# Drop of the noxious heat threshold induced by surgical incision in the rat: mediators, pharmacological modulation by analgesics and by a novel peripheral neuroregulatory mechanism

**Doctoral (PhD) Dissertation**

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# Abbreviations

| | |
|---|---|
| ANOVA | repeated measures analysis of variance |
| ATP | adenosine 5'-triphosphate |
| CGRP | calcitonin gene-related peptide |
| C-SOM | cyclosomatostatin |
| DNIC | diffuse noxious inhibitory controls |
| GDNF | glial cell line-derived neurotrophic factor |
| i.p. | intraperitoneal |
| i.pl. | intraplantar |
| L-NOARG | $N^{(G)}$ -nitro-L-arginine |
| NDGA | nordihydroguaiaretic acid |
| NGF | nerve growth factor |
| NKA | neurokinin A |
| NKB | neurokinin B |
| NO | nitric oxide |
| s.c. | subcutaneous |
| SP | substance P |
| SRIF | somatotropin release-inhibiting factor |
| TNP-ATP | 2′,3′-O-(2,4,6- trinitrophenyl) adenosine 5′-triphosphate |
| TRPA1 | transient receptor potential ankyrin 1 receptor |
| TRPV1 | transient receptor potential vanilloid 1 receptor |

# 1 Introduction

## 1.1 Basic terms used in pain research

Pain is an unpleasant sensation caused by intense stimuli that can lead to tissue damage. As pain is a subjective concept, in case of studies conducted on animals such sensations cannot be reported, thus other paradigms have been set up.

Nociception is the neural processes of encoding and processing of noxious (i.e. painful) stimuli resulting in a behavioral response. The reactions observed are called nocifensive behaviors, including avoidance of the noxious stimulus, flight and vocalization. The time elapsed between the application of the noxious stimulus and the occurrence of the nocifensive reaction is the reflex latency time. According to the evoking stimulus there is thermonociception, mechanonociception and chemonociception. Encoding takes place in specific uni- or polymodal nociceptors. Unimodal nociceptors can be activated by only one type of stimulus (e.g. high-threshold mechanoreceptors), while polymodal nociceptors respond to several types of stimuli such as noxious heat, mechanical and chemical stimuli.

Hyperalgesia is an increased pain sensation to noxious stimuli. It is characterized by a decreased pain threshold and an enhanced response to suprathreshold stimuli. Allodynia is an extremely sensitized state which is defined as pain evoked by non-noxious stimuli (e.g. touch). By the modality of the stimulus thermal hyperalgesia/allodynia, mechanical hyperalgesia/allodynia, and chemical hyperalgesia/allodynia can be distinguished. An antinociceptive effect means alleviation of nociception in hyperalgesic or non-hyperalgesic states by chemical agents or physical interventions.

## 1.2 Nociceptors and their functions

Nociceptors are specialized nerve endings of primary afferent neurons. These pseudounipolar neurons innervate the skin, mucous membranes and internal organs. Their peripheral axons form the sensory nerve fibers and the cell bodies are located in the dorsal root ganglia of the spinal cord or in the trigeminal ganglion. The central axons conduct the signal to the dorsal horn of the spinal cord and to the trigeminal nucleus. Nociceptors are sensitive to stimuli that are endangering the cellular integrity of the innervated tissues. These stimuli cause depolarization of the plasma membrane of the nerve ending. If the intensity of the stimulation reaches the threshold, an action potential is elicited, and is conducted to the central nervous system.

Sensitization of the peripheral nociceptive nerve endings means an increase in responsiveness to noxious stimuli, thus the threshold for activation becomes lower. Sensitization may occur as a consequence of presence of inflammatory mediators released at the site of tissue injury or inflammation[1].

Nociceptors can be classified in different ways. Based on the characteristics of their axons there are Aδ type nociceptors with myelinated fibers with a conduction velocity of 12–30 m/s and C type nociceptors with non-myelinated fibers with a conduction speed of 0.5–2 m/s.

Contrary to other sensory receptors the majority of nociceptors are polymodal i.e. they can be activated by noxious heat and mechanical stimuli as well as by a variety of endogenous and exogenous chemical substances. The polymodal nociceptors, especially those of C type, are crucial in the sensation of noxious heat because there are no other types of fibres that can be activated by this stimulus[1]. Another characteristic feature of the polymodal nociceptors is that they express in their membrane the pharmacological receptor of capsaicin, the pungent agent of hot pepper. This receptor is called TRPV1 (transient receptor potential vanilloid 1) and is a non-selective cation channel consisting of six transmembrane domains. It can be activated by temperatures above 43 ℃, low pH, lipoxygenase products,

cyclooxygenase (COX) products, nitric oxide (NO) and other chemical stimuli [2]. The activation of the receptor leads to the opening of the ion channel and influx of $Na^{+}$ and $Ca^{2+}$ ions, resulting in depolarization of the membrane and development of an action potential.

Another TRP channel expressed by nociceptive primary afferent neurons is TRPA1 (transient receptor potential ankyrin 1)[3]. It can be activated by environmental irritants and cold stimuli. Its endogenous activator is 4-hydroxynonenal (α,β-unsaturated aldehyde) that accumulates in membranes during inflammatory reaction and oxidative stress. Exogenous substances include allyl isothiocyanate, the pungent substance of mustard oil, wasabi and horseradish, cinnamaldehyde, formalin and hydrogen peroxide, the later can be endogenous or exogenous.

From a neurochemical aspect non-peptidergic and peptidergic polymodal nociceptors can be distinguished. Both types contain glutamate, a ubiquitous amino acid neurotransmitter. Non-peptidergic nociceptors express isolectin B4 in their plasma membrane. These neurons require the presence of glial cell line-derived neurotrophic factor (GDNF) for growth and functioning. In contrast, peptidergic nociceptors lack isolectin $B_4$, depend on nerve growth factor (NGF), and contain neuropeptides, such as substance P (SP), neurokinin A and B (NKA, NKB), calcitonin gene-related peptide (CGRP) and somatostatin, which are released upon activation[4].

Peptidergic nociceptors, besides their afferent function (conducting action potentials in response to adequate stimulation to the central nervous system) have efferent functions, too (Fig. 1). These latter functions are local tissue responses evoked by neuropeptides released from nociceptors. Two main types of these responses are neurogenic inflammation and smooth muscle reactions. Neurogenic inflammation is characterized by vasodilatation and plasma extravasation mediated by CGRP and substance P. Smooth muscle reaction can be contraction, predominantly evoked by substance P and neurokinin A (NKA), or relaxation mediated by CGRP[5].

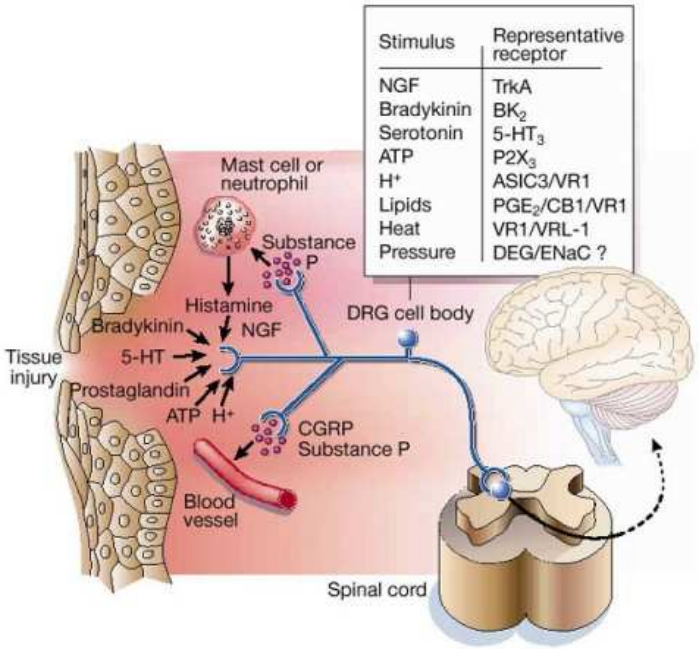


**Fig. 1.** Scheme for the local efferent function of peptidergic nociceptors (from reference [5])

At our department Pintér and Szolcsányi discovered that following bilateral transection of the $4^{th}$-$6^{th}$ dorsal roots of anesthetized rats and guinea pigs, stimulation of the peripheral stumps resulted in marked neurogenic inflammation of the ipsilateral hind paw skin which was strongly reduced by stimulation of the contralateral stump 5 min before[6]. This inhibition was not observed if the two stimulations took place at the same time or the second stimulation was carried out 60 min later. These results suggested that anti-inflammatory mediator(s) were also released from the sensory nerve endings and reached distant parts of the body via systemic circulation. It was also observed that degeneration of capsaicin-sensitive

fibers by perineural capsaicin pretreatment prevented the antiinflammatory effect of stimulation. In another series of experiments stimulation of the peripheral stump of cut sciatic nerves of the anesthetized rats was performed (Fig. 2). Stimulation of the right sciatic nerve was followed by stimulation of the left sciatic nerve 5 min later. The secondary response was inhibited by 45 % compared to the primary reaction. Somatostatin-like immunoreactivity increased 3.3-fold in plasma, compared to the sham-operated animals. Perineural pretreatment with capsaicin abolished the orthodromic stimulation-induced release of somatostatin. Pretreatment with a polyclonal anti-somatostatin antibody prevented the remote anti-inflammatory effect, while systemic administration of exogenous somatostatin reduced neurogenic inflammation by 30 %[7,8]. This study provided evidence for the first time that excitation of capsaicin-sensitive sensory nerve fibres elicited not only a local effector response, but a systemic anti-inflammatory effect as well, via somatostatin release to the plasma from the activated nerve endings. This phenomenon was termed as the sensocrine function of peptidergic nociceptors[9].

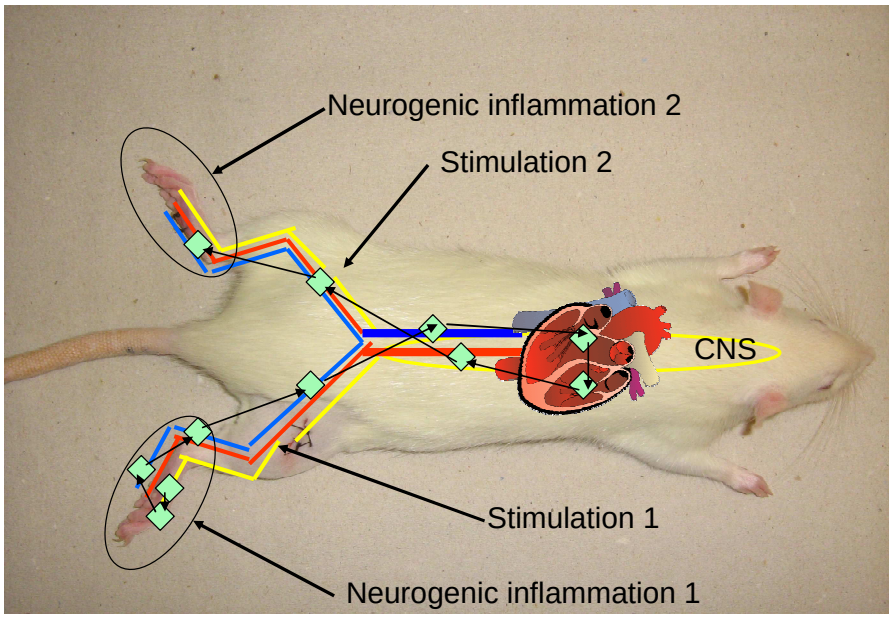


**Fig. 2.** Experimental arrangement for investigation of the remote anti-inflammatory effect of sciatic nerve stimulation

## 1.3 Methods for measuring thermonociception in conscious animals

The conventional methods for assesment of thermonociception in awake animals, such as the hot plate, tail flick and paw withdrawal (plantar) test are based on the same principle[10]. One or more extremity of the animal is exposed to a constant, suprathreshold heat stimulus, and the latency of the nocifensive behavior is measured. In case of the hot plate or water bath, a constant temperature is set, in case of the Hargreaves method a focused light beam (visible or infrared) is aimed at the plantar surface or tail, and then the reflex latency time is measured. The accepted nocifensive reaction (lifting, licking, shaking of the extremity, and jumping) is determined before the commencement of the experiment. These methods have become popular in the pharmaceutical industry as time measurement can easily be automated.

Another approach to measure thermonociception is based on measurement of the lowest temperature evoking a nocifensive reaction, the so-called noxious heat threshold. The first published implementation of noxious heat threshold measurement dates back to about 25 years ago when Szolcsányi determined the approximate noxious heat threshold of rats by immersing one of their hind paws into a thermostatic water bath whose temperature was increased stepwise by 1℃ at a time until the animal withdrew its paw[11,12]. Recently an increasing-temperature water bath has been developed in our department[13]. The equipment consists of a water-filled water container and a controlling unit. The equipment provides a homogenous and fast increase in water temperature. One of the hind paws of a conscious animal is immersed into the water before the heating process is started. After heating the animal withdraws its paw and the actual water temperature is considered the noxious heat threshold of the paw. The heat threshold shows little intra- and interindividual variability. This is advantageous, compared to classical methods, as reflex latencies determined in the hot plate or tail flick tests may vary upon repeated determinations. The increasing-temperature water bath was validated in experiments in which a mild heat injury was applied to the hind paw to induce heat hyperalgesia. The mild heat injury induced a marked drop of noxious

heat threshold that lasted for at least 1 h. The antihyperalgesic effect of systemic or local (intraplantar) administration of morphine, diclofenac or ibuprofen administered 20 min after heat injury were all sensitively detected with noxious heat threshold measurements.

## 1.4 The endogenous antinociceptive neural systems

In mammals various endogenous antinociceptive systems have evolved that can promote survival of the animal following injuries. The spinal gate control theory was proposed by Melzack and Wall in 1965. The theory suggests that in the spinal cord there is a neuronal gate that can be open or closed, thus modulating the sensation of pain. The term refers to a phenomenon describing that stimulation of the dorsal horn wide dynamic range neurons innervating a well defined area of the body can inhibit the procession of noxious stimuli applied to another location corresponding to the same spinal segment. The inhibition was supposed to take place in the central nervous system, and was thought to affect the functioning of both wide dynamic range and nociception-specific neurons of the dorsal horn. The major concept stated that the stimulation of large diameter fibres closed the gates, while the activation of smaller diameter fibres opened the gates. The closed state of the gates was hypothesized to prevent signals from nociceptors to enter the central nervous system. It was also suggested that descending pathways from brain could also influence the gates.

Another antinociceptive mechanism is based on descending pathways that originate from the periaqueductal gray matter of the reticular formation and receive input from the spinothalamic and the spinomesencephalic tract. Stimulation of these pathways leads to activation of encephalin-producing neurons that project to the raphe nuclei, from where a pathway runs to the dorsal horn releasing norepinephrine and serotonin onto inhibitory interneurons. These inhibitory neurons release opioids, enkephalin and dynorphin, binding to μ opioid receptors of afferent C and Aδ fibers terminating in the dorsal horn, thus inhibiting propagation of nociceptive signals. Endogenous opioids are peptide molecules able to bind to opioid receptors. These peptides are assigned into families: endorphins, enkephalins, dynorphins and endomorphines.

The third revealed endogenous antinociceptive neural system is diffuse noxious inhibitory controls (DNIC) [14]. Briefly, DNIC refers to a modulatory pathway which is activated in response to a painful stimulus and decreases perception of a second noxious stimulus. It is suggested that noxious stimuli activate the C and Aδ nerve fibres, which transmit the action potential wide dynamic range neurons of the spinal cord. The inhibition is hypothesized to originate in the brain, and is thought to affect both wide dynamic range and nociception-specific neurons in the dorsal horn in more than one spinal segment. In chronic pain DNIC function is suggested to be impaired, being responsible for central sensitization.

The exact mode of action of the above-mentioned endogenous antinociceptive systems has only been partly cleared to date but an important common feature of them is that they are activated by innocuous or noxious input to the central nervous system i.e. propagation of action potentials along afferent nerve fibers into the spinal cord and also possibly into the brainstem.

# 2 Aims

**1.** The behavioral noxious heat threshold measured with an increasing-temperature water bath was previously shown to decrease in acute thermal hyperalgesia induced by heat injury. Surgical incision of the hind paw in rats and mice has been introduced as a new animal model of human postoperative pain which is a lasting hyperalgesic state. The aim of our first study was to examine whether the surgical incision-induced sustained hyperalgesia involves a drop of the heat threshold too, and to assess the effects of conventional opioid and non-opioid analgesics in this model.

**2.** The different time course of heat hyperalgesia induced by heat injury and surgical incision raised the possibility that different mediators take part in these two responses. Therefore the aim of the second study was to compare the peripheral mediator background of heat hyperalgesia in mild heat injury and surgical incision by locally applied test substances**.** We assessed the possible contribution of bradykinin $B_2$ and $B_1$, purinergic P2X and TRPV1 receptors as well as formation of lipoxygenase products and NO to these two types of thermal hyperalgesia by measurement of the noxious heat threshold.

**3.** Previous studies demonstrated that stimulation of capsaicin-sensitive nerve endings resulted in a remote, systemic anti-inflammatory effect, mediated by somatostatin released into systemic circulation and referred to as sensocrine effect of peptidergic nociceptors. Somatostatin was shown to have antinociceptive effect in numerous experimental arrangements including ones in which a peripheral site of action can be assumed. Therefore the aim of the third study was to decide whether stimulation of polymodal nociceptors can evoke an antinociceptive effect in a distant part of the body, i.e. whether the sensocrine effect of peptidergic nociceptors involves an antinociceptive action as well. We assessed whether chemical stimulation of nociceptors can influence the incision-induced hyperalgesia in a remote part of the body.

# 3 Methods

## 3.1 Ethics

All experiments were carried out according to the Animals (Research Procedures) Act of 1998 (Hungary) and complied with the ethical guidelines of the International Association for the Study of Pain[15]. The Ethics Committee on Animal Research of the University of Pécs approved the studies.

## 3.2 Animals

Female Wistar rats (Charles River Hungary Ltd, Budapest, Hungary) weighing 150–200 g were used. The animals were kept in the Animal House of the University of Pécs, in a specific pathogen-free, temperature-controlled room providing a 12 hour light–dark cycle. Animals were brought to the air-conditioned laboratory the day before the experiment and were provided with food and water *ad libitum*. Throughout all the experiments the same assistant handled all the animals. The observer was blind as to the drug treatment of animals.

## 3.3 Materials

Solvent used for dissolution of compounds and dilution of their stock solutions, if not mentioned otherwise, was physiological saline (obtained from Department of Pharmaceutics and Central Clinical Pharmacy of the University of Pécs).

Pentobarbital sodium for veterinary use was purchased from CEVA Inc. Budapest, Hungary.

Diethyl ether (pharmaceutical grade, Ph. Hg. VII) was obtained from Department of Pharmaceutics and Central Clinical Pharmacy.

Povidone iodine (Betadine ®) was obtained from Department of Pharmaceutics and Central Clinical Pharmacy.

Morphine hydrochloride salt (pharmaceutical grade, Ph. Hg. VII) was obtained from Department of Pharmaceutics and Central Clinical Pharmacy.

Diclofenac sodium was purchased from Research Biochemicals International, Natick, MA, USA.

Paracetamol (acetaminophen, pharmaceutical grade, Ph. Hg. VII) was obtained from Department of Pharmaceutics and Central Clinical Pharmacy. The solvent was 1,2-propanediol and the initial solution was diluted with saline resulting in a stock solution for the highest applied dose (300 mg/kg) containing 12.5% 1,2-propanediol.

HOE 140, [des-Arg$^{10}$]-HOE 140, trinitrophenyl-ATP (TNP-ATP) and N$^{(G)}$ -nitro-l-arginine (L-NOARG) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Nordihydroguaiaretic acid (NDGA) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The stock solution of 10 mM was made with dimethylsulfoxide.

AMG9810 and SB-366791 were purchased from Tocris Bioscience (Bristol, UK). Stock solutions of 50 and 100 mM, respectively, were made with dimethylsulfoxide.

Capsaicin was purchased from Sigma-Aldrich (St. Louis, MO, USA). The stock solution contained 1 % capsaicin, 10 % Tween-80 and 10 % ethanol (96 %), and it was further diluted with saline.

Mustard oil (allyl isothiocyanate) was purchased from Merck (Darmstadt, Germany). The pure mustard oil was further diluted with paraffin oil.

Somatostatin, cyclosomatostatin, naloxone hydrochloride dehydrate, and naloxone methiodide were purchased from Sigma-Aldrich (St. Louis, MO, USA).

AM-251 was purchased from Tocris Bioscience (Bristol, UK). The stock solution (100 mM) was made with dimethylsulfoxide.

## 3.4 Measurement of the noxious heat threshold with an increasing-temperature water bath

The increasing-temperature water bath, developed in our department in cooperation with Experimetria Ltd. (Budapest), consists of a tap water-filled plastic container and a controlling unit (Fig. 3). The cylindric container (120 mm inner diameter, 140 mm height) is equipped with a built-in heating unit in its bottom that provides a homogenous and fast increase in the water temperature. The controlling unit serves for setting different starting temperatures (30 or 40 ℃) and heating rates (6, 12 or 24 ℃/min), and has a display continuously showing the actual water temperature measured by a thermocouple at the middle position 35 mm below the water level. A foot switch can interrupt heating and the actual bath temperature remains on the display to be recorded. After each measurement, the water bath is cooled back to the starting temperature by pumping cold water into the container controlled by a feedback. The homogeneity of the temperature distribution in a given layer of the water bath was verified[13].

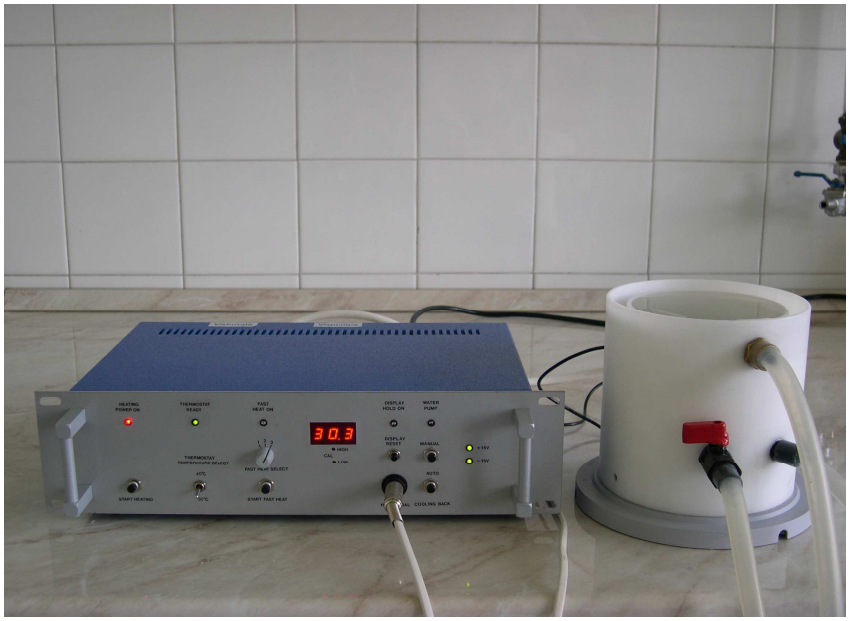

**Fig. 3.** The control unit and the water container of the increasing-temperature water bath.

The lightly restrained rats were held in an upright position above the water bath allowing free movement of the hind limbs (Fig. 4). After one of the hind paws was immersed into the water the heating process was started. When the animal withdrew its paw, heating was immediately stopped and the corresponding temperature was recorded as the behavioral noxious heat threshold of the examined paw.

The day before the experiment, animals were habituated to the heat threshold measurement procedure by performing two heat threshold determinations whose results were not included in the analysis. On the day of experiment, two heat threshold measurements separated by a 30 min interval were performed for the same paw of each animal and the mean of the two values was used as control (baseline) heat threshold.

In all series of experiments the starting temperature was 30 ℃ and the heating rate was 24 ℃/minute. The cut-off temperature was set to 53 ℃.

**Fig. 4.** Handling of the animal during noxious heat threshold measurement.

## 3.5 Induction of heat hyperalgesia

### 3.5.1 Induction of heat hyperalgesia by heat injury

After determination of the control heat threshold of both hind paws, rats were anesthetized with diethyl ether, the left paw was immersed into a constant, 51 ℃ hot water bath for 20 s. These parameters for the heat injury were empirically established in a series of preliminary measurements in order to evoke a substantial drop of heat threshold without spontaneous nocifensive behavior. Following recovery from anesthesia, heat threshold determinations for the injured paw were repeated 10 and 20 min after heat injury to confirm the development of thermal hyperalgesia.

### 3.5.2 Induction of heat hyperalgesia by plantar incision

The control noxious heat threshold of both hind paws was determined before the operation which was performed according to previous descriptions with minor modifications[16]. Rats were anesthetized with 50 mg/kg pentobarbital sodium i.p., and the plantar surface of the left hind paw was prepared in a sterile manner. Starting 1 cm from the proximal edge of the heel, the incision extended longitudinally 1 cm towards the toes, intersecting the skin, fascia and plantar muscle by using a number 10 scalpel (Fig. 5, panel A). The skin was opposed with two single interrupted sutures using 4-0 nylon (Fig. 5, panel B). The wound site was anointed with povidone iodine solution. The animals recovered in their home cages for 18 h. The duration of the recovery period was the shortest period of time needed to completely eliminate the behavioral consequences of general anesthesia.

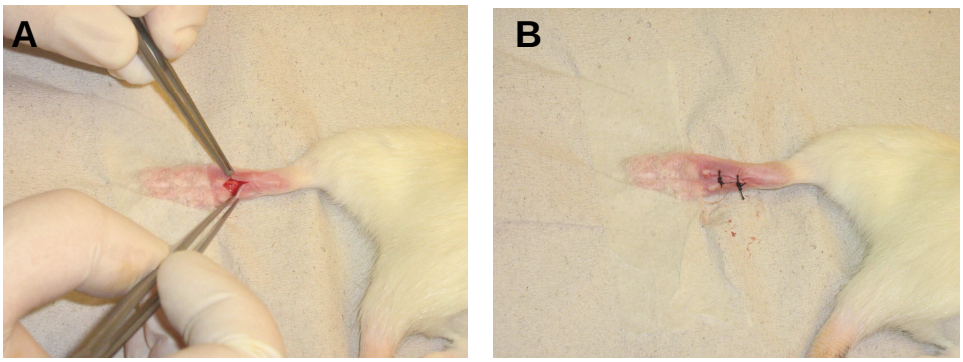


**Fig. 5.** Intersection of skin, fascia and plantar muscle during plantar incision (panel A). The skin was opposed with two single interrupted sutures using 4-0 nylon (panel B)**.**

In a series of experiments the time course of incision-induced heat hyperalgesia was investigated by daily noxious heat threshold measurements for a week. No serious incision-related wound healing complications were noted.

In the series of experiments in which the effect of conventional analgesics on the plantar incision-induced hyperalgesia was investigated, only animals showing a threshold drop greater than 3 ℃ 18 h after surgery were included. After the postoperative noxious heat threshold measurement (18 h after surgery) drugs or their solvents were administered intraperitoneally (i.p., 1 ml/kg) or intraplantarly (i.pl., 0.1 ml/paw), followed by repeated heat threshold measurements at 10 min intervals. The effect of each dose of drugs was examined by comparison to an actual solvent control meaning that after randomization, one half of the group was treated with the drug and the other half with its solvent. The minimum effective dose of drugs for the thermal antihyperalgesic action was defined as the lowest dose applied causing a statistically significant inhibition of the incision-induced drop of heat threshold. The percentage inhibition of hyperalgesia was calculated on the basis of the sum of threshold drops (for details see chapter 3.9) measured 20, 30 and 40 min after heat injury.

## 3.6 Experimental paradigm for investigation of the remote thermal antihyperalgesic effect evoked by chemical activation of peripheral nociceptors

The experimental paradigm used was based on previous studies conducted in our department on the remote anti-inflammatory effect of stimulation of capsaicin-sensitive nerve endings[7, 8].

To detect a potential remote antihyperalgesic effect we stimulated nociceptive nerve endings of the acutely denervated right hind limb of conscious rats, and measured incision-induced heat hyperalgesia on the contralateral (i.e. left) hind paw (Fig. 6). Denervation prevented peripheral impulses generated by nociceptor stimulation from entering the central nervous system.

After determination of preoperative noxious heat threshold of both hind paws, animals underwent two operations in one session under general anesthesia (pentobarbital sodium 50 mg/kg i.p.). First, acute denervation of the right hind limb was carried out by transection of sciatic and saphenous nerves (Fig. 7), then the above-described surgical incision was performed on the left plantar surface.

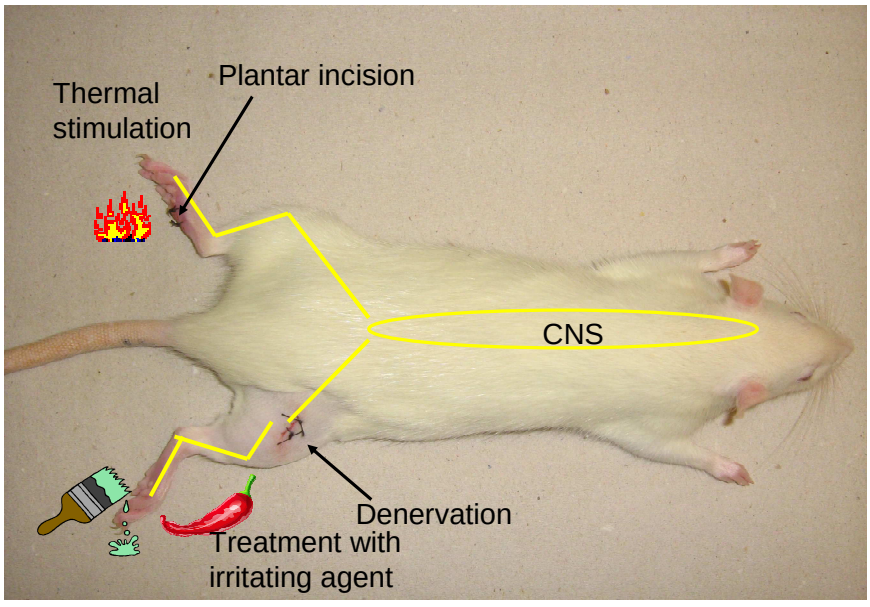


**Fig. 6.** Schematic demonstration of the experimental setup. See details in text.

Regarding acute denervation, after depilation of both the extensor and flexor side of the thigh, the operation site was prepared in a sterile manner. The skin was incised with number 10 scalpel and the nerves were exposed by blunt dissection, then transected with sharp scissors (Fig. 7, panels A and B). The skin was opposed with 4-0 nylon with interrupted stitches (Fig. 7, panels C and D), and anointed with povidone iodine. The animals recovered in their home cages for 18 h.

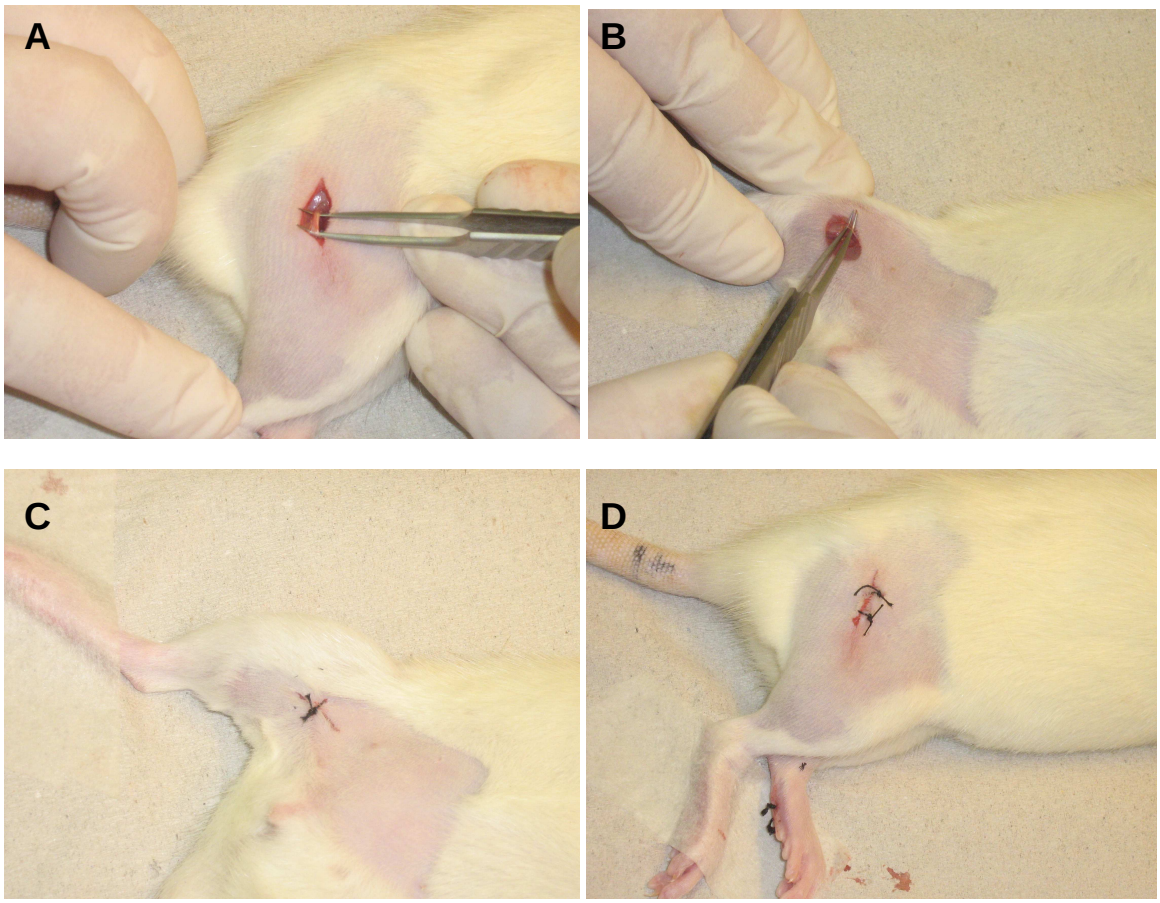


**Fig.7.** The denervation of the hind limb. The exposure of the sciatic (panel A) and saphenus (panel B) nerve and the postoperative state (panels C and D).

For chemical stimulation of peripheral nociceptors irritant substance or its solvent was administered to the denervated right hind paw either intraplantarly (0.1 ml/paw) with a 26-gauge hypodermic needle or percutaneously by anointing the skin with cotton swab. The needle was inserted into the paw at approximately 7–8 mm from the heel and advanced subcutaneously another 5–6 mm before injection.

Thereafter, heat threshold measurements were performed at 10 min intervals for 40 min. The overall effect of chemical stimulation on hyperalgesia was assessed by comparison of the sum of threshold drops measured at the above mentioned time points in the drug- and solvent-treated groups.

## 3.7 Desensitization of capsaicin-sensitive sensory nerve endings

A sustained exposure to high concentration of capsaicin can cause desensitization of the TRPV1-expressing nociceptive nerve endings rendering them unable to respond to further stimulation by capsaicin or any other painful stimuli. The desensitization was carried out 3 days prior to the actual experiment by intraplantar administration of 100 µg capsaicin to the right hind paw that was not denervated.

## 3.8 Induction of neuropathic mechanical hyperalgesia by partial sciatic nerve ligation (Seltzer's model)

The animals were brought to the air-conditioned laboratory 48 h before the experiment. For habituation, two series of measurements of the mechanonociceptive threshold of each hind paw with a Randall–Selitto apparatus (Ugo Basile, Milan, Italy) were carried out 24 h apart. These results were not taken into account for analysis. Right before the operation two series of measurements (10 min apart) on the left hind limb were performed, and their mean was considered the control mechanonociceptive threshold.

The operation was carried out according to Seltzer[17], under general anesthesia (50 mg/kg pentobarbital sodium i.p.). The operation site was depilated, then prepared in sterile manner. The skin was opened with number 10 scalpel, at high thigh level. The sciatic nerve was exposed by blunt dissection, then the 1/3 of the nerve was ligated with 6-0 nylon using atraumatic needle. The skin was opposed with two interrupted stitches and wound was anointed with povidone iodine.

Acute denervation of the right hind limb was carried out in the same session prior to the Seltzer' operation. After the operations the animals recovered for 18 h in their homecages.

## 3.9 Statistical analysis

One-way repeated measures analysis of variance (ANOVA) followed by Newman–Keuls post hoc test was used for comparison of thresholds determined upon repeated measurements in the same group of animals.

Two-way ANOVA followed by Newman–Keuls post hoc test was used for comparison of threshold values determined in solvent and drug-treated animals at various time points.

Student's t-test for unpaired samples was used for statistical comparison of the sums of threshold drops at repeated measurements (10, 20, 30 and 40 min after treatment in case of nociceptive heat threshold and 10 and 20 min after treatment in case of mechano-nociceptive threshold measurements. The overall effect of each drug was assessed on the basis of this parameter.

With all tests, a value of $P<0.05$ was considered statistically significant.

The percentage inhibition of hyperalgesia was calculated according to the following formula:

$$[(Drop_{solv}-Drop_{drug})/Drop_{solv}]\times 100$$

where $Drop_{solv}$ and $Drop_{drug}$ refer to the average of the sum of threshold drops measured at the examined post-treatment time points in either the heat injury or plantar incision paradigms in the solvent- and drug-treated animals, respectively.

# 4 Results

## 4.1 Effect of plantar incision on the noxious heat threshold

The noxious heat threshold of animals was measured daily, for seven consecutive days. There was no significant change in the noxious heat threshold, compared to the baseline value, in case of uninjured hind paws, while in case of operated hind paws there was a 5 to 7 ºC decrease in the noxious heat threshold (Fig. 8). This decrease was statistically significant, compared to the threshold values of the contralateral uninjured hind paw and the preoperative heat threshold, respectively. This heat hyperalgesia, manifesting itself as a drop of the noxious heat threshold, was sustained lasting for at least seven days (Fig. 8). After the sixth postoperative day, the heat threshold started to increase approaching the baseline value. The experiment was terminated after the seventh postoperative day to reduce unnecessary animal discomfort.

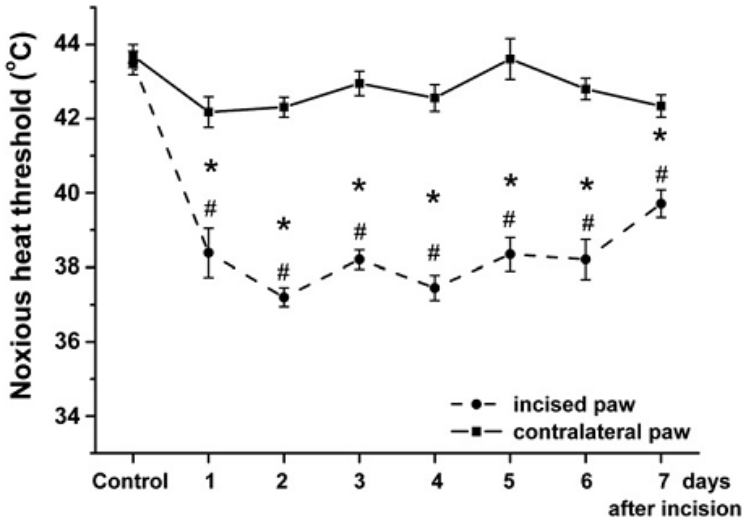


**Fig. 8.** Effect of surgical incision performed on the plantar surface of the rat hind paw on the noxious heat threshold. In this and all following figures, data are means ± S.E.M. of 12 animals. Statistically significant differences ($P<0.05$, two-way repeated measures ANOVA followed by Neumann−Keuls post hoc test) are indicated as follows. Pounds: differences compared to the initial (preoperative) control value of the incised paw; asterisks: differences compared to the corresponding values of the contralateral uninjured hind paw.

## 4.2 Effects of conventional analgesics on the plantar incision-induced drop of heat threshold

### 4.2.1 Morphine i.p.

After confirming the development of post-incision thermal hyperalgesia by measurement of the noxious heat threshold, morphine (0.1 to 3 mg/kg) or its solvent was administered systemically i.p.. Subsequently, threshold measurements were repeated at 10 min intervals.

Morphine dose-dependently reduced the incision-induced drop of heat threshold (Fig. 9) as compared to the solvent-treated group. Its minimum effective dose was 0.3 mg/kg. The 3 mg/kg dose produced a reversal of hyperalgesia by about 60 % without affecting the overall behavior of the animals. The percentage inhibition of hyperalgesia was calculated according to the formula $[(Drop_{solv}-Drop_{drug})/Drop_{solv}]\times100$, where $Drop_{solv}$ and $Drop_{drug}$ refer to the average of the sum of threshold drops measured at the examined post-treatment time points.

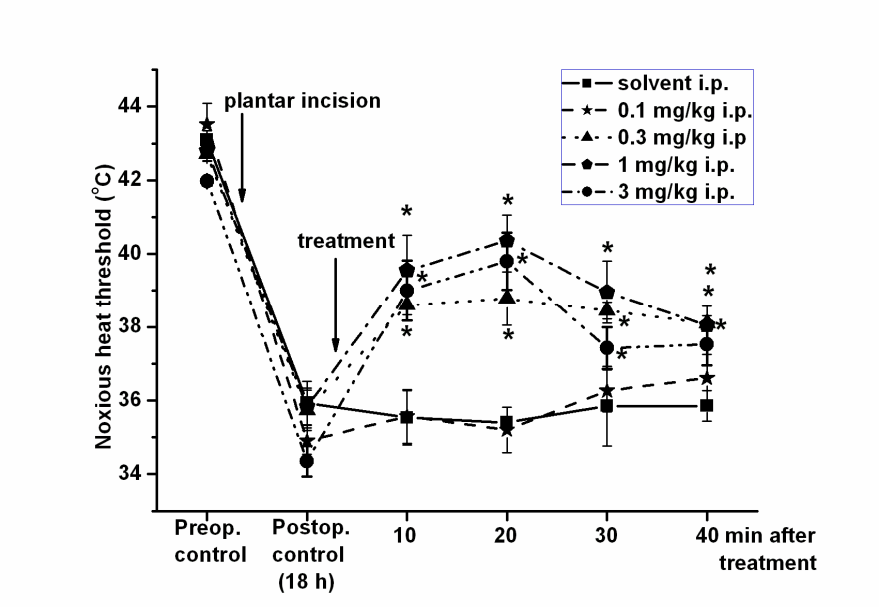


**Fig. 9.** Effect of morphine on the drop of heat threshold induced by plantar incision. Morphine was administered intraperitoneally just after the first postoperative heat threshold measurement as indicated by the treatment arrow. Solvent treatment refers to the effect of vehicle in the series of experiments in which the highest dose of the drug was examined. The effects of the other doses were also compared to actual solvent controls, these data are not shown to avoid confusion.

### 4.2.2 Diclofenac i.p.

Diclofenac administered systemically in doses from 0.3 to 10 mg/kg i.p. was able to mitigate the postoperative drop of heat threshold in a dose-dependent manner (Fig. 10). Its minimum effective dose was 1 mg/kg and its maximal effect ranged to about 40 % inhibition of hyperalgesia. The percentage inhibition of hyperalgesia was calculated according to the formula $[(Drop_{solv}-Drop_{drug})/Drop_{solv}]\times100$, where $Drop_{solv}$ and $Drop_{drug}$ refer to the average of the sum of threshold drops measured at the examined post-treatment time points.

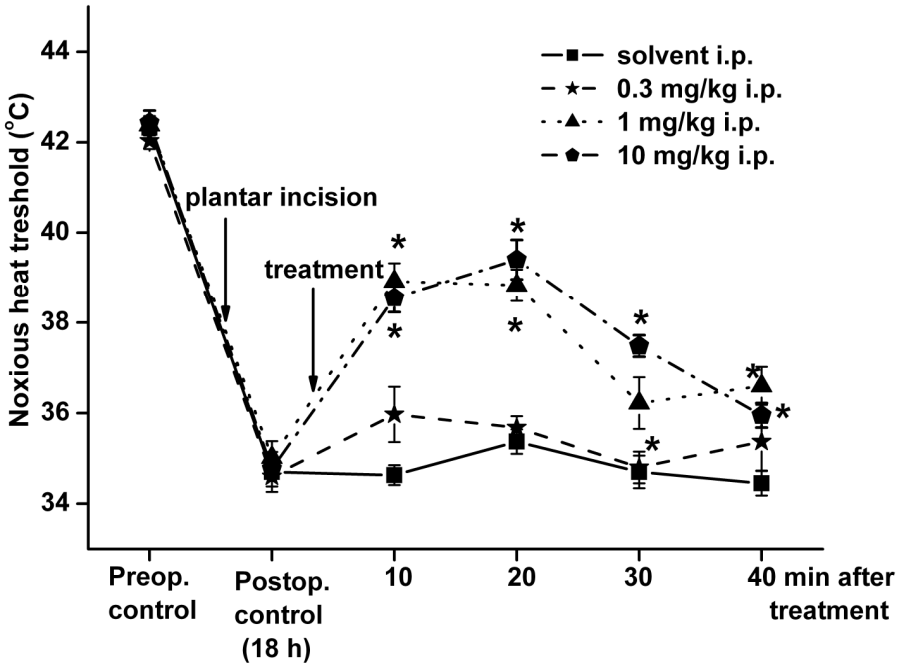


**Fig. 10.** Effect of diclofenac on the drop of heat threshold induced by plantar incision. Diclofenac was administered intraperitoneally just after the first postoperative heat threshold measurement as indicated by the treatment arrow. Solvent treatment refers to the effect of vehicle in the series of experiments in which the highest dose of the drug was examined. The effects of the other doses were also compared to an actual solvent controls, these data are not shown to avoid confusion.

### 4.2.3 Paracetamol (acetaminophen) i.p.

Systemically administered paracetamol also inhibited the incision-induced thermal hyperalgesia in a dose-dependent manner (Fig. 11). The minimum effective dose was found to be 100 mg/kg. The maximum inhibition of thermal hyperalgesia was 52 % at the highest dose applied. The percentage inhibition of hyperalgesia was calculated according to the formula $[(Drop_{solv}-Drop_{drug})/Drop_{solv}]\times100$, where $Drop_{solv}$ and $Drop_{drug}$ refer to the average of the sum of threshold drops measured at the examined post-treatment time points.

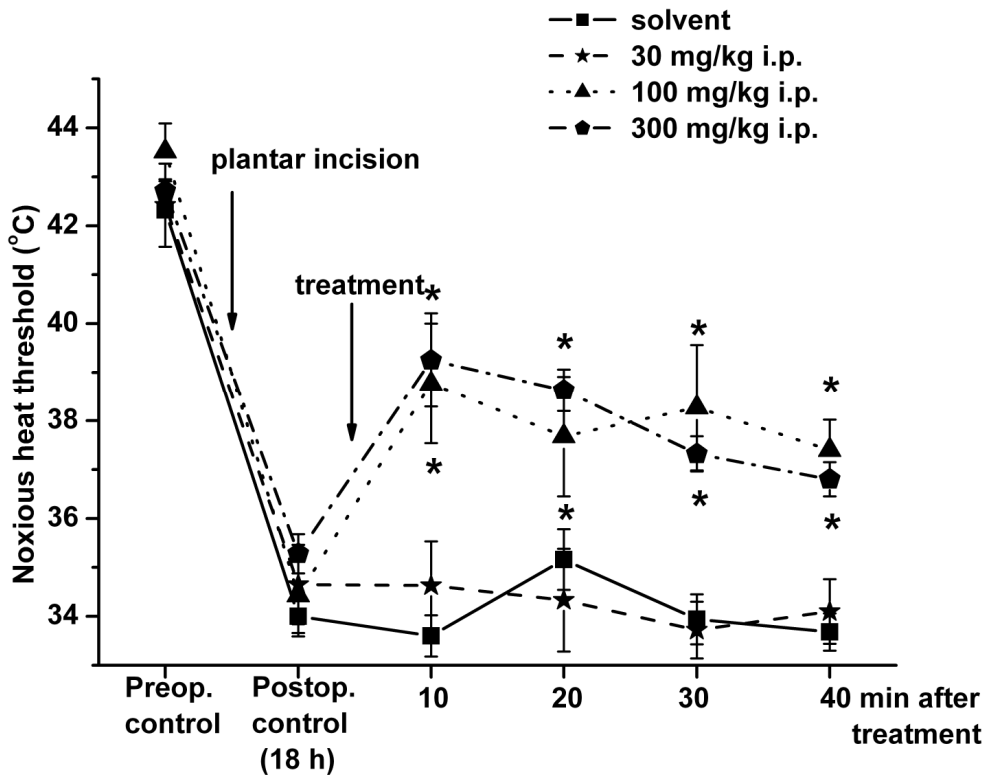


**Fig. 11** Effect of paracetamol on the drop of heat threshold induced by plantar incision. Paracetamol was administered intraperitoneally just after the first postoperative heat threshold measurement as indicated by the treatment arrow. Solvent treatment refers to the effect of vehicle in the series of experiments in which the highest dose of the drug was examined. The effects of the other doses were also compared to an actual solvent controls, these data are not shown to avoid confusion.

### 4.2.4 Dose−response relationship for systemically administered analgesics

The dose−response relationship for the antihyperalgesic effects of the reference analgesic drugs is shown in Fig. 12. Morphine proved the most potent and paracetamol the least potent in this experimental model as assessed on the basis of the minimum effective doses. Morphine and paracetamol showed similar efficacy, (i.e. maximum inhibition of the threshold drop) while diclofenac was less efficacious.

In a series of experiments in previously anesthetized but uninjured animals it was clarified that the highest doses of morphine, diclofenac or paracetamol used in this study failed to alter the baseline noxious heat threshold or to have influence on the overall behavior of the animals.

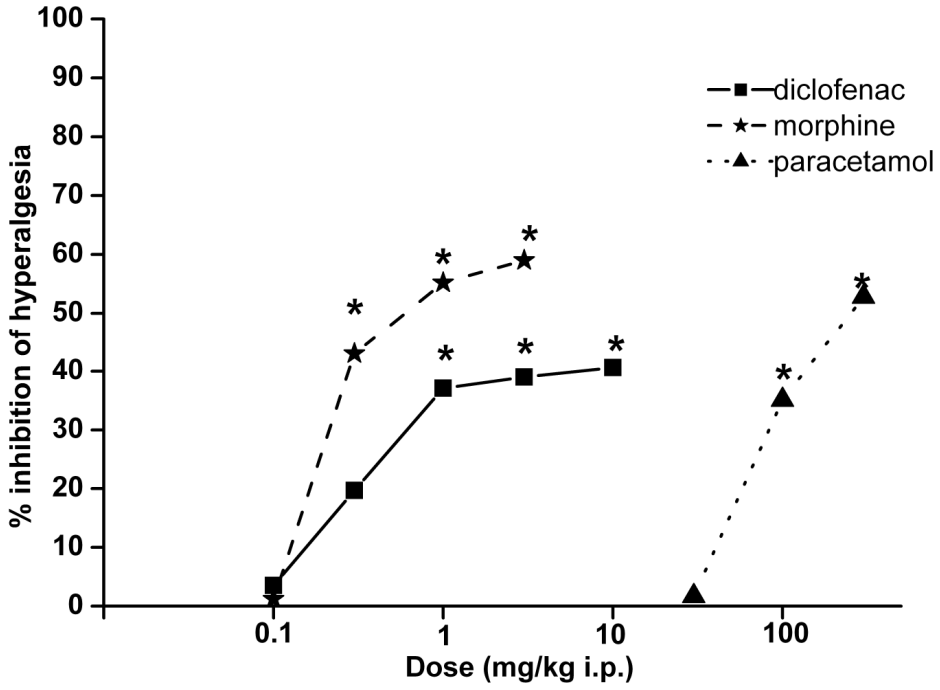


**Fig. 12.** Dose−response relationship for intraperitoneally applied morphine, diclofenac and paracetamol for inhibition of the plantar incision-induced drop of heat threshold. The percentage inhibition of heat hyperalgesia was calculated according to the following formula: [($Drop_{solv}$ −$Drop_{drug}$) /$Drop_{solv}$]×100, where $Drop_{solv}$ and $Drop_{drug}$ refer to the average of threshold drops measured at the 20, 30 and 40 min time points in the solvent- and drug-treated animals, respectively.

### 4.2.5 Effects of locally applied analgesics on the plantar incision-induced drop of noxious heat threshold

The local effect of analgesics on the incision-induced heat hyperalgesia was examined by intraplantar (i.pl.) administration of doses below the minimum systemic effective doses to avoid any systemic effect. Solvent treatment refers to administration of 0.1 ml saline. The effect of each drug was compared to an actual solvent control. The volume administered was 0.1 ml. Drug or solvent was administered just after the first postoperative heat threshold measurement.

Intraplantar injection of 10 µg morphine or 100 µg diclofenac significantly decreased subsequent drop of heat threshold (Fig. 13). In contrast, locally administered paracetamol failed to have a statistically significant effect (data not shown).

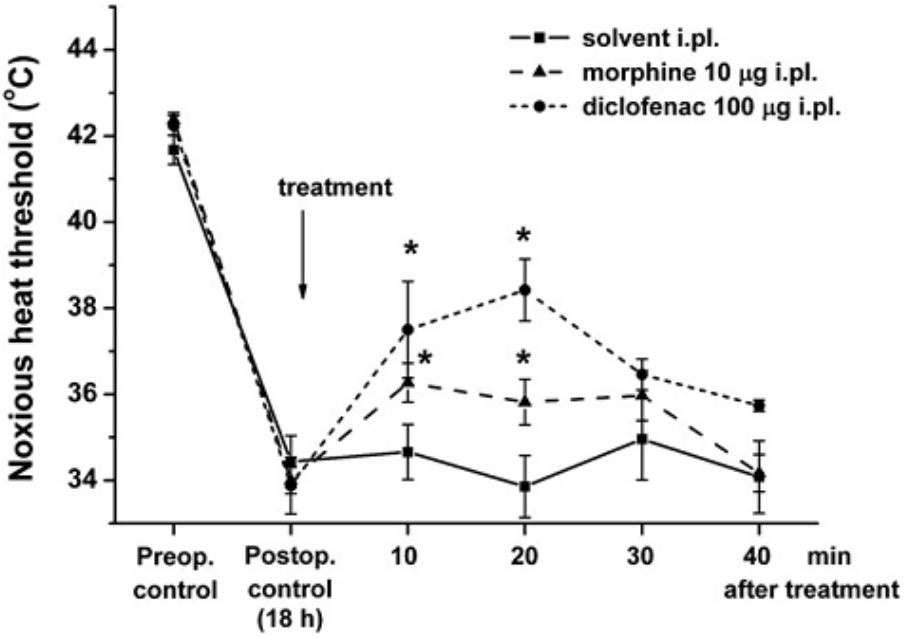


**Fig. 13.** Effect of local treatment with morphine or diclofenac on the drop of heat threshold induced by plantar incision. Drugs were administered intraplantarly just after the first postoperative heat threshold measurement as indicated by the treatment arrow. Solvent treatment, only displayed once, refers to administration of 0.1 ml saline, although in all experiments there was an actual solvent control.

## 4.3 Comparison of mediators involved in the drop of noxious heat threshold induced by heat injury or plantar incision

### 4.3.1 Effect of a bradykinin $B_2$ receptor antagonist on thermal hyperalgesia induced by heat injury or plantar incision

Intraplantar administration of 10 µM HOE 140, a selective bradykinin $B_2$ receptor antagonist, significantly inhibited heat injury-induced noxious heat threshold drop 10 and 20 min after treatment (Fig. 14). The overall effect of the drug assessed on the basis of the sum of threshold drops (see chapter 3.9) proved to be significant.

In the case of plantar incision, a statistically significant inhibition of the noxious heat threshold drop was observed at all time points of measurement in animals receiving intraplantar HOE 140 compared to the solvent-treated group (Fig. 14). In accord, the antagonist's overall inhibitory effect was statistically significant.

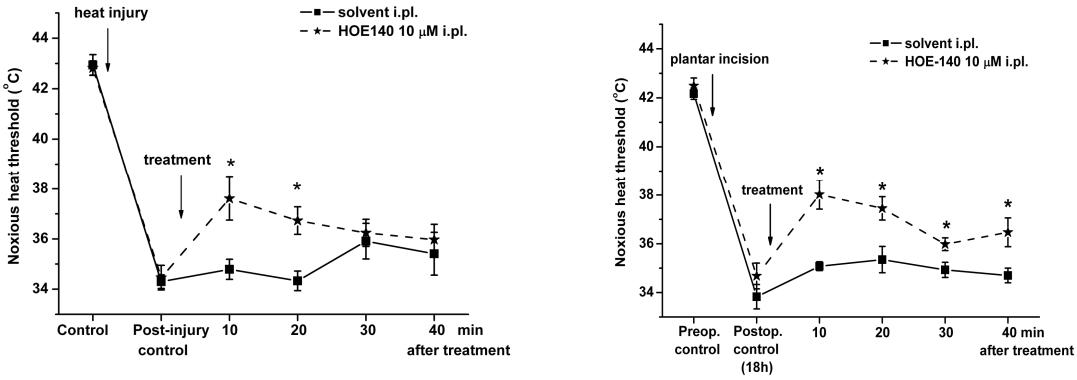


**Fig. 14.** Effect of the selective bradykinin $B_2$ receptor antagonist HOE 140 on the heat threshold drop induced by heat injury or plantar incision performed at time indicated by the arrow. Drug or its solvent was administered intraplantarly (i.pl.) as indicated by the treatment arrow.

### 4.3.2 Effect of a bradykinin $B_1$ receptor antagonist on thermal hyperalgesia induced by heat injury or plantar incision

Intraplantar treatment with 10 µM [des-Arg$^{10}$]-HOE 140, a bradykinin $B_1$ receptor antagonist, following heat injury resulted in a statistically significant difference in threshold drop between the substance- and solvent-treated groups at only one time point, 20 min after treatment (Fig. 15). Accordingly, this antagonist failed to produce a significant overall inhibitory effect.

In contrast, a local [des-Arg$^{10}$]-HOE 140 treatment after plantar incision evoked a marked and statistically significant inhibition of the noxious heat threshold drop at all time points of measurement compared to the solvent-treated group (Fig. 15). In accord, the overall inhibitory effect was highly significant.

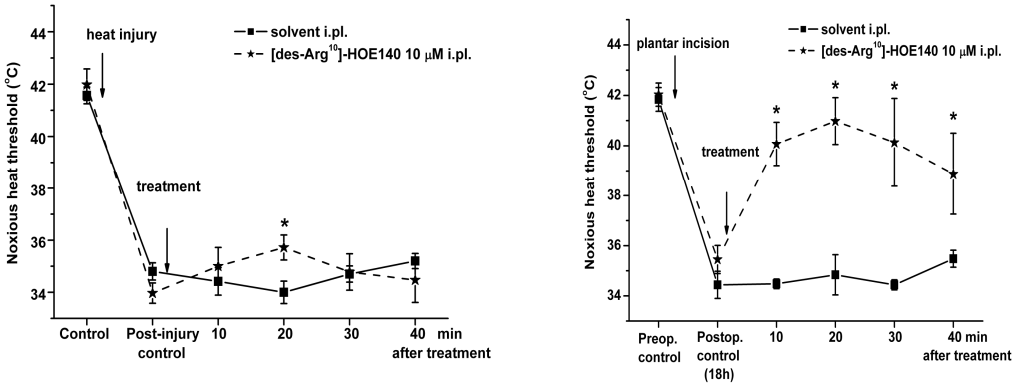


**Fig. 15.** Effect of the selective bradykinin $B_1$ receptor antagonist [des-Arg $^{10}$]-HOE 140 on the heat threshold drop induced by heat injury or plantar incision performed at time indicated by the arrow. Drug or its solvent was administered intraplantarly (i.pl.) as indicated by the treatment arrow.

### 4.3.3 Effect of a lipoxygenase inhibitor on thermal hyperalgesia induced by heat injury or plantar incision

After heat injury, intraplantar treatment with the non-selective lipoxygenase inhibitor nordihydroguaiaretic acid (NDGA) led to a statistically significant inhibition of the heat threshold drop 10 and 20 min after treatment at both applied concentrations (Fig. 16), with the overall effect being significant in both cases.

After plantar incision, intraplantar NDGA treatment at the lower concentration (10 µM) caused no statistically significant difference compared to the solvent-treated group at any time points. With the higher concentration a statistically significant difference between heat threshold drop in the drug- and solvent-treated animals was observed only 10 min after treatment (Fig. 16). The overall effect of the drug was not statistically significant at either concentration.

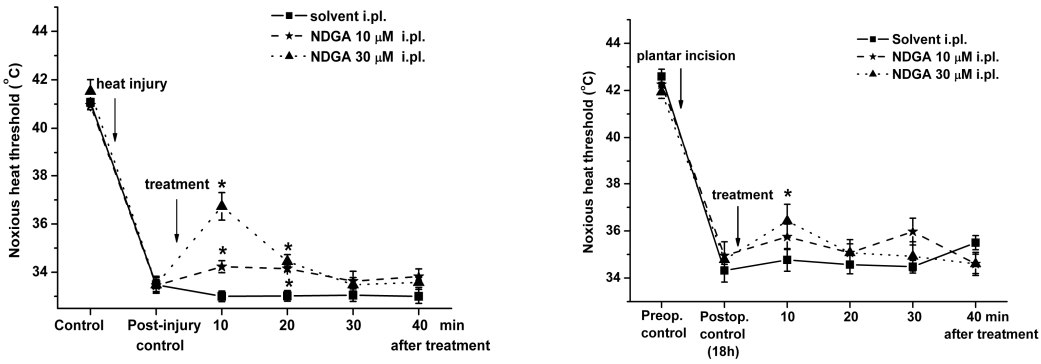


**Fig. 16.** Effect of the non-selective lipoxygenase inhibitor NDGA on the heat threshold drop induced by heat injury or plantar incision performed at time indicated by the arrow. Drug or its solvent was administered intraplantarly, as indicated by the treatment arrow.

### 4.3.4 Effect of a nitric oxide synthase inhibitor on thermal hyperalgesia induced by heat injury or plantar incision

The heat injury-induced drop of the noxious heat threshold was significantly decreased by intraplantar treatment with 100 µM L-NOARG, a nitric oxide synthase inhibitor, at all time points of measurement except for the last one, when the difference was marked but not statistically significant (Fig. 17). The overall inhibitory effect of the substance was statistically significant.

In case of plantar incision, the intraplantar administration of 100 µM L-NOARG reduced the drop of noxious heat threshold significantly at all time points of measurement compared to the solvent treated group (Fig. 17). The overall inhibitory effect of the drug was significant.

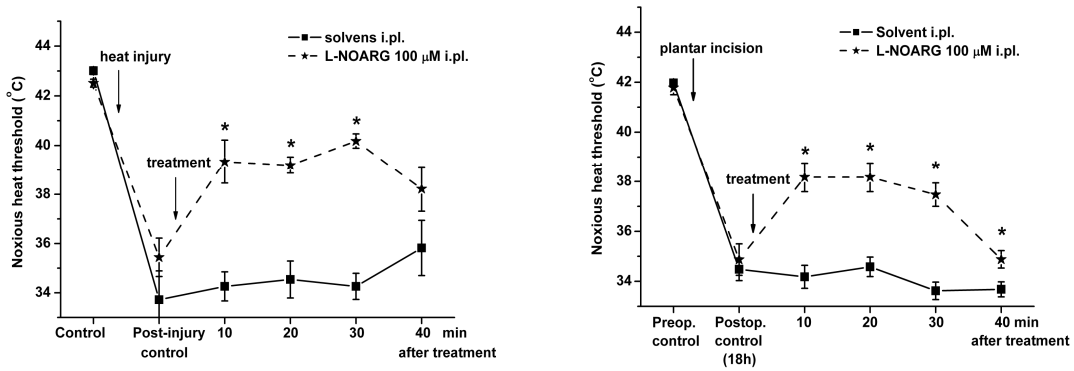


**Fig. 17.** Effect of the nitric oxide synthase inhibitor L-NOARG on the heat threshold drop induced by heat injury or plantar incision performed at time indicated by the arrow. Drug or its solvent was administered intraplantarly as indicated by the treatment arrow.

### 4.3.5 Effect of a P2X purinoceptor antagonist on thermal hyperalgesia induced by heat injury or plantar incision

Heat injury-induced drop of noxious heat threshold was significantly attenuated at all time points of measurement by an intraplantar treatment with 0.3 µM TNP-ATP, a P2X purinoceptor antagonist (Fig. 18). Accordingly, the overall inhibitory effect of the substance was significant.

In the case of plantar incision-induced thermal hyperalgesia, the drop of noxious heat threshold was significantly diminished by intraplantar TNP-ATP treatment (0.3 µM) at the first three time points of measurement (Fig. 18). The overall inhibitory effect of the substance was significant.

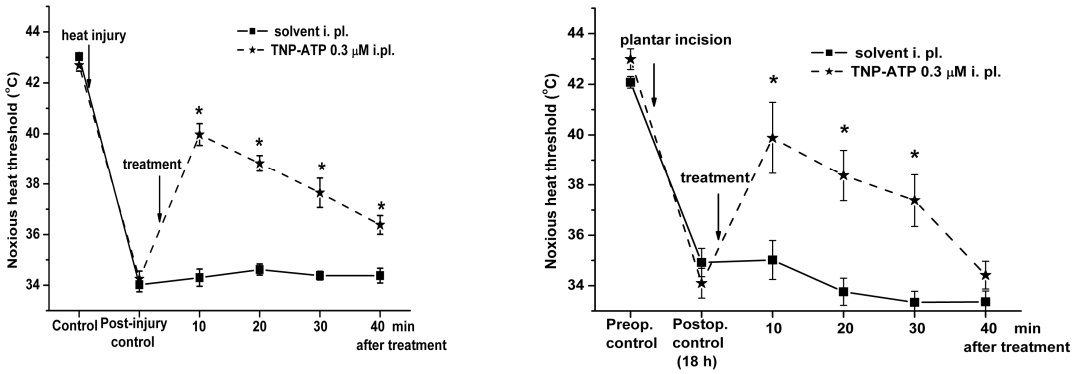


**Fig. 18.** Effects of the P2X purinoceptor antagonist TNP-ATP on the heat threshold drop induced by heat injury or plantar incision performed at time indicated by the arrow. Drug or its solvent was administered intraplantarly as indicated by the treatment arrow.

### 4.3.6 Effects of TRPV1 receptor antagonists on thermal hyperalgesia induced by heat injury or plantar incision

The heat injury-induced drop of noxious heat threshold was diminished by intraplantar treatment with the TRPV1 receptor antagonist AMG9810 in a concentration-dependent manner. 1 µM and 10 µM of the antagonist both significantly reduced the heat hyperalgesia at all time points of measurement (Fig. 19). The overall inhibitory effect of the lower concentration proved non-significant but that of the higher one was significant.

In the case of plantar incision-induced thermal hyperalgesia, intraplantar treatment with the TRPV1 receptor antagonist SB-366791 produced no statistically significant decrease in the noxious heat threshold drop at 10 µM, while at 100 µM it had a significant inhibitory effect at all time points of measurement (Fig. 19). Accordingly, the overall inhibitory effect of the lower concentration was not significant but that of the higher one was significant.

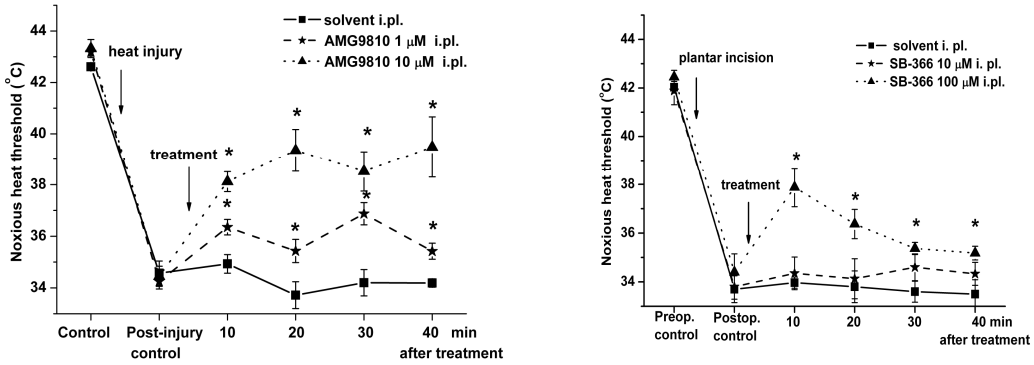


**Fig. 19.** Effects of the selective TRPV1 receptor antagonists AMG9810 and SB366791 on the heat threshold drop induced by heat injury or plantar incision performed at time indicated by the arrow. Drugs or their solvents were administered intraplantarly as indicated by the treatment arrow.

### 4.3.7 Summary of the results concerning peripheral mediators involved in thermal hyperalgesia induced by heat injury or plantar incision

The detailed results of the above-described series of experiments are shown in the Table Table 1.

| Substance | Concentration | Heat injury | | Plantar incision | |
|---|---|---|---|---|---|
| | | Overall effect P value | Percentage inhibition | Overall effect P value | Percentage inhibition |
| | | | | | |
| HOE 140 | 10 µM | < 0.05 | 32.6 % | < 0.05 | 31.6 % |
| [des-Arg$^{10}$]-HOE 140 | 10 µM | Nonsignifi-cant | 10 % | <0.01 | 79 % |
| NDGA | 10 µM | < 0.001 | 18 % | Nonsignificant | 13.6 % |
| | 30 µM | < 0.05 | 28 % | Nonsignificant | 17.9 % |
| L-NOARG | 100 µM | < 0.001 | 62.4 % | <0.01 | 52.4 % |
| TNP-ATP | 0.3 µM | < 0.05 | 61.6 % | < 0.05 | 54.5 % |
| AMG9810 | 1 µM | 0.073 | 25.8 % | - | - |
| | 10 µM | < 0.05 | 48.2 % | - | - |
| SB-366791 | 10 µM | - | - | Nonsignificant | 6.1 % |
| | 100 µM | - | - | < 0.05 | 46.9 % |

**Table 1.** Comparison of the effects of locally applied test substances on the drop of noxious heat threshold induced by heat injury or plantar incision.

## 4.4 Investigation of a remote antinociceptive effect induced by chemical stimulation of nociceptors

### 4.4.1 Effect of intraplantar capsaicin administration into the acutely denervated hind limb on the incision-induced heat hyperalgesia of the contralateral hind paw

In these experiments, peripheral nociceptors of the acutely denervated right hind paw were stimulated by chemical agents applied intraplantarly. The effect of this procedure on thermonociception in the contralateral, i.e. left hind paw was investigated. Because intraplantar administration of 1 μg, 10 μg or 100 μg capsaicin into the acutely denervated right hind paw failed to alter the baseline (i.e. non-conditioned) noxious heat threshold of the contralateral hind paw, thermal hyperalgesia was induced by plantar incision in order to detect subtle changes in thermonociception. Intraplantar injection of increasing doses of capsaicin into the acutely denervated right hind paw diminished the incision-induced noxious heat threshold drop of the contralateral hind paw in a dose dependent manner i.e. it evoked a remote thermal antihyperalgesic effect (Fig. 20). The lowest capsaicin dose was without a significant effect while the highest dose had an antihyperalgesic effect comparable to that of 3 mg/kg morphine administered i.p. (Fig. 9).

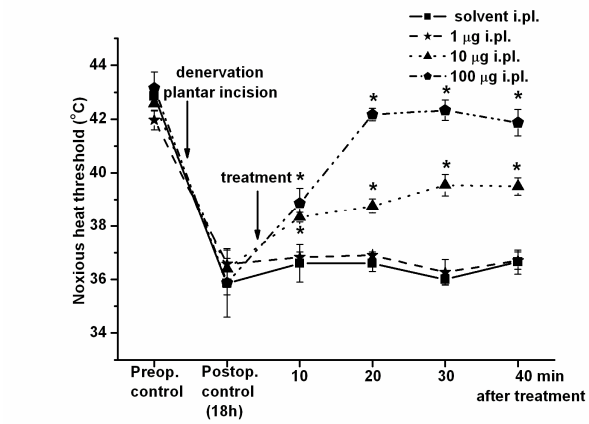


**Fig. 20.** Effect of intraplantar capsaicin injection into the denervated hind paw on the heat threshold drop induced by plantar incision on the contralateral hind paw performed at time indicated by the arrow. Capsaicin or its solvent was administered intraplantarly as indicated by the treatment arrow.

### 4.4.2 Effect of intraplantar administration of capsaicin into the chronically denervated hind limb on plantar incision-induced heat hyperalgesia of the contralateral hind paw

Chronic denervation leads to irreversible destruction of nerve endings including capsaicin-sensitive ones rendering the denervated area unresponsive to capsaicin (and any other stimulus) but sparing possible systemic effects of capsaicin.

Five days after denervation performed by section of both the sciatic and saphenous nerves intraplantar administration of highest dose of capsaicin into the denervated hind paw failed to have an effect on plantar incision-induced at hyperalgesia of the contralateral hind paw (Fig. 21).

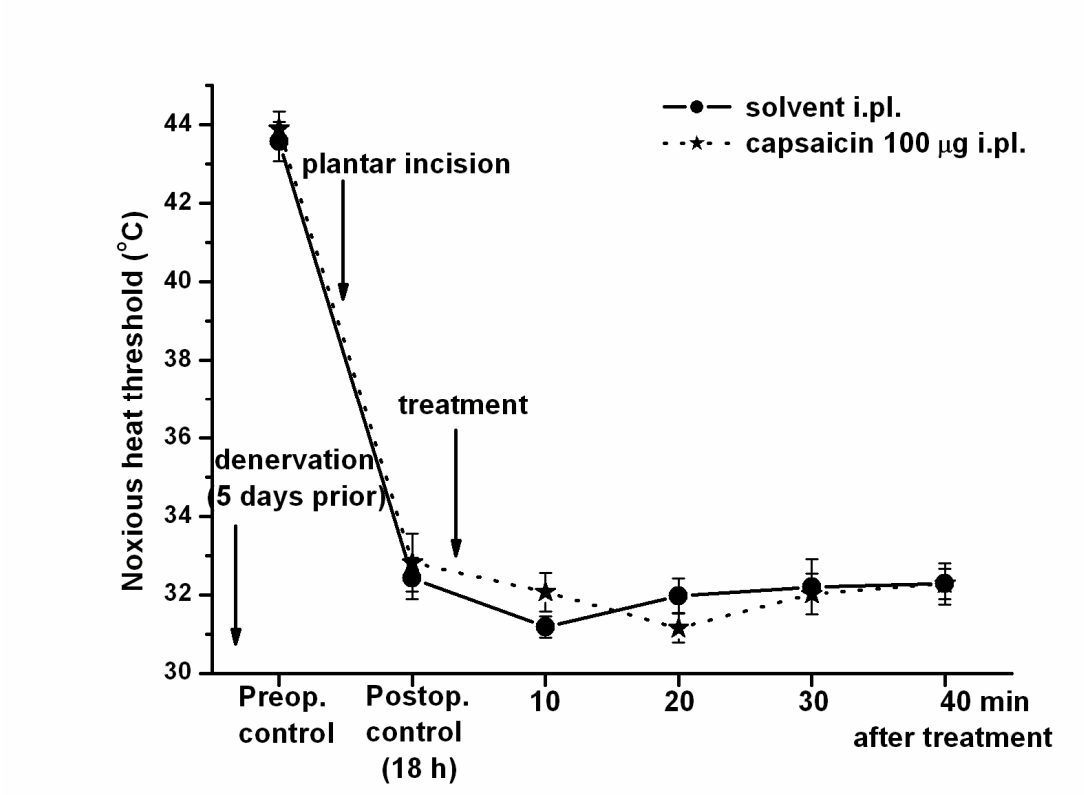


**Fig. 21.** Effect of intraplantar capsaicin injection into the chronically (5 days) denervated hind paw on the heat threshold drop of the contralateral hind paw induced by plantar incision performed at time indicated by the arrow. Capsaicin or the solvent was administered intraplantarly as indicated by the treatment arrow.

### 4.4.3 Effect of intraplantar capsaicin administration following local capsaicin desensitization on plantar incision-induced heat hyperalgesia of the contralateral hind paw

A sustained exposure to high concentration of capsaicin can cause desensitization of the TRPV1-expressing polymodal nociceptive nerve endings rendering them unable to respond to further stimulation by capsaicin and other stimuli.

Pretreatment with intraplantar injection of a single high dose of capsaicin (100 μg) into the intact i.e. not denervated hind paw was carried out 3 days prior to the experiment. After this capsaicin desensitization, intraplantar injection of capsaicin failed to have any effect on plantar incision-induced heat hyperalgesia of the contralateral hind paw (Fig. 22). Pretreatment with the solvent of capsaicin did not alter the inhibitory effect of subsequent capsaicin administration on contralateral thermal hyperalgesia (Fig. 22).

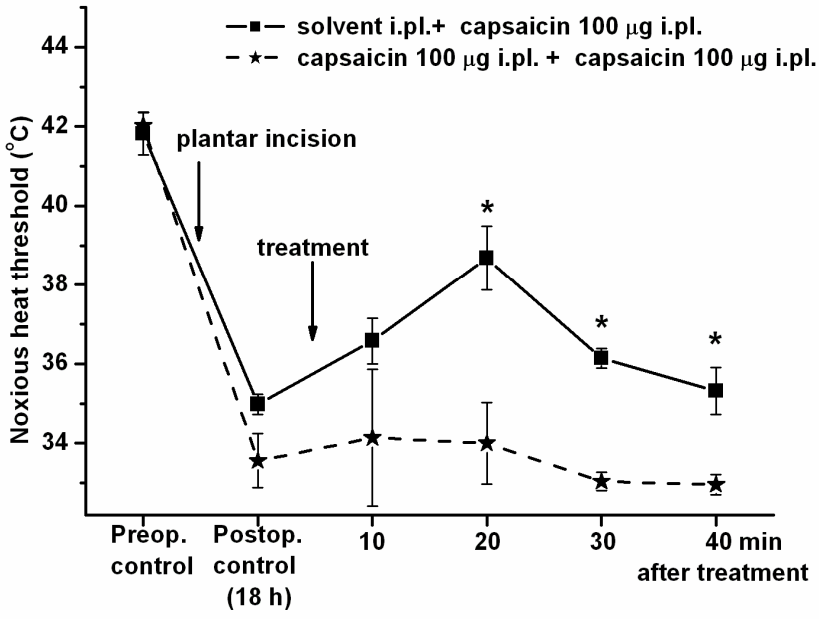


**Fig. 22.** Effect of intraplantar capsaicin injection following pretreatment (3 days prior) with intraplantar capsaicin (100 μg) or its solvent on the heat threshold drop induced by plantar incision of the contralateral hind paw performed at time indicated by the arrow. Capsaicin or its solvent was administered intraplantarly as indicated by the treatment arrow.

### 4.4.4 Effect of percutaneous mustard oil administration to the acutely denervated hind paw on plantar incision-induced heat hyperalgesia of the contralateral hind paw

Percutaneous treatment with 5 % mustard oil of the denervated hind paw significantly reduced the plantar incision-induced heat threshold drop of the contralateral hind paw. The solvent failed to affect the noxious heat threshold drop induced by incision (Fig. 23). The extent of the mustard oil effect was comparable to that of capsaicin (Fig. 20).

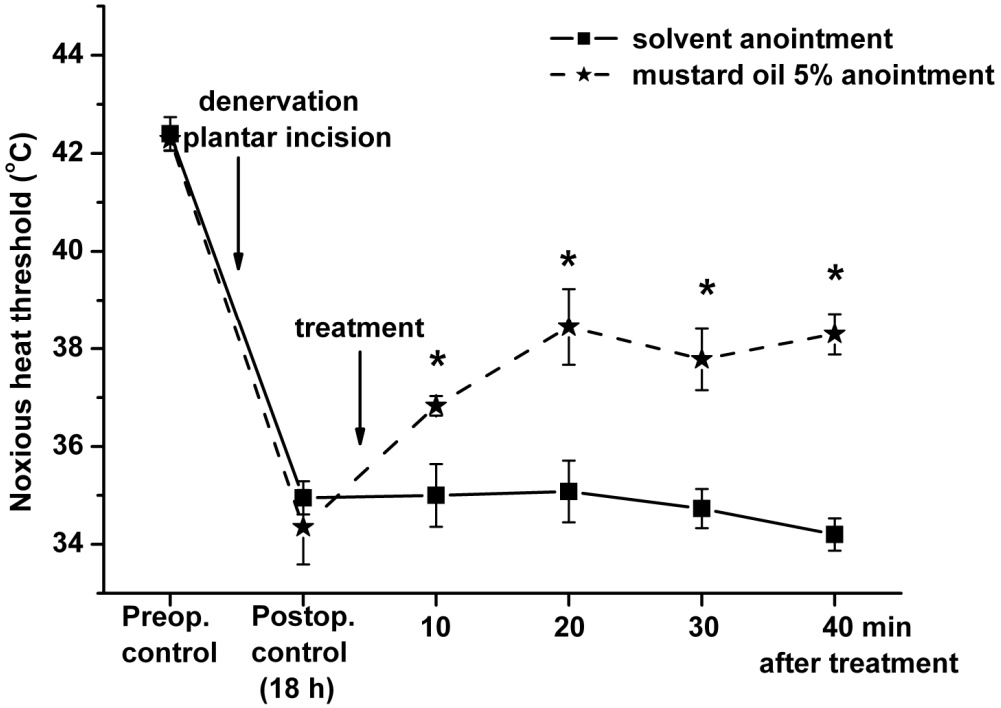


**Fig. 23.** Effect of percutaneous mustard oil treatment of the denervated hind paw on the noxious heat threshold drop of the contralateral hind paw induced by incision performed at time indicated by the arrow. Mustard oil or its solvent was administered topically as indicated by the treatment arrow.

### 4.4.5 Possible mediators of the remote thermal antihyperalgesic effect of chemical stimulation of peripheral nociceptors

#### 4.4.5.1 Effect of intraplantar capsaicin treatment of the denervated hind paw on plantar incision-induced heat hyperalgesia of the contralateral hind paw following systemic pretreatment with the somatostatin receptor antagonist cyclosomatostatin

Systemic pretreatment with cyclosomatostatin (C-SOM), 20 min before administration of capsaicin, inhibited its remote antihyperalgesic effect as it diminished the capsaicin-evoked reduction of the incision-induced drop of the heat threshold in the contralateral hind paw. Its solvent did not have any effect on the heat threshold (Fig. 24). Compared to the pretreatment with the solvent of C-SOM the difference was significant at all time points (Fig. 24).

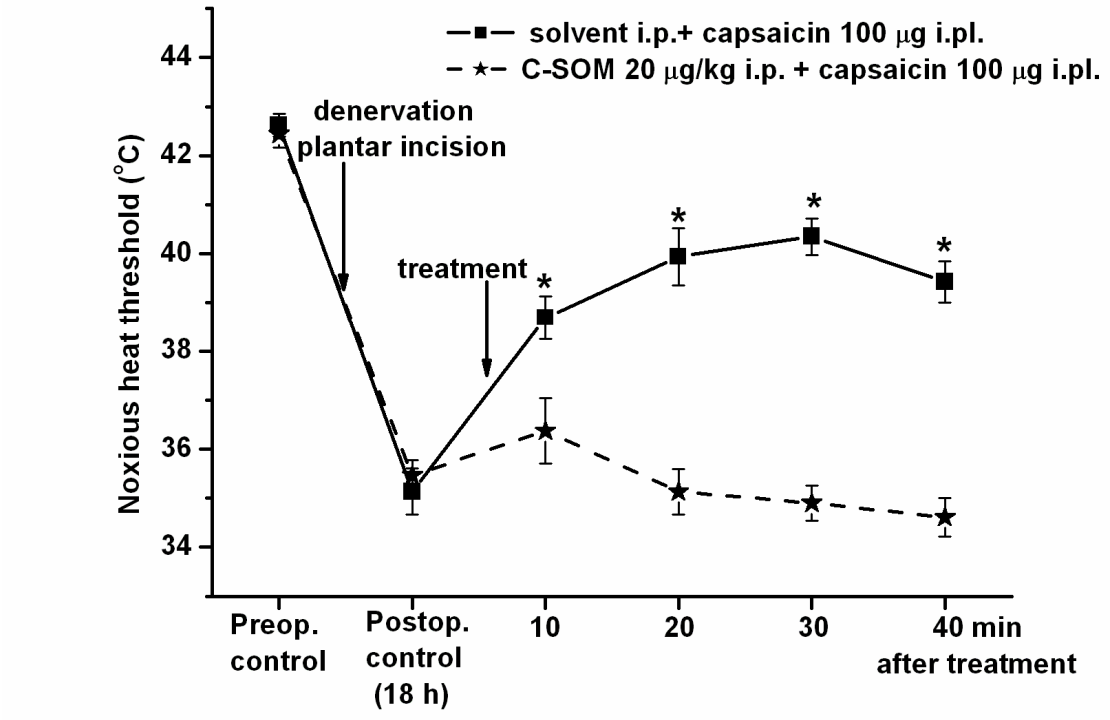


**Fig. 24.** Effect of intraplantar capsaicin (or its solvent) treatment (at time point indicated by treatment arrow) of the denervated hind limb on the incision-induced (indicated by the arrow) noxious heat threshold drop of the contralateral hind paw following systemic pretreatment with cyclosomatostatin (C-SOM) 20 min prior.

#### 4.4.5.2 Effect of percutaneous mustard oil treatment of the denervated hind paw on plantar incision-induced heat hyperalgesia of the contralateral hind paw following systemic pretreatment with the somatostatin antagonist cyclosomatostatin

Systemic pretreatment with cyclosomatostatin (C-SOM), 20 min before mustard oil anointment, diminished the remote antihyperalgesic effect as it diminished the mustard oil-evoked reduction of the incision-induced drop of the heat threshold in the contralateral paw. Its solvent was without effect (Fig. 25). Compared to the pretreatment with the solvent of C-SOM the difference was significant at the 30 and 40 min time points (Fig. 25).

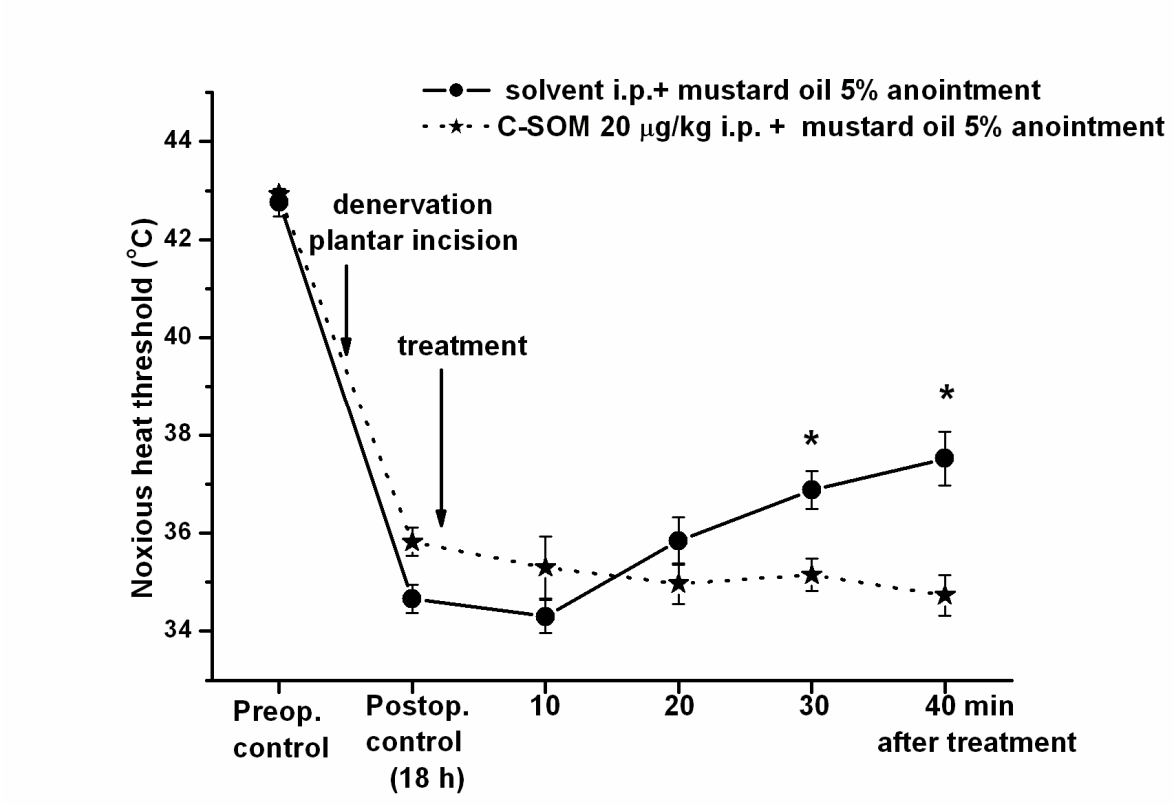


**Fig. 25.** Effect of percutaneous mustard oil (or its solvent) treatment (at time point indicated by treatment arrow) of the denervated hind limb on the incision-induced (indicated by the arrow) noxious heat threshold drop of the contralateral hind paw following systemic pretreatment with cyclosomatostatin (C-SOM) 20 min prior.

#### 4.4.5.3 Effect of systemically applied exogenous somatostatin on plantar incision-induced heat hyperalgesia

In order to further clarify the role of somatostain in the remote antihyperalgesic effect of nociceptor stimulation, the effect of exogenous somatostatin on incision-evoked heat hyperalgesia was studied. Compared to actual solvent treatment, i.p. applied somatostain significantly reduced the plantar incision-induced noxious heat threshold drop at all measurement points (Fig. 26).

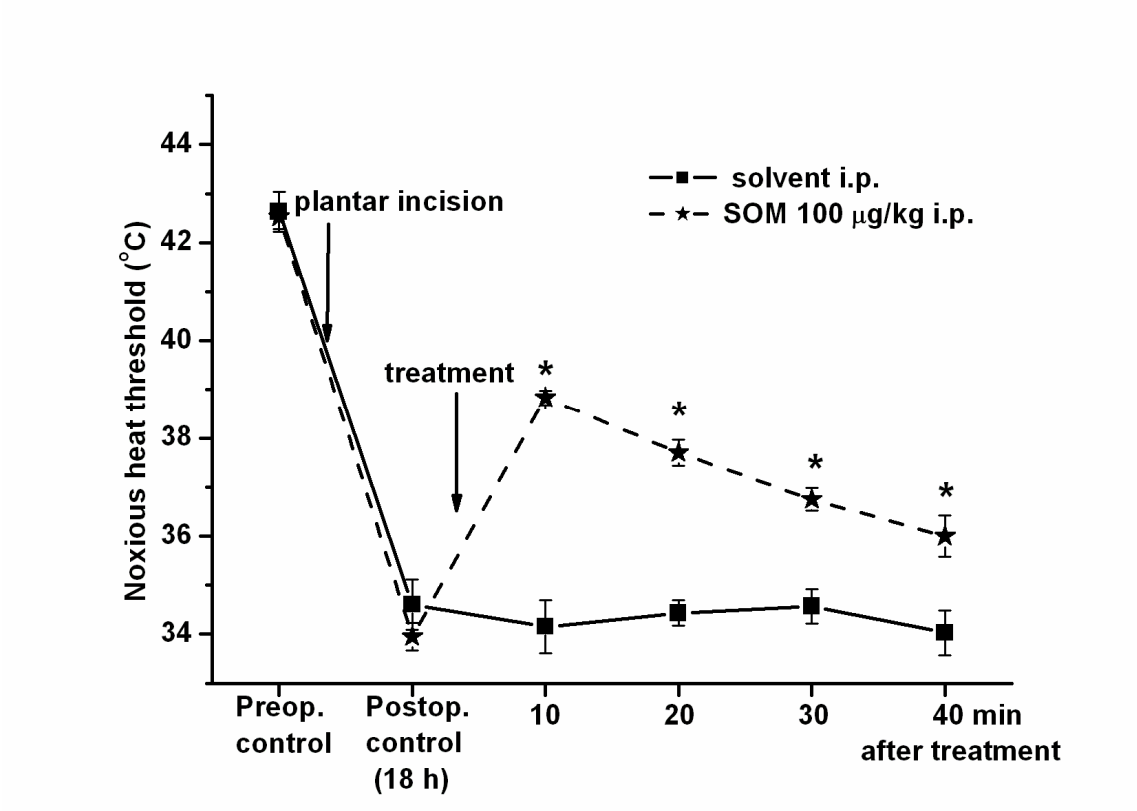


**Fig. 26.** Effect of systemically applied somatostatin (SOM) on the noxious heat threshold drop induced by plantar incision performed at time indicated by the arrow. Time point of somatostatin administration is indicated by the treatment arrow.

#### 4.4.5.4 Effect of systemically applied somatostatin on incision-induced heat hyperalgesia following systemic pretreatment with the somatostatin receptor antagonist cyclosomatostatin

In order to verify the antagonistic ability of C-SOM against somatostatin, the effect of exogenous somatostatin after pretreatment with C-SOM was also examined. Pretreatment with C-SOM, 20 min before administration of somatostatin, strongly reduced (apparently abolished) the antihyperalgesic effect of exogenous somatostatin at all time points of measurement (Fig. 27). Pretreatment with solvent had no effect (Fig. 27).

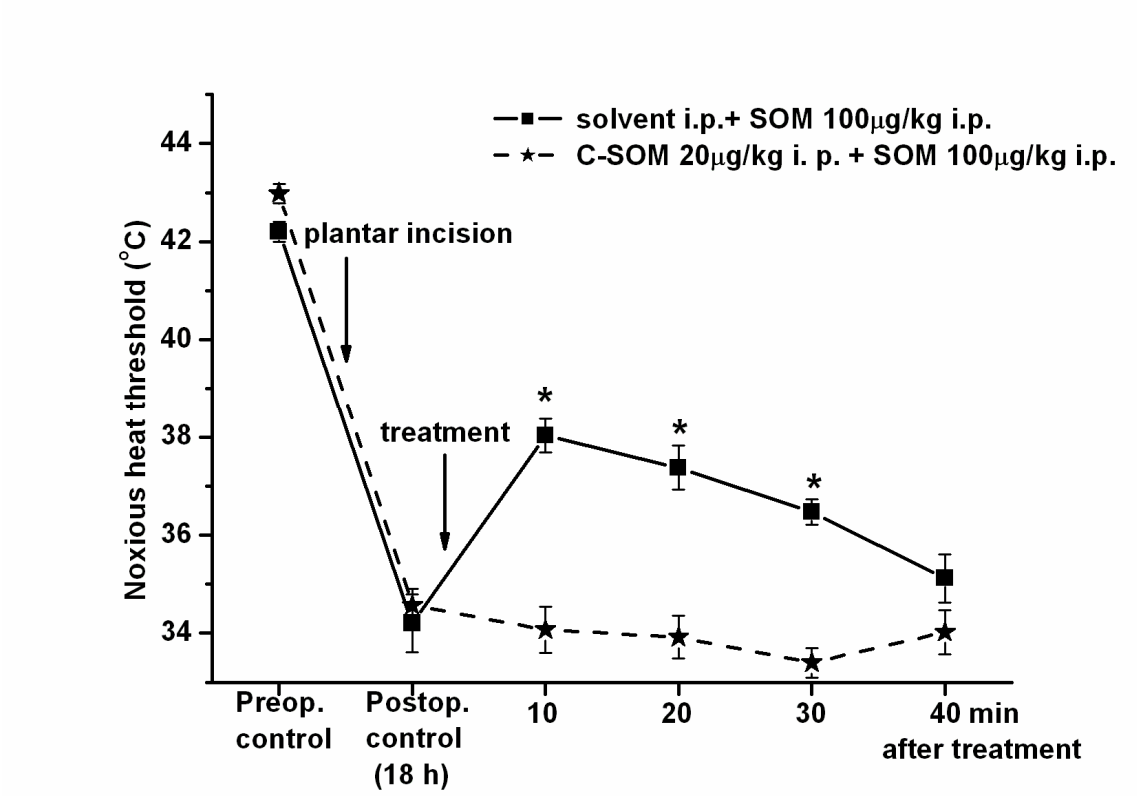


**Fig. 27.** Effect of systemic somatostatin treatment (indicated by treatment arrow) on incision-induced (performed at time indicated by the arrow) drop of the heat threshold following systemic pretreatment with cyclosomatostatin (C-SOM) 20 min prior.

#### 4.4.5.5 Effect of intraplantar capsaicin treatment of the denervated hind paw on incision-induced heat hyperalgesia of the contralateral hind paw following systemic pretreatment with the opioid receptor antagonists

Systemic pretreatment with the opioid antagonist naloxone, 20 min before administration of capsaicin into the denervated hind paw, significantly reduced, but did not abolish the analgesic effect of intraplantar capsaicin treatment on the heat threshold drop of the contra lateral, incised hind limb (Fig. 28) at all time points, compared to solvent treatment. Pretreatment with peripherally acting naloxone methiodide did not led to reduction in the antihyperalgasic effect of the capsaicin treatment (Fig 28).

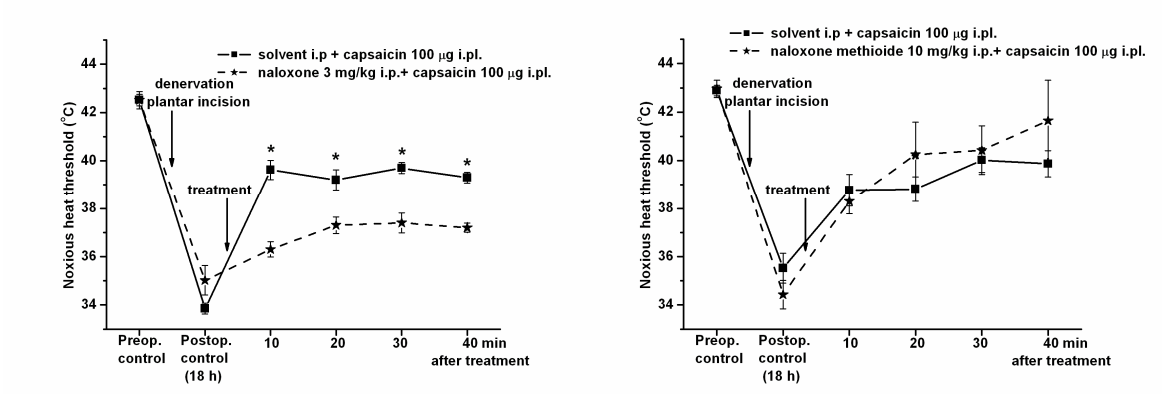


**Fig. 28.** Effect of intraplantar capsaicin treatment (indicated by the treatment arrow) of the denervated hind limb, on the noxious heat threshold drop of the contralateral hind paw induced by plantar incision performed at time indicated by the arrow, following systemic pretreatment with naloxone, (left panel) or naloxone methiodide (right panel) 20 min prior.

### 4.4.5.6 Effect of intraplantar capsaicin treatment of the denervated hind paw on incision-induced heat hyperalgesia of the contralateral hind paw following systemic pretreatment with the $CB_1$ cannabinoid receptor antagonist AM 251

Pretreatment with $CB_1$ cannabinoid receptor antagonist AM 251 was performed 20 min before administration of capsaicin. The cannabinoid antagonist significantly decreased the incision-induced heat hyperalgesia of the contralateral hind paw 10 and 20 min after treatment (Fig. 29).

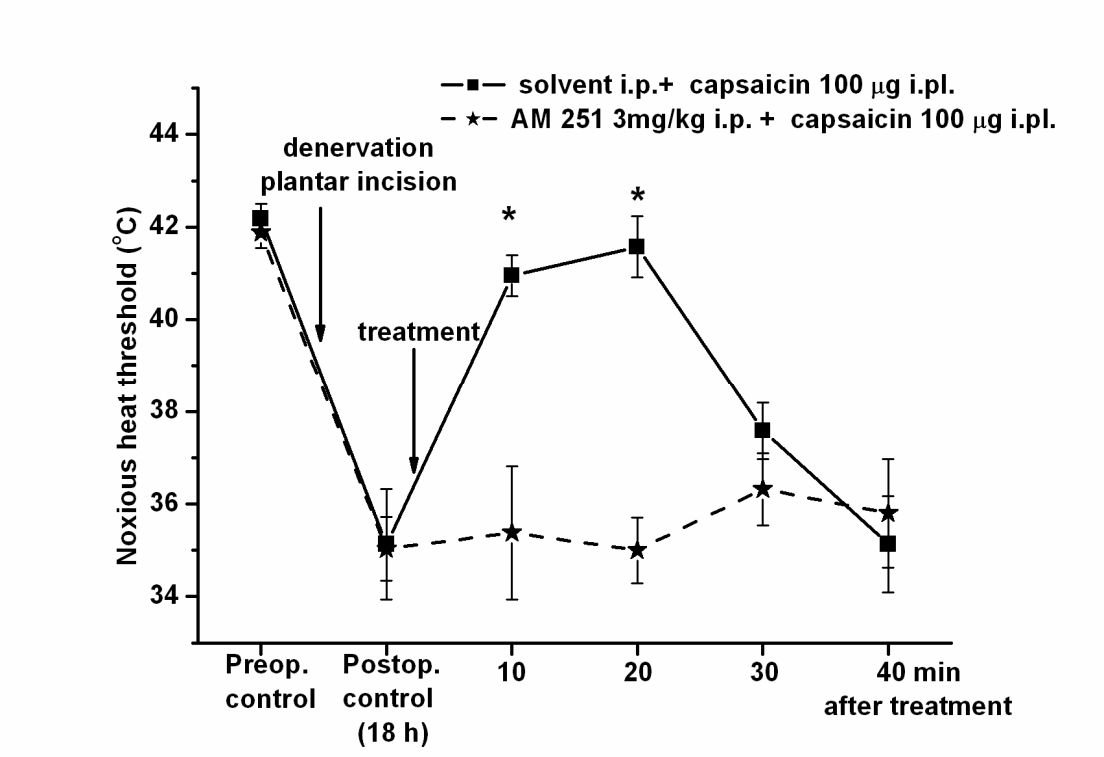


**Fig. 29.** Effect of intraplantar capsaicin treatment (indicated by the treatment arrow) of the denervated hind limb on the heat threshold drop of the contralateral hind paw induced by plantar incision (performed at time indicated by the arrow) following systemic pretreatment with the CB1 cannabinoid receptor antagonist AM 251 20 min prior.

### 4.4.6 Remote mechanical antihyperalgesic effect of chemical stimulation of peripheral nociceptors

#### 4.4.6.1 Effect of intraplantar capsaicin treatment of the denervated hind paw on neuropathic mechanical hyperalgesia of the contralateral hind paw induced by partial sciatic nerve ligation

Administration of capsaicin into the denervated hind paw significantly diminished the mechanical hyperalgesia of the contralateral paw induced by partial sciatic nerve ligation (Seltzer's operation performed 48 h prior to the denervation) at both time points of measurement with a Randall–Selitto apparatus (Fig. 30).

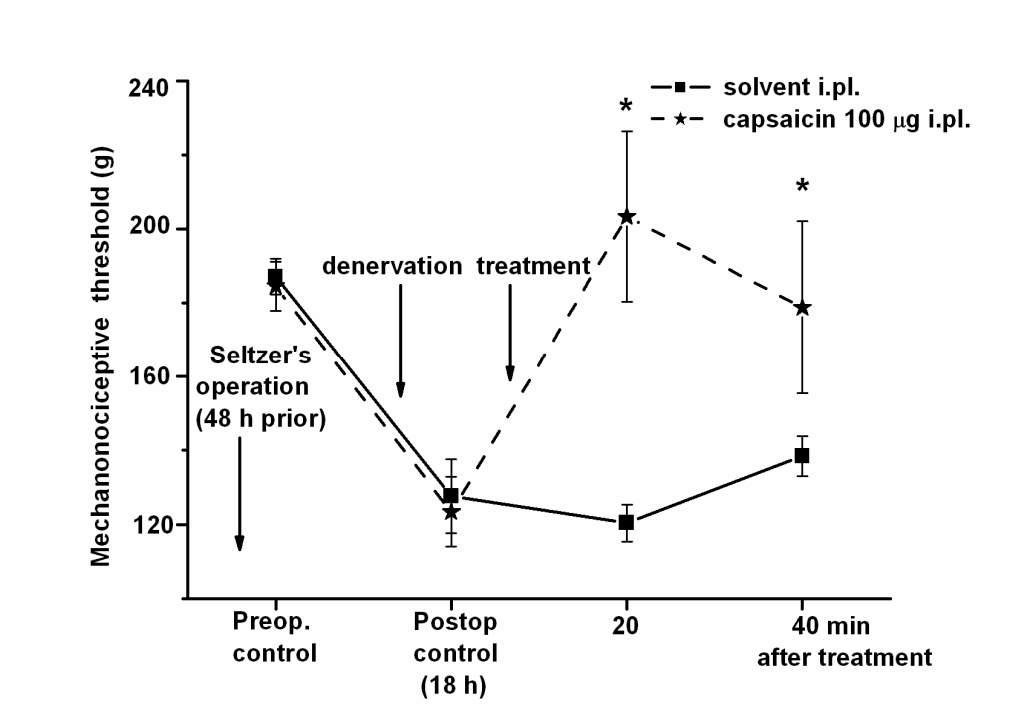


**Fig. 30.** Effect of intraplantar capsaicin treatment of the denervated hind limb on the mechanical hyperalgesia induced by partial sciatic nerve ligation (48 h before denervation) on the contralateral hind paw (performed at time indicated by the arrow). Capsaicin or the solvent was administered intraplantarly as indicated by the treatment arrow.

#### 4.4.6.2 Effect of percutaneous administration of mustard oil to the denervated hind paw on neuropathic mechanical hyperalgesia of the contralateral hind paw induced by partial sciatic nerve ligation

Percutaneous application of mustard oil to the denervated hind limb significantly reduced mechanical hyperalgesia of the contralateral paw, at each time point of measurement, induced by Seltzer's operation 48 h before denervation. The solvent treatment was without effect.

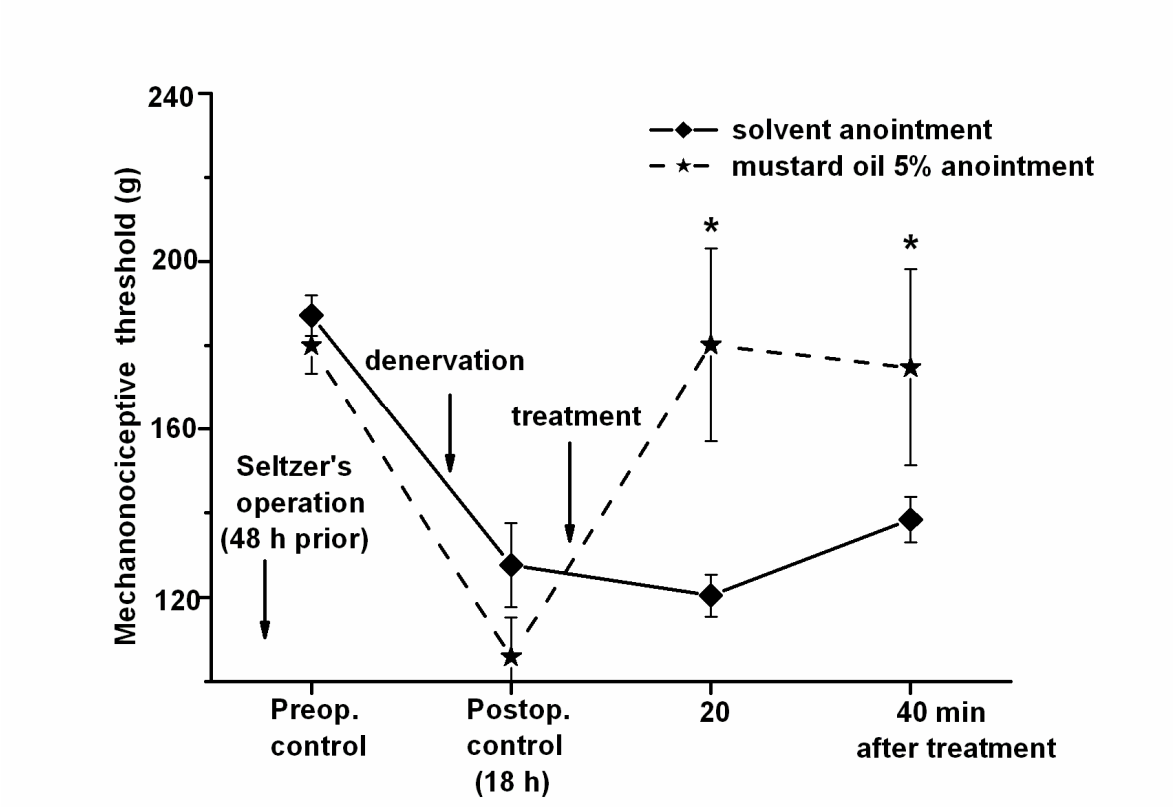


**Fig. 31.** Effect of topical mustard oil treatment of the denervated hind paw on the mechanonciceptive threshold drop of the contralateral hind paw induced by partial sciatic nerve ligation performed at time indicated by the arrow. Mustard oil or the solvent was administered topically as indicated by the treatment arrow.

### 4.4.7 Effect of intraplantar capsaicin treatment of the intact (not denervated) hind paw on plantar incision-induced heat hyperalgesia of the contralateral hind paw

Intraplantar injection of the lower of the two effective doses of capsaicin (10 µg) into the intact (not denervated) right hind paw reduced the noxious heat threshold drop induced by plantar incision on the contralateral, left hind paw (Fig. 32). Capsaicin injection induced an intense nocifensive reaction consisting of paw licking and shaking, which lasted for approximately 5 minutes. The higher capsaicin dose was not tested for ethical reasons. The antihyperalgesic effect was found to be significant upon all measurements following treatment.

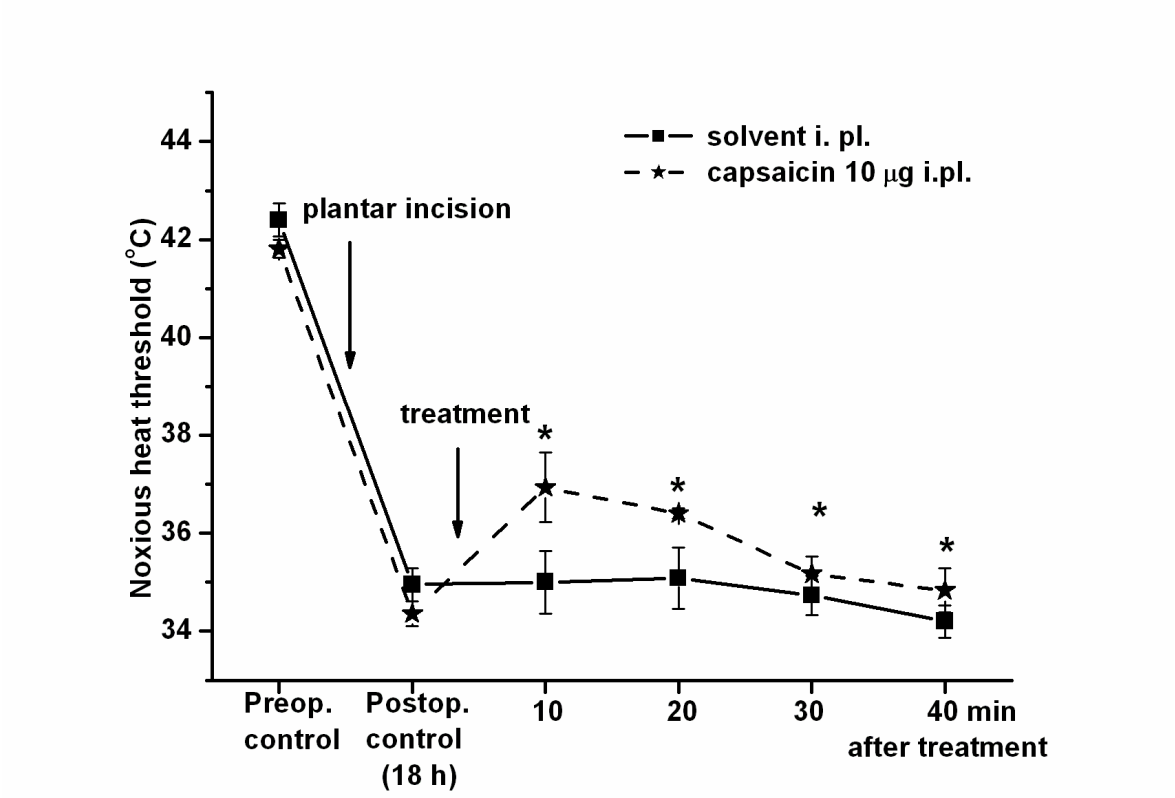


**Fig. 32.** Effect of intraplantar capsaicin injection into the intact hind paw on the heat threshold drop induced by plantar incision on the contralateral hind paw performed at time indicated by the arrow. Capsaicin or its solvent was administered intraplantarly as indicated by the treatment arrow.

# 5 Discussion

In our experiments a novel approach to the study of thermonociception was employed: measurement of the noxious heat threshold temperature with an increasing-temperature water bath. As mentioned in more detail in the Introduction, conventional methods of thermonociception in animals are based on determination of the withdrawal latency, i.e. measurement of time. The heat threshold measurement paradigm, albeit technically more complicated than latency determination, have proved to be suitable for different experimental purposes in previous studies conducted in our department[7, 8]. With this method, heat hyperalgesia manifests itself as a drop of heat threshold.

In our first series of experiments surgical incision on the plantar surface of the rat hind paw was used to evoke a thermal hyperalgesic state. Surgical incision resulted in a marked, relatively constant drop of heat threshold lasting for several days. The heat threshold drop had a similar time course as the withdrawal latency time reduction measured with the standard plantar test indicating that heat threshold measurement is as reliable as latency determination for revealing incision-induced thermal hyperalgesia[18].

As postoperative pain relief is a major use of analgesics and a widely studied field of clinical research, it was conspicuous to study the effects of conventional analgesics (morphine, diclofenac and paracetamol) in our plantar incision model. These drugs were administered following the post-incision heat threshold measurement confirming development of thermal hyperalgesia and to simulate clinical use of analgesics. Due to the excellent reproducibility of heat threshold measurement even with short intervals it was possible to assess the degree and time course of the effect of the substances within the same animal[13]. All analgesics studied exerted a dose-dependent inhibitory effect on incision-evoked heat threshold drop. As the heat threshold of the intact, uninjured hind paw was not altered even by the highest tested doses of the analgesics, the heat threshold changes were considered true antihyperalgesic effects not secondary to a direct threshold-elevating action.

Even the lowest tested dose (0.3 mg/kg) of morphine had an antihyperalgesic action. This dose is equal to or smaller than those (0.3 and 1mg/kg) that inhibited the withdrawal latency reduction measured in the paw withdrawal test[20]. These findings indicate that the noxious heat threshold is an at least as sensitive parameter as the paw withdrawal latency for revealing the thermal antihyperalgesic action of morphine in the incision model. Morphine's efficacy to reduce the incision-induced heat threshold drop was the highest (60 %) among the analgesics tested. This is in accordance with the well-known high clinical analgesic efficacy of opioids.

The non-steroidal anti-inflammatory drugs are widely used non-opioid analgesics that exert their effects through inhibition of cyclooxygenase enzymes (COX-1 and COX-2) which are responsible for formation of prostanoids including prostaglandins, prostacyclin and thromboxane. The non-selective COX inhibitor diclofenac had a dose-dependent inhibitory effect on the heat hyperalgesia following plantar incision. The minimum effective dose (1 mg/kg) was found to be higher compared to that of morphine. One previous study revealed the thermal antihyperalgesic effect of a single dose (5 mg/kg) of diclofenac measured with the paw withdrawal test[19]. The maximum inhibition achieved by diclofenac was 40 %, similarly to its inhibitory effect on the resiniferatoxin- or heat injury-induced heat threshold drop[20].

Paracetamol (acetaminophen), a non-opioid analgesic with a largely unknown mode of action[21], was the least potent (i.e. requiring the highest administered dose) among the examined analgesics, but its maximum effect was higher than that of diclofenac but less than that of morphine. As to our knowledge, no other study has examined paracetamol in the incision model.

The antihyperalgesic action of either morphine or diclofenac administered intraplantarly was similar to that observed in our previous studies examining the heat injury- or resiniferatoxin-induced heat threshold drop [13, 20].The locally applied doses were substantially lower than the respective systemic minimum effective doses, ascertaining that the drugs acted locally without any systemic effect. The

local antinocicptive effect of diclofenac can be explained by inhibition of the formation of nociceptor-sensitizing prostaglandins in inflamed or damaged tissues while in case of opioids a peripheral site of action at the level of peripheral nociceptors was revealed, especially under inflammatory conditions[22].

The second series of experiments aimed at comparing peripheral mediators of the heat threshold drop (thermal hyperalgesia) evoked by mild heat injury and plantar incision. As selective receptor antagonists and enzyme inhibitors were used influence of possible secondary changes caused by gene deletion in knockout animals could be avoided. All these test substances were applied intraplantarly at doses that have previously been employed in several other studies making likely that their effects were restricted to the periphery. While in case of incision both injury and local drug treatment were confined predominantly to the plantar part of the paw, with heat injury both plantar and dorsal surfaces of the hind paw were injured but test substances were applied intraplantarly leaving the dorsal part of the paw untreated. This area of the paw certainly contributed to the animal's nociceptive behavior also, thus the inhibitory effect of test substances have most likely been underestimated. For this reason the percentage inhibition values obtained in the two models were not compared.

Regarding the mechanisms (receptors and enzymes) evaluated, relatively small differences have been revealed between mediators of heat threshold drop induced by either plantar incision or heat-injury. Activation of the $B_2$ bradykinin receptor, P2X purinoceptors and the TRPV1 receptor as well as formation of NO were observed in both models. In mild heat injury-induced heat hyperalgesia formation of lipoxygenase products was shown as well, while incision-evoked heat hyperalgesia involved activation of $B_1$ bradykinin receptors, too.

Bradykinin is a 9 amino acid peptide that is produced by kallikrein in pathophysiologic conditions such as inflammation, trauma, burns, shock and allergy. It has two receptor types, $B_1$ and $B_2$ which are G-protein-coupled receptors. $G_q$ protein stimulates phospholipase C to increase intracellular free calcium and diacylglycerol while $G_i$ protein inhibits adenylate cyclase and activates phospholipase $A_2$, resulting in formation of prostanoids.[23,24]. $B_2$ receptors are

constitutively and ubiquitously expressed in various cells types including nociceptive primary afferent neurons, smooth muscle cells, endothelium, macrophages and postganglionic sympathetic nerve fibers[25]. In contrast, $B_1$ receptors are inducible, meaning that they are de novo synthesized in response to tissue injury[26]. An involvement of both $B_2$ and $B_1$ receptor activation in the incision-evoked drop of heat threshold was revealed in our experiments in contrast to a previous study that found no participation of either subtype of bradykinin receptors in this model of heat hyperalgesia at a similar time point after injury[27]. It must be considered that in that study paw withdrawal latency to radiant suprathreshold heat stimulation was measured while in the present study the noxious heat threshold temperature was determined, thus our methodology was proven to be more sensitive. The role of bradykinin in incision-induced heat hyperalgesia is plausible as tissue injury is known to activate the kallikrein–kinin system. In our experiments the incision-evoked heat hyperalgesia was tested 18 h after injury when $B_1$ receptor induction is likely to have already occurred. In case of heat injury-evoked heat hyperalgesia only $B_2$ receptor activation was found possibly because the measurements took place early, 20–50 min after injury when induction of $B_1$ expression was not yet likely. Previously only one (clinical) study examined the role of bradykinin in hyperalgesia evoked by heat injury. The heat injury was induced on the thenar eminence of a patient with kininogen deficiency and the thermal hyperalgesia was measured to be within the range observed in age-matched control subjects[28]. In addition, an increase in bradykinin content was observed in the mouse ear after burn injury[29]. Involvement of $B_2$ receptors in the edema response following burn injury to the rat paw, back of rabbits and 40 % body surface of sheep was also revealed[30,31,32].

During the validation process of the increasing-temperature water bath, it was shown that intraplantar application of COX inhibitors exerted an antihyperalgesic effect following heat injury or plantar incision, in both models decreasing the injury-evoked heat threshold drop[13.] These results also provide evidence for an involvement of COX products i.e. prostanoids in both experimental paradigms. Supporting our observation in the incision model, prostaglandin $E_2$ was detected in wound exudate following Caesarean section in women[33]. These findings suggest that COX products play role in development of hyperalgesia following tissue injury

which is reflected by the well-known clinical efficacy of non-steroidal anti-inflammatory analgesics in such clinical conditions.

Leukotrienes, products of the 5-lipoxygenase enzyme, are important mediators of the inflammatory response acting principally on a subfamily of G protein-coupled receptors. 5-lipoxygenase catalyzes oxidation of unsaturated fatty acids with oxygen to form peroxides, and facilitates the synthesis of leukotrienes by converting arachidonic acid to 5-hydroperoxyeicosatetraenoic acid via an oxidative process[34]. In accordance with the described pro-nociceptive role of lipoxygenase products, leukotriene $B_4$ and other lipoxygenase products were demonstrated to exert pro-nociceptive actions in a variety of animal models[35,36,37,38,39;40]. Our results suggest that activation of the lipoxygenase pathway takes place following both heat injury and plantar incision, but its effect is more pronounced following heat injury.

Nitric oxide (NO) is formed from L-arginine, oxygen and NADPH by NO synthase (NOS). There are three isoenzymes of NOS: the endothelial NOS (eNOS) and neuronal NOS (nNOS) are constitutively expressed and regulated by calcium and calmodulin, the third type is inducible NOS (iNOS). NO stimulates the soluble guanylate cyclase to form cyclic GMP that activates protein kinase G which can phosphorylate various target proteins. NO is capable of inducing acute pain in humans and plays an important role in hyperalgesia caused by inflammation and injury, partly via activation of the TRPV1 receptor (for rev. see Pethő and Reeh, 2012[34]). For example, NO solutions injected intracutaneously, paravascularly or perfused through a vascularly isolated hand vein segment evoked pain[41,42]. Intraarticular injection of a NOS inhibitor reduced secondary heat hyperalgesia in rats with kaolin-induced arthritis[43]. Our results are in accordance with these data revealing the mediator role of locally formed NO in both models because an intraplantarly applied NOS inhibitor diminished the heat threshold drop evoked by burn injury or incision. The isoenzyme involved cannot be determined because L-NOARG is a non-specific inhibitor of NOS. In the heat injury model, however, the short time window argues against the involvement of the iNOS.

P2X purinoceptors are a family of cation-permeable ligand-gated ion channels that open in response to the binding of extracellular ATP. P2X receptors are expressed predominantly in nociceptive nerve endings but they can be found in a variety of other cells types[1]. P2X receptors have been shown to modulate synaptic transmission[1] on presynaptic and postsynaptic nerve terminals throughout the central, peripheral and autonomic nervous systems. Other research groups reported that a selective P2X receptor antagonist diminished the nocifensive reaction during the late phase of the formalin test in the rat[44] and similar results were obtained in P2X3 receptor null-mutant mice[45,46]. It was also known that high levels of ATP persist in heat-damaged skin of guinea-pigs[47]. In accordance with these data, an involvement of P2X receptor activation was demonstrated both in plantar incision and heat injury model by local administration of TNP-ATP, a selective P2X receptor antagonist.

An involvement of TRPV1 receptor activation in the heat threshold drop induced by heat injury and plantar incision was revealed using two selective TRPV1 receptor antagonists, AMG9810[48] and SB-366791[49], respectively. The contribution of TRPV1 to the heat threshold drop in these models was expected as thermal hyperalgesia was shown to critically depend on activation of this ion channel in several models of acute/subacute inflammation[50,51,52,53,54]. In our previous study in which the noxious heat threshold of rats was measured with increasing-temperature hot plate, the mild heat injury-evoked drop of the noxious heat threshold was reduced in TRPV1 knockout mice compared to wild-type animals[55]. An involvement of TRPV1 activation in surgical incision-induced thermal hyperalgesia was previously shown in vivo by measurement of paw withdrawal latency in response to suprathreshold heat stimulation in mice and rats[56] and in vitro by single fiber recording in mice[56]. It is worth mentioning that TRPV1 also can be activated by low pH and after plantar incision in rats a decrease in plantar tissue pH was revealed[57].

Considering that the sum of the percentage inhibition values obtained with the various mediator blockers exceeds 100 % (Table 3), it is unlikely that these mediators act independently in a parallel fashion, rather, their action may converge on common pathway(s). Such a role can be proposed for TRPV1 which can be

sensitized through phosphorylation by various intracellular protein kinases such as protein kinase C (PKC) and A (PKA)[58]. Both bradykinin and prostaglandins were shown to sensitize TRPV1 to heat through activation of PKC and PKA[59,60]. Therefore the revealed contribution of bradykinin and prostanoids to the heat injury- and incision-evoked heat hyperalgesia may be mediated by PKC- and PKA-mediated phosphorylation of TRPV1. Certain lipoxygenase products directly activated TRPV1 channels in both sensory neurons and TRPV1-transfected host cells[61] offering a possible mode of action for lipoxygenase products in the heat injury-evoked heat hyperalgesia. Finally, NO was also shown to activate TRPV1 through S-nitrosylation of cysteine residues[62] possibly explaining its involvement in both hyperalgesia models studied.

In summary, relatively small differences were revealed between the examined peripheral mediators of heat hyperalgesia evoked by mild heat injury and plantar incision despite the different nature and time course of hyperalgesia in these conditions. Involvement of the $B_2$ bradykinin receptor, P2X purinoceptors and the TRPV1 receptor as well as formation of NO and COX products were revealed in both models implying that pharmacological blockade of these targets may theoretically be used in treatment of both burn-induced and postoperative pain. Lipoxygenase products were found to have major effect in heat injury-induced hyperalgesia compared to that in plantar incision. The involvement of $B_1$ receptor activation in the incision-induced sustained heat hyperalgesia (18 h after injury) but not in heat injury-evoked acute hyperalgesia (20–50 minutes after injury) suggests that subacute or chronic hyperalgesia models are more likely to detect the antinociceptive effect of $B_1$ receptor antagonists than the acute ones. This might have relevance for the clinical testing of the analgesic effects of these drugs in that more sustained pain models should be incorporated into the human methodology.

In the third series of experiments we examined a remote antihyperalgesic effect induced by chemical stimulation of peripheral endings of nociceptive primary afferent neurons. The experiments were designed according to a previous model in which Pintér and Szolcsányi revealed a novel neuroregulatory mechanism by discovering a remote, systemic anti-inflammatory effect induced by chemical

(capsaicin) or electrical stimulation of peripheral nociceptive nerve endings (Fig. 2) [7, 8] . They showed that activation of the peripheral nociceptors leads to release of somatostatin that is absorbed into systemic circulation and exerts anti-inflammatory actions in different parts of the body behaving as a hormonal agent derived from sensory nerve endings. As somatostatin is known to have antinociceptive action, our hypothesis was that the above-described systemic, sensocrine effect of peptidergic polymodal nociceptors involves a systemic antinociceptive action, too. It should be stressed that the essence of this novel sensocrine effect is its peripheral origin from nociceptive nerve endings. It explains why the chemically stimulated hind paw had to be acutely denervated in order to exclude possible neural compensatory mechanisms in response to propagation of action potentials to the central nervous system. It should be also noted that acute denervation does not impair the mediator-releasing ability of peptidergic nociceptors.

The finding that in our experiments capsaicin-induced stimulation of the denervated hind paw failed to affect the noxious heat threshold of the contralateral hind paw that was not incised led us to induce heat hyperalgesia on the contralateral side by plantar incision in order to increase the sensitivity of our paradigm. (Fig. 6).

The injection of capsaicin into the denervated right hind paw resulted in a dose-dependent reduction in the noxious heat threshold drop induced by prior surgical incision of the contralateral, left hind paw. This remote antihyperalgesic effect was substantial i.e. the degree of reduction of the threshold drop was comparable to that seen with morphine (3 mg/kg i.p.) in the first series of experiments. As capsaicin is known to act on a subset of primary afferent neurons we hypothesized that neuronal elements are involved. To test our hypothesis capsaicin challenge was repeated following chronic denervation of the stimulated right hind paw. Surgical transection of the peripheral nerves innervating the right hind limb was made five days prior to the stimulation to ensure that no viable nerve fibers or endings are present. Administration of capsaicin to the chronically denervated paw failed to alter the noxious heat threshold of the incised left hind paw, thus we concluded that neuronal elements of the paw should be involved in the

phenomenon. In addition, this finding argues against an involvement of a possible systemic antinociceptive effect of capsaicin. As the only type of nerve endings known to be responsive to capsaicin are the TRPV1-expressing polymodal nociceptors, we decided to test their involvement by performing local capsaicin desensitization. An intermediate dose of capsaicin was injected into the right hind paw of the animal three days before the actual experiment to allow sufficient time for inhibition of capsaicin-sensitive nerve endings. As capsaicin injected after local desensitization failed to alter the heat threshold on the contralateral incised paw, we concluded that capsaicin-sensitive nerve endings participate in the remote effect of nociceptor stimulation. Moreover, this finding also supports the view that capsaicin challenge acted locally in the injected paw and not by systemic desensitization. As mustard oil applied to the denervated hind paw was able to exert a similar remote antihyperalgesic effect, we concluded that the examined phenomenon is not restricted to TRPV1 receptor activation. Mustard oil acts by activation of TRPA1 receptor and does not have effect on the previously tested TRPV1 receptor[63]. It is worth noting, however that TRPV1 and TRPA1 receptors are both expressed by polymodal nociceptive primary afferents.

Previous studies at our department demonstrated that in a similar experimental arrangement the remote anti-inflammatory effect evoked by chemical stimulation of the denervated hind paw was mediated by somatostatin released into the systemic circulation[6]. In addition to functional evidence, a marked elevation of plasma somatostatin-like immunoreactivity could be detected by radioimmunoassay following chemical stimulation of the nerve endings of the denervated hind paw. As somatostatin is known to exert a peripheral antinociceptive effect it was plausible to examine its role in the remote antihyperalgesic effect, too[6]. Following systemic pretreatment with the somatostatin receptor antagonist cyclosomatostatin, the reduction in noxious heat threshold drop of the contralateral incised hind paw was prevented. In a further experiment the animals went through the same operations but received systemic somatostatin treatment instead of peripheral nerve ending stimulation. The effect of i.p. administered somatostatin on the noxious heat threshold drop of the incised hind paw was similar to the antihyperalgesic effect of local capsacin administration into the denervated hind paw. These findings support the role of somatostain

release from peripheral nerve endings into the systemic circulation in the remote antihyperalgesic effect. As somatostatin is known to be unable to penetrate the blood brain barrier[64] the likely site of action for the antihyperalgesic effect are the somatostatin receptors located on peripheral nociceptors. Carlton and his coworkers studied the role of peripheral somatostatin receptors in counter-irritation-induced analgesia. Rats were either pretreated with s.c. administration of 0.1 % capsaicin into the tail or muzzle, followed by i.pl. administration of 2 % solution of formalin 10 minutes later, or the i.pl. administration of 2 % formalin was followed by subcutaneous (s.c.) administration of 0.1 % capsaicin solution into the tail. The nocifensive behavior, defined as time spent with lifting and licking of the treated hind paw, was registered. The effect of counter-irritation was also studied on inflammatory mechanical hyperalgesia induced by Freund's Complete Adjuvant. The results indicated that activation of nociceptors (either before or after the second noxious stimulus) in a distant body part can result in marked systemic antihyperalgesic effect, in both hyperalgesic states. Pretreatment with somatostatin receptor antagonists (1.3 mM C-SOM i.pl.) prevented the antihyperalgesic effect in case of spontaneous nocifensive behavior or mechanonociceptive threshold. The naloxone pretreatment (3 µM i.pl.) did not result in a significant decrease of hyperalgesia. The authors hypothesized that release of SOM from peripheral primary afferent terminals results from afferent input reaching to the spinal cord, then antidromic impulses in the sciatic nerve and its branches lead to release of SOM from primary afferent terminals[65]. In our experiments the denervation of the stimulated hind paw prevented neuronal impulses from primary afferents from reaching the spinal cord, suggesting that release of SOM from capsaicin sensitive nerve endings is possible without generation of antidromic impulses[65].

While studying endogenous antinociceptive mechanisms the role of endogenous opioids was inevitable to examine. Therefore in another experiment animals received a systemic pretreatment with the opioid receptor antagonist naloxone before stimulation of the denervated hind paw’s nerve endings. This pretreatment resulted in inhibition of the decrease in noxious heat threshold drop of the contralateral, incised hind paw supporting a mediator role of opioids in the remote antihyperalgesic effect. The peripherally acting naloxone methiodide did not have

any effect on the antihyperalgesic action of capsaicin, therefore a central site of action of the released endogenous opioids was confirmed. The source of the opioids cannot be determined on the basis of our results, a plausible possibility is that they are also released from the stimulated nerve endings as endomorphine is present in primary afferents[66].

The recently described endocannabinoid system is also recognized as an endogenous antinociceptive mechanism of the body. Endocannabinoids are neuromodulatory lipid products, such as arachidonoylethanolamide (anadamide), involved in processes of pain perception, appetite regulation, emotional state and learning[67]. They bind to cannabinoid G-protein coupled receptors $CB_1$ and $CB_2$, present both in the central and peripheral nervous system. The starting molecule for endocannabinoids is arachidonic acid. As pretreatment with a selective $CB_1$ cannabinoid receptor antagonist resulted in a similar decrease in the remote antihyperalgesic activity as in case of naloxone, we can conclude that endocannabinoids also contribute to the remote antihyperalgesic activity. Regarding the site of action of endogenous opioids and endocannabinoids one must consider the following. Although there is evidence for the presence of both opioid and $CB_1$ receptors on peripheral terminals of primary afferent neurons[1] a central site of action for them cannot be excluded on the basis of the present results.

According to the findings gathered with specific antagonists we can conclude that at least three mediators (somatostatin, endogenous opioids, endocanabinoids) contribute to the remote antihyperalgesic effect of stimulation of the peripheral nerve endings following acute denervation. The cellular source(s) of these mediators and their possible interconnections cannot be determined on the basis of the present results necessitating further investigations in this direction.

A plausible question was whether the remote antihyperalgesic effect is operational against non-thermal e.g. mechanical hyperalgesia. As neuropathic conditions are often accompanied by a marked mechanical hyperalgesia, we decided to examine whether chronic, neuropathic mechanical hyperalgesia elicited by partial sciatic nerve ligation (Seltzer’s operation) can be influenced by the above-described

remote antinociceptive mechanism. The denervation was carried out as previously but instead of plantar incision, partial sciatic nerve ligation was performed on the contralateral side to induce a decrease of the mechanonociceptive threshold as measured with the Randall–Selitto method. Stimulation of the denervated, right hind paw either with capsaicin i.pl. or mustard oil percutaneously resulted in a reduction of neuropathy-induced mechanical hyperalgesia. These results indicate that the discovered remote antihyperalgesic is effective not only against heat hyperalgesia of traumatic origin but against neuropathic mechanical hyperalgesia too, suggesting that it can be exploited in a variety of painful conditions including neuropathic pain.

To simulate a more physiological and clinically more relevant situation, in the last experiment we stimulated by capsaicin the nerve endings of the right hind paw without denervation. The hyperalgesic state on the contralateral side was induced by incision. To avoid unnecessary animal discomfort, only the lowest effective dose of capsaicin (producing nocifensive behavior and such as lifting and licking) was administered i.pl. The reduction in noxious heat threshold drop was similar to that found in animals with a denervated hind leg suggesting that denervation, as expected, is not a "sin equa non" of the remote antihyperalgesic effect. Nevertheless, denervation was an important experimental tool for revealing the mechanism of the phenomenon.

The experimental setup used in our study can be considered as a specialized animal model of counter-irritation, a procedure during which noxious or innocuous stimulation of one part of the body leads to an analgesic effect in other parts of the organism Counter-irritation is one of the oldest analgesic methods and is still in clinical and veterinary use[68]. Although the precise mechanism of the counter-irritation-evoked analgesia is not known, most authors explain it by a central nervous system processing of the neural impulses i.e. action potentials coming from the irritated tissue[65]. DNICs have been proposed as a possible mechanism[69]. Our present results raise the possibility that counter-irritation-evoked analgesia may be mediated by a peripheral mechanism, too, by the remote antihyperalgesic effect of nociceptor stimulation. Moreover, it can also hypothetized that a sensed irritation is not a prerequisite for the phenomenon.

Our third study has provided evidence for a conceptually novel peripheral neuroregulatory mechanism of pain. Chemical stimulation of the peripheral endings of peptidergic capsaicin-sensitive nociceptive primary afferent neurons can exert a remote, most likely systemic antinociceptive effect that is mediated by release from the stimulated nerve endings of somatostatin (and other mediator(s) which after entering the systemic circulation exerts an antinociceptive action throughout the body. The novelty of this mechanism is that peripheral nociceptors, so far thought to be developed for only detecting painful stimuli, are also a source for antinociceptive mediator(s) functioning as hormone-like agents. This mechanism is a good example for a bidirectional control of a physiological function. In addition, this sensocrine antinociceptive effect can provide alternative explanation for phenomena (counter-irritation, acupuncture) that have so far been explained predominantly by central mechanisms. Last but not least, the sensocrine antinociceptive mechanism provides potential new targets for developing novel analgesics with a peripheral site of action thereby promising fewer side effects. Not only somatostatin analogs can represent this new pathway for drug development but also stimuli and procedures that activate this endogenous antinociceptive machinery.

# 6 Novel findings

**1.** Using an increasing-temperature water bath, a sustained decrease (lasting for days) of the noxious heat threshold following incision of the plantar surface of the rat hind paw was revealed as a part of heat hyperalgesia.

**2.** In the incision model, the antihyperalgesic actions of systemically and/or locally applied morphine, diclofenac and paracetamol could be demonstrated making the paradigm suitable for preclinical testing of conventional analgesics.

**3.** Relatively small differences between peripheral mediators of heat hyperalgesia evoked by mild heat injury and plantar incision were revealed despite the different time course of heat threshold drop. $B_1$ bradykinin receptors were shown to be involved in the incision-evoked drop of heat threshold whereas an involvement of lipoxygenase products was revealed only in the heat injury evoked hyperalgesia. The $B_2$ bradykinin receptor, P2X purinoceptors and the TRPV1 receptor activation as well as NO formation were revealed in both models.

**4.** Chemical stimulation of peripheral endings of capsaicin-sensitive nociceptors in the acutely denervated rat hind paw not connected to the central nervous system can evoke a remote thermal antihyperalgesic effect manifesting itself as an inhibition of incision-induced heat threshold drop in the contralateral hind paw. This novel manifestation of counter irritation-evoked antinociception is peripherally initiated and is mediated by somatostatin, endogenous opioids and endocannabinoids acting on $CB_1$ receptors.

**5.** Chemical stimulation of peripheral endings of capsaicin-sensitive nociceptors in the acutely denervated rat hind paw can also lead to a diminishment of mechanical allodynia induced by partial sciatic nerve ligation on the contralateral side demonstrating the broad spectrum of the sensocrine antinociceptive mechanism revealed in the present studies.

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# 9 Publications related to the dissertation

## 9.1 Published papers

**Furedi R**, Bolcskei K, Szolcsanyi J, Petho G

Effects of analgesics on the plantar incision-induced drop of the noxious heat threshold measured with an increasing-temperature water bath in the rat.

EUROPEAN JOURNAL OF PHARMACOLOGY 605:(1-3) pp. 63-67. (2009)

**IF: 2.778**

Independent citations: 5, All citations: 9

**Furedi R**, Bolcskei K, Szolcsanyi J, Petho G

Comparison of the peripheral mediator background of heat injury- and plantar incision-induced drop of the noxious heat threshold in the rat.

LIFE SCIENCES 86:(7-8) pp. 244-250. (2010)

**IF: 2.704**

Independent citations: 2 All citations: 4

# 10 Publications not releated to the dissertation

## 10.1 Published papers

Varga E, Simon M, Tényi T, Schnell Z, Hajnal A, Orsi G, Dóczi T, Komoly S, Janszky J, Füredi R, Hamvas E, Fekete S, Herold R.
Irony comprehension and context processing in schizophrenia during remission - A functional MRI study.
BRAIN AND LANGUAGE;126:231-242 (2013)

**IF: 3.841**

Muhl D, **Furedi R**, Gecse K, Ghosh S, Falusi B, Bogar L, Roth E, Lantos J
Time course of platelet aggregation during thrombolytic treatment of massive pulmonary embolism.
BLOOD COAGULATION & FIBRINOLYSIS 18:(7) pp. 661-667. (2007)

**IF: 1.248**
Independent citations: 1 All citations: 2

Muhl D, **Furedi R**, Cristofari J, Ghosh S, Bogar L, Borsiczki B, Gasz B, Roth E, Lantos J
Evaluation of oxidative stress in the thrombolysis of pulmonary embolism.
JOURNAL OF THROMBOSIS AND THROMBOLYSIS 22:(3) pp. 221-228. (2006)
**IF: 1.985**
Independent citations: 3 All citations:: 7

## 10.2 Oral presentations and posters

**Füredi R**., Mühl D., Kiss T., Cristofari J., Gecse K., Rőth E., Lantos J.
Predictive role of oxidative stress in sepsis and multiple organ failure
19 th Annual Congress of European Society of Intensive Care Medicine

Barcelona, Spaim 24-27 September 2006, Intensive Care Medicine **32** Suppl:1, S12

Gecse K., Mühl D., Cristofari J., Bogár L., **Füredi R**., Lantos J.
Masszív pulmonális emblólia streptokinase és alteplase kezelésének hatására bekövetkező thrombocytaaggregáció válltozás
Magyar Kardiológusok Társasága Tudományos Ülése, Cardiologia Hungarica, 2006; Suppl:a, **36**: A37

Cristofari J., Mühl D., Gecse K., **Füredi R**., Lantos J.
Masszív pulmonális embólia streptase és alteplase kezelése és az oxidatív stressz
Magyar Kardiológusok Társasága Tudományos Ülése, Cardiologia Hungarica, 2006; Suppl:a, **36**: A33

Mühl D., **Füredi R**., Gecse K., Bogár L., Gasz B., Lantos J.
Hogyan változik a thrombocyta aggregáció és a fibrinogén szint különböző thrombolytikumok hatására pulmonalis embóliában?
M A I T T XXXIII. Nemzeti Kongresszusa és V. Duna Kongresszus, Budapest, 2005. október 13-15. Aneszteziológia és Intenzív *Terápia* **35**, 2005; Suppl: 2, 14.

**Füredi R.**, Mühl D., Lantos J., Gasz B.
A pulmonális embólia trombolítikus kezelésére adott szisztémás gyulladásos válasz a leukocita aktiváció tükrében M A I T T XXXIII. Nemzeti Kongresszusa és V. Duna Kongresszus, Budapest, 2005. október 13-15. Aneszteziológia és Intenzív Terápia **35**, 2005; Suppl: 2, 14.

D. Mühl, **R. Füredi**, K. Gecse, L. Bogár, J. Lantos
Platelet function (PF) and thrombolytics in massive pulmonary embolism (PE)
4th International Meeting, Intensive Cardiac Care Tel Aviv, Israel September 27-29, 2005

**R. Füredi**, D. Mühl, J. Cristofari, B. Gasz, J. Lantos
Leukocyte inflamation markers during reperfusion due to thrombolysis of pulmonary embolism (PE)

4th International Meeting, Intensive Cardiac Care, Tel Aviv, Israel September 27-29, 2005

Lantos J., Mühl D., Gasz B., Borsiczky B., **Füredi R.**, Bogár L., Rőth E.,
Leukocita gyulladásos markerek változása pulmonális embólia trombplítikus kezelése során
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Pulmonalis embólia trombolízisét kísérő oxidatív stressz és leukocita aktiváció vizsgálata
4.Magyar Mikrokeringési Kongresszus, Balatonkenese, 2005. 04. 1-2. Érbetegségek, 2005 (Suppl 1):

J.Lantos, B.Gasz, D.Mühl**, R.Füredi**, E.Rőth
Monitoring of oxidative stress in the thrombolytic treatment of pulmonary embolism
Mikrosympózium a Slávnostnu Pracovnú Schodzu, Bratislava, Slovak Republik, 2004.11.16

D.Mühl**, R.Füredi**, P.Szabó, L.Bogár
How does platelet function (PF) alterate during thrombolysis?
Rudolf Kucher Lecture 4$^{th}$ International Danube Symposium, Austrian Internat. Congr. (Ö G A R I), Linz, 2004. 09. 08-10., Anaesthesiology and Intensive Care news, 2004 (Suppl 2); **54**: 86

**R.Füredi**, D.Mühl**,** J.Lantos, B.Gasz, E.Rőth, L.Bogár
Pulmonary embolysm (PE), thrombolysis and oxidative stress

Rudolf Kucher Lecture 4th International Danube Symposium, Austrian Internat. Congr. (Ö G A R I), Linz, 2004. 09. 08-10., Anaesthesiology and Intensive Care news, 2004 (Suppl 2); **54**: 84

***Best of Abstracts award***

Mühl D., Lantos J., **Füredi R.**, Szabó P, Gasz B

A subtotalis pulmonalis embólia (PE) thrombolyticus kezelése és az oxidatív stressz

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