Spotting pain in the brain. Towards a useful animal model of pain

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LUND UNIVERSITY
Faculty of Medicine

Spotting pain in the brain

Towards a useful animal model of pain

by

Tanja Jensen

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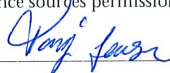
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Spotting pain in the brain - Towards a useful animal model of pain

Abstract

To understand how the brain and spinal cord processes nociceptive information and how this processing changes during long term nociceptive input, valid animal models of pain are needed. The main aim of the present thesis was to develop a translational animal model based on cortical recordings for assessing pain related mechanisms. To this end, focus was put on evaluating changes in nociceptive transmission to the primary somatosensory cortex (SI). A comparison with changes in nocifensive responses, commonly used to assess pain related mechanisms, was also made.

It is demonstrated that both a sedative and an analgesic compound can inhibit CO_2 laser C fibre evoked potentials (LCEPs) in SI and that by adjusting for effects on the electroencephalogram, sedative effects can be differentiated from the analgesic effects in anaesthetized rats.

Following induction of hyperalgesia with UVB-light, LCEPs from both the primary and the secondary hyperalgesic skin was significantly increased in awake animals. In anaesthetised animal, LCEPs increased from secondary hyperalgesic skin. The opiate tramadol counteracted the UVB induced changes. From recordings in awake animals using implanted multichannel electrodes this hyperalgesia was found to peak the first day and then decline over 2-14 days. Nocifensive responses increased from the primary hyperalgesic skin and occurred later than the changes of LCEP, indicating that pathways to motor circuits and sensory circuits differ substantially. These findings suggests that multichannel electrodes implanted in rat SI offer a more valid test for hyperalgesia in rats than conventional models based on behavioural measurements.

Key words:	Analgesics, Freely moving rat, Microelectrode, Nociception, Pain, Primary somatosensory cortex, Sedatives

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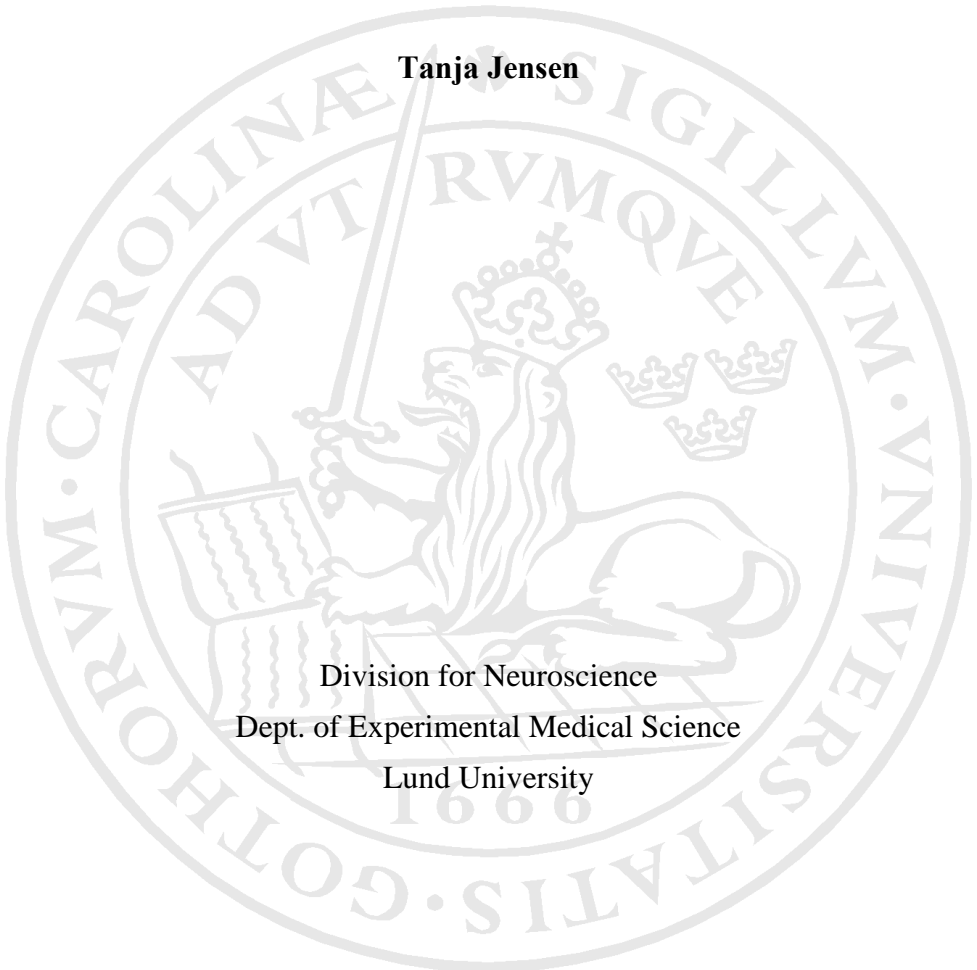
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Spotting pain in the brain
Towards a useful animal model of pain

By

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"There´s a crack in everything
- that´s how the light gets in"
Leonard Cohen

Table of Content

Original papers

This thesis is based on the following papers, which are referred to by their roman numerical in the text:

I   Granmo M., Jensen T., Schouenborg J. The effect of sedation and analgesia on nociceptive transmission to the rat primary somatosensory cortex (SI). *Submitted to Eur J Pain*

II   Jensen T., Granmo M., Schouenborg J. (2010) Altered nociceptive C fibre input to primary somatosensory cortex in an animal model of hyperalgesia. *Eur J Pain*, **15**, 368-375

III   Jensen T., Ljungquist B., Etemadi L., Lind G., Garwicz M., Thelin J., Petersson P., Schouenborg J. Nociceptive input to primary somato-sensory cortex in awake rats during development of hyperalgesia – a comparison to behavioural readouts. *Preliminary manuscript*

Populärvetenskaplig sammanfattning

På apoteket hittar vi den ena burken efter den andra med olika sorters smärtstillande medicin. Hur många av dessa är verkligen effektiva mot ihållande och svår smärta?

Många receptbelagda läkemedel som lindrar smärta förändrar samtidigt vårt beteende, till exempel vår förmåga att fatta beslut. De kan dessutom också orsaka slöhet och onormal koordination. För att kunna tillverka effektiva läkemedel mot smärta behöver vi därför först förstå hur smärta fungerar. Vi måste veta att det är en smärtsignal vi mäter och även hur smärtsystemet bearbetar den.

En metod som ger information om hur hjärnbarken (den yttre delen av hjärnan där upplevelsen skapas och som bl. a ger information om var och hur mycket det gör ont) bearbetar smärtsignaler i vaket tillstånd skulle ge ett mer lämpligt mått på smärta än vanliga reflextester. Vårt mål var att utarbeta en metod, där vi kunde mäta smärtsignalen i hjärnbarken på råttor och följa den före, under och efter en skada.

Många smärtforskare använder reflexer som ett mått på att något gör ont hos djur, men även en beröring kan utlösa en reflex. Detta är ett stort problem inom smärtforskningen och läkemedelstillverkningen. Vid tillverkning av läkemedel testar man ofta den medicinska effekten på just reflexer. Med elektroder på hjärnbarken kan vi däremot mäta aktiviteten i de nervceller som mottar signalen om en stimulering på huden och skilja på om den orsakas av beröring eller smärta.

Vi ville ta reda på om det är möjligt att även kunna särskilja smärtstillande läkemedels lugnande effekt från den bedövande, genom att mäta smärtsignalen i hjärnbarken med elektroder. Smärtsignalen uppstår när vi stimulerar råttornas ena baktass med en kort laserpuls som känns som ett nålstick.

Vi visar att det sannolikt går att särskilja på läkemedels lugnande och bedövande effekt om man samtidigt tar höjd för den sänkta hjärnaktiviteten, som är en konsekvens av lugnande läkemedel. Det visade sig att vi kunde kompensera för den lugnande effekten genom att sänka narkosnivån. Observationen skapar en förutsättning att skräddarsy läkemedel till att ge önskade smärtstillande egen-skaper.

Därefter undersökte vi om det är möjligt att mäta smärtsignalen i hjärnbarken från skadad hud. Vi undrade om signalerna i hjärnbarken återspeglar reflextester. Hälen

på råttornas ena baktass strålade vi med UV ljus för att skapa en solbränna med rodnad hud. För att kunna mäta smärtsignalen i vakna djur under en längre tid opererade vi in en elektrod i hjärnan. Elektroderna består av flera 18 mikrometer tunna vajrar. Det betyder att ungefär fem vajrar motsvarar tjockleken på ett hårstrå.

För första gången synliggör vi en läkningsprocess i hjärnbarken. Vi visar att signalerna från en stimulering på solbränd hud är större första dagen efter UV-strålningen än före skadan. Den ökade känsligheten sjunker kraftigt efter andra dagen och efter två veckor har signalen återgått till ursprungsläget. Skadan orsakar också större smärtsignaler i hjärnbarken från en stimulering på huden bredvid den solbrända. Där såg vi den största förändringen och tidsförloppet liknande det i skadad hud. Det är alltså inte bara skadan i sig som kan göra ont, utan även området vid sidan om.

En annan intressant upptäckt var att våra reflextester inte uppvisade samma tidsförlopp för förändringarna som signalerna från hjärnbarken gjorde. Dessutom såg vi den största skillnaden i det strålade området. Detta tyder på att reflexbanorna och de uppåtgående banorna troligtvis är organiserade på olika sätt med olika funktion.

Från ryggmärgen löper grovt sett två nervbanor - reflexbanor och uppåtstigande banor. De uppåtgående banorna gör oss medvetna om att något gör ont. När vi skadar oss, t ex trampar på en spik, skickas en signal i smärtnerverna via ryggmärgen till hjärnbarken. Men innan vi känner att det gör ont har vi reflexmässigt dragit undan foten.

Smärta är ett viktigt varningssystem som talar om att något kan skada kroppen. Men ibland fortsätter smärtsignaleringen även om skadan har läkt ut. Idag lider var femte svensk av långvariga smärtor pga. kroniska sjukdomar eller skador, som försvårar deras liv och kostar samhället 87 miljarder kronor om året. För att kunna hjälpa dessa personer behöver smärtforskare ett tillförlitligt sätt som kan mäta graden av smärta.

Studierna i denna avhandling är startskottet till en ökad förståelse för hur smärtsystemet signalerar i vakna individer. De har öppnat upp för möjligheter att följa de föränderliga förlopp som ligger till grund för långvarig smärta. Det i sin tur kan leda till framställningen av effektiva smärtstillande läkemedel och bättre behandlingar.

Abbreviations

AUC	area under curve
EEG	electroencephalogram
LCEP	CO_2 laser C fibre evoked potentials
LFP	local field potentials
SI	primary somatosensory cortex
UV	ultraviolet

Glossary and definition

- **Allodynia**

  Pain resulting from a stimulus that does not normally cause pain

- **Hyperalgesia**

  An increased response to a stimulus which is normally painful

    o **Primary hyperalgesia**

      Hyperalgesia at the site of injury

    o **Secondary hyperalgesia**

      Hyperalgesia in an area adjacent or remote of the site of injury

- **Nociception**

  Response to a noxious stimulus

- **Noxious stimulus**

  An incident of actual or potential tissue damage

- **Pain**

  "Unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such", as defined by International Association for the Study of Pain (IASP)

Summary

Current models of pain in conscious animals usually scores nocifensive responses. However, it is still unclear to what extent these responses are related to, for instance, the sensory discriminative or affective aspects of pain. This touches upon an intriguing question on how the nervous system processes nociceptive information in the conscious brain, a matter of which little is known. In order to illuminate how the nociception is processed, a suitable animal model for analysis on the conscious brain is essential.

In this thesis, we pursued to develop an animal model to illuminate how nociception is processed in primary somatosensory cortex (SI), which is likely to play an important role in processing sensory aspects of pain. As part of this, differentiating the antinociceptive outcome of drugs would clarify confounding sedative properties of drugs when assessing analgetic effects. Surface electrodes or ultrathin implantable electrodes were used to record the transmission to SI.

We show that both a sedative and an analgesic compound can inhibit nociceptive transmission to the cortex. Furthermore, by adjusting for effects on the electroencephalogram, CO_2 laser C fibre evoked potentials (LCEP) may be used to distinguish between the sedative and analgesic effect of a drug in anaesthetized rats.

To clarify the implications whether LCEP can provide information about central changes in anaesthetized and conscious rats, hyperalgesia was induced by partially irradiating the hind paw of rats with UVB-light. Changes were monitored during 14 days after induction of hyperalgesia in conscious animals, whereas changes from anaesthetised animals were collected one day after irradiation.

A clear increase in LCEPs from both the primary and the secondary hyperalgesic skin, peaking the first day and declining over 14 days, was demonstrated. Also later onset latencies were observed the first day after exposure in awake rats.

Additionally in anaesthetised rats, the LCEPs in forelimb SI elicited from forelimb skin displayed unaltered magnitude. This area was not monitored in conscious rats. Furthermore, tactile poke evoked potentials were also collected and displayed no change in anaesthetised rats, however, increased from secondary hyperalgesic skin day one in conscious rats.

To further evaluate hyperalgesia in anaesthetised rats, tramadol was administered, which counteracted the changes induced by UVB exposure.

This suggests that altered sensory processing related to hyperalgesia is reflected in altered LCEPs in SI. Comparing the time course and spatial

characteristic of the changes in transmission to SI and the behavioural responses in the same animals, it is clear that there are prominent differences. Behavioural responses increased preferentially from the primary hyperalgesic skin. Moreover, the significant changes in nociceptive transmission to SI occurred earlier than those of motor responses. In view of this, it is conceivable that pathways to motor circuits and sensory circuits differ markedly. Together these findings show that multichannel electrodes implanted in SI may offer a more sensitive test for hyperalgesia in conscious, behaving rats than conventional models.

The improvement of ground breaking neural interfaces has the potential to lay fundamentally new grounds for our understanding of how the nervous system processes nociceptive information in the long run.

Introduction

What is pain?

Pain is essential for our survival. It is usually caused by the activation of a special class of receptors in the tissue called nociceptors [1-3]. Without the ability to detect noxious stimuli we would not be able to protect ourselves sufficiently from the heat of a flame, piercing from sharp objects or even the discomfort of bruising. This is evidenced by the rare occurrence of congenital insensitivity to pain, in which a person is born without the ability to detect pain. Their life expectancy is greatly reduced, as they fail to engage in protective behaviour against injuries they inflict on themselves [4].

Following the activation of nociceptors by a noxious stimulus, alterations at several levels in the nociceptive pathways occur that serve to protect the injured area. For example, through local mechanisms in the tissue, peripheral nerves release neuropeptides that increase blood flow and vascular permeability which contribute to the healing process. In the central nociceptive pathways, the excitability may increase temporarily, causing enhanced pain and focused attention to the injured tissue [3].

From a clinical point of view, acute pain can usually be relieved in an adequate way. The situation is very different for long lasting (chronic) pain, whether caused by malignant or a non-malignant conditions, which is not satisfactorily treated, with troublesome side effects and inadequate relief [5-7]. Although there has been considerable progress on the molecular and cellular basis for nociception, the development of analgesics over the last decades has been a scarcity [3,8-10]. Conceivably, this may be due to underestimating the complexity of the nociceptive system. Indeed, poorly understood functional changes of the nociceptive circuits have been implicated in the development and maintenance of pain [11,12]. It may thus be that chronic pain conditions cause maladaptive alterations in the nociceptive system, the understanding of which may lead to effective therapies to prevent or even reverse these alterations. To allow significant advances on this urgent matter, valid animal models for pain are essential. As will be described below, current models of pain in the awake animals usually scores nocifensive responses [13-17] as a measure of pain. However, it is still unclear to what extent these responses are related to, for instance, the sensory discriminative or affective aspects of pain.

Pain cannot be perceived until it reaches the brain, where the location and the intensity of the pain are sensed and associated to an unpleasant emotion and cognitive memory. This touches upon an intriguing question on how the nervous system processes nociceptive information in the conscious brain. At present, very little is known about this, as much of the information available comes from anaesthetized animals.

The nociceptive pathways – a brief overview

Pain is normally caused by the activation of a subpopulation of peripheral nerve fibres called nociceptors. These can detect mechanical, thermal and chemical noxious stimuli [18] and send a message about the damage or threat of damage to the brain. Nociceptors can further be divided into two major classes [3], thin myelinated Aδ afferents and small diameter, unmyelinated C fibres, which in turn can be divided in many subgroups on the basis of response characteristic and surface receptors [19,20].

Nociceptive signals from the body are conveyed to the dorsal horn of the spinal cord, where neuronal processing leads to e.g. the execution of reflexes and interactions between different modalities [21]. Processed nociceptive information is then distributed by projection neurones, mainly located in laminae I and V in the dorsal horn [22,23], to supraspinal centres [18,24] in the brainstem, cerebellum, hypothalamus, thalamus and cerebral cortex [25-27]. These different targets are involved in different aspects of pain and pain modulation. For example, the spinothalamo-primary somatosensory cortical pathway is held to be the major contributor to sensory discriminatory aspects of pain [24], such as intensity and location of pain [28,29]. Other pathways project to hippocampus and amygdala which appear to be engaged in emotional responses relevant to aversive properties of pain [30] and the anterior cingulate cortex which appear to be associated with emotional aspects of pain [31,32]. In addition, many supraspinal centres, in particular the periaqueductal gray (PAG) and rostral ventromedial medulla (RVM) [33] engage in descending control of the spinal nociceptive processing, which can be either antinociceptive or pronociceptive [34,35]. The widespread distribution of nociceptive information to different targets in the brain thus provides the basis for the complexity of the pain experience including its sensory, emotional, and motor components [25,36].

This thesis focuses on the sensory aspects of nociception. SI is a major receiving area of somatotopically organized somatosensory information in the rat [37,38]. SI also receives somatosensory information in other species, such as monkey [28,39], cat [40] and human [11,12,26]. Additionally, from animal studies, it is clear that a population of neurons receiving nociceptive input are present in SI [28,37,38] and that these show a graded response to graded nociceptive input from restricted receptive fields [38,41], similar to many neurons in, for example, the ventral posterior lateral

nucleus of the thalamus and the dorsal horn of the spinal cord. Furthermore, SI also contains neurons that have convergent input from nociceptors and other afferents [38]. These findings strongly implicate SI in the sensory discriminative aspect of pain.

Sensitization of nociceptors and the phenomenon of hyperalgesia and pain

Injury commonly results in sensitization of primary nociceptive afferents [42,43] leading to decreased pain threshold and an increase in pain to suprathreshold stimuli from the injured tissue. This phenomenon is termed primary hyperalgesia. Moreover, the activation of nociceptors also causes an increased excitability of nociceptive neurons in the central nervous system, often termed central sensitization [44,45], leading to enhanced transmission of nociceptive messages. Numerous spinal mechanisms are believed to be implicated in central sensitization [46,47], which is expressed as lowered mechanical and thermal activation thresholds [48], facilitation of responses to innocuous stimuli [49], increased response to peripheral nociceptive stimuli [50], and expanded receptive fields of convergent neurons [51]. Importantly, secondary hyperalgesia may also arise from the tissue outside the innervation territory of the nociceptors in the injured area due to central mechanisms [52]. Whether or not this latter phenomenon is mainly a sensory phenomenon or also expressed in sensorimotor systems, such as nocifensive reflex systems, is not entirely clear. Nociceptive processing in the brain may likely follow enhanced neuronal activity in the dorsal horn [53], but it is still unclear to what extent hyperalgesia is reflected in enhanced nociceptive transmission to SI.

There are several methods to induce and study hyperalgesia, such as diet or stress induced hyperalgesia and drug or irritant induced hyperalgesia [47]. Many established and commonly used animal models evoke inflammation, which contributes to hyperalgesia, by injecting irritant substances (such as mustard oil, formalin, Complete Freund´s Adjuvant) [47]. An important aspect of inflammatory pain is human relevance. Intensely studied models of cutaneous hyperalgesia, which are also used on humans, are paradigms like thermal burn [54,55] and capsaicin (the pungent ingredient of chilli pepper) application [44,56]. Both models produce primary and secondary hyperalgesia. This has also been shown after other cutaneous injuries such as ultraviolet (UV) irradiation (i.e. sunburn), which produces pronounced primary mechanical and thermal hyperalgesia in humans [57,58] as well as rats [15,16]. Furthermore, cutaneous UVB irradiation is a sterile injury, restricted to cutaneous tissue, which to date has emerged as an important inflammatory pain model [15,16,59], suitable for analogous studies on humans [60].

Current pain assessments in animals

Understanding how the spinal cord and brain process nociceptive information in the awake individual and how this processing change during long term nociception has for a long time been hampered by the pain models available. Spinal withdrawal reflexes or other nocifensive behaviours, such as innate (e.g. flinching or licking) or operant (e.g. learned escape), are extensively used as an index of pain [17,61]. Yet, it is still unclear to what extent these responses are related to the sensory discriminative or affective aspects of pain. Also, the behaviour can be elicited by stimuli provoking painful sensation, although, non-noxious stimuli may also elicit a withdrawal reflex [62], which is therefore not specific for nociception. In fact, spinal sensorimotor circuits mediating nocifensive reflexes do not appear to be part of ascending pathways mediating nociceptive information to the relevant nociceptive areas of thalamus and cortex [63]. Instead, there is a clear link between reflex circuits and ascending spino-olivo-cerebellar pathways [64].

It is not obvious to recognize pain in rats, neither by looking at them [65] nor by interpreting their behaviour [66]. Furthermore, behavioural measures can be distorted by the effect of analgesics or sedatives on motor coordination [67,68], since they depend on a functional motor system. The frequent failure of animal models of pain in predicting analgesic effects of drugs [69,70] provide additional support for the notion that motor responses are subserved by at least partly different systems than those involved in pain perception. Consequently, animal models of pain based on other measurements than motor responses are needed for our full understanding of the mechanisms underlying nociception. A promising model would be one in which the pain condition occurred naturally and in which cerebral events are measured, rather than reflexes.

Studies show that, in humans, nociceptive evoked potentials in the SI correlate with pain sensation in the normal situation [25] and after induction of hyperalgesia [71]. Further, the potentials change with the level of analgesia [72]. In the rat, nociceptive C fibre evoked potentials in SI [73], have been suggested to be a useful model to monitor pain related ascending transmission under various conditions [74]. However, so far, this model has only been used in anaesthetized animals and it is not clear if the model can be used to differentiate between sedative and analgesic treatments and whether or not it can be used to monitor hyperalgesia.

How pain better could be measured in animals

There remain crucial gaps in our knowledge of nociceptive processing in the conscious brain. While imaging studies on awake humans have contributed importantly to identify several cortical areas that subserve nociceptive functions [11,12,26], such techniques do not provide the spatial and temporal resolution

necessary to reveal detailed neuronal and network mechanisms underlying pain. Such information can only be obtained from animal studies. However, much of the current knowledge on the functional organisation of the nociceptive system comes from electrophysiological studies performed in anaesthetized animals [37,38,48].

Implanted multichannel electrodes serving as a brain computer interface, which enables long term measurements of neural information processing, has pushed the limits and opened new fields of research. As the technique of recording neural activity has progressed, new areas of application have emerged such as monitoring the simultaneous activity of hundreds of neurons or individual neurons in behaving animals. Implanted electrodes have successfully been used to study nociceptive transmission in conscious animals for short periods [31,75,76] but due to that they cause substantial tissue responses and provide unstable recordings [77,78] they need to be improved before being used in long term studies of pain. Nevertheless, improved brain computer interfaces have the potential to lay fundamentally new grounds for our understanding of how the nervous system processes nociceptive information in the long run. Clearly, analysing neural processes in conscious animals during prolonged periods will also lay new grounds for novel drug candidates to reach the light of the market.

Aims

The general aim of this thesis was to develop a useful animal model for studies on the sensory aspects of pain. The specific goals were:

1. Develop a method to differentiate between the sedative and analgesic effect of drugs on nociceptive transmission to SI
2. Evaluate if primary and secondary hyperalgesia is reflected in altered transmission to SI
3. Develop a method for long term measurements of nociceptive transmission to SI in conscious unrestrained rats using chronically implanted multichannel electrodes
4. Evaluate the relation between nociceptive behavioural responses and transmission to SI in the conscious animal during development of hyperalgesia

Method

Animals used

Sprague-Dawley rats of both sexes were used. All rats received food and water *ad libitum* and were kept in a 12-hour day–night cycle at a constant environmental temperature of 21°C and 65% humidity. Approvals for the experiments were obtained in advance from the Lund/Malmoe local ethical committee on animal experiments.

Experimental procedure

Indices of pain and pain related phenomena

The experimental protocol was devised so as to allow a quantitative characterization and comparison of how nociceptive transmission is affected by different indices of pain related phenomena and/or drug administration.
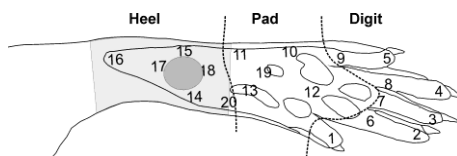
Figure 1. Stimulated sites, irradiated area and behavioural test area of the right hind paw. The sites, subjected to CO_2 laser and mechanical stimulation with the tapping device, are indicated with numbers. The UVB exposed area is shaded in light grey (III). In II the UV irradiated area consisted of sites 14-18. From this area laser Doppler measurements were taken. The dark grey ellipse indicates where the stimulation of the behavioural tests was done. Dotted line divides the paw into three areas; heel, pad and digit.

Induction of hyperalgesia using UVB narrowband irradiation (I & III)

UVB irradiation produces a skin inflammation and a dose-dependent hyperalgesic state [16]. The rats were anaesthetized with isoflurane (1.4-1.8 %) in a mixture of 40 % oxygen and 60 % nitrous oxide. They were then covered with an UV opaque material, exposing the proximal plantar part of the right hind paw (Figure 1), which was irradiated with 1.2-1.3 J/cm^2. A calibrated UVB narrowband lamp (λ = 300-320 nm, Philips) was used.

Behavioural tests

Assessment of behavioural responses to mechanical stimulation (III)

Withdrawal responses to mechanical stimuli were tested from the proximal part of the right hind paw using a dynamic plantar aesthesiometer (Ugo Basile). Rats were placed in a clear cubicle (19.5 x 19.5 x 14 cm) on top of a metal grid and left to acclimatize. A linear force ramp of 0.4 g/s was applied and the paw withdrawal thresholds were collected during six weeks.

Assessment of behavioural responses using noxious thermal stimulation (II & III)

Behavioural hyperalgesia was assessed before surgery in awake rats (II) by measuring the threshold of the withdrawal reflex of the irradiated and contralateral heel. Further, nocifensive withdrawal responses (III) from 20 sites of the hind paw were determined. A radiant heat CO_2 laser (Irradia, Sweden; model 315M Superpulse, wavelength 10.6 μm, output power 10 W, beam diameter 3.0 mm) was used.

Also, the presence of thermal nociception was determined (III), by measuring paw withdrawal latency to a thermal stimulation system (Hargreaves apparatus, Ugo Basile). Rats were placed in a clear cubicle (17 x 22 x 14 cm) on top of a glass floor and left to acclimatize, whereafter data was collected during six weeks.

Assessment of inflammation (III)

To determine the degree of inflammation, rats were anesthetised with isoflurane (1.2-1.5 %) in a mixture of 40 % oxygen and 60 % nitrous oxide, whereafter blood flow in the proximal part of plantar right hind paw was measured with a laser Doppler flow meter (MoorVMS-LDF™).

Surgery and preparation for electrophysiology

Acute experiment (I, II)

Rats were anaesthetized with isoflurane (1.8–2.0 % during surgery and 0.6–1.1 % during recordings) in a mixture of 40 % oxygen and 60 % nitrous oxide. The trachea was cannulated and the animals were artificially ventilated. An infusion of 5 % glucose in Ringer's acetate was given through the right jugular. Mean arterial blood pressure was monitored continuously and the rectal temperature was kept at 36.5–38.5 °C. The head was fixed by a nose ring and ear bars, after administration of local anaesthesia (EMLA® salve). The spinous process of a thoracal vertebra was clamped and the chest lifted to facilitate ventilation. A craniotomy partly exposing the left parietal and frontal cortex was made, whereafter the dura mater was cut and the surface covered with paraffin oil. Infiltration of local anaesthetic (Xylocaine®) was made during all surgery to reduce the nociceptive input. After completed surgery, muscle relaxant (Pavulon®) was given repeatedly to enable stable recordings, whereafter surface electrodes (~0.3 mm diameter) were placed on SI.

Long term experiment (III)

All rats were anaesthetised i.p. with a solution containing an analgesic (fentanyl) and a sedative (Domitor® vet.). The head was placed in a stereotactic frame and the skull was exposed. Thereafter a reference screw was mounted and inserted to the depth of the dura mater. Additionally two more screws were mounted for anchoring the implant to the skull. The centre of the stereotactic coordinates for the craniotomy (3 x 2 mm) was 1 mm caudal of Bregma and 2.4 mm lateral to the midline, corresponding to hind paw representation area. The exposed dura mater was removed and the surface covered with artificial cerebrospinal fluid. The electrodes were implanted in the left hemisphere and dental cement (FujiCEM™ Automix) covered the hole in the skull and embedded the wire bundle. After the surgery, an antidote (Antisedan® vet.) to the anaesthesia and an analgesic (Temgesic®) was injected s.c.

Electrodes (III)

In I and II ball tipped platinum electrodes were used to record surface potentials. In III, in house developed multichannel electrodes were used. Twenty eight 12 µm wires (platina-irridium) insulated with a polymer (3 µm thick parylene C) and embedded in gelatine, were implanted. In total, the microelectrode was 200-300 µm in diameter. One of the electrodes was uninsulated 2 mm at the tip and used as

a reference. The electrodes and a 150 µm platina ground wire were soldered to a chip and the connections were covered with epoxiharts (bisfenol F).

Electrophysiological recordings and sequence of stimulations

In experiments I and II, recordings of mechanical and laser evoked potentials started after completed surgery.

Anaesthetized rats were irradiated (II) with UVB-light 20-24 hours prior to recordings. At this time, an inflammatory process had begun [16]. Furthermore, moderate redness, but no skin lesions or scarring, was seen on the irradiated skin.

In experiments III, electrophysiological recordings in the awake animal started > 1 week after implantation of multichannel electrodes. About two weeks after implantation, the heel was irradiated with UVB. Recordings were made on days 1, 2, 4, 7 and 14 after UVB irradiation. These were compared to base line recordings from the same animals collected during the week before irradiation.

Assessing cutaneous representation on SI by tactile stimulation (I, II)

Nociceptive and tactile input from the skin overlaps to some extent in SI [38,73]. Therefore, to avoid input from nociceptors, which may produce excitability changes in peripheral and central pathways, tactile input was used to locate the cortical representation of the glabrous skin of the arch, heel (I, II) and digits (I) of the right hind paw and of the digits of the right forepaw (II). A hand-held electromechanical stimulator with a blunt metal probe was used. For each skin area and rat, the cortical site eliciting potentials with the highest amplitude was used for recordings.

Nociceptive heat stimulation

To elicit input from nociceptors in SI, the glabrous skin of the right hind paw or forepaw (II) were stimulated with a CO_2 laser. The stimulation energies used, corresponded to 210-330 mJ, have been shown to evoke late cortical field potentials reliably in the rat SI through the activation of cutaneous nociceptive C fibres [73,79]. Furthermore, C fibres, and also Aδ fibres, in rats [73] and humans [80] are activated by noxious CO_2 laser stimulation, without simultaneous activation of low threshold mechanoreceptors. CO_2 laser evoked potentials have furthermore been monitored in human and rat SI [75,81]. CO_2 laser pulses were delivered to different sites within a skin area, e.g. the heel, at a frequency of 1 Hz (I, II). The stimulations of an area were repeated three to five times, with an interstimulus interval of 10 minutes (I, II). Furthermore, in rats administered with a drug, an

additional set of CO_2 laser C fibre evoked potentials (LCEPs) were collected. Electroencephalogram (EEG) was also monitored every 10 minutes throughout the experiments.

In paper III, 20 sites (Figure 1) on the hind paw were stimulated 20 times in a semi random order.

Drug administration (I, II)

The effect of 2 mg/kg of tramadol (Tradolan®) *i.v.* on LCEP was evaluated on some UVB exposed rats (II).

In experiment I, either morphine (3 mg/kg) or midazolam (10 µmol/kg; Midazolam Hameln) was administered *i.v.* to evaluate the effect on LCEP. Furthermore, in some animals the level of volatile anaesthesia was also lowered after drug administration (I) to reverse the EGG frequency to that of the control level.

Statistical analysis

Analysis of motor response tests

To compare the thresholds of the nociceptive withdrawal reflex in the irradiated heel with the contralateral (II) paired t test was used. One-way ANOVA followed by a Tukey´s post hoc test was used for statistical analysis (III) of behavioural tests ($p < 0.05$ was taken as significant, 95 % confidence interval). Withdrawal responses elicited by CO_2 laser stimulation evoked during SI recordings were followed by Bonferroni´s multiple comparison test. Paired student´s t test was used for statistical analysis of blood flow measurements.

Data analysis of EEG and evoked potentials

Study I and II
The signals (10 kHz sampling frequency), were amplified and filtered using Digitimer Neurolog system (Digitimer LTD, England) with a low cut-off frequency of 1 Hz and a high cut off frequency of 700 Hz.

Fourier analysis was used to analyse the dominating EEG frequency in study I. In house scripts, created in Scilab-4.1.1, were used to calculate area under the curve (AUC), onset latency and duration (Figure 2) to characterize C fibre evoked potentials. As for tactile evoked potentials, the onset latency and peak amplitude

of the initial positive surface potential were analysed (II). Student´s t-test was used as statistical analysis, $p < 0.05$ considered significant.

Study III

Local field potential (LFPs) were recorded using a 32-channel Neuralynx system (Digital Lynx 10S, Neuralynx). The signals were pre-amplified and buffered. LFPs were sampled at 1 kHz and filtered using a low-pass (<300 Hz) FIR filter (Figure 2).

Averaged evoked LFP responses were calculated using in-house developed MATLAB (version 2010 b) scripts. Data were grouped according to: 1) Area stimulated, divided into digits (sites 1-9), secondary area (pad, sites 10-13, 19) and primary area (heel, sites 14-18, 20); 2) stimulus modality (nociceptive and tactile) and 3) days after UV exposure over all animals. Furthermore, means of LFP responses were calculated for each site and modality and then smoothened using a moving average filter.

AUC and onset latencies of CO_2 laser C fibre evoked potentials were determined as was the peak amplitudes and peak latencies of tactile evoked potentials.

A two-sample Kolmogorov-Smirnov test with Bonferroni correction ($p < 0.05$ was taken as significant) was used for analysis.
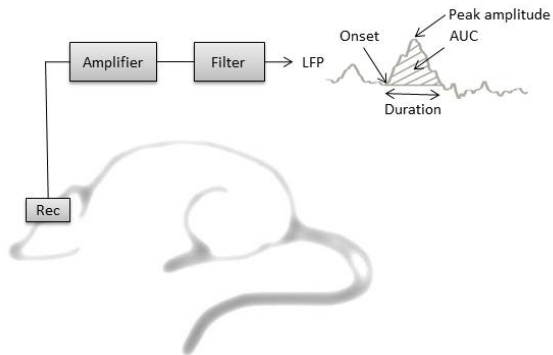
Figure 2. Illustration of the scheme to record local field potentials. Signals were recorded from the recording site (Rec). Amplified LFPs were filtered. The LFP shows the onset, duration, peak amplitude and area under curve (AUC) of the nociceptive C fibre evoked potentials.

Results at a glance

- By adjusting for effects on EEG, CO_2 laser evoked cortical potentials may be used to distinguish between the sedative and analgesic effect of a drug in anaesthetized rats.
- Secondary hyperalgesia is reflected in altered transmission to SI in the anaesthetized rat.
- Tramadol reverses much of the changes in nociceptive C fibre transmission to cortex from the primary and secondary skin regions but had little effect on transmission from distant skin
- Nociceptive C fibre transmission to SI in the awake animal is enhanced, peaking on the first day, after cutaneous UVB irradiation.
- Hyperalgesia as measured from SI and from motor responses differ in time course in the awake animal.
- Secondary hyperalgesia was prominent from cortical recordings but was not significant in reflex tests, indicating differences in topographical organisation of nociceptive motor and sensory systems.

Results and comments

Characterizing evoked potentials may indicate if a drug has sedative or analgesic properties (I)

The preceding studies on nociceptive transmission to SI indicate that nociceptive C fibre evoked potentials in SI may be useful to evaluate the overall nociceptive transmission to the brain and the analgesic properties of drugs [3,25,74]. An unresolved, but important question is, however, how to differentiate between the analgesic and sedative properties of a drug using this model. Little attention has been given in the past to this problem in animal pain models.

To clarify the antinociceptive outcome of drugs, we set out to develop a method to differentiate the sedative and analgesic effect of drugs. First we studied the effects of different depths of anaesthesia on nociceptive C fibre transmission on LCEP and EEG. We then tested whether it is possible to compensate for the sedative effects of a drug on LCEP by keeping the dominant frequency of EEG constant. Finally, we tested whether the method of keeping the dominant frequency of EEG constant could be used to differentiate between sedative and analgesic properties of two drugs.

Isoflurane and midazolam reduces LCEP and EEG frequency

Initially, the effect of different concentrations (0.8 - 1.3 %) of isoflurane on EEG and LCEP was tested. At 0.8 - 0.9 % isoflurane, the EEG was dominated by 3-8 Hz and clear LCEPs could be elicited. LCEP consisted of a late surface positive wave. Previous studies have shown that the source of this potential is in cortical layer III-IV [73]. As the anaesthetic level increased, the LCEP and the dominating EEG frequency were gradually reduced. At 1.3 % isoflurane, LCEP was nearly abolished and the mean dominating frequency of EEG was about 2.0 Hz. Under anaesthesia, a parallel reduction in EEG frequency and LCEP was also found after administration of the sedative midazolam. The produced outcome resembled that of an increase in the isoflurane concentration from 0.9 to 1.1 %. By lowering the concentration of isoflurane from 0.8-0.9 % to 0.6-0.7 %, the dominant frequency of EEG and LCEP were reversed to control level again. These findings suggest that the apparent analgesic effect of midazolam is mainly due to its sedative properties.
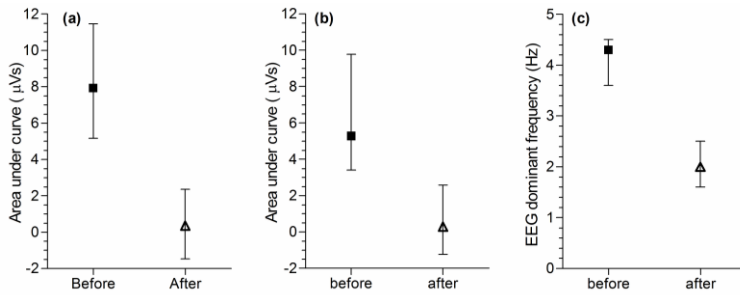
Figure 3. The effect of morphine on LCEP without and with EEG frequency compensation. Averaged median values and 25/75 % quartiles are shown. a) The effect of morphine on LCEP. b) The effect of morphine on LCEP after compensation of change in EEG. c) Changes of dominant EEG frequency after administering morphine.

Morphine reduces LCEP and EEG frequency despite reduction of anaesthesia

Morphine is an analgesic, which depress nociceptive transmission to SI [82,83]. Nevertheless, since morphine also exhibit sedative properties, in particular in higher doses [84,85], the possibility that the entire effect on LCEP is due to its sedative properties, could not be excluded. In this thesis, morphine (3 mg/kg) caused a similar effect on LCEP and EEG as midazolam, yet still caused a profound depression of the LCEP (5.6 % of the control, $p < 0.01$) after lowering the isoflurane level to keep the EEG frequency around 4-6 Hz (Figure 3). These findings indicate that at the dosage used, morphine causes both significant sedative and analgesic effects.

Taken together this study suggests that the proposed method might be a valuable tool to distinguish between analgesic and sedative effects of a drug. Importantly, the clear relationship between EEG and LCEP also indicate that the dominant frequency of EEG needs to be kept constant when assessing the analgesic effects of various drugs or other manipulations.

Secondary hyperalgesia is reflected in altered nociceptive transmission to SI in anaesthetised rats (II)

Since SI is likely to play an important role in processing sensory aspects of pain we assessed whether monitoring SI nociceptive C fibre evoked potentials can provide useful information about central changes related to primary and secondary hyperalgesia in rats. Additionally we tested whether tramadol, a centrally acting opiate, could reverse the changes noted. Local UVB irradiation was used, which has been demonstrated to cause reliable behavioural hyperalgesia in animals [16] and in humans [57,58].

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To confirm that the dosage of UVB irradiation used caused behavioural hyperalgesia, we tested the withdrawal response to CO_2 laser stimulation in awake rats and compared the UVB exposed heel to the contralateral heel. This comparison confirmed an increased sensitivity in the irradiated heel [15,16], displaying reflex thresholds significantly lower (7.2%) compared to the contralateral heel. In paper III, we also confirmed an increased blood flow in the irradiated skin.

A first step towards characterising the changes in tactile and nociceptive C fibre evoked potentials after UVB irradiation, was to monitor the spatial distribution of hyperalgesia. For this purpose, multiple surface electrodes were used to record potentials in forepaw and hind paw SI (heel and arch). The heel and arch of the hind paw were used to assess primary and secondary hyperalgesia respectively. These two areas represent skin areas where primary hyperalgesia [16] and secondary hyperalgesia may occur in human [57]. The LCEP evoked from the forepaw was studied to characterize distant effects. The evoked potentials were recorded under anaesthesia in control conditions and one day after UVB irradiation of the heel. All measurements were conducted during dominant EEG frequencies of 3-8 Hz.
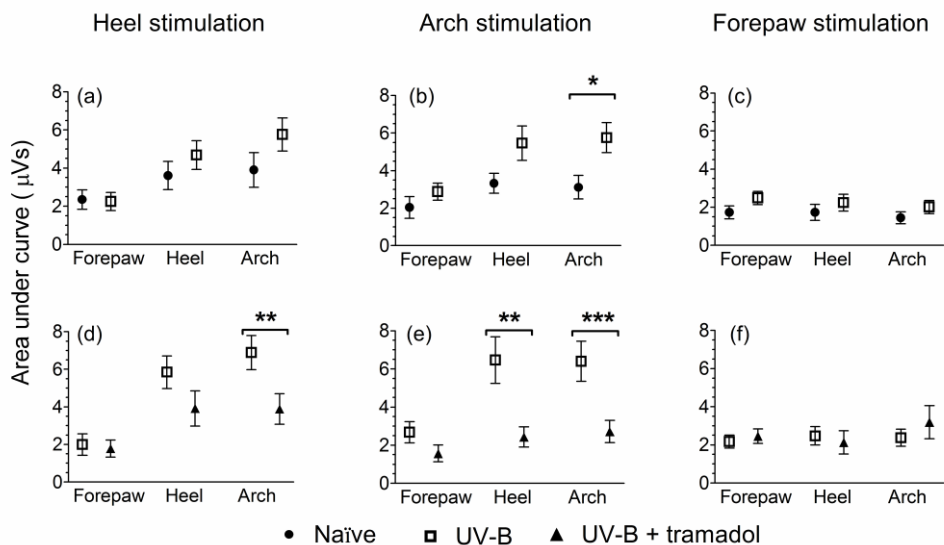
Figure 4. Magnitude of LCEPs recordings from three cortical areas. Top: The difference between means and S.E.M of area under the curve (AUC) for the naïve (black circles) and the UVB exposed (white boxes) groups are shown. Bottom: The mean of differences and S.E.M of AUC in UVB exposed rats before (white boxes) and after (black triangles) tramadol administration are shown. The x-axis depicts the recording areas on SI cortex. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Control recordings confirmed a rather crude somatotopic organisation of the nociceptive C fibre input as compared to tactile input. Hence, CO_2 laser stimulation of the hind paw also evokes significant, but smaller, potentials in the forelimb representation area of SI and vice versa [73].

Although tending to increase, the magnitude (measured as AUC) of the LCEPs in heel SI elicited from the primary hyperalgesic skin, did not reach significant levels (Figure 4). However, an increased duration of the LCEPs in heel and arch SI was found. By contrast, the magnitude, but not duration, of the LCEPs in the arch SI was strongly increased (46 % of control) upon stimulation of secondary hyperalgesic skin. Furthermore, the LCEPs on forelimb skin stimulation increased (44 ms difference) in duration in the SI forelimb area and showed delayed onset latencies (difference: heel 29 ms and arch 41 ms) in hind limb SI after irradiation. Magnitudes remained unaltered. Concerning the tactile evoked potentials, UVB irradiation induced no significant changes in onset latencies or peak amplitude.

From these studies, it can be concluded that UVB not only altered the nociceptive transmission to SI from the irradiated skin area but also from nearby and distant skin areas.

In the next step, we examined whether tramadol, a centrally acting opiate, can counteract the changes in LCEPs following UVB irradiation (Figure 4). The drug reduced the changes produced by UVB irradiation on transmission to SI from the
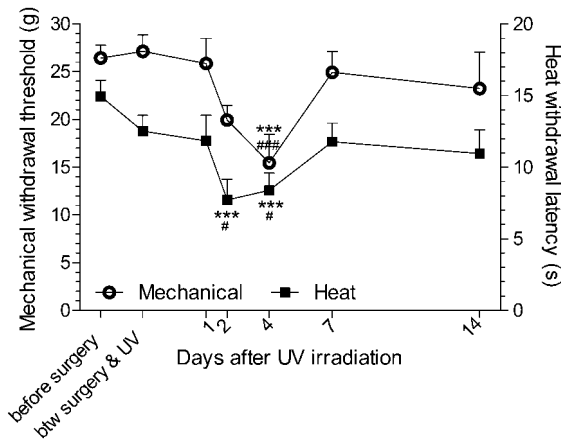
Figure 5. Time course of changes in mechanical and thermal thresholds after UVB exposure. Mean and SEM of mechanical withdrawal thresholds (mechanical; left y-axis) and the withdrawal latencies to heat (heat; right y-axis) are shown. The measurements made following exposure were compared to the measurements before surgery (*) or to measurements between surgery and irradiation (#) (*, #p < 0.05; ***, ### p < 0.001).

hind limb significantly. Notably, tramadol administration lessened both the prolonged duration of the input from the irradiated heel to the heel and arch representation in SI, as well as the increase in transmission from the secondary hyperalgesic skin to the arch and heel SI. However, no significant effect on transmission from forelimb skin was observed. Taken together, tramadol reversed much of the changes in nociceptive C fibre transmission to cortex from the primary and secondary skin regions but had little effect on transmission from distant skin. The latter finding may indicate that the effects on transmission from distant skin by UVB irradiation is produced by mechanisms other than those related to primary and secondary hyperalgesia.

These results support the notion that LCEPs in rat SI provides useful information on altered sensory processing related to hyperalgesia and in particular, provide an opportunity to monitor transmission to SI from primary and secondary hyperalgesic skin in the same rat. Therefore, this way of monitoring the pain related pathways appears to be a useful supplement to animal behavioural tests of mechanisms related to pain and analgesia.

Nociceptive transmission to SI differ from behavioural responses during development of hyperalgesia in the awake animal (III)

As mentioned in the introduction, there is a need for a new animal model of pain that can monitor pain related activity in the brain in the awake animal during a long time period. To this end a novel multichannel electrode was used. It consists of ultrathin platinum wires embedded in hard gelatine, shaped as a needle to permit implantation. The embedding technique solved the long standing problem of how to implant ultrathin electrodes in soft tissue. This construction was used, since studies have shown that gelatine embedding and ultrathin electrodes produce less tissue responses (astrocyte proliferation and microglia invasion) than large electrodes [78,86], and thus fewer effects on the normal condition of the brain. The electrode was implanted in SI and the individual wires spread out in layer V, where nociceptive neurons are present [73,76,87].

It is conceivable that surgery and the electrode in itself may cause sensitization on nocifensive behavioural tests. We therefore tested these plausible effects using two established behavioural tests before and after the implantation. No significant changes in mechanical and thermal nociceptive withdrawal thresholds were found, indicating that the surgery and the implanted probe did not cause major changes in nociceptive responses (Figure 5).

UVB irradiation of the heel was used to induce hyperalgesia. Blood flow measurements made before and one day after UVB irradiation confirmed the development of an erythema.
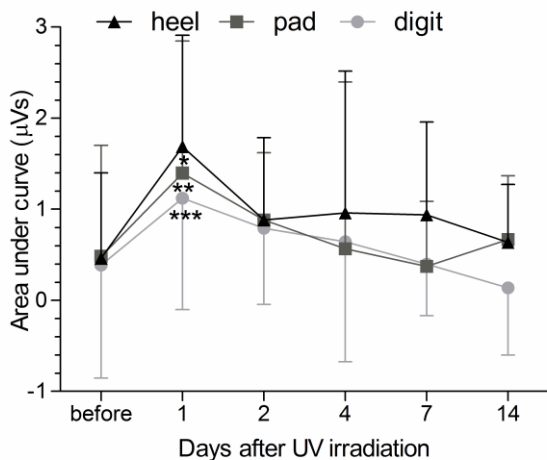
Figure 6. Time course of C fibre magnitude. Mean and SD of LCEP magnitudes are shown for digit, pad and heel before irradiation and during 14 days after. (*p < 0.05, **p < 0.01, ***p < 0.001)

Tactile and nociceptive evoked potentials in SI were studied one week before and up to 14 days after UVB irradiation of the heel. CO_2 laser stimulation of the skin evoked a short latency potential due to input from nociceptive Aδ fibres and a later potential due to input in nociceptive C fibres. These potentials resembled the potentials evoked in layer V, previously observed in anaesthetized rats [73]. There was a clear increase (188 % of the control) in the magnitude of nociceptive evoked potentials elicited from the secondary hyperalgesic skin peaking the first day after UVB exposure, whereafter a decline over the next days was shown (Figure 6). Also, primary hyperalgesic skin displayed the same trend with an increased magnitude (265 % of the control) the first day after irradiation, followed by a decrease in magnitude the subsequent days. Additionally, later onset latencies the first day following irradiation was also observed from both primary (53 ms difference) and secondary skin (41 ms difference) in awake rats compared to before.

It should be noted that the CO_2 laser intensity was kept lower in paper III than in paper II, to ensure cooperation of the awake rat. A preceding study exhibited same responses to laser heat stimuli when data was divided into reflex and no-reflex group [76], thus suggesting that the long latency evoked potential unlikely is caused or affected by secondary input from muscles or joints.

Additionally, tactile evoked responses, markedly increased from secondary hyperalgesic skin (peak amplitudes: pad 14 % of control; digit 4.2 % of control) on the first day following exposure.

Interestingly, hyperalgesia as measured from behavioural responses to mechanical and thermal stimuli in the same animals using motor response tests, changed significantly after UVB irradiation, with sensitivity peaking at days four and two, respectively (Figure 5 and 7). This increased sensitivity declined over the following days to baseline levels. Furthermore, the frequency of withdrawal reflex responses to CO_2 laser stimulation increased significantly only from the primary hyperalgesic skin peaking day two. Comparing the time course and spatial characteristic of the changes in transmission to SI and the behavioural responses in the same animals, it is clear that there are marked differences. Whereas, behavioural responses increased preferentially from the primary hyperalgesic skin, transmission to SI increased from both primary and secondary hyperalgesic skin. Moreover, the significant changes in nociceptive transmission to SI occurred earlier than those of motor responses. In view of these findings, it is conceivable that mechanisms related to hyperalgesia in pathways to motor circuits and sensory circuits differ markedly. Together, these results show that multichannel electrodes implanted in SI may offer a more sensitive test for hyperalgesia in conscious, behaving rats than conventional models.
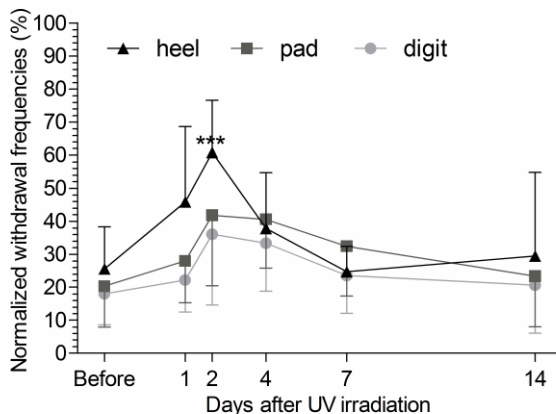
Figure 7. Time course of changes in withdrawal frequencies to CO_2 laser stimulation. Mean and SD of the normalized withdrawal responses to CO_2 laser stimulation within three areas of the hind paw are shown for elicited responses before and after UV exposure (***$p < 0.001$).

General discussion

The main aim of this thesis was to contribute to the development of a valid animal model for studies of pain related mechanisms, in particular sensory aspects of pain related to hyperalgesia. To this end, focus was put on nociceptive transmission to SI, since this region is likely to be involved in the sensory discriminative analysis of pain in both humans and animals. Moreover, we used UVB irradiation, a translational method to induce hyperalgesia. For the first time, using brand new multichannel electrodes, we have achieved long term recordings of nociceptive transmission to SI covering the entire time course of hyperalgesia in awake animals. Importantly, it is demonstrated that the recorded nociceptive C fibre evoked potentials in SI provide useful information on changes in nociceptive transmission related to both primary and secondary hyperalgesia in anaesthetized and also awake rats. Moreover, a comparison of the nociceptive input to SI and nocifensive behavioural and reflex tests in the awake animal indicates clear differences in spatial and temporal characteristics. This latter finding underlines the notion that nocifensive behavioural responses may differ substantially from pain perception and thus may provide misleading information on pain related mechanisms. It is also demonstrated that CO_2 laser evoked C fibre potentials in SI, in combination with EEG recordings, can provide information on the sedative and analgesic effects of systemically administered drugs.

Monitoring UVB induced changes in nociceptive transmission to SI in anesthetized and awake animals

Hyperalgesia is a healthy physiological reaction, which is subject to short term inflammatory responses. This means that the process of hyperalgesia is usually reversible. If, however, the painful condition indeed persists there may be more pronounced changes in central nociceptive pathways. The mechanisms of hyperalgesia are therefore commonly assumed to play an important role in the development of chronic pain [3,47] and have consequently been studied extensively [2,46,47]. As this thesis shows, these mechanisms may differ substantially for different nociceptive systems. Since hyperalgesia is primarily a sensory phenomenon, it was of particular interest to study the mechanisms of hyperalgesia in a system involved in sensory discrimination. Our studies demonstrate that changes in transmission from both primary and secondary hyperalgesic areas as well as from distant skin

areas can be monitored in SI in the anaesthetized (II) and awake animals (III). Notably, the studies on awake animals, in addition, revealed that the time course of enhanced nociceptive C fibre responses in SI after UVB, is similar to that in humans [88], which add strength to this animal model of hyperalgesia.

While the magnitude of the LCEPs elicited from the primary and secondary hyperalgesic skin increased significantly in awake animals, the LECPs elicited from the primary hyperalgesic skin, did not reach statistical significance in anaesthetised animals. Importantly, these changes were found to be reversed by the opiate tramadol, indicating that the changes after UVB irradiation were in fact due to hyperalgesia. In awake animals, changes in onset latencies were noted for LCEPs elicited from the primary and secondary hyperalgesic skin. Similar observations regarding primary hyperalgesia in anaesthetised animals, have been made in dorsal horn neurons (wide dynamic range neurons) after UVB exposure[59]. One conceivable explanation for the lack of significant potentiation of responses from the irradiated skin in anaesthetised animals, is that the nociceptive C fibre input from the primary skin and consequent activity in the dorsal horn are desynchronized somewhat by the ongoing spontaneous activity, thereby partly masking the stronger nociceptive input from the primary hyperalgesic skin. An additional possibility is that the response frequency in nociceptive C fibres on CO_2 laser stimulation of the irradiated skin, despite being sensitized [48], decreases in the hyperalgesic situation. Nociceptive C fibres fatigue easily [89], and there is evidence that, after skin inflammation, induced by injection of complete Freund's adjuvant, the response frequency on moderate to strong noxious heat stimulation decreases in C fibres [48]. However, the finding that there was a significant enhancement of nociceptive input from the primary hyperalgesic skin area to both SI cortex and reflexes in the conscious rat (III), may however suggest that these two plausible mechanisms alone cannot explain why the potentiation of nociceptive transmission from the irradiated skin to SI did not reach statistical significance in the anesthetized rat. Rather, anaesthesia has an effect on the nociceptive transmission [72]. Therefore it is tempting to speculate that the discrepancy, between LCEPs from primary hyperalgesic skin in awake and anaesthetised animals, indicates that anaesthesia may have differential effects on nociceptive pathways from the primary and secondary hyperalgesic skin areas. Indeed, it has been reported that the transmission pathways from nociceptors from primary and secondary hyperalgesic skin to the SI are under different supraspinal control [35]. Further, there is evidence for an inhibitory and excitatory supraspinal control of transmission from the primary and secondary hyperalgesic skin area, respectively, which might have contributed in the present situation. This possibility should be addressed in further studies, in which responses in cortical nociceptive neurons are studied during activation of different supraspinal control systems. Nevertheless, irrespective of which mechanisms are responsible and where they are located, it is clearly an advantage to be able to record the end result in SI, since this is likely to reflect the perception of pain intensity.

Relation between nociceptive input to SI and nocifensive responses

At present, most studies on nociceptive transmission and analgesia in awake animals are based on behavioural and reflex responses [13-17]. As mentioned in the introduction, there are reasons to suspect that nociceptive motor pathways are at least partly different from nociceptive pathways to SI. We therefore evaluated the relationship between nociceptive transmission to SI and nocifensive behaviour. In contrast to the results obtained on nociceptive input to SI, enhanced nocifensive responses were more prominent from the primary skin than from the secondary hyperalgesic skin. Moreover, we found major differences in the time course of potentiation of nociceptive input to SI and reflex output after UVB irradiation, the latter lagging behind in time. Nocifensive responses to heat stimulation were significantly altered day two, whereas responses day four following irradiation was most prominently potentiated for mechanical stimulation. These findings support the notion that the functional organization of the withdrawal reflex system does not match that of a sensory system and that different nociceptive systems exhibit different properties.

From a functional point of view, however, it is not clear why the defensive reactions should be sensitized later than sensory pathways to peripheral sensitization. One possibility that deserves to be analysed in more detail is that there may be more subtle changes in the nociceptive motor system after induction of hyperalgesia, such as alterations in which reflex modules that are active.

On the effect of sedation on nociceptive input to SI

In studies aiming at developing new centrally acting analgesic drugs, the potential contamination of sedative properties is a major concern. Ideally, an analgesic should of course produce no sedation at all, but this is rarely the case with available centrally acting analgesics. An animal model for assessment of analgesic effects should therefore also consider sedative effects. In the case of nocifensive behaviour, sedative effects are rarely accounted for despite the fact that sedative properties of a drug can have direct effects on the motor responses. Interestingly, after maturation of supraspinal centres, midazolam desensitizes flexor reflex activity and shows sedative effects as evaluated from both behavioural tests and electrophysiological recordings [90]. In contrast, midazolam sensitizes withdrawal reflex activity in rat pups, which additionally do not show signs of sedation.

In this thesis (I) it is demonstrated that LECP is clearly affected by the anaesthetic level and can be equally depressed by systemic administration of the sedative midazolam, a benzodiazepine, and the analgesic morphine. Importantly, the depressant effect of midazolam, but not morphine, on LCEP was found to be

abolished if the dominant frequency of EEG is kept constant. This suggests that the apparent analgesia produced by midazolam mainly is derived from its sedative effects [91]. The main effect of benzodiazepines is sedative [92], but for certain routes of administration it has been claimed to also exert analgesic effects [93].

In case of morphine, sedative properties are exhibited in addition to its analgesic effects, explaining its effect on EEG [84,85]. However, what is striking from the present results is that, at the dose used, the sedative effects of morphine appear to depress the nociceptive transmission equally effective as its analgesic effects.

Hence, the method of recording nociceptive evoked potentials in SI while keeping the dominant frequency constant, may prove to be useful in probing new drugs with potential analgesic effects. Although promising, a word of caution is appropriate as it is not entirely clear to what extent the effects of different sedatives is additive. Therefore, this method should at present only be considered a first test for sedative effects of a compound and be supplemented by other conventional tests for sedation based on behavioural changes.

Conclusions and future aspects

It is clear that recordings from conscious rats will offer a superior animal model for pain that, with proper study design of multichannel electrodes, will lay new grounds to reach a profound knowledge of how the central nervous system processes nociceptive input. The method has the potential to clarify the physiological role of various ascending pathways and different cortical targets. Not only will it be possible to reveal how pain occurs, but most importantly it will also be possible to ease the way of new and effective analgesics and treatments of pain. This has the potential to revolutionize the way we treat pain and will provide new strategies to develop novel pharmacological candidate drugs.

We are still left to speculate on how acute pain switches into the maladaptive changes of chronic pain. It is complicated to gain access and isolate pain related mechanisms, not the least because injury starts a chain of reactions, which depend on the context in which the pain mechanism function (e.g. target of innervation, time after injury, history of the injured tissue). Nevertheless, successively pin pointing one mechanism of nociception after the other is likely to fill the current black box between nociceptive input and perception and eventually lead to unravelling the puzzle of pain.

Once we know the lay of the land, we can use the knowledge to develop effective therapies for chronic pain.

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Paper II

Altered nociceptive C fibre input to primary somatosensory cortex in an animal model of hyperalgesia

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ABSTRACT

Evaluating potentially analgesic effects of drugs and various treatments is critically dependent on valid animal models of pain. Since primary somatosensory (SI) cortex is likely to play an important role in processing sensory aspects of pain, we here assess whether monitoring SI cortex nociceptive C fibre evoked potentials can provide useful information about central changes related to hyperalgesia in rats. Recordings of tactile and CO_2-laser C fibre evoked potentials (LCEPs) in forelimb and hind limb SI cortex were made 20–24 h after UV-B irradiation of the heel at a dose that produced behavioural signs of hyperalgesia.

LCEPs from irradiated skin increased significantly in duration but showed no significant change in magnitude, measured as area under curve (AUC). By contrast, LCEPs in hind limb SI cortex from skin sites nearby the irradiated skin showed no increase in duration or onset latency but increased significantly in magnitude after UV-B irradiation. The LCEPs in forelimb or hind limb SI cortex elicited from forelimb skin did not change in magnitude, but were significantly delayed in hind limb SI cortex. Tramadol, a centrally acting analgesic known to reduce hyperalgesia, induced changes that counteracted the changes produced by UV-B irradiation on transmission to SI cortex from the hind paw, but had no significant effect on time course of LCEPs from forelimb skin. Tactile evoked potentials were not affected by UV-B irradiation or tramadol. We conclude that altered sensory processing related to hyperalgesia is reflected in altered LCEPs in SI cortex.

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1. Introduction

To develop new analgesics, appropriate animal models of pain are crucial. The current models are based primarily on measuring the changes in motor responses (Sandkuhler and Gebhart, 1984; McMahon et al., 1991; Koltzenburg et al., 1994; Yeomans et al., 1996; Valle et al., 2000; Davies et al., 2005; Bishop et al., 2007; Munro et al., 2008; Saade et al., 2008). Because the nociceptive input to motor systems and to sensory systems are channelled through at least partly different central pathways, with different physiological and pharmacological properties (Weng and Schouenborg, 1996), the validity of motor responses in predicting sensory aspects of pain and analgesia is ambiguous (Kalliomaki et al., 1993b; Weng and Schouenborg, 1996; Palecek et al., 2002). To develop new and effective analgesics, it is therefore crucial to develop supplementary animal models that provide assessments of the activity in the brain regions involved in the sensory aspects of pain. The primary somatosensory (SI) cortex receives strong and somatotopically organized nociceptive input in humans (Apkarian et al., 2005) and animals (Lamour et al., 1983; Kalliomaki et al.,

1993a; Chang et al., 2008; Qiao et al., 2008). In humans, nociceptive evoked potentials in the SI cortex correlate with pain sensation in the normal (Schnitzler and Ploner, 2000) and analgesic situation (Kochs et al., 1990), and sometimes after induction of hyperalgesia (Treede et al., 2003). Although it is clear that other cortical areas also contribute to pain processing, SI cortex plays an important role in this aspect (Apkarian et al., 2005; Lee et al., 2008). Also, animal studies reveal the presence of a population of neurons, receiving nociceptive input, in the SI cortex. These show a graded response to graded nociceptive input from restricted receptive fields (Treede et al., 2003), similar to many neurons in, for example, the ventral posterior lateral nucleus and the dorsal horn of the spinal cord.

Monitoring cortical potentials evoked by electrical or cutaneous CO_2 laser stimulation in animals has shown that nociceptive C fibres provide powerful input to SI cortex (Schouenborg et al., 1986; Kalliomaki et al., 1993b; Qiao et al., 2008). This is mediated by multiple parallel spinal pathways in the rat (Schouenborg et al., 1986; Kalliomaki et al., 1993b). Notably, CO_2 laser C fibre evoked potentials (LCEPs) are reduced following morphine-induced spinal analgesia (Kalliomaki et al., 1998) and increased in an NMDA-dependent way after spinal wind-up (Kalliomaki et al., 2003). It is thus conceivable that rat LCEPs can be used to monitor pain related ascending transmission under various conditions. If this

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notion proves to be correct, LCEPs may provide a useful animal model for the assessment of potentially analgesic drugs.

Our aim was to evaluate whether hyperalgesia, induced by UV-B irradiation of the skin (Davies et al., 2005; Bishop et al., 2007; Saade et al., 2008), is reflected in altered LCEPs in SI cortex. In addition, we examined whether tramadol hydrochloride, a centrally acting analgesic drug known to reduce hyperalgesia (Munro et al., 2008), can counteract the changes in LCEPs following UV-B irradiation.

2. Methods

2.1. Ethical approval

Approval for the experiments was obtained in advance from the Lund/Malmoe local ethical committee on animal experiments, regulated by the code of regulations of the Swedish Board of Agriculture. These regulations, including directives from the European Union, follow the law on animal welfare legislated by the Swedish parliament. The County Administrative Board governs the implementation of the rules. Further, the experiments were in accordance with the policies and guidelines reported by Drummond (Drummond, 2009) and IASP (Zimmermann, 1983).

2.2. Animals used

Twenty-seven Sprague–Dawley (Taconic, Denmark) rats weighing 215 ± 55 g were used whereof 17 were used in the analysis of LCEPs. All rats received food and water *ad libitum* and were kept in a 12-h day–night cycle at a constant environmental temperature of 21 °C and 65% humidity. The animals were kept in the animal facilities of the Biomedical Center at Lund University and the experiments were carried out at the Section for Neuroscience. The facilities are approved by the Swedish Board of Agriculture.

2.3. Induction of hyperalgesia using 1.3 J cm^{-2} UV-B narrowband irradiation

The right hind paw, exposing the heel, was covered with a UV-blocking film from an FR-4 clad board (ELFA, Sweden) and tin foil covered with paper to protect the rat from UV exposure. Sixteen animals were irradiated with 1.3 J cm^{-2} on the right heel (exposure area 8 mm × 9 mm), using a Philips UV-B TL/01 narrowband lamp (PL-S 9 W/01, λ = 300–320 nm). This intensity has been reported to be just below the threshold for blistering (Bishop et al., 2007). Moreover, Bishop et al. (2007) show that UV-B irradiation produces a skin inflammation and a dose-dependent hyperalgesic state. Before every exposure, the lamp was left on for 3 min to allow the UV-B intensity to stabilize. UV intensity was measured before every exposure using a Varicontrol UV/PDT meter and skin tester (Herbert Waldmann GmbH & Co. KG, Germany). Recordings of laser evoked potentials were commenced 20–24 h after irradiation. At this time, discrete to moderate redness of the irradiated skin, but no skin lesions or scarring, was seen on the irradiated skin in each rat, confirming an inflammatory process as has been shown by Bishop et al. (2007). However, one rat later on developed a small blister on the UV-B irradiated skin and was therefore excluded from further analysis. Animals did not exhibit any obvious signs of distress while being handled or observed and appeared to groom normally.

2.4. Nociceptive withdrawal reflex

In order to verify that an effective dose of UV-B irradiation had been given and that the UV-B irradiated rats showed similar changes as have been reported previously at a reflex level (Bishop et al., 2007), behavioural hyperalgesia was assessed before surgery by measuring the threshold of the nociceptive withdrawal reflex of the irradiated and contralateral heels in awake animals (n = 14). A radiant heat CO_2 laser (Irradia, Sweden; model 315 M Superpulse, wavelength 10.6 μm, output power 10 W, beam diameter 3.0 mm; pulse length of 18–24 ms stimulation) was used. Loosely embedded in a towel, the rat rested calmly, with no signs of distress, in the hands of our experienced laboratory technician while the experimenter operated the laser. The duration of the CO_2 laser pulse was increased in steps of 2 ms until the threshold, defined as a response in three of five trials, was reached.

2.5. Surgery and preparation for electrophysiology

The rats were anaesthetized with isoflurane (1.8–2.0% during surgery) in a mixture of 40% oxygen and 60% nitrous oxide. The adequacy of the depth of anaesthesia was assessed throughout the surgery by applying noxious pinch to check for reflexes or by monitoring blood pressure. The trachea was cannulated and the animals were artificially ventilated. The end-expiratory PCO_2 was monitored continuously. An infusion of 2.5–4.5 ml h^{-1} of 5% glucose (50 mg ml^{-1}) in Ringer's acetate was given through the right jugular vein. Mean arterial blood pressure was monitored continuously in the left femoral artery. The rectal temperature was kept at 36.5–38.5 °C, using a feedback-regulated heating system. The spinous process of T11 was clamped and the chest lifted to facilitate ventilation. The rat's head was fixed by ear bars after administration of local analgesia (EMLA® salve 5%; eutectic mixture of 2.5% prilocaine and 2.5% lidocaine, AstraZeneca, Södertälje, Sweden) and a nose ring. Cerebrospinal fluid was drained between the base of the skull and the first cervical vertebra to reduce the risk of cortical oedema (Kalliomaki et al., 1993a). A craniotomy exposing the left parietal cortex was made. The dura mater was cut and the surface covered with paraffin oil. Local infiltration of lignocaine (Xylocaine® 20 mg ml^{-1} + 12.5 μg ml^{-1} adrenaline, Dentsply Ltd., Addlestone, Weybridge, England) was made during all surgery to reduce the nociceptive input. After completed surgery, the muscle relaxant pancuronium bromide 0.2 ml (Pavulon® 2 mg ml^{-1}, Organon AB, Göteborg, Sweden) was given and thereafter 0.15 ml once every hour. Also, the isoflurane level was lowered to 0.8–0.9% in the same gas mixture as before. This anaesthetic level was characterized by an EEG dominated by 4–6-Hz waves. The EEG was recorded (recording sites same as used for recording of potentials below) for periods of 90 s using fine silver ball-tipped electrodes (∼0.3 mm diameter) and analysed with Signal 3.05 software (Cambridge Electronic Design Limited, Cambridge, England). The sampling rate for EEG recordings was 500 Hz. Experiments were terminated after any signs of deterioration such as cortical oedema, hind paw oedema or a precipitous decline in expiratory PCO_2 (five rats were discarded on these criteria). At the end of the experiment, the rats were killed with an overdose of isoflurane in a mixture of 40% oxygen and 60% nitrous oxide. When the PCO_2 and blood pressure was 0, air was injected i.v.

2.6. Mappings of cutaneous representation on the SI cortex

The SI representations of tactile and nociceptive input from the same skin area overlap to a large extent, with the tactile input being more (Kalliomaki et al., 1993a). Moreover, it is known that neurons receiving nociceptive input in SI cortex often also receive a tactile input from the same area on the skin (Lamour et al., 1983; Kalliomaki et al., 1993a). To avoid input from nociceptors as much as possible, which in itself may produce excitability changes in peripheral and central pathways, tactile input was used to locate the cortical representation of the glabrous

skin of the arch and heel of the right hind paw (Fig. 1a) and of the digit area of the right forepaw. A hand-held electromechanical stimulator with a blunt metal probe (0.8 mm diameter) attached to a coil was used for tactile stimulation. The probe was displaced 1 mm by a current pulse (10 ms) generated by a Grass stimulator. The stimulation was adjusted to cause a light touch of the skin activating tactile Aβ fibres, without any visible joint movement. The recordings of the tactile evoked potentials were amplified and monitored using Signal 3.05 software. Evoked potentials were sampled from 6 to 18 cortical sites in hind paw and forepaw areas respectively (coordinates: hind paw −0.6 to −3.3 mm rostro-caudal to bregma and 1.3–2.7 mm lateral to the midline; forepaw 1.0 to −2.14 mm rostro-caudal to bregma and 3.0–4.3 mm lateral to the midline). For each skin area/rat, the cortical site eliciting potentials with the highest amplitude was used for recordings. In total, three electrodes were placed on SI cortex. Fig. 1b shows the distribution of the recording sites used.
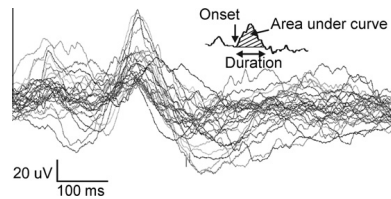
2.7. Nociceptive stimulation

To elicit C fibre evoked potentials, the glabrous skin of the right hind paw/forepaw areas were stimulated using a CO_2 laser with a pulse duration of 21–33 ms. These stimulation energies have been shown to evoke late cortical field potentials reliably in the rat SI cortex through the activation of cutaneous nociceptive C fibres (Kalliomaki et al., 1993a,b). Based on previous latency measurements (Kalliomaki et al., 1993a) we here classify evoked potentials with an onset latency exceeding 120 ms and 180 ms for forepaw and hind paw, respectively, as C fibre evoked. Superimposed averaged recordings can be seen in Fig. 2. During the stimulation of a given skin area, e.g. the heel, a train of 16 CO_2 laser pulses with a frequency of 1 Hz was used. The stimulation site, within the skin area, was shifted between the pulses to avoid repeated stimulation of the same site, as this could reduce LCEPs (Kalliomaki et al., 1993a). No visible damage to the skin was observed.
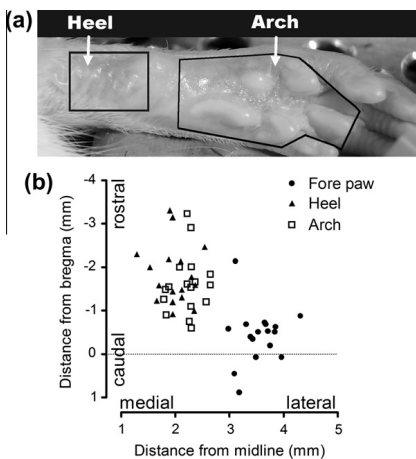
Fig. 1. (a) Hind paw stimulation areas. The heel and arch areas of the rat hind paw that were subjected to stimulation are indicated. (b) Recording sites on SI cortex. All animals (n = 17) used in the LCEPs analysis are included. Coordinates in mm are given with respect to bregma and midline for forepaw digits, heel (heel of the hind paw), arch (arch of the hind paw).

Fig. 2. Superimposed recordings of LCEPs. The raw data recordings from naïve (n = 7) animals (28 averaged recordings) after nociceptive stimulation illustrates the variation of the onset, duration and AUC of the LCEPs. The inset shows a single averaged (n = 16) recording.

2.8. Electrophysiological recordings and sequence of stimulations

LCEPs elicited in the contralateral SI cortex representation area of the forepaw digits (forepaw), the heel and the arch of the hind paw were recorded simultaneously (Fig. 1b). Rats (n = 5) that did not show clear LCEPs from forelimb stimulation, which served as a control of transmission of nociceptive input to cortex, were excluded from analysis of LCEPs. The time interval between trains of stimulations per cutaneous area and individual site was set to 10 min. Each train consisted of 16 stimulations. These recordings were averaged and the trains repeated five times for each cutaneous area. All data on LCEPs reported in this study are based on averaged LCEPs. From animals receiving tramadol (Tradolan® 50 mg ml^{-1}, tramadol hydrochloride, Nordic Drugs, Limhamn, Sweden), an additional five LCEPs were collected after drug administration. The first LCEPs recording per SI cortex area was not used in the analysis, as the controls showed a stable baseline after the first train. Since it is necessary to establish a stable baseline before testing the effect of a drug and the LCEP resulting from the first train tended to be larger than the subsequent LCEP, the first train of stimulation was excluded. Notably, there was no trace of potentiation of LCEP resulting from the first train of stimulation. The sequence of stimulations was at time (t in minutes) t_0 for heel, t_5 for arch of the hind paw (arch) and t_7 for forepaw. EEG was monitored 90 s every 10 min five times, starting at t_3.

2.9. Drug administration

In the UV-B irradiated group, 2 mg kg^{-1} of tramadol was administered i.v. 5–10 min after the fifth completed cycle. Tramadol at this dose is known to be analgesic (Kayser et al., 1991). Twenty minutes after tramadol administration, the sequence of stimulations was repeated five times. The first recording used in the analysis with tramadol was commenced 30 min after drug injection.

2.10. Data analysis of EEG and evoked potentials

The signals (10 kHz sampling frequency), were amplified and filtered using Digitimer Neurolog system (Digitimer Ltd., England) with a low cut-off frequency of 1 Hz and a high cut-off frequency of 700 Hz. The epoch length was 0.7 s with a pre-stimulus interval of 10 ms for evoked potentials and 90 s for EEG recordings. Fourier analysis was used to analyse the EEG.

CO_2 laser Aδ evoked potentials (onset 20–45 ms), occurred irregularly and were therefore not analysed in detail. In case of C fibre input, due to their slow conduction velocity the impulses arrive to the spinal cord during a relatively long time period. Therefore, to obtain a representative measure of the magnitude of the activity evoked by nociceptive C fibres following a laser stimulus the area under the curve (AUC) (inset in Fig. 2) was calculated using in-house scripts created in Scilab-4.1.1 (INRIA, France). The

AUC was defined as the sum of amplitudes between the baseline level and LCEPs, with a maximum duration of 300 ms. Baseline was set to the amplitude at the onset latency of each LCEP.

As for tactile evoked potentials, the onset latency and peak amplitude of the initial positive surface potential, defined as the maximal amplitude of the averaged ($n = 16$) recording within an interval of 10–33 ms from the onset of the stimulus, was measured. Since the tactile input is much more synchronized and short lasting than the nociceptive input and the decay phase of the first tactile potential overlaps with subsequent more variable potentials, AUC was not used as a measure for the tactile potential.

2.11. Statistical analysis

Four averaged recordings from each cutaneous area were collected from each animal. In animals receiving tramadol an additional four CO_2 laser evoked potential trials were collected after drug administration. The amplitude of tactile evoked potentials and the AUC, duration and latency of LCEPs were used to compare the differences between the groups. The examined data was assumed to be normally distributed and Student's *t* test was used for statistical analysis. The unpaired two-tailed *t* test was used to compare the difference between naïve and irradiated rats. Paired two-tailed *t* test was used to analyse the thresholds of the nociceptive withdrawal reflex in irradiated rats. Furthermore, paired two-tailed *t* test was used in the analysis of UV-B irradiated rats before and after tramadol administration. Seven rats from the UV group were administered tramadol and used in the analysis of LCEPs. A *p* value < 0.05 was considered significant.

3. Results

3.1. General findings

Tactile evoked potentials with peak latencies between 10 and 33 ms were reliably evoked in all rats (Kalliomaki et al., 1993a).

Early potentials from Aδ-fibres (onset latency 20–45 ms) were on some occasions evoked in homotopic SI cortex (e.g. in forelimb representation on forelimb stimulation), but not in heterotopic SI cortex (e.g. hind limb representation on forelimb stimulation), and can be seen in the grand mean recordings (Figs. 3 and 5). It is plausible that the stimulus intensity must be higher than used here in order to reliably activate nociceptive Aδ fibres (LaMotte et al., 1982). Since these potentials were too variable they were not analysed further. Similar observations have been made in previous studies using a similar stimulation paradigm (Kalliomaki et al., 1993a).

In all included rats, a late surface-positive field potential was evoked with onset latencies starting around 160–200 ms on forelimb stimulation and 200–240 ms on hind limb stimulation with a duration exceeding 110 ms, corresponding to input from nociceptive C fibres (Figs. 3 and 5). This potential has previously been found to be due to C fibre input (Kalliomaki et al., 1993a). The energy level (30–47 mJ mm^{-2}) of the CO_2 laser pulse to elicit LCEPs corresponds to C nociceptive activation in rats (Kalliomaki et al., 1993a) and humans (Bromm et al., 1984). The LCEPs exhibited the largest amplitude in the homotopic SI cortex but were also seen, albeit at lower magnitude, in heterotopic SI cortex in accordance with previous findings (Kalliomaki et al., 1993b). The LCEPs from the forepaw were used to provide information on heterosegmental effects, whereas the heel and arch of the hind paw were used to test the segmental effects on transmission from the UV-B irradiated skin and adjacent non-irradiated skin. These two latter areas represent skin areas where primary hyperalgesia (Bishop et al., 2007) and secondary hyperalgesia, in human (Gustorff et al., 2004), may occur.

3.2. Behavioural effects of UV-B irradiation

The effect of UV-B irradiation on the reflex threshold was evaluated by comparing the withdrawal responses from the irradiated heel on CO_2 laser stimulation with those evoked from the contralateral heel in awake animals. This comparison confirmed an increased sensitivity in the irradiated heel (Davies et al., 2005; Bishop et al.,
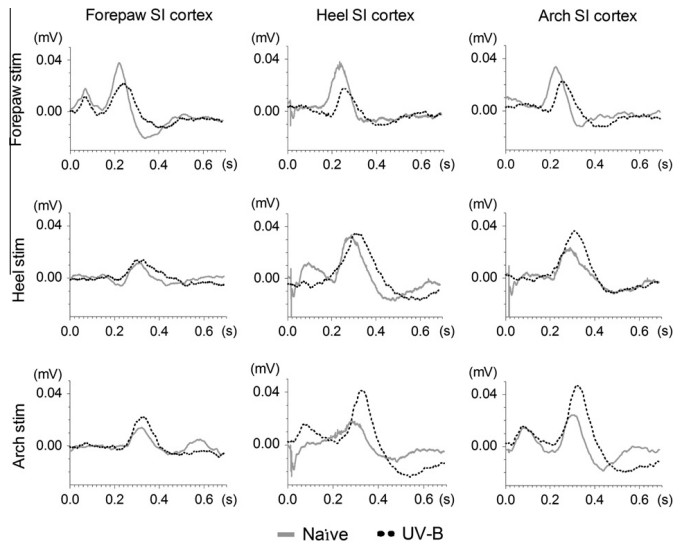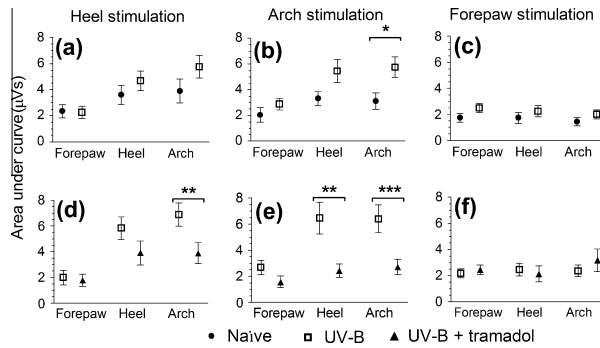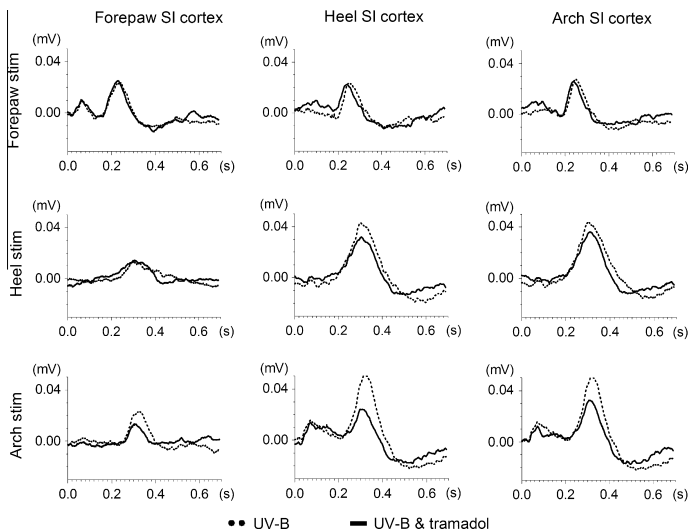

Fig. 3. Grand mean of LCEPs recordings from three cortical areas. The grand mean of recordings from naïve ($n = 7$) and UV-B irradiated rats ($n = 10$) are plotted. CO_2 laser stimulation (stim) evoked potentials starting at ~200 ms for the hind paw areas and at ~160 ms for the forepaw digits (forepaw). Arch denotes the arch of the hind paw.

Fig. 4. Magnitude of LCEPs recordings from three cortical areas. Top: The difference between means and S.E.M. of area under the curve (AUC) for the naïve (black circles) and the UV–B exposed (white boxes) groups are shown. Bottom: The mean of differences and S.E.M. of AUC in UV–B exposed rats ($n = 7$) before (white boxes) and after ($n = 7$) (black triangles) tramadol administration are shown. The x-axis depicts the recording areas on SI cortex. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig. 5. Grand mean of LCEPs recordings from three cortical areas. The grand mean of recordings from UV–B irradiated rats before ($n = 7$) and after ($n = 7$) tramadol administration are shown. CO_2 laser stimulation (stim) evoked potentials starting at ~200 ms for the hind paw areas and at ~170 for forepaw digit (forepaw) stimulation. Arch denotes the arch of the hind paw.

2007) with reflex thresholds significantly lower (7.2%, mean of differences −15 mJ, $p < 0.01$) compared to the contralateral heel.

3.3. Effects of UV–B irradiation on cortical evoked potentials

LCEPs in homo- and heterotopic SI cortex in rats irradiated on the heel ($n = 10$) were compared to naïve rats ($n = 7$).

3.3.1. Responses from UV–B irradiated skin
On stimulation of the UV–B irradiated skin (heel), LCEPs in SI cortex for the corresponding area displayed a significantly longer duration (difference between means 60 ms, $p < 0.01$) (Table S1, online only) compared to naïve animals ($n = 7$) but showed no signif-

icant change in onset latency (Table S2, online only), although there was a tendency towards shorter onset latency in heel SI cortex. Similarly, the duration (Table S1) of the LCEPs in the SI cortex area corresponding to nearby non-irradiated skin displayed a longer duration (difference between mean 91 ms, $p < 0.01$) in the UV group and showed no significant change in onset latency or magnitude (Fig. 4a). The LCEPs in the forepaw SI cortex of UV–B irradiated rats did not differ from naïve rats.

3.3.2. Responses from non-irradiated nearby skin
On stimulation of the arch, the magnitude (Fig. 4b) of the LCEPs in the arch SI cortex area was significantly higher (46%, $p < 0.05$) compared to that in naïve rats. LCEPs onset latency (Table S2)

and duration (Table S1) did not differ. Furthermore, the magnitude, onset latency and duration of the LCEPs in heel SI cortex did not differ from naïve rats, although the magnitude of LCEPs in the heel SI cortex tended to increase. Likewise, the LCEPs in the forepaw SI cortex area did not differ significantly from naïve rats.

3.3.3. Responses from forepaw skin area

On forepaw stimulation, the magnitude (Fig. 4c) and onset latency (Table S2) of the LCEPs in the forepaw SI cortex did not differ between irradiated and naïve rats, although the duration (Table S1) was significantly longer in irradiated rats (difference between means 44 ms, $p < 0.05$). LCEPs in the heel and arch SI cortex in the irradiated rats exhibited later onset latency (difference between means 29 ms and 41 ms respectively, $p < 0.001$) compared to the naïve rats on forepaw stimulation.

3.3.4. Effect of UV-B irradiation on tactile evoked potentials

UV-B irradiation elicited no significant changes in tactile evoked potentials in homotopic cortical areas. See Table S3 (online only) for details on onset latencies and peak amplitude.

3.4. Effects of tramadol on rats exposed to UV-B irradiation

LCEPs in homo- and heterotopic SI cortex in rats irradiated on the heel were compared before ($n = 7$) and after ($n = 7$) administration of 2 mg kg^{-1} tramadol (i.v.). Detailed data on the effects are shown in Fig. 4d–f and Tables S4 (online only) and S5 (online only). Further, the mean of the averaged recordings for all groups are shown in Fig. 5. Tramadol had no obvious effect on the EEG.

3.4.1. Responses from irradiated skin after tramadol administration

Tramadol did not affect the magnitude (Fig. 4d) of LCEPs evoked in homotopic SI cortex, but did reduce the magnitude of LCEPs in arch SI cortex (44%, $p < 0.01$). Further significant change noted was a reduction in the duration (Table S5) of LCEPs in the heel SI cortex (mean of differences 57 ms, $p < 0.05$) and in the arch SI cortex (mean of differences 100 ms, $p < 0.001$). Also, the onset latency (Table S4) of LCEPs in the arch SI cortex was delayed (mean of differences 33 ms, $p < 0.05$). These changes thus reversed the changes noted above after UV-B irradiation.

3.4.2. Responses from non-irradiated nearby skin after tramadol administration

Tramadol decreased the magnitude (Fig. 4e) of the LCEPs in arch (56%, $p < 0.001$) and heel (62%, $p < 0.01$) SI cortex areas on stimulation of non-irradiated nearby skin. In these SI cortex areas, also the duration of LCEPs decreased (mean difference in arch SI cortex 69 ms and heel SI cortex 72 ms, $p < 0.05$). Furthermore, the onset latencies were unaffected. No change of LCEPs in forelimb SI cortex was seen (Fig. 4e, Tables S4 and S5). These changes thus reversed the changes noted above after UV-B irradiation.

3.4.3. Responses from distant skin after tramadol administration

Tramadol had no significant effect on the transmission in pathways originating from the forepaw. Detailed information is shown in Fig. 4f and Tables S4 and S5.

3.4.4. The effect of tramadol on tactile evoked potentials

Tramadol administration had no effect on tactile evoked potentials in UV-B irradiated rats. See Table S3 for details on onset latencies and peak amplitude.

4. Discussion

In the present study, we recorded nociceptive C fibre evoked potentials from the SI cortex. These potentials arise from depolarization of the deeper layers of the cortex on synchronous input from several ascending pathways, mediating input from nociceptive C fibres (Kalliomaki et al., 1993b). For this reason, they may provide information on the overall ascending activity, as they reflect the summed activity in many ascending pathways. From previous studies it is known that spinal opioidergic analgesia or potentiation can be monitored by recording these potentials (Kalliomaki et al., 1998). The present data indicate, in addition, that it is possible to monitor changes in central nociceptive transmission after UV-B induced hyperalgesia. By recording the representations of the skin area of inflammation, an adjacent skin area not exposed directly to UV-B and a skin area distant from the affected regions, we were able to monitor changes that are reminiscent of primary hyperalgesia and secondary hyperalgesia in the same animal. An analogous method to produce hyperalgesia and record transmission to the cortex is possible in humans, making translational research conceivable. The present study also indicates that tramadol, a centrally acting opiate, reverses hyperalgesia. While nociceptive Aδ fibre evoked potentials were too irregular to allow detailed statistical analysis, it may be worth commenting that these potentials only occurred in the area exhibiting maximal tactile potentials in the control animals, whereas the nociceptive C fibre evoked potentials were much more widespread. It is tempting to speculate that this difference in cortical activation between Aδ and C nociceptive input underlies the well known difference in spatial characteristics of first and second pain in humans (Lewis and Pochin, 1937). This remains to be tested using evoked potentials in humans.

4.1. Hyperalgesia—features and mechanisms

Transmission from the UV-B irradiated skin area to the target area in the SI cortex showed signs of primary hyperalgesia such as longer duration and a tendency towards shorter latency of the response. Similar observations have been made in dorsal horn neurons (wide dynamic-range (WDR) neurons) after UV-B exposure (Urban et al., 1993). In particular, after UV irradiation of their receptive fields, WDR neurons responded to both tactile and nociceptive input with increased duration in response to heat stimulation as well as exhibiting expanded receptive fields. Nociceptive-specific neurons also exhibit a lower threshold and increased response on cutaneous stimulation in the hyperalgesic situation (Hedo et al., 1999; Sandkuhler, 2009). These changes are known to involve both central and peripheral mechanisms, although in the case of primary hyperalgesia, the peripheral sensitization appears to play a major role (LaMotte et al., 1982, 1992). The reason why the magnitude, i.e. area under curve, of the LCEPs did not increase in the pathway from the irradiated skin area to its primary projection area in the SI cortex is not clear. Because evoked potentials are dependent on relatively synchronous input, one conceivable explanation is that the nociceptive C fibre input from the primary skin area and consequent activity in the dorsal horn are desynchronized somewhat by the ongoing spontaneous activity, thereby partly masking the stronger nociceptive input from the primary hyperalgesic skin area. An alternative, but not mutually exclusive, possibility is that the response frequency in nociceptive C fibres on CO_2 laser stimulation of the irradiated skin area, despite being sensitized (Andrew and Greenspan, 1999), decreases in the hyperalgesic situation. Nociceptive C fibres fatigue easily (Torebjork et al., 1984), and there is evidence that, after skin inflammation, induced by injection of complete Freund's adjuvant, the

response frequency on moderate to strong noxious heat stimulation decreases in C fibres (Andrew and Greenspan, 1999). A third possibility is that the transmission pathways from nociceptors from UV-B irradiated and adjacent skin areas (secondary hyperalgesic) to the SI cortex are under different control. There is evidence for a differential descending inhibitory control of transmission from primary hyperalgesic skin areas and excitatory control of secondary hyperalgesic areas (Vanegas and Schaible, 2004). Further studies will be necessary to resolve this issue. Notably, the potentiation of the LCEPs elicited from the arch, assumed to be related to mechanisms of secondary hyperalgesia, was surprisingly stronger than the changes in the transmission from the primary hyperalgesic skin area. The mechanisms underlying secondary hyperalgesia have for a long time been assumed to arise mainly from central mechanisms (Torebjork et al., 1984; Sandkuhler, 2009). It is conceivable, but remains to be tested, that NMDA dependent mechanisms triggered by ongoing spontaneous input from sensitized nociceptors are involved, since it is known that MK-801 (an NMDA antagonist) blocks frequency dependent potentiation of LCEP (Kalliomaki et al., 2003). We stimulated a skin area on the arch located about 5–15 mm away from the border of the UV-B irradiated skin area. This distance is greater than the known expansion of receptive fields caused by inflammation (Andrew and Greenspan, 1999). Nevertheless, if the spread of hypothetical algogenic chemicals, caused by inflammation, to nearby skin areas sensitize mechano-insensitive C nociceptors at a distance, then such changes may contribute to the enhanced evoked potentials from the arch. As mentioned above, there is evidence for an excitatory supraspinal control of transmission from the secondary hyperalgesic skin area, which, if operative, might have contributed in the present situation. An additional central mechanism underlying the increased transmission from the adjacent skin area is the expansion of receptive fields of dorsal horn neurons (Hylden et al., 1989; Urban et al., 1993). Also, thalamic neurons exhibit enhanced responses to heat and mechanical stimuli in parts of their receptive field remote from the injury site (Guilbaud et al., 1986).

4.2. Effects of tramadol

Our data are consistent with a previous report showing that UV light-induced hyperalgesia can be reduced with opioids and non steroidal anti-inflammatory drugs (NSAIDs) (Bishop et al., 2007). Our present data suggest that tramadol ($2 \, \text{mg} \, \text{kg}^{-1}$) to a large extent reverses the changes noted after UV-B irradiation, but had no effect on transmission from forepaw. Notably, tramadol administration lessened both the increase in transmission from the arch (nearby non-irradiated skin) to the arch and heel SI cortex, and the prolonged duration of the input from the heel (irradiated skin) input to the heel and arch representation. Interestingly, tramadol has been reported to affect hyperalgesia as measured using reflex responses (Munro et al., 2008). Interestingly, a significantly increased duration of forelimb LCEP evoked by forelimb stimulation after UV-B irradiation and increased latencies for the arch and heel LCEP evoked on forelimb stimulation was found, supporting a report (Kayser and Guilbaud, 1987) showing that hyperalgesia in one body part may affect nociceptive transmission from distant body parts. That tramadol did not affect this distant effect may indicate that it is produced by mechanisms other than those related to primary and secondary hyperalgesia.

We emphasize that our aim was not to characterize the effects of tramadol per se, but rather to evaluate the hypothesis that the LCEPs can be used to monitor the changes occurring after induction of hyperalgesia. That it was possible to reduce the changes in the LCEPs occurring after UV-B exposure adds strength to this hypothesis.

4.3. Animal models for hyperalgesia

Several animal models are used to study hyperalgesia, and different agents can be used to induce inflammation. In both humans and rats, cutaneous UV exposure has emerged as an important inflammatory pain model because it causes hyperalgesia (Urban et al., 1993; Davies et al., 2005; Bishop et al., 2007; Saade et al., 2008) by releasing endogenous substances, which evoke inflammation and sensitize the peripheral nociceptive terminals (Treede et al., 1992). This model has several advantages, and most importantly, the model can be used in analogous human studies safely to permit translational studies. UV-B irradiation elicits both primary hyperalgesia and secondary hyperalgesia in humans (Gustorff et al., 2004).

At present, most animal studies of nociceptive transmission and analgesia are based on measuring different types of behavioural changes (Sandkuhler and Gebhart, 1984; McMahon et al., 1991; Koltzenburg et al., 1994; Yeomans et al., 1996; Valle et al., 2000; Davies et al., 2005; Bishop et al., 2007; Munro et al., 2008; Saade et al., 2008); in particular, reflex responses. Because the reflex pathways and the ascending pathways to SI cortex differ at least partly, it is not always clear to what extent information derived from reflex tests is a valid predictor of the sensory aspects of pain related activity. LCEPs thus have the potential to be a useful complement to behavioural and reflex tests when screening potential analgesic drugs.

4.4. Conclusion

In summary, changes in transmission in nociceptive pathways to primary somatosensory cortex induced by UV-B irradiation of the skin can be monitored by recording CO_2 laser C fibre evoked potentials in SI cortex. Therefore, this way of monitoring the pain related pathways appears to be a useful supplement to animal behavioural tests of mechanisms related to pain and analgesia.

Acknowledgements

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejpain.2010.09.009.

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Duration: difference between means of UV-B and naive group			
stimulation site / SI recording area	(s)	(%)	significance
heel / heel	0,060	34	**
heel / arch	0,091	54	**
heel / forepaw	0,014	8	ns
arch / arch	0,028	16	ns
arch / heel	0,040	25	ns
arch / forepaw	0,003	2	ns
forepaw / forepaw	0,044	38	*
forepaw / heel	0,030	24	ns
forepaw / arch	0,014	12	ns

Table S1. Difference in duration of the LCEPs between naïve and UV-B exposed rats. The mean of the duration in the naïve group (n = 7) is subtracted from the mean value in the UV-B exposed group (n = 10). Significant differences are indicated in grey. * p < 0.05,** p < 0.01, using unpaired t test.

Onset: difference between means of UV-B and naive group			
stimulation site / SI recording area	(s)	(%)	significance
heel / heel	-0,016	-7	ns
heel / arch	-0,022	-10	ns
heel / forepaw	0,004	2	ns
arch / arch	0,002	1	ns
arch / heel	0,007	3	ns
arch / forepaw	0,005	2	ns
forepaw / forepaw	0,000	0	ns
forepaw / heel	0,029	17	***
forepaw / arch	0,041	25	***

Table S2. Difference in onset latency between naïve and UV-B exposed rats. The mean of the onset latency in naïve rats (n = 7) is subtracted from the mean value of the UV-B exposed rats (n = 10). Values are shown in seconds (s) and percentage. Significant differences are indicated in grey. *** p < 0.001, using unpaired t test.

stimulation site/SI recording area:		heel / heel	arch / arch	forepaw / forepaw
A: Onset latency:	**naïve (n=7)**	0.011 /0.0014/	0.011 /0.0014/	0.009 /0.0017/
mean /±SEM/ (s)	**UV-B (n=10)**	0.008 /0.0003/	0.009 /0.0004/	0.007 /0.0003/
	significance	ns	ns	ns
	UV-B (n=7)	0.0009 /0.0005/	0.009 /0.0004/	0.008 /0.0004/
	UV-B+t (n=7)	0.0009 /0.0003/	0.009 /0.0001/	0.007 /0.0004/
	significance	ns	ns	ns
B: Peak amplitude:	**naïve (n=7)**	105 /13/	106 /9/	132 /18/
mean /±SEM/ (µV)	**UV-B (n=10)**	101 /5/	106/12/	127 /6/
	significance	ns	ns	ns
	UV-B (n=7)	102 /7/	101 /9/	129 /8/
	UV-B+t (n=7)	93 /9/	82 /10/	113 /12/
	significance	ns	ns	ns

Table S3. Mean and SEM of onset latencies and peak amplitudes of tactile evoked potentials recorded from the cortical surface. The mean and SEM of averaged tactile evoked potentials are shown between naïve (n=7), UV-B irradiated rats (n=10) as well as UV-B irradiated rats before (n=7) and after (n=7) tramadol administration (UV-B+t). Ns depicts no significant difference.

Onset: mean of differences before and after tramadol administration			
stimulation site / SI recording area	(s)	(%)	significance
heel / heel	0,008	4	ns
heel / arch	0,033	17	*
heel / forepaw	-0,006	-2	ns
arch / arch	0,017	7	ns
arch / heel	0,016	7	ns
arch / forepaw	0,004	2	ns
forepaw / forepaw	0,004	2	ns
forepaw / heel	-0,014	-7	ns
forepaw / arch	-0,007	-4	ns

Table S4. The effect of tramadol on the LCEPs onset latency in irradiated rats. The mean of the onset latency in UV-B exposed rats before (n = 7) tramadol administration is subtracted from the value of the onset latency in UV-B exposed rats after (n = 7) tramadol administration. The values are shown in seconds (s) and percentage. Significant differences are indicated in grey. * p < 0.05, using paired t test.

Duration: mean of differences before and after tramadol administration			
stimulation site / SI recording area	(s)	(%)	significance
heel / heel	-0,057	-24	*
heel / arch	-0,100	-37	***
heel / forepaw	-0,019	-10	ns
arch / arch	-0,069	-33	*
arch / heel	-0,072	-34	*
arch / forepaw	-0,019	-11	ns
forepaw / forepaw	-0,009	-6	ns
forepaw / heel	-0,043	-27	ns
forepaw / arch	0,012	8	ns

Table S5. The effect of tramadol on the LCEPs duration. The mean of the duration in the UV-B exposed group before (n = 7) tramadol administration is subtracted from the mean in the UV-B exposed group after (n = 7) tramadol administration. The values are shown in seconds (s) and percentage. Significant differences are indicated in grey. * p < 0.05, *** p < 0.001, using paired t test.