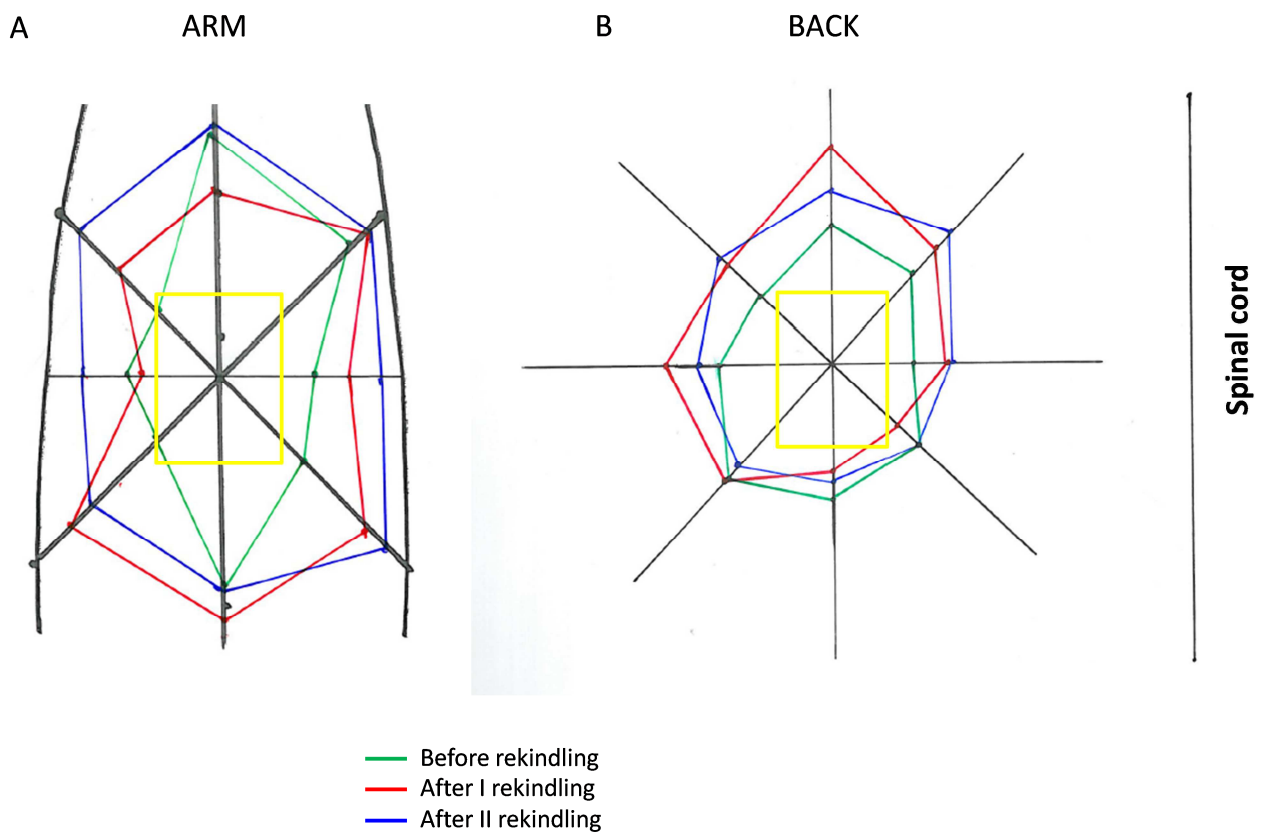
Figure 13. Examples of mapping of area of secondary hyperalgesia (A) and allodynia (B) at baseline (72 h after irradiation) and after the I and the II heat rekindling in the arm and back respectively. The yellow square indicates the irradiated area. The areas of hyperalgesia have been mapped using a pin prick corresponding to the subject threshold, whereas the area of allodynia has been mapped using a single 23 g von Frey filament. The data refers to a single subject and has been taken from Study III.

Figure 14. Examples of expected timing regarding the increase of the allodynic area after heat rekindling combined with UVB irradiation (A), NGF injection (B) or both models (C), respectively.

6.8 Summary and conclusions

Ultraviolet-B irradiation model has been used for many years as a translational model since it is known to induce cutaneous inflammatory pain in both animals and humans. The aim of this PhD-project was to evaluate time course and sensory changes following 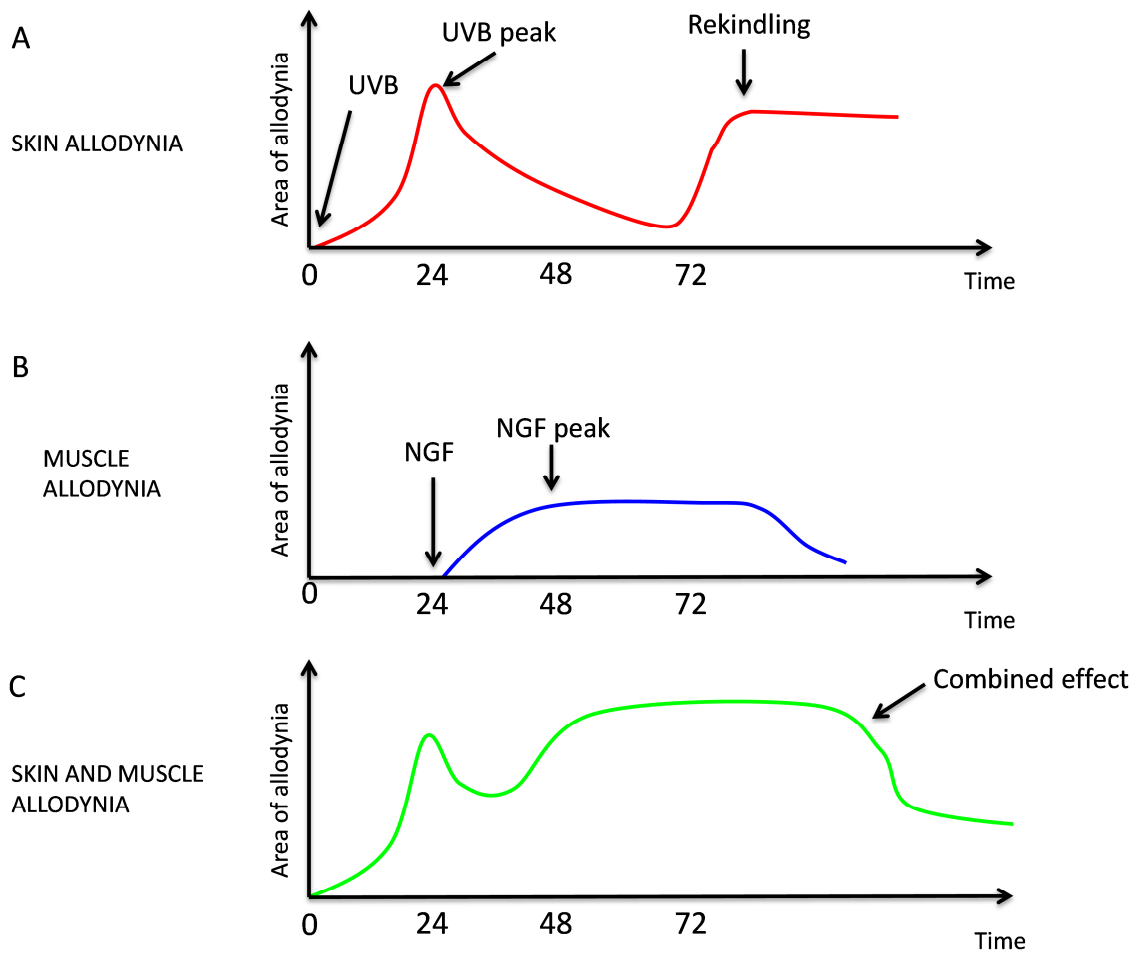UVB irradiation by addressing the following points: 1) evaluate the change in vasomotor response and pain sensitivity inside and outside the irradiated skin, 2) investigate the existence of cumulative effects of cutaneous and deep-tissue hyperalgesia, 3) investigate the UVB-induced mechanical hyperalgesia and allodynia after application of additional heat stimuli and if the response of the UVB-model to heat may be modified by sensitization of deep tissues.

The results of this PhD project demonstrated that UVB irradiation of the skin provokes cutaneous primary hyperalgesia to mechanical and pressure stimulations, probably due to sensitization of peripheral nociceptors caused by the inflammatory mediators released after irradiation. Moreover, UVB irradiation induced an area of secondary hyperalgesia. The cutaneous-UVB model also induced an inflammatory reaction leading to an increase in cutaneous blood flow (Studies I and II). The same studies also confirmed that UVB irradiation induced a left-shift of the stimulus-response curve, relating pressure stimulation to pain intensity, and facilitation of temporal summation to repetitive pressure stimuli. Administration of topical anesthetic cream 24 h after irradiation did not have any effect on mechanical hyperalgesia on the UVB-irradiated area and this lacking effect could be due to the fact that the anesthetic used, namely the EMLA cream, might not block the skin receptor once they have been sensitized (Study I).

In Study III, it was found that the UVB model induces long and stable primary areas of allodynia and hyperalgesia up to 72 h after skin irradiation. Both these areas can be also enhanced and prolonged by application of mild heat (heat rekindling, Study III). Moreover, it was also found that muscle hyperalgesia induced by NGF injection, does not suppress or potentiate the effect of UVB irradiation or heat rekindling on the areas of allodynia and hyperalgesia (Study III).

The combination of cutaneous hyperalgesia (UVB model) and muscle hyperalgesia (DOMS) in the same area showed that muscle hyperalgesia may enhance the UVB-induced vasodilatation (Study II). The same study also confirmed that muscle hyperalgesia induced by DOMS does not induce any change in UVB-induced cutaneous hyperalgesia.

In conclusion, the UVB-model can be considered a long-lasting and non-invasive experimental model of primary and secondary hyperalgesia, and a valid tool for screening of new analgesic

medications, active at both peripheral and central level. Moreover, it can also be used in pain research as a translational model, from animals to healthy subjects.

6.9   Perspectives and Implications

The results of this PhD project will help in validating the UVB model as a non-invasive and prolonged human experimental model that can be used for development and testing of new analgesic medications. Moreover, the finding related to the induction of primary and secondary hyperalgesia underline the importance of the UVB model as a tool for screening of new drugs having different mechanisms of action. In the longer perspectives it may lead to the development of better treatment regimens and thus a more effective treatment of patients with acute and chronic pain. In order to test the prophylactic effects of an anti-inflammatory drug, the measurement of biomarkers in serum samples could represents a valid tool in the future for testing the mechanism of action of tested drugs. Moreover, in order to clarify the involvement of central mechanisms in the UVB irradiation model, the future studies are recommended to test whether the administration of an anti-inflammatory drug prior irradiation can block the development of allodynia and hyperalgesia in skin.

7 Dansk Sammenfatning

I mange år har ultraviolet-B (UVB) bestrålingsmodellen været anvendt som en translatorisk model, idet den er kendt for at kunne inducere inflammatorisk kutansmerte hos både dyr og mennesker. Formålet med denne ph.d.-afhandling var at vurdere tidsforløbet og de sensoriske ændringer efter UVB-bestråling ved hjælp af følgende: 1) Evaluere ændringen i det vasomotoriske respons og smertefølsomheden i og uden for det bestrålede hudområde, 2) Undersøge akkumulerede effekter af hyperalgesi og i overfladisk og dybt væv, 3) Undersøge UVB-induceret mekanisk hyperalgesi og allodyni efter påføring af yderligere varmestimuli samt om UVB-modellens respons til varme kan modificeres ved hjælp af sensibilisering af dybt væv.

Resultaterne af afhandlingen viste, at UVB-bestråling af huden udløser primær kutan hyperalgesi til mekanisk stimulation og trykpåføring; sandsynligvis grundet den sensibilisering af de perifere nociceptorer, der forårsages af de inflammatoriske mediatorer, som frigives efter bestrålingen. Endvidere resulterede UVB-bestrålingen i et område med sekundær hyperalgesi. Den kutane UVB-model forårsagede også en inflammatorisk reaktion, som førte til en stigning i den kutane blodgennemstrømning (Studie I og II). Disse studier bekræftede også, at UVB-bestråling forårsager en venstre-forskydning i stimulusrespons-kurven der relaterer trykstimulation til smerteintensitet og facilitering af temporal summation af smerte til gentagne trykstimuli. Påføring af bedøvende creme 24 timer efter bestrålingen havde ingen virkning på den mekaniske hyperalgesi i det UVB-bestrålede område; denne manglende virkning kan skyldes, at den anvendete creme, nemlig EMLA-creme, måske ikke blokerer receptorerne i huden tilstrækkeligt, når de er sensibiliseret (Studie I).

Studie III viste, at UVB-modellen påfører stabile primære områder med allodyni og hyperalgesi i op til 72 timer efter bestråling af huden. Virkningen i begge disse områder kan også forstærkes og forlænges ved påføring af let varme (genfremkaldes vha. varme, Studie III). Endvidere viste det sig, at eksperimentel muskelhyperalgesi induceret vha. NGF-injektioner hverken dæmper eller forøger effekten af UVB-bestråling eller genfremkaldelsen i områder med allodyni og hyperalgesi (Studie III).

Kombinationen af kutan hyperalgesi (UVB-modellen) og muskulær hyperalgesi (DOMS) i samme område viste, at muskelhyperalgesi kan forstærke den UVB-inducerede kardilatation (Studie II). Dette studie bekræftede også, at muskelhyperalgesi, som induceres vha. DOMS, ikke forårsager ændringer i UVB-induceret hyperalgesi i hud.

Det konkluderes, at UVB-modellen kan ses som en langtidsvirkende og non-invasiv eksperimentel model for primær og sekundær kutan hyperalgesi og som en interessant model til screening af ny smertestillende medicin virkende på både perifert og centralt niveau, herunder som en translatorisk model fra dyr til raske forsøgspersoner.

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