Melanocortins and Neuropathic Pain

Melanocortines en neuropathische pijn

(met een samenvatting in het Nederlands)

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Dorien Henriëtte Vrinten
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Promotoren:
Prof. dr. W.H. Gispen
Prof. dr. R.A.H. Adan
Prof. dr. C.J. Kalkman

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'Ik heb wel eens pijn in mijn snor,' zei de walrus. 'Een soort doffe pijn. Alsof mijn snor bonst. Zo'n soort pijn.'
'En ik heb soms schildpijn,' zei de schildpad. 'Vooral als ik op reis moet, 's ochtends vroeg.' Hij zweeg even. 'Het beste is om dan maar niet te gaan,' zei hij toen.
Het hert vertelde over de pijn in zijn gewei: 'Mijn hele gewei lijkt wel in brand te staan, als ik dat heb.'
De slak zei dat hij nogal eens kramp in zijn steeltjes had, en de kameel vertelde over onaangename tintelingen in zijn bulten.
Het nijlpaard zei: 'Ik heb hier pijn.' Hij deed zijn mond wijd open en wees naar binnen. Iedereen boog zich voorover om die pijn te zien, maar het was te schemerig en te ver om iets te kunnen onderscheiden.
'Dat is jammer,' zei het nijlpaard. 'Want het is wel een interessante pijn.'

(Toon Tellegen. Uit: Misschien wisten zij alles)

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Abbreviations

ACTH adrenocorticotrophic hormone

CCI chronic constriction injury

βEP β-endorphin

MC melanocortin

MC-R melanocortin receptor

μ-OR μ-opiate receptor

δ-OR δ-opiate receptor

α-MSH α-melanocyte stimulating hormone

POMC pro-opiomelanocortin

S.E.M. standard error of the mean

Definitions

The following definitions of terms for the description of neuropathic pain syndromes are used according to the task force on taxonomy of the International Association for the Study of Pain , the IASP

Allodynia: pain due to a stimulus that does not normally provoke pain.

Hyperalgesia: an increased response to a stimulus that is normally painful

Neuropathic pain: pain initiated by a primary lesion or dysfunction in the nervous system.

Nociceptor: a receptor preferentially sensitive to a noxious stimulus or to a stimulus that would become noxious if prolonged.

Noxious stimulus: a stimulus that is damaging to normal tissues.

Pain: an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage.

1 General introduction

Dorien H.Vrinten
Cor J.Kalkman
Roger A.H.Adan
Willem Hendrik Gispen

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1 Neuropathic pain: symptoms and classification

Probably the best definition of pain was formulated by the International Association for the Study of Pain: "Pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage"[1]. Acute pain, elicited by the activation of nociceptors in the skin or other tissues of the body, may function as a warning of potential or ongoing tissue damage, and thus the ability to experience pain serves to protect the organism. Typically, pain resolves as the injury heals. However, in some cases, pain can become persistent, either as a result of injury so extensive that it surpasses the healing ability of the body, or as a result of damage to the nervous system itself. The latter type of – maladaptive - pain is known as neuropathic pain, and may result from injury to the peripheral (e.g. peripheral nerves, plexus, nerve roots) or central (e.g. spinal cord, brain) nervous system.

Although the causes of nervous system injury may vary, symptoms of neuropathic pain are common and include both negative and positive symptoms. Negative symptoms consist of diminished sensitivity to stimuli (hypoalgesia and hypoesthesia). Positive symptoms include: 1) Spontaneous sensations: stimulus-independent pain, which can be continuous (often described as burning, stabbing, cutting, prickling) or paroxysmal (described as shooting, electric-like), and spontaneous abnormal sensations, such as numbness, tingling, prickling or itching feelings (dysthesias and paresthesias). 2) Evoked sensations: increased responses to painful stimuli, such as skin heating and cooling, or strong mechanical stimuli (hyperalgesia) and pain due to normally non-painful stimuli such as mild warming, cooling or touch (allodynia). These symptoms are amongst the most serious and invalidating symptoms of neuropathic pain. They can occur alone or in combination. In clinical practice, it is very difficult to differentiate between allodynia and hyperalgesia. Also, because the stimuli are painful, the patient is often difficult to examine. All symptoms share in common that their distribution pattern is consistent with the underlying neural dysfunction, that is, pain is experienced in the area innervated by a nerve root (radicular) or peripheral nerve (dermatome, glove and stocking area).

Because of its heterogeneity, neuropathic pain is difficult to classify. In gen-

eral, neuropathic pain syndromes are classified according to the aetiology and anatomical distribution of the underlying injury. In table 1 various disorders that may give rise to neuropathic pain syndrome are listed, this list being far from complete [2-4]. Recently, Dellemijn[4] defined three different groups of syndromes, based on the underlying mechanisms. 1) Nociceptive nerve pain, pain due to an inflammatory process activating the 'nervi nervorum', innervating the nerve sheath, epi- and perineurium. This type of pain always originates in the peripheral nervous system. 2) Complex regional pain syndromes and dystrophies, which may be sympathetically maintained. 3) Deafferentation pain, pain due to damage to the nervous system, without an inflammatory component, stemming from either the peripheral or central nervous system. Other terms used to define central pain are "thalamic pain" or "dysesthetic pain" [5].

Not every injury to the nervous system has the same effect, that is, not every patient with a similar nerve injury develops neuropathic pain, and currently there are no predictors which patients will be affected. Therefore, at present there is a tendency towards a more "symptom-and-sign" based approach [6-7] instead of the "cause-and-effect" classification based on aetiology. Similarly, the efficacy of drugs in different types of human pain syndromes generates another possible classification of neuropathic pain, based upon the response to different pharmacological agents[8]. For instance, mechanical allodynia, irre-

Table 1. Some disorders in which neuropathic pain may appear

Peripheral	Central
traumatic nerve injury	spinal cord injury
nerve or root compression	brain injury
plexus injury	infarction /hemorrhage
post-amputation pain	syringomyelia
herpes zoster	neoplasms
neoplasms	multiple sclerosis
ischemic neuropathy	
polyneuropathy; e.g. toxic, metabolic (e.g. diabetic), hereditary, inflammatory	

spective of its cause, may be reduced by NMDA receptor antagonists[9], whereas lancinating pain may be best treated with anti-convulsants[10]. Such an approach might not only improve our understanding of neuropathic pain, but might also give us a tool to establish the pharmacological treatment that best fits the individual patient's needs[11]. However, in the search for potential new treatment strategies, it is important to understand the pathophysiological mechanisms involved in neuropathic pain.

2 Animal models for neuropathic pain

In the investigation of human neuropathic pain it is inevitable to make use of animal models. In the past decades, numerous animal models with various lesions to the nervous system have been developed and studied. Here we give an overview of several of these models.

2.1 Central nervous system lesions

An important cause of chronic central pain syndromes is spinal cord injury (SCI). Following SCI, neuropathic pain syndromes develop in the majority of patients[12-14]. It usually presents as a burning or shooting pain and mechanical allodynia in the dermatomes at or below the level of injury. As the majority of human SCI is due to mechanical trauma, several animal models have been developed in which a mechanical injury is inflicted upon the spinal cord, parallel with the clinical situation. The spinal cord contusion model, in which the injury is inflicted by dropping a weight on the spinal cord, has been used extensively to study regeneration and return of function after SCI. Siddall and co-workers have shown that rats with a weight-drop injury develop signs of neuropathic pain, as indicated by a mechanical allodynia in the dermatomes close to the level of injury[15-16]. Similarly, hemisection of the spinal cord at the thoracic level elicited mechanical and thermal allodynia in all four limbs[17-18].

Watson and colleagues[19] developed a method to produce an ischemic spinal cord lesion by laser-irradiation of the dorsal horn after systemic injection of a photopigment, thus causing a photochemical reaction and occlusion of blood vessels in the radiated area. This type of lesion bears a resemblance to

stroke, which is an important cause of chronic central neuropathic pain in humans[20]. Indeed, animals with such a photochemically-induced injury develop a mechanical allodynia that lasted for months[21-22].

Although the mechanical and ischaemia induced SCI-models share certain characteristics with the human situation, the vast extent of tissue damage associated with these type of lesions make it difficult to study the role of specific neuronal substrates involved in the related sensory disturbances. Therefore, Yezierski and Park followed a different approach by simulating injury-induced elevations of excitatory amino acids and excitotoxic cell death[23]. After intraspinal injection of the excitatory amino acid agonist quisqualic acid (QUIS), animals acquire a hypersensitivity to thermal and mechanical stimulation. Also, increased responsiveness to peripheral input and higher background activity of spinal sensory neurons have been recognized after QUIS injection[24], indicating a central hyperexcitability. A different approach of mimicking the central sensitized state present in neuropathic pain is the pharmacological removal of inhibitory systems. Intrathecal administration of pertussis toxin (PTX) inactivates inhibitory G-proteins, thus blocking inhibitory control and leading to a predominantly excitatory state. Another means to remove inhibitory control is intrathecal application of strychnine (STR), thus blocking spinal glycinergic transmission. In rats or mice, both PTX and STR cause hyperalgesia and allodynia[25-27], indicative of a neuropathic pain state through a direct central mechanism.

2.2 Peripheral nerve lesions

2.2.1 Complete nerve damage

Originally, much insight into the mechanisms of nerve-injury induced pain has come from Wall's neuroma model, or experimental anaesthesia dolorosa[28], in which the sciatic nerve is transected and ligated. After cutting the nerve, the animals develop a tendency to bite or automutilate the affected limb, a phenomenon referred to as autotomy. Although very rare, there are some reports mentioning autotomy following deafferentation injuries in humans[29-31]. The question whether this behaviour really is an index of chronic pain, or reflects dys- or anesthesia associated with the nerve injury, has however met with considerable controversy[32-33]. By applying neurotoxins prior to a deafferenting nerve lesion, Rodin and Kruger[34] provide evidence

that autotomy merely reflects a tendency of the animal to get rid of a denervated, anaesthetic body part, rather than being a response to pain. However, Kingery and Vallin[35] report a high degree of correlation between the onset of autotomy behaviour and the development of mechanical hyperalgesia following sciatic nerve section, suggesting that the autotomy might reflect a response to pain. The observation that functional deafferentation by chronic local lidocaine application to the sciatic nerve does not induce autotomy subscribes this view[36].

Another nerve-transection or neuroma model has been described, involving the inferior alveolar nerve, which is a sensory branch of the trigeminal nerve[37]. In humans, this nerve often gets damaged, e.g. after trauma or surgery on the jaw or mouth, sometimes leading to chronic sensory disturbances indicative of neuropathic pain. Yet, in the animal model the main outcome parameters are electrophysiological measurements , making this model less manageable.

2.2.2 Partial nerve damage

To date, there are three widely used animal models of peripheral neuropathic pain, all involving partial lesioning of the rat sciatic nerve. The chronic constriction injury (CCI), described by Bennet and Xie[38] is produced by tying four loosely constrictive ligatures around the sciatic nerve. This evokes intraneural edema, thus causing the nerve to strangulate within the ligatures. The animals develop symptoms similar to those found in patients with neuropathic pain, including signs of ongoing, spontaneous pain, and abnormal responses to both noxious and non-noxious stimuli, e.g. mechanical and cold allodynia and heat and mechanical hyperalgesia. Although this model proved to be highly reproducible, attempts have been made to adapt it to a more standardized level of constriction, by means of applying polyethylene cuffs of variable diameter around the nerve[39-40]. There was, however, no correlation between the extent of axonal fiber loss and pain scores, suggesting that merely changes in the nerve's properties or its local microenvironment play a key role in the generation of pain, rather than the degree of axonal alterations.

Two similar animal models, involving partial lesioning of the sciatic nerve are the spinal nerve transsection model of Kim and Chung[41] and the partial nerve ligation model of Seltzer et al.[42] The former entails transsection (by

tight ligation) of the L5-L6 spinal nerves, close to the intervertebral foramen and in the latter a suture is inserted through the sciatic nerve and approximately ⅓ to ½ of the nerve is tightly ligated. The CCI model results in a differential axonal fiber loss, since the ligatures predominantly affect myelinated fibers. Thus most of the large myelinated Aβ and a large portion of the thinner myelinated Aδ fibers are lost, whereas a large percentage of the unmyelinated C-fibers remain intact[43-44]. In contrast, both the spinal nerve transsection and the partial nerve ligation model produce a partial, non-differential axonal loss, leaving the hind paw innervated by a reduced number of all fiber types. These three models produce similar signs of both spontaneous and stimulus-evoked pain, although the magnitude of the different components could differ between models[45].

Next to the above-mentioned models, other, less frequently used models involving partial lesioning of a peripheral nerve have been developed. Sciatic cryoneurolysis, in which the proximal sciatic nerve is subjected to a cryoprobe freeze-thaw-freeze sequence, produces autotomy, signs of spontaneous pain and touch-evoked allodynia[46]. A crush injury of the sciatic nerve has also been used to study neuropathic pain. Allodynia and hyperalgesia following this type of injury have been reported, but with very dissimilar time-courses[47-49]. Inferior caudal trunk injury[50], produced by resection of the left inferior caudal trunk between the S3 and S4 spinal nerves, leads to signs of neuropathic pain in the rats tail. An advantage of this model is the possibility of blind behavioural testing, since there is no visible difference between the tails of neuropathic and sham-operated animals, whereas models involving lesions of the sciatic nerve usually lead to deformities of the hind paw.

All these models share in common that the nerve lesion occurs in a more or less acute fashion, whereas in certain human clinical conditions, such as sciatica due to a herniated disc or stenosis of the intervertebral foramen, nerve compression takes place more gradually. By placing a gradual compresion-onset constrictor around a spinal nerve, Cornefjord and colleagues[51] have mimicked this condition in the pig. Also, by by placing a silastic tube around a dog spinal nerve root with a diameter slightly larger than that of the nerve root, histological and electrophysiological changes develop gradually, over a period of months[52].

By loosely ligating the L4 and L5 dorsal root ganglia (DRG), Chatani et al[53]

have developed a model in which the role of the DRG in sciatica can be investigated. Also chronic DRG compression produced by insertion of a small stainless steel rod into the L5 intervertebral foramen[54], may serve as a model for human low back pain and sciatica. A recent study by Omarker an Myers[55] showed that combining chronic displacement of the L4 nerve root and DRG with a herniated nucleus pulposus results in a transient decrease in thermal threshold, indicative of neuropathic pain.

An animal model resembling human post-herpetic neuralgia is created by

Table 2 . Animal models of neuropathic pain

1. central nervous system lesions	2. peripheral nervous system lesions
spinal cord contusion[15,16]	**complete nerve transsection**
spinal cord hemisection[17,18]	sciatic nerve transsection: experimental anaesthesia dolorosa[28]
photochemically induced spinal cord ischaemia[19,22]	inferior alveolar nerve transsection[37]
spinal quisqualic acid injection[23,24]	**partial nerve injury**
intrathecal administration of pertussis toxin or strychnin[25-27]	chronic constriction injury of the sciatic nerve[38]
	spinal nerve transsection[41]
	partial sciatic nerve ligation[42]
	polyethylene cuffs around sciatic nerve[39, 40]
	sciatic cryoneurolysis[46]
	sciatic nerve crush[47-49]
	inferior caudal trunk injury[50]
	gradual onset spinal nerve compression[51]
	spinal nerve root tubing[52]
	dorsal root ganglion injury
	DRG ligation[53]
	intervertebral foramen stenosis[54]
	herniated nucleus pulposus[55]
	polyneuropathy
	streptozotocin-induced diabetes[58,59]

injecting varicella-zoster virus-infected cells into the footpad of the rat's hindpaw[56], leading to allodynia and hyperalgesia in the injected, but not the contralateral paw[57].

Whereas the aforementioned models produce a mono-neuropathic pain, in humans, clinical conditions associated with polyneuropathy, e.g. diabetes or chemotherapy, can also lead to chronic pain states. Experimental animal models, giving rise to such a polyneuropathy can also produce a similar symptomatology. A widely used model to study the pathophysiology of human diabetes is streptozotocin-induced diabetes in rats. Several reports have been published demonstrating signs of hyperalgesia and allodynia associated with this condition[58-59].

All these aforementioned animal models thus have many similar features but also differences and may represent different populations of clinical patients. They serve as valuable tools in the investigation of pathophysiological mechanisms and pharmacology of human neuropathic pain. In table 2 a summary of the different animal models is presented.

3 Pathophysiology of neuropathic pain

It is beyond the scope of this chapter to give a detailed overview of the currently known mechanisms underlying neuropathic pain (for review, see Woolf and Mannion[6] and Attal and Bouhassira[60]). In summary, they include both peripheral and central systems, as described below.

3.1 Peripheral mechanisms

3.1.1 Primary afferent sensitisation and recruitment of silent nociceptors

In tissue injury, nociceptors may become sensitised, resulting in a decrease in the threshold for stimuli and an increased response to suprathreshold stimuli. The neurochemical basis for primary afferent sensitisation involves the release of inflammatory mediators such as amines, prostaglandins, leukotrienes and bradykinins[61].

In addition, there are nociceptors that normally have a very high threshold for activation (so-called "silent" or "sleeping" nociceptors[62]). These receptors

can become sensitised, and thus recruited, upon prolonged noxious stimulation, as is the case in tissue damage with the local release of inflammatory mediators, for which a number of receptors are found on peripheral nerve fibres[63].

3.1.2 Sympathetically induced discharges

An interaction between primary afferent terminals and sympathetic postganglionic efferent terminals also plays an important role. Noradrenaline released from sympathetic terminals via the activation of autoreceptors causes the production of eicosanoids, which diffuse to the sensory neuron, resulting in sensitisation[64]. Moreover, damaged sensory neurons become sensitive to catecholamines by expressing α-adrenoceptors[65]. Nerve injury also induces sprouting of sympathetic axons into the dorsal root ganglion, forming baskets around the cell bodies of primary afferents[66]. Together, this might represent a mechanism by which sympathetic activity maintains discharge in primary afferent fibres.

3.1.3 Spontaneous discharges in damaged primary afferents

Injured axons start discharging spontaneously. After axotomy, the regenerating nerve forms sprouts. In these regenerating sprouts ectopic discharges are observed. These can be purely spontaneous (due to instability of the membrane potential) or caused by occult stimuli such as circulating catecholamines or light mechanical stimulation (e.g. the beating of an nearby arteriole), because these sprouts also become more sensitive to mechanical, thermal, ionic or catecholamine stimulation[67]. An ongoing local inflammatory process could also contribute to these ectopic discharges, because local application of eicosanoids to injured primary afferents can cause C-fibre discharge[68]. These ectopic discharges are also observed in the dorsal root ganglion, where the cell bodies of the axotomized primary afferents lie[69].

3.1.4.Collateral sprouts

Neighbouring undamaged primary afferents form new sprouts that innervate denervated areas of skin. These areas of skin are shown to become hyperalgesic and allodynic to mechanical stimuli[35,70]. It is not clear whether

this is a peripheral (these newly formed sprouts can exhibit some of the abnormalities seen in neuroma sprouts) or central mechanism, or a combination of both.

Taken together, these changes result in an increased afferent barrage, which in turn can lead to hyperexcitability of dorsal horn neurons (see below)

3.2 Central mechanisms

3.2.1 Excitotoxicity

A high rate of discharge in damaged small afferent fibres can produce a state of central hyperexcitability. The abnormal discharge causes an excessive release of excitatory amino acids (glutamate, aspartate), resulting in high levels of activity of glutaminergic synapses (mediated by the NMDA type receptor)[71]. Subsequently, small-to-medium-sized neurons in laminae I-III, presumably inhibitory interneurons, undergo degenerative changes (and become so-called 'dark neurons'). This excitotoxicity results in a state of increased excitability, due to disinhibition[72,73].

3.2.2 Wind-up

Another mechanism inducing central sensitisation is the so-called "action potential wind up" or simply "wind up" in which spinal cord neurons receiving small-fibre input generate more action potentials after each successive stimulus of a pulse-volley. The key receptor in this process is the NMDA receptor[74]. There is evidence that after nerve injury, large myelinated afferents (of the Aβ type, or low-threshold mechanoreceptors) may also begin to induce such central sensitization. A switch in the Aβ fibre chemical phenotype, causing them to adopt a C fiber-like phenotype, is thought to underlie this phenomenon[75].

3.2.3 Reorganisation of dorsal horn synaptic connectivity

After peripheral nerve injury, Aβ fibers begin to sprout into lamina II of the dorsal horn, where postsynaptic targets usually receive only small afferent fibre (nociceptor) input[76]. This reorganisation may contribute to the touch-evoked pain that can follow nerve injury, because an area that normally only receives noxious information now obtains input from non-noxious tactile

stimuli. The misinterpretation of this information by the nervous system as noxious input thus provides an anatomical basis for mechanical allodynia.

3.2.4 Spontaneous discharge and altered thresholds

Spinal neurons that connect with axotomised primary afferents (thus lacking input from the periphery) often discharge spontaneously. Cells receiving input from uninjured neighbouring axons may show abnormal responses and lowered thresholds. Similar abnormal responses and altered thresholds are also observed at higher levels, such as in spinothalamic tract neurons and in the thalamus[77-79]. It thus appears that central homeostatic mechanisms detect the failure of input and increase the excitability of central cells in an attempt to compensate for the diminished input (for review, see Wall[80]).

3.2.5 Neuropeptide plasticity

In addition to the above-mentioned changes, the levels of several neuropeptide genes and their products are regulated in response to nerve injury. This neuropeptide plasticity can also contribute to the altered transmission of sensory information observed in neuropathic pain, because neuropeptides modulate neuronal activity in conjunction with the neurotransmitter with which they are colocalized. Thus by acting as neuromodulators they fine-tune the direct communication between neurons. A main event in neuropeptide plasticity is the so-called phenotypic switch of primary afferents, as reflected by an altered expression of neuropeptides, which is often accompanied by changes in their receptor levels in the dorsal horn (reviewed in Hokfelt et al.[81]).

Substance P and calcitonin-gene-related peptide (CGRP) are normally expressed by thin primary afferents conveying information from nociceptors. CGRP in sensory primary afferent neurons has an excitatory effect on postsynaptic neurons and potentiates the effect of substance P in the rat spinal dorsal horn[82]. After peripheral nerve injury, the levels of these peptides are markedly decreased in primary afferents and in the dorsal horn[83-85]. Thus the two main excitatory peptides involved in the transmission of nociceptive information to the dorsal horn are down-regulated in a neuropathic state. In adjacent intact nerves, however, CGRP is upregulated, which could contribute to the hyperexcitability of dorsal horn neurons[86]. Morever, a subpop-

ulation of dorsal root ganglion neurons associated with large myelinated fibres starts to synthesise these peptides[82,87].

Different response patterns have been described for a group of peptides that are normally almost non-detectable in small-diameter primary afferents. After nerve injury there is a marked increase in the level of galanin, in both injured and spared dorsal root ganglion neurons[88-90]. Galanin has a predominantly inhibitory effect[91] and reduces the activity of wide-dynamic range neurons[92]. However, galanin is also reported to be involved in the facilitation of nociceptive transmission[93] and increases the release of substance P evoked by stimulation[94]. Vasoactive intestinal polypeptide (VIP) and pituitary adenylate cyclase-activating peptide (PACAP) are also up-regulated after peripheral nerve injury[83-95]. Studies of nociceptive responses have revealed an excitatory role for VIP [96,97], and it has been suggested that this peptide can take over the role of substance P, which is down-regulated in nerve injury, and thus maintain nociceptive transmission in the dorsal horn[98]. PACAP, which belongs to the same peptide superfamily as VIP[95], has an excitatory effect on spinal cord function as well[99,100], similar to that of the above-mentioned peptides.

This list of neuropeptides that undergo up-regulation or down-regulation after nerve injury is far from complete; e.g. alterations in neuropeptide Y [101,102], cholecystokinin [103,104], and somatostatin[105] have been reported as well. These changes in the levels of neuropeptide messengers reflect a complex adaptive response of the organism to neuropathic conditions, and might contribute to the hyperalgesia seen in patients with neuropathic pain.

Another group of neuropeptides not mentioned until now is the melanocortins. These peptides might too play a role in nociception, as explained below.

4 The melanocortin system in pain

The melanocortins comprise a group of natural peptides all derived from the precursor molecule pro-opiomelanocortin (POMC), and various synthetically derived related peptides. In the anterior lobe of the pituitary gland POMC is processed to form the melanocortin adrenocorticotropic hor-

mone (ACTH), the effects of which on the adrenal gland have been long known. POMC is also expressed in the pituitary intermediate lobe, where it is processed to form the melanocortins ACTH, β-MSH and α-MSH, a peptide which plays an important role in skin pigmentation. However, aside from these effects on peripheral tissues, direct effects of melanocortins on the nervous system have been described as early as the late 1950's [106,107]. Demonstration of immunoreactivity for α-MSH[108] and ACTH[109], as well as the expression of POMC in the brain[110] indicated that the nervous system has its own melanocortin system, distinct from that in the pituitary. Since then, a wide variety of effects of melanocortin has been described, including effects on inflammation, fever, nerve regeneration, grooming, social behaviour and regulation of body weight (for review see Adan[111], de Wied[112]).

An additional intriguing effect of the melanocortin system is its functional interaction with the opiate system, as suggested by several lines of evidence. Electrophysiological studies have demonstrated that melanocortins can block morphine-induced depression of evoked potentials in frog and cat nervous tissue[113,114]. Indications that melanocortins interfere with the effects of opiates also come from pharmacological studies. Intracerebroventricular (i.c.v.) or peripheral administration of melanocortins, prior to or simultaneously with morphine, attenuates the analgesia induced by morphine[115-118]. Repeated administration of morphine leads to a reduction of its analgesic potency, and an apparent increase in the potency of naloxone to block the effects of morphine. The development of this opiate tolerance can be inhibited by melanocortins[118,119]. Also, melanocortins can attenuate the acquisition of heroin self-administration[120], and counteract opiate addiction (for review see Alvaro et al.[121]), as demonstrated by the induction of withdrawal-like symptoms in morphine-dependent[122] and drug-naïve[123] rats.

Apart from their interactions with the opiate system, melanocortins are also known to have direct effects on nociception. I.c.v. administration of 0.1-10 μg α-MSH in rats induced hyperalgesia in the tail-flick test, an effect lasting for 20 (0.1-1 μg) to 80 (10 μg) min[124]. Similar results were obtained with 20-50 μg i.c.v. administered ACTH, which induced hyperalgesia in the hot-plate and tail-shock tests in the rat, an effect which lasted for 80 min[125]. In the hedgehog, high doses of peripherally administered ACTH also produced hyperalgesia[126]. Much lower doses of ACTH (0.5 and 1.0 μg) also caused

hyperalgesia, as indicated by decreases in ear withdrawal latency from heat in the rabbit[127], although a similar dose (1.0 μg) had no effect on tail-flick latency in the rat[117]. Also i.c.v. α-MSH (0.25-2.0 μg) had no effect on ear withdrawal latency in the rabbit[127]. A few reports claimed that melanocortins can also produce hypoalgesia. Ohkubo and colleges showed that i.c.v. administration of 0.1-10 μg α-MSH in mice induced analgesia in the hot-plate test, an effect lasting 20 min[128]. Microinjection of α-MSH in the periaqueductal grey matter also significantly reduced responsiveness to pain[129].

In spite of the growing list of the biological actions of melanocortins, the molecular mechanisms underlying these effects were largely unknown. Only since the 1980s have brain binding sites for the melanocortins been demonstrated[130,131]. Soon thereafter, the first melanocortin receptors were cloned[132,133], which started a new era in the field of melanocortin research. So far, five melanocortin receptor subtypes have been identified, all members of the G-protein-coupled receptor superfamily (for review see Cone[134],Tatro[135]). Of these five subtypes, the melanocortin MC_3 and melanocortin MC_4 receptors are expressed in the nervous system. The melanocortin MC_3 receptor has a limited distribution in the brain, and is found mainly in the hypothalamus, thalamus, brainstem and cortex. Compared to the melanocortin MC_3 receptor, the melanocortin MC_4 receptor has a much more widespread distribution in virtually every region of the brain[136-140]. Moreover, it is the only subtype to be expressed in the spinal cord[138]. Binding of a radioactively labelled α-MSH analogue to rat spinal cord demonstrated that melanocortin MC_4 receptors are expressed most abundantly in the superficial dorsal horn (lamina I and II) and in the grey matter surrounding the central canal (lamina X), areas that are important in nociceptive transmission[141]. In addition, mRNA encoding for POMC has been demonstrated in spinal cord[141,142] and the nucleus tractus solitarius has been suggested as a possible source of POMC projections to the spinal cord[143]. Interestingly, electrical stimulation of the nucleus tractus solitarius has been shown to produce pronounced analgesia[144], suggesting that the POMC system may play a role in modulating nociceptive transmission.

Immunoreactivity for the POMC-derived peptides ACTH and α-MSH has been detected in the dorsal horn and lamina X[142,143]. Together, the presence

of melanocortin MC_4 receptors, mRNA and cleavage products of POMC strongly suggest the presence of a functional melanocortin system in the rat spinal cord. The expression of the melanocortin MC_4 receptor in nociception-associated areas in the spinal cord suggests that this spinal melanocortin system might play a role in pain, and that the melanocortin MC_4 receptor might be a potential target in the ongoing search for new analgesics.

None of the natural melanocortin peptides show distinct selectivity for one of the melanocortin receptors, although γ-MSH is relatively more selective for the melanocortin MC_3 than the melanocortin MC_4 receptor[145]. The synthetic peptide [Nle^4,D-Phe^7]α-MSH is a very powerful agonist for all melanocortin-receptor subtypes and can be used as a radioligand when labelled with iodine. Another strong synthetic melanocortin receptor agonist is cyclo-[Nle^4,Asp^5,D-Phe^7,Lys^{10}]α-MSH (melanotan II). A ligand with a significant higher affinity and potency at the melanocortin MC_4 receptor than at the melanocortin MC_3 receptor is cyclo-[Nle^4,Asp^5,D-Tyr^7,Lys^{10}]α-MSH (D-Tyr-melanotan II). In contrast, Ac-[Nle^3]γ_2-MSH (Nle-γ-MSH) is a ligand with higher selectivity for the melanocortin MC_3 receptor[146].

A derivative of melanotan II, cyclo-[Nle^4,Asp^5,D-Nal(2)^7,Lys^{10}]α-MSH (SHU9119), has been shown to be a potent competitive melanocortin receptor antagonist[147]. With the availability of these ligands, it became possible to investigate in further detail the role of the melanocortin MC_4 receptor in various melanocortin actions.

5 Aims and outline of the thesis

As summarized in the previous paragraphs, much progression has been made in the understanding of factors precipitating neuropathic pain. However, despite extensive clinical and experimental research, we are still limited in our ability to successfully treat patients suffering from this condition. Further research directed towards a better understanding of the pathophysiological mechanisms underlying this condition is warranted, and will hopefully lead to new treatment strategies for neuropathic pain. The main objectives of the studies presented in this thesis are to investigate whether the MC system is

involved in neuropathic pain and how MCs can modulate neuropathic pain symptoms.

Much of our insight into the mechanisms of neuropathic pain has come from studies employing various animal models. The most commonly used models include spinal nerve transsection[41], partial ligation of the sciatic nerve[42] and a chronic constriction injury (CCI) of the sciatic nerve[38] (see also table 2).

In the experiments described in this thesis the CCI model is used. In chapter 2 we extensively characterize this model with respect to the time course of different motor and sensory disturbances. We studied the same outcome parameters after a crush injury to the sciatic nerve, a model often used to study nerve regeneration. We demonstrate that after a sciatic nerve crush neuropathic pain symptoms, comparable to those in the CCI model, develop. Amongst these symptoms is an exaggerated response to light mechanical stimuli, referred to as mechanical allodynia. Assessment of mechanical allodynia is usually performed by means of von Frey filaments. In chapter 3 we present a study describing an alternative way of assessing mechanical allodynia, by CatWalk automated gait analysis. We demonstrate a high degree of correlation between different CatWalk parameters and withdrawal thresholds to von Frey stimulation.

As outlined in the previous section, melanocortins have been demonstrated to play a role in nociception and to functionally interact with the opiate system. In chapter 4 and 5 we investigate the involvement of the spinal MC system in neuropathic pain. We therefore administered different MC-R agonists and antagonists to CCI rats, both in an acute and chronic administration paradigm. Based upon the results of these studies we conclude that the spinal MC system undergoes plastic changes in neuropathic pain and that antagonism of the MC_4-R can reduce the associated cold and mechanical allodynia. We hypothesize that these effects are explained by an interaction between the opiate and MC system, whereby blockade of the MC system reveals the anti-allodynic effects of tonic endogenous opiate receptor activation.

In chapter 6 we studied such a possible interaction between the MC and opiate system at the spinal level. The main findings and implications of these studies are summarized and discussed in chapter 7.

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2

A comparison of two sciatic nerve lesions producing neuropathic pain in the rat: The chronic constriction and crush injury

Dorien H. Vrinten
Roger A.H. Adan
Jan H. Brakkee
Willem-Hendrik Gispen

Abstract

This study provides an extensive comparison of the motor and sensory disturbances following two types of lesion of the sciatic nerve: the chronic constriction injury (CCI), which serves as a model for neuropathic pain, and the crush injury, a model widely used to study nerve regeneration.

Initially, there was a severe loss of motor function with both lesions, as measured by the sciatic function index, toe spreading and open field locomotor behaviour. In the CCI group, motor function remained impaired for over 70-80 days, whereas in the crush group, motor locomotion gradually recovered within the first 28-35 days post-injury.

CCI rats developed signs of hyperalgesia and allodynia to cold, mechanical and chemical stimulation already within a few days after lesioning, whereas in the crush group there were no signs of sensory abnormalities at this time. However, after 29-35 days, they developed sensory abnormalities similar to those in the CCI group, this onset coinciding with the return of sciatic motor function. This suggests that, analogous to the CCI lesion, a neuropathic pain syndrome develops after sciatic nerve crush, which can only be detected after regeneration and re-establishment of a functional contact between the periphery and the central nervous system, allowing the animal to perceive stimuli applied to the hindpaw.

The above data suggest that the sciatic nerve crush might provide an additional useful tool in the study of neuropathic pain.

Introduction

Following a peripheral nerve injury, in humans sometimes a painful syndrome develops. This neuropathic pain is characterised by spontaneous pain in combination with allodynia and hyperalgesia. A widely used animal model to study this condition is the chronic constriction injury (CCI)[1], in which 4 ligatures are loosely tied around the sciatic nerve. The model induces severe sensory abnormalities, closely mimicking the clinical symptoms of neuropathic pain, as well as motor deficits[1,2]. The pathogenic mechanisms underlying neuropathic pain in the CCI model have been studied extensively, and comprise dysfunctions both in the peripheral as well as in the central nervous system[3]. Histologically, associated with this lesion is an extensive axonal loss, predominantly of the Aβ-type and , to a lesser extent, the Aδ type[4,5]. Thus the periphery is innervated by C fibers and a largely reduced number of Aδ fibers[6].

Another experimental nerve lesion producing extensive fibre loss is the sciatic nerve crush[7-9]. As with the CCI lesion, the surrounding nerve sheath is not disrupted, thus allowing guidance of the outgrowing axons during regeneration and reconnection of distal targets. The sciatic nerve crush is a much used animal model to study nerve regeneration, of which the extent and speed of regeneration is usually defined by the return of sensory and motor function[10,11]. However, only few studies using the crush model specify the different sensory modalities or focus on possible pain-related behaviours associated with the lesion[12-14].

The similarities between the CCI and crush model raised the question to what extent the crush lesion produces symptoms of neuropathic pain, as seen in the CCI model. Here we systematically compare the two models with regard to the temporal correspondence between motor function recovery and changes in thresholds to different painful and non-painful stimuli.

Materials and methods

All procedures in this study were performed according to the Ethical Guidelines of the International Association for the Study of Pain[15] and approved of by the Ethics Committee on Animal Experiments of Utrecht University.

Animals

Fifty male Wistar rats weighing 250-300 g at the start of the study were used. Animals were housed in groups of 2-3 in plastic cages on sawdust bedding. They were kept at a 12/12hr light/dark cycle, with food and water available ad libitum. Animals were randomly divided into 4 groups (N=12-13).

Surgery

Animals were anaesthetised with a single subcutaneous injection of Hypnorm (Janssen Pharmaceutical Ltd., Beerse, Belgium), containing 0.315 mg/mL fentanyl citrate and 10 mg/mL fluanisone, diluted in saline (1:2, 0.3 ml/100 g bodyweight).

In 38 animals the right sciatic nerve was exposed at mid-thigh level by blunt dissection. Hereafter, in one group of animals (N=13) the nerve was chronically constricted by placing 4 loose ligatures of 4-0 chromic catgut around the nerve, as previously described by Bennet and Xie1 (CCI-group). In the second group (N=13) the nerve was crushed by tightly squeezing it with a forceps for 30 seconds (the distance between the lesion site and the mid-plantar skin was 7.5 cm), as described by de Koning et al10 (crush-group). In the third group (N=12) the nerve was exposed for 5 minutes, without ligating or crushing it (sham-group).

Subsequently the incision was closed with silk sutures and the animals were allowed to recover for a 2-3 day period. A group of animals (N=12) which underwent no surgery was also included (control group).

General observations

As a measure of general health, bodyweight was monitored twice weekly for the first 5 weeks, and weekly thereafter. Autotomy was scored as follows: 0; no autotomy, 1; autotomy of one or more toenails, >1; autotomy of one or more toes (adapted from Wall et al.[16]). Animals showing signs of autotomy were closely monitored and excluded from testing in case of severe damage to the paw.

Test procedures

Testing started at post-operative day (p.o.d.) 3 and continued until p.o.d. 84. The following tests were performed:

1 Locomotor testing

1.1 Sciatic Function Index

In order to quantify disturbances in walking patterns we used the Sciatic Function Index (SFI). In short, after dipping their hind paws in developing fluid we let the animals walk over a strip of photographic paper, thus creating black footprints on the paper. Four measurements were taken from both the normal (N) and the experimental (E) paw: IT (distance between the 2nd and 4th toe), TS (distance between the outer toes), PL (foot print length) and TOF (distance to opposite foot). The SFI was calculated as follows:

$$SFI = (EIT\text{-}NIT/NIT) + (ETS\text{-}NTS/NTS) + (NPL\text{-}EPL/EPL) + (ETOF\text{-}NTOF/NTOF) \star 55$$

A score of 0 ± 10 indicates a normal function of the sciatic nerve, whereas a score of -100 or less indicates a complete loss of function (for further details, see de Medinacelli et al.[17]).

1.2 Open field locomotor performance

To analyse locomotor recovery animals were observed in an open field. We used a scoring system in which individual symptoms were scored together with their frequency of occurrence. Sets of these symptoms were ranked according to severity of disability, analogous to the Basso, Beattie, Bresnahan (BBB) locomotor rating scale[18]. Possible scores ranged from 0 (paralytic dragging of the operated hind limb) to 14 (normal gait) (see Table 1).

1.3 Toe spreading

To quantify the extent of spontaneous toe spreading the animals were held in a vertical position that causes them to flex the hind paw and spread their toes. The difference in distance between the outer toes from the right (experimental) foot was measured in mm, as described by Bijlsma et al.[19]

2 Sensory testing

2.1 Cold stimulation test

Withdrawal latency to a cold stimulus was measured by immersing the hind paws into a 4.5°C or a 10°C water bath, as previously described[20]. Upon immersion of the paw an electronic circuit including a clock was closed. Withdrawal of the paw resulted in a discontinuation of the circuit, which

Table 1. Scoring of open field locomotor performance

Status of operated hind paw	Score
Paralytic dragging	1
Plantar placement **without** weight support	2
Plantar placement **with** weight support	3
Occasional* plantar stepping **and** frequent* *or* continuous* heel placement	4
Occasional plantar stepping **and** occasional heel placement	5
Frequent plantar stepping **and** frequent *or* continuous heel placement	6
Frequent plantar stepping **and** occasional heel placement	7
Continuous plantar stepping **and** frequent *or* continuous heel placement	8
Continuous plantar stepping **and** occasional heel placement	9
Toe clearance **and** continuous plantar stepping **without** heel placement	10
Parallel placement *or* toe spreading **and** toe clearance **and** continuous plantar stepping **without** heel placement	11
Parallel placement **and** toe spreading **and** toe clearance **and** continuous plantar stepping **without** heel placement	12
Heel of the ground in stance **and** parallel placement **and** toe spreading **and** toe clearance **and** continuous plantar stepping **without** heel placement	13

* occasional: < 50%, frequent: 51-94%, continuous: ≥95% of the observation time

stopped the clock, thus allowing a careful registration of the withdrawal latency. Cut-off time was set at 10 sec. Interval time between consecutive tests was at least 10 min. to allow restoration of original foot temperature.

2.2 Mechanical stimulation test

Foot withdrawal threshold in response to a mechanical stimulus was determined using a series of von Frey filaments (Stoelting, Wood Dale, IL), ranging from 1.12 to 52.15 g. Animals were placed in a plastic cage with a metal mesh floor, allowing them to move freely. They were allowed to acclimatise

to this environment prior to the experiment. Von Frey filaments were applied to the mid-plantar surface of the both feet through the mesh floor. Each probe was applied to the foot until it just bent, and kept in this position for 6-8 s[21]. The smallest filament that elicited a foot withdrawal response was considered the threshold stimulus.

2.3 Chemical stimulation test

A 5 µl drop of 50% mustard oil (n-allyl-isothiocyanate (Sigma) in ethanol) with 0.1% methylene blue added to visualise spreading was applied to the lateral dorsum of the right hind paw, as described by Bennet and Xie[1]. The animal was then placed in an open field and observed for two minutes. During this time the total number of the following responses to the mustardoil was recorded: shaking the paw, tapping it on the floor and licking the paw.

Statistical analysis

All data are plotted as mean ± standard error of the mean (S.E.M.), for visualisation purposes.

Differences in bodyweight, SFI, toe spreading and number of responses to mustardoil application were analysed using a repeated measures analysis of variance. For post-hoc analysis of group differences a Students t-test (SFI) or Student-Newman-Keuls test (other) was performed.

All other data were analysed using non-parametric tests. For the von Frey stimulation absolute applied forces in g are plotted. In order to obtain a linear scale of applied force the logarithm of the applied force was calculated and statistical analysis was performed on the transformed data. Overall group differences in von Frey and temperature stimulation tests were analysed by a Kruskall Wallis test. For analysis of differences in open field locomotor performance scores and mechanical or cold withdrawal responses, a Mann-Whitney U test was performed, with Bonferroni-correction when necessary. Results were considered significant when $p<0.05$.

For open field locomotor performance scores 95% confidence intervals (CI) were calculated and scores were considered significantly different from maximum possible score if 14 was not within the 95% CI.

Results

Exclusion of animals

One sham animal died during anesthesia, thus rendering a sham-group of N=11. On p.o.d. 17 one animal in the crush-group was excluded due to severe autotomy and inflammation of the right hindpaw. On p.o.d. 51, five animals of each group were sacrificed for purpose of histology (manuscript in preparation).

General observations

Bodyweight gradually increased throughout the study. There were no significant differences in bodyweight between groups at any time point.
Autotomy of the affected hindpaw was observed in both the crush and CCI group. The time of onset as well as the total number of animals showing autotomy differed between the two groups, with the crush group showing earlier and more autotomy. In this group the first signs were seen already at p.o.d. 7 and the number of animals displaying autotomy reached a maximum at p.o.d. 14 (8 out of 13 animals). In the CCI group onset was at p.o.d. 14 and the maximum (5 out of 13 animals) was reached only at p.o.d. 31 (fig. 1). Overall, autotomy in both groups was mild. Only a few animals reached a score of >1, two in the CCI group and two in the crush group, of which one was sacrificed because of the severity of autotomy.

1 Locomotor testing

There were no significant differences between control and sham animals at any time point in any of the locomotor tests.

1.1 Sciatic Function Index

Already from the first testing day after surgery (p.o.d. 3) SFI in the CCI group was significantly lower than in the crush group (-101.0 ± 2.3 vs. -93.4 ± 1.4, respectively) and remained lower until p.o.d. 73. In contrast to the CCI group, recovery in the crush group was much faster and already from p.o.d. 30 SFI in the crush-group was within normal limits (fig. 2A).

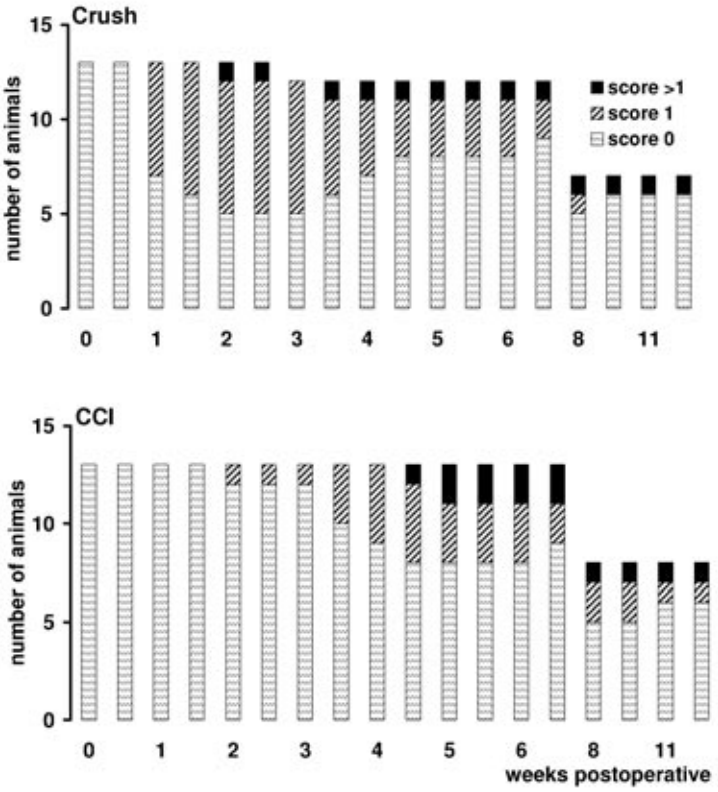

Figure 1.
The onset and extent of autotomy behaviour after crush or CCI of the scia-tic nerve. The total number of animals showing different levels of autotomy is depicted. Autotomy scores are adapted from Wall et al [16].

1.2 Open field locomotor performance

P.o.d. 7 was the first day the animals' locomotor performance was observed in an open field. Locomotor performance scores in the CCI and crush groups were decreased to 4.6 ± 0.6 and 7.2 ± 0.6, respectively. Starting at p.o.d. 35 crush scores did no longer significantly differ from maximum score (14). CCI values remained significantly lower until p.o.d. 70 (fig. 2B)

1.3 Toe spreading

Toe spreading in the control and sham animals displayed a gradual increase during the first 10 days, due to growth of the animals. At p.o.d. 3 toe spreading in CCI and crush group was decreased to 13.2 ± 0.3 and 14.2 ± 0.3 mm, respectively. After p.o.d. 17 toe spreading in the crush group rapidly increased and from p.o.d. 28 no longer differed from control values. In contrast, in the CCI group, toe spreading remained significantly smaller until p.o.d. 63 (fig. 2C).

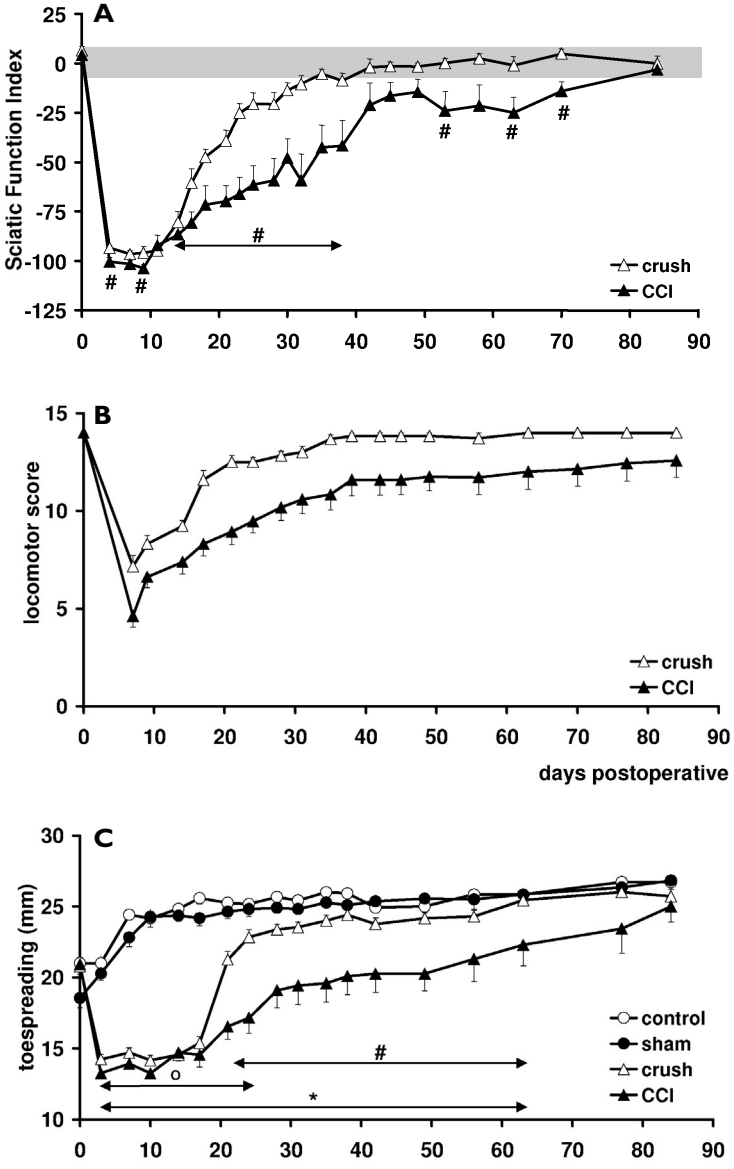

Figure 2. Locomotor testing

(A) Sciatic Function Index. The grey area indicates the range of SFI in normal rats (0 ± 10). (for details, see de Medinacelli et al.,17). (B) Open field locomotor performance scores (see Table 1). Maximum possible score was 14, indicated by the dotted line. (C) Toe spreading. The distance between the outer toes of the experimental hindpaw. Measurements were taken in rats held in vertical position and actively spreading their toes.

Locomotor recovery in the crush-group took place between post-operative days 28 (toe spreading) and 35 (open field). In the CCI group functional recovery occurred later and scores remained below control level until p.o.d. 63 (toe spreading) through 73 (SFI).

Data represent mean ± S.E.M. of 12 (control), 11 (sham) or 13 (crush and CCI) rats each. (° $p < 0.05$ crush vs. sham; * $p < 0.05$ CCI vs. sham, # $p < 0.05$ CCI vs. crush).

2 Sensory testing

There were no significant differences between control and sham animals at any time point in any of the sensory tests. Also there were no differences in withdrawal latencies of the left, unoperated hind paw from a mechanical or cold stimulus between groups at any time point.

2.1 Temperature stimulation test

Already from the first testing day after surgery (p.o.d. 4) CCI animals developed a cold allodynia, as indicated by a significant decrease in withdrawal latency to a 4.5 °C stimulus (3.2 ± 1.0 s, cut-off value was 10 sec). Withdrawal latencies in this group remained lower when compared to sham values until p.o.d. 77. Starting at p.o.d. 29 crush animals also developed a significant cold-allodynia. Withdrawal latencies decreased to a minimum of 2.4 ± 1.1 s and gradually returned to normal level towards the end of the testing period (fig. 3A).
Similar results were obtained when temperature was increased to 10 °C (data not shown).

2.2 Mechanical stimulation test

Mechanical allodynia developed in the CCI group, as demonstrated by a large decrease in withdrawal threshold to von Frey stimulation starting at p.o.d. 8 and reaching a minimum of 10.9 ± 1.8 g. Also in the crush group a mechanical allodynia developed. Thresholds were significantly lower than sham values starting at p.o.d. 29 and reached a minimum of 12.8 ± 1.6 g.
In both CCI and crush animals the mechanical allodynia gradually resolved over the 12 week postoperative period (fig. 3B).

2.3 Chemical stimulation test

Both sham and control groups showed a small, gradual increase in the number of responses during the first 2 weeks. No mustardoil was applied between p.o.d. 49 and 77 and hereafter the number of responses was again reduced to initial levels in these groups. Already at p.o.d. 3, the first testing day after surgery, CCI animals exhibited significantly more behavioural responses upon application of mustardoil as compared to the sham group. Also in the crush group a hyperalgesia developed. The total number of

responses in this group was significantly increased at a single time point (p.o.d. 17) and thereafter from p.o.d. 35 until p.o.d. 49. From p.o.d.77 on, the number of responses had normalised in both CCI and crush animals (fig. 3C).

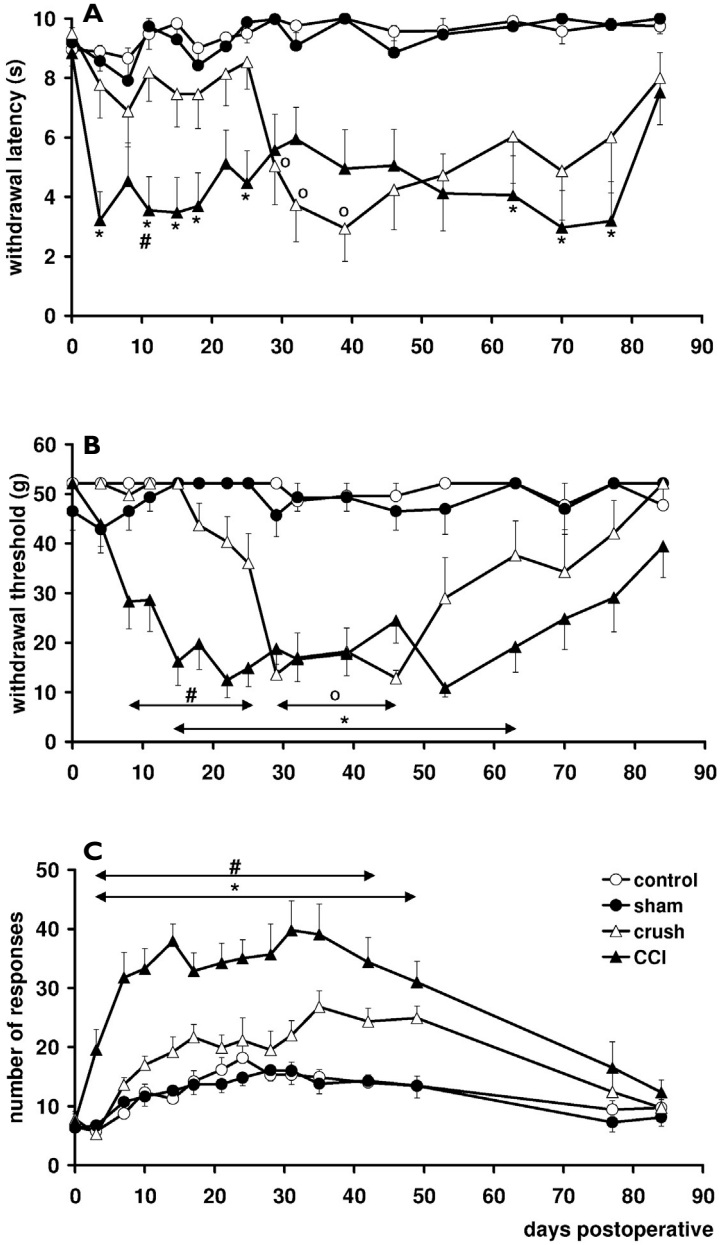

Figure 3.
Sensory testing
(A) Cold stimulation. Withdrawal latencies of the experimental hindpaw upon immersion in a 4.5 °C waterbath. Cut-off latency was 10 s. (B) Mechanical stimulation. Withdrawal thresholds of the experimental hindpaw to von Frey filaments (ranging from 1.12 to 52.15 g). (C) Chemical stimulation. The total number of behavioural responses upon application of 5 ml of 50% mustardoil to the dorsum of the experimental hindpaw was determined. Responses consisted of shaking, licking and tapping the paw and were counted during two minutes.
CCI rats showed cold and mechanical allodynia and chemical hyperalgesia already from the first testing day after lesioning. Animals in the crush group developed similar sensory abnormalities between p.o.d. 29 (cold and mechanical stimulation) and 35 (mustardoil). Data represent mean ± S.E.M. of 12 (control), 11 (sham) or 13 (crush and CCI) rats each (° $p < 0.05$ crush vs. sham; * $p < 0.05$ CCI vs. sham, # $p < 0.05$ CCI vs. crush).

Discussion

In the study presented here we describe the time course of different sensory and locomotor functions, as well as the development of a neuropathic pain syndrome, after a crush injury to the sciatic nerve.

At the first experimental day after surgery, the SFI in crush rats had decreased to approximately –95%, indicating that an effective nerve injury had been accomplished[22]. During regeneration, return of function is reflected in an increase in SFI towards 0%[10,23]. Toe spreading and the open field locomotor performance test (which contains an evaluation of toe spreading with the higher scores) both include an assessment of the function of the interosseus muscles, innervated by distal branches of the sciatic nerve. Normalization of these functional tests thus indicates a successful regeneration of the most distal parts of the sciatic nerve[19,24]. In our rats, normalisation of these locomotor function tests occurred between postoperative days 28 and 35, which is in good agreement with previous studies[22,23,25,26]. Since in the present study autotomy was limited and usually confined to biting off one ore more toenails without damage to the skin or flesh (except in one animal which was sacrificed for this reason), it had no influence on the outcome of the functional tests we used.

Upon a sciatic crush injury a profound sensory loss occurs, as has been demonstrated by using electrical stimulation of the foot sole[10,23], or mechanical or thermal stimulation[26,27]. In the present study there were no differences in responses to cold and mechanical stimulation between control, sham and crush rats, early after surgery. Most animals in these groups did not respond to the highest filament tested (52.15 g) or demonstrate a positive withdrawal upon cold stimulation within 10 s (cut-off value). Thus, we were unable to detect a decreased sensitivity to these stimuli in crush animals compared to controls. Since a positive response consists of a flexion of the limb, away from the stimulus, an absence of withdrawal in crush animals cannot be subscribed to the severe motor damage associated with the lesion, as the femoral nerve which is needed for hip flexion is still intact. There were also no significant differences in the number of responses to mustard oil application between crush and control rats early after surgery. The fact that the number of responses was not lower in the crush group compared to control values,

as would be expected with a complete lesion of the sciatic nerve, might be explained by a spread of the oil to skin areas innervated by the adjacent saphenous nerve. Also, mustard oil has a very strong smell which could cause the animal to shake and lick its paw in an attemp to get rid of the oil. In contrast to early after surgery, the crush animals demonstrated increased responses to all sensory tests after postoperative day 29, temporally corresponding with the restoration of locomotor function.

Through analysis of the return of positive reactions to electrical stimulation of the sole of the foor after a sciatic nerve crush, de Koning et al.[10] estimated axonal growth to be 2.8-2.9 mm/day. By using immunohistochemical methods, Verdu and Navarro[9] have estimated similar regeneration rates, for both sensory and alphamotor fibers They reported an interval of about 2-3 days between histological signs of reinnervation of target sites and the onset of functional responses, due to the formation of functional end-organs such as Meissner corpuscles and neuromuscular junctions, this interval also being similar for different nerve fiber types. Our data correspond well with these findings, since the distance that the regenerating nerve had to span was 7.5 cm, which would take about 27 days. This indicates that the sensory abnormalities we observed in animals with a crush lesion occur after regeneration of the sciatic nerve and re-establishment of a functional contact between the periphery and the central nervous system. Although few studies have demonstrated nociceptive behavior after a crush injury[12-14], the disorders they described are moderate and short-lasting, and occurred within the first week after lesioning of the nerve. A possible explanation for this might be an incomplete lesion of the sciatic nerve, thus still allowing stimuli applied to the nerves peripheral territory being perceived by the animal. In contrast, we show that only late after crush, animals develop severe and long-lasting allodynia and hyperalgesia to cold, mechanical and chemical stimuli. A recent study by Bester et al.[27] reports similar pain-related behaviors late after crush. Here we compare the time course and magnitude of these sensory abnormalities to those observed in a generally accepted model for neuropathic pain, the chronic constriction injury of the sciatic nerve[1].

Immediately after surgery, the magnitude of motor function loss was comparable in crush and CCI animals as demonstrated by similar SFI and toe spreading scores (testing for open field locomotor behaviour was started only

at postoperative day 7). In contrast to the crush animals, in CCI animals signs of allodynia and hyperalgesia were already present at this time, consistent with previous reports[1,28]. These responses are mediated through surviving fibers, that are predominantly of the unmyelinated and small myelinated class[29,30].

To date, many neurobiological mechanisms which may contribute to the pathogenesis of neuropathic pain have been identified, including changes in both the peripheral and central nervous system[3,31,32]. One of the pathological changes following nerve injury is an alteration in the level of several neuropeptide genes and their products involved in pain processing. These changes include increases in the level of galanin (GAL), vasoactive intestinal polypeptide (VIP) and neuropeptide Y (NPY), and decreases in substance P (sP) and calcitonin gene-related peptide (CGRP) levels in DRG neurons[33,34]. Many of these plastic changes, which can contribute to to the altered sensory processing present in neuropathic pain, also occur following nerve crush, such as increased NPY, VIP and GAL and decreased sP levels[35]. There is also a considerable degree of reorganization of synaptic connectivity in response to nerve injury. Sprouting of large myelinated fibers into lamina II of the dorsal horn, an area that normally receives only small fiber input, has been described both in sciatic nerve crush[36] and chronic constriction injury[37], and has been suggested to be play a role in the mechanical allodynia that is present in neuropathic pain. Also, both in complete and partial nerve injuries, sympathetic postganglionic fibers start to sprout into the DRG[38,39]. These sprouted neurones form synaptic varicosities within the DRG[40] and could form an anatomical basis for a sympathetic dependency of neuropathic pain[40,41]. Thus similar phenomena, contributing to the pathogenesis of neuropathic pain, occur with both lesion types.

Moreover, as has been suggested for the CCI model, the development of increased nociception is related to the preferential loss of large-diameter myelinated fibers, thus resulting in a loss of inhibitory control[30,42,43]. After a crush lesion, the myelin sheath of regenerating nerve fibers is thinner than in an unlesioned nerve up to 12 months after lesioning[7,44]. Also, both sensory and motor nerve conduction velocities remain decreased for over 6 months after crush[25]. Functionally, as in the CCI model, these phenomena would result in a diminished large-diameter fiber mediated inhibition. On

the other hand, the increased sensitivity after a crush injury might also be in part explained by hyper-responsiveness of regenerated afferent fibers, as suggested by Andrew and Greenspan[45].

In the experiments described here, mechanical allodynia was assessed by von Frey probing of the mid-plantar region and chemical hyperalgesia was measured by applying mustardoil to the lateral dorsum of the hindpaw, both areas normally innervated by the sciatic nerve. In contrast, cold allodynia was quantified by measuring the withdrawal latencies to thermal stimulation upon immersion of the whole hind-paw in a 4.5 °C waterbath. In this way the exact nociceptive territory could not be determined, since the sensory innervation from the hind paw is provided by both the sciatic and the saphenous nerve. Thus we cannot rule out the possibility that the saphenous nerve is involved in mediating this cold allodynia, consistent with a previously suggested role for the saphenous nerve in pain-related disorders following sciatic nerve injury[13,46,47]. This is however not likely, since Kaupilla and Xu[48] found no saphenous nerve mediated cold hypersensitivity following sciatic nerve section. Kingery et al.[12] reported a temporal correspondence between recovery of motor function after a crush lesion and resolution of saphenous nerve-mediated heat and pressure hyperalgesia. However, in this present study the sensory abnormalities only occurred when the different locomotor function tests returned to normal, suggesting that these altered responses are mediated by sciatic reinnervation rather than through the adjacent saphenous nerve. Since Kingery and colleagues used larger rats and a different method to determine sciatic motor function it is difficult to compare their time course for sciatic nerve regeneration with our present data. Moreover, they focussed on saphenous nerve mediated hypersensitivity in the period preceding sciatic motor function recovery, thus making it possible that later sciatic nerve mediated sensory abnormalities remained undisclosed.

At present, the sciatic nerve crush is an animal model often used to study nerve regeneration. A consequence of the development of hyperalgesia and allodynia with this type of lesion might be a disturbance of the outcome of functional and sensory recovery tests. Moreover, we have recently demonstrated that melanocortins, often used in regeneration studies because of their positive effects on functional recovery[49-51], can worsen neuropathic pain[52,53].

Therefore, in interpreting results of these studies one should bear in mind the presence of possible confounding factors in sensory recovery, such as an increased response to a normally non-noxious stimulus. However, this does not hold true for the beneficial effects of melanocortins on locomotor recovery after sciatic nerve crush.

Next to the CCI model we used here, several other experimental animal models are available to study the mechanisms underlying the symptomatology and possible therapeutic strategies of neuropathic pain. They include partial tight ligation of the sciatic nerve[54] and segmental spinal nerve ligation[55]. Although the main symptoms produced by these three models (allodynia and hyperalgesia to heat, cold, and mechanical stimuli) share a high degree of similarity, there are also substantial differences, such as the extent and time course of the abnormalities and the response to sympathectomy[41]. In the present study the sensory abnormalities in CCI and crush rats are of comparable magnitude but greatly differ in their time course. In the clinical situation there is a complex relationship between the aetiology, mechanisms and symptoms of the neuropathic pain syndrome[31], and possibly the different rodent models demonstrating contrasting features involve different pathophysiological mechanisms[56] and might represent different populations of human neuropathic pain[41].

Since it is not likely that a single animal model will cover the complete range of possible mechanisms, it is valuable to study additional animal models producing symptoms of neuropathic pain, such as the sciatic nerve crush model we present here, since this might provide further insight into human neuropathic pain.

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3

'CatWalk' automated quantitative gait analysis as a novel method to assess mechanical allodynia in the rat; a comparison with von Frey testing

Dorien H. Vrinten
Frank P.T.Hamers

Pain, in press

Abstract

A characteristic symptom of neuropathic pain is mechanical allodynia. In animal models of neuropathic pain, mechanical allodynia is often assessed by using von Frey filaments. Although the forces applied with these filaments are highly reproducible, there are various disadvantages with this method. Testing paradigms and definitions of withdrawal threshold are not standardised. Moreover, measurements may be influenced by various conditions, such as ambient temperature, humidity, weight bearing of the limb and stress. We have therefore investigated another technique to assess mechanical allodynia, the "CatWalk" automated quantitative gait analysis. With this computer-assisted method of locomotor analysis it is possible to objectively and rapidly quantify several gait parameters, including duration of different phases of the step cycle and pressure applied during locomotion. We tested rats with a chronic constriction injury of the sciatic nerve, a model of neuropathic pain, both with von Frey filaments and the CatWalk method. We demonstrate that these rats minimise contact with the affected paw during locomotion, as demonstrated by a reduction in stance phase and pressure applied during stance. Moreover, these parameters show a high degree of correlation with mechanical withdrawal thresholds as determined by von Frey filaments. We therefore suggest that the CatWalk method might serve as an additional tool in the investigation of mechanical allodynia.

Introduction

Neuropathic pain is a condition caused by a lesion to the peripheral or central nervous system. Clinically it is characterized by the presence of spontaneous as well as different types of evoked pain. The latter include exaggerated responses to both noxious and non-noxious stimuli, referred to as hyperalgesia and allodynia, respectively. Allodynia to tactile stimuli is a common symptom[1,2], and amongst the most problematic clinical phenomenon, since physical contact with the environment is difficult to avoid. To study the pathophysiological mechanisms underlying neuropathic pain symptoms such as allodynia, several animal models of neuropathic pain have been developed in the past decades. The most widely used models are the chronic constriction injury (CCI)[3], partial ligation of the sciatic nerve[4] and tight ligation of the L5 and L6 spinal nerves[5]. Although there are differences between these models, they all produce behavioural signs of neuropathic pain, including mechanical allodynia[6].

To date, the most commonly used method to assess mechanical allodynia in such animal models is application of a series of von Frey filaments (also known as Semmes-Weinstein filaments) to the paw. The animal's response to these filaments is used to determine mechanical withdrawal thresholds. Usually, a paw withdrawal upon probing or immediately upon release of the filament is considered a positive response[2]. However, responses are not always clear-cut and their interpretation may vary between investigators. Most commonly, the filaments are applied to the plantar surface of the hind paw while the animal is standing on a metal grid floor, but also probing of the lateral or dorsal surface of the paw has been performed[7]. Also, various paradigms to determine withdrawal thresholds have been employed. In a frequently used testing paradigm filaments are applied according to the up-and-down method described by Dixon[8]. Here probing is initiated with a filament in the middle of the series, and depending on the presence or absence of a positive response the next smaller or larger filament is tested, respectively. From the resulting pattern of positive and negative responses a 50% withdrawal threshold can be calculated[2]. Alternatively, filaments can be used in order of increasing stiffness, starting with the smallest filament. With this testing in ascending order, each filament can be applied a different number of

times, at various intervals, and held in position for different periods. Also, different definitions of withdrawal threshold have been employed; for example the smallest filament eliciting at least one positive response[9], or three out of three[10], three out of five[7], or four to six out of ten positive responses[11]. Yet another way to quantify mechanical allodynia is by testing either a complete series of filaments, or a selection of two, and calculating the total number of responses as a percentage of total number of applications[2,6]. Thus, by using von Frey probes, assesment of mechanical allodynia can be performed in numerous ways, and may be influenced by subjectivity, thus making it difficult to compare results obtained from different studies.

In the present study we investigated whether mechanical allodynia can also be measured in a more objective way. We produced a mechanical allodynia by subjecting rats to a chronic constriction injury of the sciatic nerve. It is to be expected that an increased sensitivity to mechanical stimuli will cause the animal to exert less pressure on the affected limb during walking, and will minimize contact of this paw with the floor. We tested CCI rats on the "CatWalk", an automated quantitative gait analysis method recently developed in our laboratory[12]. Here we compare von Frey withdrawal thresholds in these animals with relevant data obtained from the CatWalk analysis.

Materials and methods

Animals and surgery

All procedures in this study were performed according to the Ethical Guidelines of the International Association for the Study of Pain[13] and approved of by the Ethics Committee on Animal Experiments of Utrecht University.

Twelve male Wistar rats weighing 250-300 g at the start of the study were used. Animals were housed in groups of 2-3 in plastic cages on sawdust bedding. They were kept at a 12/12hr light/dark cycle, with food and water available *ad libitum*.

Animals were anaesthetised with a single subcutaneous injection of Hypnorm (Duphar, the Netherlands) diluted in saline (1:2, 0.3 ml/100 g bodyweight).

Four loose ligatures were placed around the right sciatic nerve as described previously[3]. Subsequently the incision was closed with silk sutures and the animals were allowed to recover for a 2-3 day period.
Since we previously observed that sham-surgery does not induce any changes in withdrawal thresholds to von Frey stimulation or locomotor function (unpublished results, see also Kupers et al.[14], Vrinten et al.[15]), in this experiment we used only CCI animals.

Test procedures

Before surgery and at 2, 5, 8 and 10 weeks after surgery, the following tests were performed:

1 Mechanical withdrawal thresholds (von Frey)

Paw withdrawal threshold in response to a mechanical stimulus was determined using a series of von Frey filaments (Stoelting, Wood Dale, IL), ranging from 1.08 to 21.09 g. Animals were placed in a plastic cage with a metal mesh floor, allowing them to move freely. They were acclimatised to this environment for approximately 10 minutes prior to testing, to allow for behavioural accomodation. Von Frey filaments were applied to the mid-plantar surface of the operated hind paw through the mesh floor. Probing was only performed when the animals paw was in contact with the floor. Each probe was applied to the foot until it just bent, and kept in this position for 6-8 s[2]. Interval between consecutive filaments was at least 5 s. Filaments were applied in ascending order, and the smallest filament that elicited a foot withdrawal response was considered the threshold stimulus.

2 "CatWalk" automated gait analysis

The animals traverse a walkway (plexiglass walls, spaced 8 cm apart) with a glass floor (100 X 15 x 0.6 cm) located in a darkened room. Light from an otherwise completely encased white fluorescent tube (length 110 cm, 30W) enters the distal (from the observer) long edge of this glass floor. Sufficiently far from the edge it strikes the surface below the critical angle and is entirely internally reflected. Only at those points where a paw touches the glass, light exits the floor and scatters at the paw, illuminating the points of contact only (fig. 1A). Via a mirror, the corridor's floor is monitored by a Pulnix

TM-765E CCD camera (Pulnix Inc., UK) equipped with a wide angle objective (Cosimar 8.5mm) (fig. 1B). The intensity of the signal depends on the degree of paw floor contact and increases with pressure applied[16]. The camera detects the average intensity within a rectangular area (pixel) in which total skin-floor contact may differ. The more pressure is exerted, the larger the total area of skin-floor contact and thus the brighter the pixel (fig. 1C).

The signal is digitized by a PCImage-SG frame grabber board (MatrixVision GmbH, Oppenheimer, Germany). The CatWalk program acquires, com-

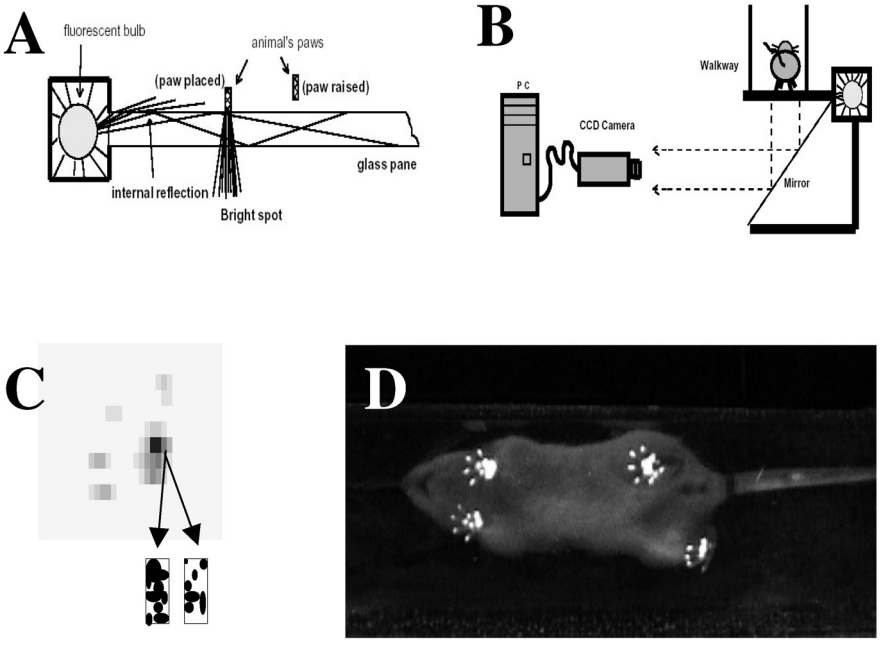

Figure 1.
Priciple of the CatWalk setup

(A) Light from a fluorescent bulb is sent through a glass plane. Light rays are completely reflected internally, except where a paw is placed on the glass surface. This results in a sharp image of the paw. (B) Images are reflected by a mirror placed at a 45° angle, and recorded by a CCD videocamera connected to a computer. (C) Example of a paw print during walkway crossing. The footpad and toes are clearly visible. For visualisation purposes, intensity is inverted, i.e. the darker the print on this figure, the brighter it appeared on the walkway. Influence of skin-floor contact on pixel intensity in two neighbouring pixels is shown (insets). For details see text. (D) A faint image of the rats body is visible over the paw prints, thus making it possible to identify the different paws (adapted from Hamers et al.12).

presses, stores and eventually analyzes the "videotapes" of animals crossing the walkway. All areas containing pixels brighter than a preset threshold value are also stored uncompressed, in order to circumvent compression related artefacts in the eventual analysis. Note that a very faint image of the animal crossing the walkway is present, unless all background illumination is eliminated completely (fig. 1D). However, this image is so faint in comparison with paw prints that it does not interfere with the measurement. In effect, without the shape of the animal moving through the corridor interactive labeling of prints is far more difficult.
Data analysis is performed by first (automatically) labeling all areas containing one or more pixels above a certain analysis threshold. In a second interactive pass these areas are assigned to one of the paws; user intervention is in most cases only required at initial contact of each paw, enabling analysis of a few seconds of walkway crossing within less than a minute. Ordered data are eventually output in ASCII-format which is human readable and which can be used as input of almost every spreadsheet program.
Animals are not pre-trained to cross the walkway as the Wistar rats used in our laboratory have no hesitation in crossing the walkway spontaneously with sufficient speed. A typical crossing contains 6 step cycles and averaged data from all step cycles in a crossing are used in the analysis.

Analysis of these recordings yields many parameters[12] of which the following are of most interest in the CCI model:
step sequences (see Table 1) with their respective frequencies;
regularity index (RI); a measure of interlimb coordination. Interlimb coordination is complete if only normal step sequences are used during uninterrupted locomotion. The RI grades the degree of interlimb coordination as follows: RI = (NSSP * 4 / PP) * 100(%) wherein NSSP represents the number of normal step sequence patterns and PP the total number of paw placements. Both extra paw placements and loss of certain paw placements (irregular walking on 3 paws) will decrease RI;
intensity; a measure for the mean pressure exerted by the paw during floor contact;
duration of stance phase; and
duration of swing phase.

Since the absolut duration of stance or swing phase depends on the animals walking speed, these parameters are transformed to a fraction of total step duration according to the following formula:
fraction stance or swing phase = stance or swing phase / (stance phase + swing phase)

Data analysis and statistics

For each animal, stance or swing phase, mean intensity during stance and von Frey withdrawal thresholds are calculated as a percentage of respective pre-operative values. As such each animal serves as its own control. Von Frey withdrawal thresholds are transformed logarithmically, in order to obtain a linear scale of increasing intensities with increasing filament size. Data from stance or swing phase and mean intensity are represented as mean ± standard error of the mean (s.e.m.). Because of the discrete nature of the von Frey data, these are represented as median and 25^{th}-75^{th} percentiles.
To compare differences in stance phase and mean intensity at different time points, ANOVA's were performed, followed by paired samples T-tests (comparisons with pre-operative values). Von Frey data were analysed using a Kruskal-Wallis test, followed by Mann-Whitney U tests. Bonferroni corrections were performed.
To analyse correlations between von Frey withdrawal thresholds and mean intensities, stance or swing phase, Pearsons correlation coefficients were calculated. Data are presented as X-Y scatter plots with either % change in mean intensity, stance or swing phase on the Y-axis and corresponding von Frey thresholds on the X-axis. Regression lines were also calculated and plotted. A probability level of 0.05 was considered significant.

Results

Mechanical withdrawal thresholds (von Frey)

With preoperative testing, none of the animals responded to the largest von Frey filament tested (21.1 g), on either of the hindpaws. After chronic constriction of the sciatic nerve mechanical allodynia developed, as demonstrated by a large decrease in withdrawal threshold of the operated hind paw at 2 weeks postoper-

ative, to 31.0 (31.0-46.0) % of preoperative thresholds (median and 25th-75 th percentiles).

Thereafter thresholds gradually increased to 100 (84.8-100) % of preoperative thresholds at 10 weeks postoperatively, which is not significant from baseline (fig. 2). Withdrawal thresholds at the contralateral (non-lesioned) side remained constant throughout the experiment..

"CatWalk" analysis

1 General walking pattern

Before surgery the predominant step pattern is alternate (forelimb and contralateral hindlimb in sequence). After placement of the ligatures this remains the main step pattern, although other patterns also occur (cruciate or rotary; for description of step patterns, see Table 1). Regardless of these changes in step patterns, the Regularity Index remains constant, indicating that there is no significant loss of interlimb coordination in CCI animals. Thus placement of the lesioned paw, no matter how short, is present in almost all step cycles at all time points used.

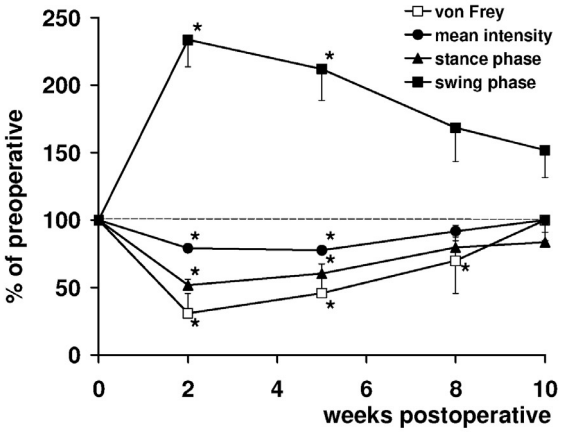

Figure 2.

Time course of different parameters obtained in rats with a chronic constriction injury (CCI). Measurements were taken preoperatively (t = 0), and at 2, 5, 8 and 10 weeks after CCI. Mechanical withdrawal thresholds as determined by application of von Frey filaments, mean intensity during stance, as a measure of paw pressure, and duration of stance and swing phase are plotted as percentages of respective preoperative values. Data on intensity, stance and swing phase are obtained by the CatWalk method. Data are presented as mean ± s.e.m. (mean intensity and stance phase) or median and 25th-75th percentile (von Frey) of 12 rats. (* $p < 0.05$ compared to baseline)

Table 1. Limb sequences in regular stap patterns[a]

Category	Sequence
Cruciate	RF-LF-RH-LH or LF-RF-LH-RH
Alternate	RF-RH-LF-LH or LF-RH-RF-LH
Rotary	RF-LF-LH-RH or LF-RF-RH-LH

[a] RF: right forelimb, RH: right hindlimb, LF: left forlimb, LH: left hindlimb (adapted from Cheng et al.[28])

2 Intensity of the right hind limb

Data analysis was performed with a threshold value of 40 (arbitrary units, a.u., possible range 0-255), i.e. all pixels brigher than 40 are used. The mean intensity with which the paw is placed is computed over the whole stance period. Mean baseline intensity of the right hind limb was 86.6 ± 3.2 (mean ± s.e.m.) (a.u.). Two weeks after placement of the ligatures, intensity was significantly reduced to 79.6 ± 3.6 % of preoperative value ($p<0.05$) and thereafter gradually normalized to 100.3 ± 3.4 % (not significant) at ten weeks postoperatively (fig. 2).

When these % changes in mean intensities were plotted against % changes in von Frey thresholds, there was a very high degree of correlation between these two parameters (Pearsons = 0.63, $p < 0.001$) (fig. 3A).

There was no consistent change in mean intensities at the contralateral side, indicating that pressure of the contralateral hindpaw does not change. Since von Frey thresholds remained constant in this paw, correlations could not be calculated.

3 Duration of stance and swing phase of the right hind limb

Preoperatively, duration of the stance phase was 0.71 ± 0.02 (fraction of total step duration). Two weeks after surgery the time that the lesioned paw was in contact with the floor was significantly reduced to 51.9 ± 4.4% of preoperative values ($p < 0.05$). In the following weeks this gradually increased

to 83.6 ±7.5 % (not significant from preoperative values) (see figure 2). There was also a very high degree of correlation between this parameter and von Frey withdrawal thresholds, as demonstrated in fig. 3B (Pearsons = 0.67, $p < 0.001$). Initial swing phase duration was a fraction of 0.29 ± 0.02 of total step duration. At two weeks postoperative this increased to 233.7 ± 19.9 % of preoper-

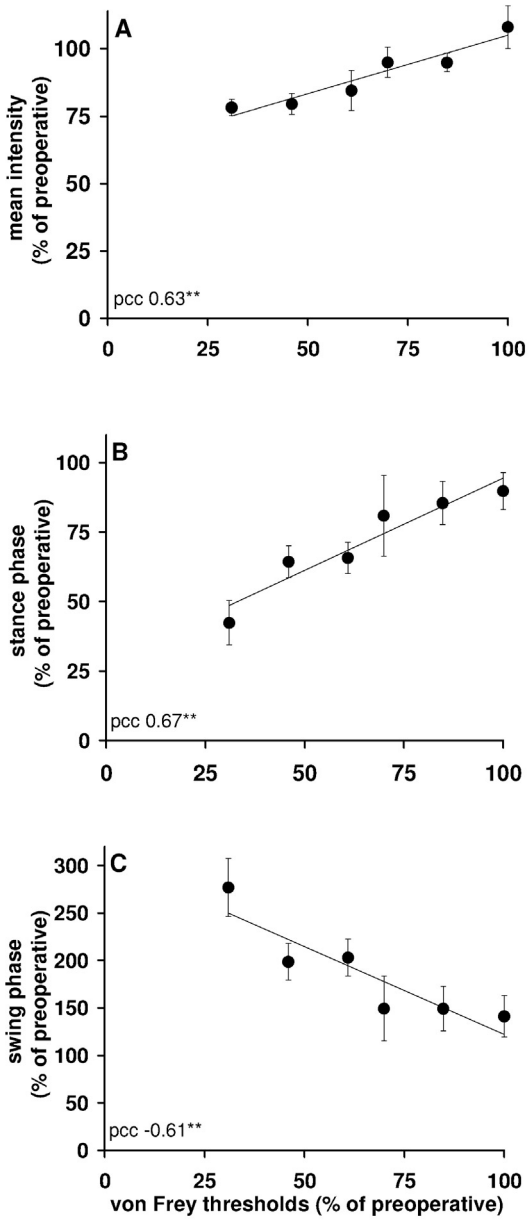

Figure 3.
Correlation between mean intensity during stance (A), stance phase duration (B), swing phase duration (C) and von Frey withdrawal thresholds in CCI rats. Data are presented as mean ± s.e.m. Linear regression lines are plotted. Pearson correlation coefficients (pcc) are calculated over 62 individual datapoints (** $p < 0.001$).

ative value, ($p < 0.05$). At ten weeks postoperative swing phase was again decreased to 151.8 ± 20.1 %, (not significant, see figure 2). Correlation between swing phase and von Frey thresholds was again very high (Pearsons = -0.61, $p < 0.001$, see figure 3C).,

There was no significant change in duration of stance or swing phase in the contralateral paw. As with the mean intensities, correlations with von Frey thresholds could not be calculated.

Discussion

In this paper we describe a possible novel approach to quantify mechanical allodynia in a rat model for neuropathic pain. We found a strong correlation between von Frey mechanical withdrawal thresholds and parameters obtained from CatWalk gait analysis.

Currently, von Frey probing is one of the most frequently used methods to measure mechanical allodynia in various animal models. Although the application forces of the von Frey filaments have been shown to be objective and reproducible[17] there are various setbacks. One of the problems is that there are many different ways to assess and define mechanical withdrawal thresholds using von Frey probing. Moreover, bending forces of the filaments are influenced by ambient humidity and, to a lesser extent, temperature. Finally, filaments may wear off with extensive use[18,19]. Since testing is usually done in non-restrained animals, the experimenter has to wait for the animal to hold its paw in the right position, which then should remain the same, as weight bearing of the limb might be a confounding factor in determining von Frey withdrawal thresholds[10]. In order to determine thresholds, multiple filaments have to be applied, which also takes time. Moreover, repetitive testing with short intervals may bias mechanical threshold determinations[2] and possibly disclose wind-up like pains[20].

The CatWalk method for automated gait analysis we used here allows for investigation of various walking parameters within a single run. Measurements are performed in freely moving, non-restrained animals, with minimal intervention, thus reducing the potentially confounding effects of stress[7]. Since the time needed for an animal to cross the walkway is in the order of several seconds it is a very rapid method, allowing relatively large groups of animals to be tested in a

short time span. Moreover, data acquisition is performed by computer which allows analysis of several parameters simultaneously. In the present study we looked at changes in mean signal intensity and the duration of different phases of a complete step cycle in rats with a chronic constriction injury (CCI). We hypothesized that these parameters would be affected as a result of the increased sensitivity to mechanical stimulation in these rats.

The mean signal intensity during placement of the paw when crossing the CatWalk walkway is an estimate of paw pressure, since signal intensity decreases when less pressure is applied with the paw[16]. There was a high degree of correlation between intensity measurements and von Frey withdrawal thresholds of the experimental hind paw. This suggests that lowered mechanical withdrawal thresholds, indicative of mechanical allodynia, are paralleled by decreased pressure applied with the paw during walking. In mice subjected to sciatic nerve crush, an injury also associated with neuropathic pain[21], we also observed this decrease in applied pressure during locomotion by using CatWalk analysis (unpublished results) . These observations are in agreement with earlier studies reporting a decrease in standing weight bearing of the affected limb associated with mechanical allodynia in different pain models, such as bone cancer pain[22], carrageenan-induced inflammation[23] and urate arthritis[24]. Also, in arthritic rats, a decrease in weight load during locomotion was observed, presumably reflecting pain[24,25].

Duration of stance and swing phase in these CCI rats were decreased and increased, respectively, and these parameters also demonstrate a high degree of correlation with von Frey thresholds. The obervation that in a rat model of inflammatory pain, i.e. carrageenan injections in the ankle joint, stance phase duration was also decreased (Angeby-Möller, personal communication) supports our findings. The use of other neuropathic or inflammatory pain models displaying mechanical allodynia[4,5,26] might further confirm this.

Together, our data imply that the CCI rats minimize contact with floor during walking, as demonstrated by an increased swing phase and shorter stance phase of the neuropathic hind paw and a decrease in pressure applied during this stance phase. Moreover, the time courses of these changes in intensity and step phases are similar to that of the von Frey withdrawal thresholds, indicating that these CatWalk parameters parallel variations in mechanical allodynia in these rats.

Since increased mechanocsensitivity might be parallelled by an increase in

touch-evoked responses of spinal α-motorneurones[27], it is possible that the observed changes in gait reflect changes in motorneuron activity, rather than direct responses induced by physical contact with the floor. In this way, alterations in intensities and stance phase might represent an indirect measure of allodynia. However, since these parameters correlate very well with the response evoked by direct application of von Frey filaments, we suggest that the CatWalk method might serve as an alternative tool to assess mechanical allodynia in CCI rats. Although we have not performed any pharmacological interventions in the present study, it is very likely that future studies will reveal pharmacological sensitivity of the CatWalk method, considering the high degree of correlation between von Frey probing and this method.

A possible drawback of the CatWalk method is that it appears to be less sensitive than von Frey probing. At 8 weeks postoperative, von Frey values are significantly different from preoperative, wherease CatWalk parameters do not reach significance (see figure 2). The magnitude of changes between pre- and postoperative intensities and stance phase durations was smaller than those of von Frey thresholds. However, the increase in swing phase duration is much larger. Moreover, at ten weeks postoperative this parameter is still increased (over 150% of preoperative, although not significant), whereas von Frey thresholds at this time point have completely normalised. This suggests that the CatWalk method might detect small changes in mechanical sensitivity that are not detected by von Frey probing. Furthermore, the CatWalk method has several advantages over the von Frey method, such as the convenience and objectivity of data collection, and the possibility to store and review the images of rats crossing the walkway.

In summary, the CatWalk method allows for rapid and objective analysis of many locomotor parameters. In CCI rats, displaying mechanical allodynia, we demonstrated that measurements of paw pressure and duration of stance and swing phase obtained with the CatWalk method show a high degree of correlation with mechanical withdrawal thresholds as determined by application of von Frey filaments. This suggests that the CatWalk method might provide an alternative means to quantify mechanical allodynia. Moreover, since the CatWalk method is not limited to rats, it might become a practical tool to study mechanical allodynia in other animal models, in which von Frey probing can be more difficult.

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4

Antagonism of the melanocortin system reduces cold and mechanical allodynia in mononeuropathic rats

Dorien H. Vrinten
Willem Hendrik Gispen
Gerbrand J. Groen
Roger A.H. Adan.

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Abstract

The presence of both pro-opiomelanocortin derived peptides and melanocortin (MC) receptors in nociception-associated areas in the spinal cord suggests that, at the spinal level, the MC-system might be involved in nociceptive transmission. In the present study we demonstrate that a chronic constriction injury (CCI) to the rat sciatic nerve, a lesion that produces neuropathic pain, results in changes in the spinal cord MC system, as shown by an increased binding of ^{125}I-NDP-MSH to the dorsal horn. Furthermore, we investigated whether intrathecal administration (in the cisterna magna) of selective MC receptor ligands can affect the mechanical and cold allodynia associated with the CCI. Mechanical and cold allodynia were assessed by measuring withdrawal responses of the affected limb to von Frey filaments and withdrawal latencies upon immersion in a 4.5°C waterbath, respectively. We show that treatment with the MC receptor antagonist SHU9119 has a profound anti-allodynic effect, suggesting that the endogenous MC system has a tonic effect on nociception. In contrast, administration of the MC4 receptor agonists MTII and D-Tyr-MTII largely increases the sensitivity to mechanical and cold stimulation. No antinociceptive action was observed after administration of the selective MC3 receptor agonist Nle-g-MSH. Together, our data suggest that the spinal cord MC-system is involved in neuropathic pain and that the effects of MC receptor ligands on the responses to painful stimuli are exerted through the MC4 receptor. In conclusion, antagonism of the spinal melanocortin system might provide a new approach in the treatment of neuropathic pain.

Introduction

In humans, damage to the nervous system - a peripheral nerve, dorsal root ganglion, dorsal root or the central nervous system - can lead to a pain state referred to as neuropathic pain. This syndrome is characterised by spontaneous pain in combination with allodynia (pain evoked by normally non-painful stimuli) and hyperalgesia (an increased response to painful stimuli). In current clinical practice, several drugs are used to control neuropathic pain, including tricyclic antidepressants (for review see Ollat and Cesaro[1], Kingery[2]), anticonvulsants[3], systemic administration of local anesthetics[4,5] and NMDA receptor antagonists[6,7]. In spite of this wide range of drugs, the treatment of neuropathic pain is often unsatisfactory and limited by the occurrence of adverse side-effects.

Over the past decade a number of animal models of neuropathic pain have become available, producing symptoms that closely resemble those observed in human neuropathic pain. Research using these preclinical models has yielded an array of potential new analgesics, including different enzyme inhibitors, ion channel blockers and ligands for various receptors (for review, see Chizh et al.[8], Yaksh[9]). Another potential target in the control of pain that has received very little attention is the melanocortin system. It has been previously reported that central administration of the melanocortins ACTH (adrenocorticotropic hormone) and α-MSH (α-melanocyte stimulating hormone) causes hyperalgesia in various pain tests[10-12]. Furthermore, these peptides have also been shown to antagonize the analgesic effects of morphine and β-endorphin[13-15]. The mechanisms through which these effects were exerted, however, remained unclear since no receptors for these peptides were identified. Only in recent years, 5 melanocortin (MC) receptors subtypes have been identified (for review, see Cone et al.[16], Tatro[17]), of which the MC3 and MC4 receptor are expressed in the nervous system. Compared to the MC3 receptor, the MC4 receptor has a much more widespread distribution throughout the brain. Moreover, it is the only subtype of which expression has been demonstrated in the spinal cord[18]. Binding of ^{125}I-NDP-MSH, a synthetic α-MSH analogue, to rat spinal cord demonstrated that the most abundant MC receptor expression is present in the superficial dorsal horn (lamina I and II) and in the grey matter surrounding the central canal

(lamina X), areas that are important in nociceptive transmission[19]. Furthermore, POMC mRNA was also demonstrated in spinal cord[19], and immunoreactivity for the POMC-derived peptides β-endorphin, ACTH and α-MSH has been described in the dorsal horn and lamina X[20,21]. Together, these findings suggest the presence of a functional MC-system in the rat spinal cord. Considering the localization of ^{125}I-NDP-MSH binding in nociception-associated areas in the spinal cord, and the fact that the MC4 receptor is the only MC receptor subtype for which mRNA has been detected in the spinal cord, the spinal MC4 receptor might be a potential target in the ongoing search for new analgesics.

As recently selective ligands for the MC receptors became available, it is now possible to study a putative role for the MC4 receptor in the control of neuropathic pain. The aim of the present study was to investigate whether changes in the spinal cord MC-system play a role in neuropathic pain. Therefore *in situ* binding of ^{125}I-NDP-MSH to rat lumbar spinal cord sections was quantified. The chronic constriction injury (CCI)[22] was chosen because of its wide acceptance as a reliable and reproducible model for neuropathic pain. In addition, we investigated whether selective MC receptor ligands can alter the response of control and mononeuropathic rats to painful stimuli.

We demonstrate that a CCI results in an increase in ^{125}I-NDP-MSH binding to the spinal cord, suggesting an increase in MC receptor levels. We also show that in CCI rats intrathecal administration of the MC receptor antagonist SHU9119 induced a decreased sensitivity to cold and mechanical stimulation, whereas the strong MC receptor agonist MTII or the more selective MC4 receptor agonist D-Tyr MTII had the opposite effect. In contrast, in control rats these ligands had no effect on sensitivity. Furthermore we show that treatment with the selective MC3-R agonist Nle-γ-MSH had no effect on sensitivity.

Materials and Methods

Peptides

For *in vivo* administration MTII (Melanotan-II or cyclo-[Nle^4, Asp^5, D-Phe^7, Lys^{10}]α-MSH-(4-10)), SHU9119 (cyclo-[Nle^4, Asp^5, D-Nal(2)^7, Lys^{10}]α-MSH-(4-10)), D-Tyr-MTII (cyclo-[Nle^4, Asp^5, D-Tyr^7, Lys^{10}]α-MSH-(4-10)) and Nle-γ-MSH (Ac-[Nle^3]-γ_2-MSH-NH_2) were used. MTII was purchased from Bachem Feinchemicalien (Buberdorf, Switzerland), SHU9119, Nle-γ-MSH and D-Tyr-MTII were synthesized using Fmoc solid phase synthesis as reported elsewhere[23]. Peptides were purified using reversed phase preparative HPLC (high-pressure liquid chromatography) to a purity of ± 90%, estimated after analysis by analytical HPLC at 215 nm. Molecular weight was confirmed by mass spectrometry performed on a Micromass Quattro sq. Potencies and affinities of these four peptides for the rat MC3 and MC4 receptor are shown in table 1.

For *in situ* melanocortin binding to spinal cord cryosections ^{125}I-NDP-MSH was used. NDP-MSH (Melanotan-I or [Nle^4, D-Phe^7]α-MSH) was purchased from Bachem Feinchemicalien (Buberdorf, Switzerland) and iodinated using bovine lacto-peroxidase (Calbiochem) and ^{125}I-Na (ICN) as described elsewhere[24], followed by HPLC-purification on a C18 column (μBondapak 3.9 x 300 mm, Waters).

Table 1.
Affinity (Ki) and potency (EC50) of melanocortin receptor ligands for the rat MC3 and MC4 receptor

	Rat MC3		Rat MC4	
Ligand	Ki (nM)	EC50 (nM)	Ki (nM)	EC50 (nM)
MTII	4.77 ± 2.13	0.78 ± 0.17	1.74 ± 0.77	0.01 ± 0.004
D-Tyr-MTII	204 ± 87.2	20.3 ± 7.1	3.84 ± 0.84	0.47 ± 0.19
Nle-γ-MSH	1.44 ± 0.26	1.26 ± 0.10	77.5 ± 37.7	11.0 ± 3.92
SHU9119	0.879 ± 0.170		0.238 ± 0.060	

Affinities and potencies are determined on HEK 293 cells expressing either the rat MC3 or rat MC4 receptor, using ^{125}I-NDP-MSH as radioligand (for Ki) or the LacZ reporter gene (for EC50). Data are expressed as mean ± 95% confidence interval (adapted from Adan et al.[38])

Animals

59 Male Wistar rats weighing 200-240 g at the start of the study were used. Animals were housed in groups of 2-3 in plastic cages on a sawdust bedding. They were kept at a 12/12hr light/dark cycle, with food and water available *ad libitum*. All testing procedures in this study were performed according to the Ethical Guidelines of the International Association for the Study of Pain[25] and approved of by the Ethics Committee on Animal Experiments of the Utrecht University.

Surgery

Animals were anesthetized with a single subcutaneous injection of Hypnorm (Janssen Pharmaceutical LTD., Grove, Oxford) containing 0.315 mg/ml fentanyl citrate and 10 mg/ml fluanisone, at a dose of 0.3 ml/kg bodyweight.
In 33 animals the right sciatic nerve was exposed at mid-thigh level by blunt dissection and a CCI was made by placing 4 loose ligatures of 4-0 chromic catgut (Ethicon INC., Norderstedt) around the nerve, as previously described by Bennett and Xie[22]. In 4 animals the same procedure was performed except for placement of the ligatures (sham surgery). After this the incision was closed with silk sutures and the animals were allowed to recover. The remaining 22 animals only received a cisterna magna cannula (control animals).
Placement of the cannulae was performed 2 weeks after the sham or CCI lesion. Rats were again anesthetized and placed in a stereotactic frame. The skull was exposed by a midline incision. A steel cisterna magna cannula was inserted through a burr hole just before the squama occipitalis and two small screws were placed lateral to the midline for extra fixation. Cannula and screws were fastened with dental acrylic. The animals were allowed a four-day recovery period before testing was initiated.

In situ ^{125}I-NDP-MSH binding to spinal cord

Seven animals that remained naive to treatment (4 sham and 3 CCI animals) were used for *in situ* ^{125}I-NDP-MSH binding.
Tissue preparation: Four weeks after placement of the ligatures or sham surgery the rats were sacrificed by decapitation. The lumbar spinal cord was rap-

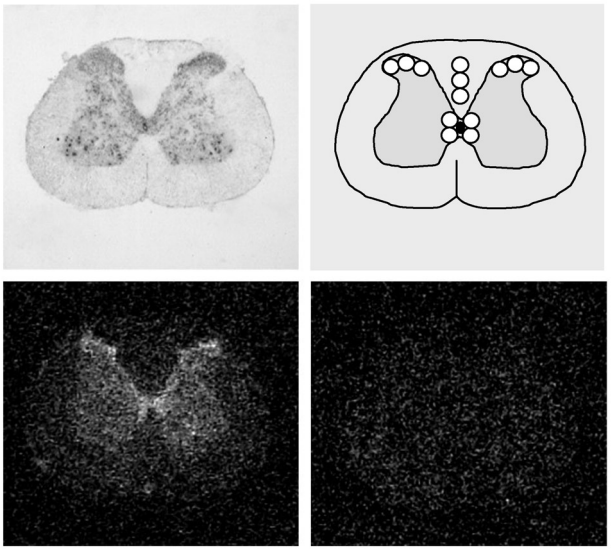

Figure 1.
^{125}I-NDP-MSH binding to rat spinal cord sections.
A: Nissl staining of a representative spinal cord section, demonstrating the neuroanatomy. B: Diagram representing the sampling template used for determining ^{125}I-NDP-MSH binding in X-ray film autoradiograms of rat spinal cord sections. a: superficial dorsal horn (left and right); b: lamina X; c: dorsal white matter column used for determining background value. For each region the mean value of 3 (superficial dorsal horn and background) or 4 (lamina X) samples was calculated. C,D: X-ray film autoradiogram of ^{125}I-NDP-MSH binding to a representative rat spinal cord section. Sections were incubated with ^{125}I-NDP-MSH in the absence (C) or presence (D) of 3 μM non-iodinated NDP-MSH. Specificity of binding present in C is demonstrated by its inhibition in D.

idly removed and frozen by submersion in 2-methyl-butane (Fluka Chemika, Buchs, Switzerland) on dry ice. Spinal cords were stored at –80 °C until further processing. From lumbar segments L4 - L6 cryostat sections (16 μm) were prepared and mounted on gelatin-coated slides (2 sections from each segment per slide).

In situ binding assay: From each animal 1 slide was incubated with ^{125}I-NDP-MSH as described previously[26]. In short, the sections were prewashed, incubated with ^{125}I-NDP-MSH (106 cpm/ml) in binding buffer for 1 hr, washed 6 times to stop binding reactions and rapid air dried. A second slide from each animal was incubated with ^{125}I-NDP-MSH in the presence of 3 μM non-iodinated NDP-MSH to determine the specificity of tracer binding. All binding assays were done on the same day, in one experimental session.

In order to visualize the neuroanatomy more clearly an adjacent section was Nissl stained (fig. 1A).
Autoradiography and analysis: Autoradiography was performed by exposing an X-ray film (BioMax MR, Kodak) directly to the slides for 1 week. All slides were run on the same, single film, with CCI and sham samples randomly divided over the film. Autoradiograms were digitized and quantitatively analyzed using the MCID (Microcomputer Imaging Device, Imaging Research Inc.). For each section, binding was measured in three anatomic regions, using a sampling template as depicted in fig. 1B. Within each region 3 or 4 samples were measured and the mean value was calculated. Specific binding was calculated by subtraction of the mean background value, determined within the dorsal white matter column of the same section. Absorbance values were converted into cpm using a linear calibration curve.

Drug administration

30 CCI and 22 control animals were used to study the effects of the different peptides on nociception. Peptides were dissolved in 10 µl of saline and injected through the cisterna magna cannula by means of a Hamilton syringe.
On each testing day CCI rats were randomly divided into 3 groups (N =10 each), each group randomly and blindly receiving one of the following doses: vehicle, SHU9119: 0.15, 0.5 or 1.5 µg (0.140, 0.466 or 1.40 nmol respectively), MTII: 15, 30, 100 or 500 ng (14.6, 29.2, 97.6 or 488.2 pmol respectively), D-Tyr-MTII: 0.3, 1.0 or 3.0 µg (0.289, 0.962 or 2.885 nmol respectively), Nle-γ-MSH: 5 µg (3.22 nmol) or a combination of 15 ng MTII and 0.5 µg SHU9119. Thus in total, 13 groups of 10 CCI animals were tested.
Similarly, on each testing day control rats were randomly divided in 2 groups (N=11 each), each group randomly and blindly receiving one of the following doses: vehicle, 1.5 µg SHU9119, 500 ng MTII or 3 µg D-Tyr-MTII (corresponding to the highest doses tested in CCI animals). Thus in total 4 groups of 11 control animals were tested.
Using this experimental setup, animals received only a single injection with a single dose on each testing day. The study was continued until all doses of all drugs were tested. Animals were given at least two days rest between drug

injections to minimize any possibility of drug interactions or development of tolerance.

Testing procedures

1 Temperature stimulation test

Withdrawal latency to a temperature stimulus was measured by immersing the right (experimental) hind paw into a 4.5°C or 47.5°C water bath. Upon immersion of the paw an electronic circuit including a timer was closed. Withdrawal of the paw resulted in a discontinuation of the circuit, which stopped the timer, thus allowing a precise registration of the withdrawal latency time. Cut-off time for both temperatures was set at 10 s. to avoid skin damage.

2 Mechanical stimulation test

Foot withdrawal threshold in response to a mechanical stimulus was determined using a series of von Frey filaments (Stoelting, Wood Dale, IL), ranging from 1.08 to 21.09 g. Animals were placed in a plastic cage with a metal mesh floor, allowing them to move freely. They were allowed to acclimatize to this environment prior to the experiment. The filaments were presented to the midplantar surface as described by[27], starting with the smallest filament. Each probe was applied to the foot until it just bent and the smallest filament eliciting a foot withdrawal response was considered the threshold stimulus.

For both mechanical and temperature stimulation tests baseline values were determined and measurements were repeated 15, 30 and 60 min after drug or vehicle administration.

Grooming assay

In 8 CCI animals a grooming assay was performed as described by Gispen et al[28]. In short, animals were placed in a plastic observation cage immediately after injection of 500 ng MTII (N=4) or saline (N=4) through the cisterna magna cannula. Starting 10 minutes after injection, grooming (face washing, genital grooming, body licking and grooming, scratching and paw licking) was scored every 15 seconds. Observation was stopped 50 minutes after injection.

Data analysis and statistics

All data are expressed as mean ± standard error of the mean (S.E.M) for visualization purposes only.

For *in situ* ^{125}I-NDP-MSH binding to spinal cord, the overall mean of levels L4-L6 and 1-2 sections per rat (thus rendering one datapoint per anatomic region per rat) were used to calculate group means and S.E.M. Differences between sham and CCI groups were analyzed using an independent Students T-test.

For the temperature stimulation test the difference between baseline and post-injection withdrawal latency was calculated for each animal at each time point.

To obtain a linear scale of perceived intensity in the mechanical stimulation test the logarithm of the withdrawal thresholds was plotted. As for the temperature stimulation test, differences between post- and pre-treatment withdrawal thresholds were calculated.

For mechanical and temperature stimulation, differences in baseline values between between control and CCI groups, and differences between drug treatment groups were analyzed using the Kruskall-Wallis test because of the non-parametric nature of the data. When appropriate, post hoc analysis was performed using the Mann-Whitney U test, comparing each treatment dose with vehicle, and for each treatment comparing the highest dose with the intermediate and lowest dose, respectively. A Bonferroni-correction was performed.

Where possible, dose-response curves were generated. Therefore, post-injection values were expressed as a percentage of baseline value. Mean ± S.E.M. of these percentages were plotted against the administered dose. Dose-response curves are reported for the time of peak effect (30 min post-injection for MTII and D-Tyr-MTII, and 15 min post-injection for SHU9119). Differences in grooming scores were analyzed using an independent Students T-test.

For all tests, a probability level of ≤ 0.05 was the criterion for a significant difference.

Results

In situ ^{125}I-NDP-MSH binding to spinal cord

Specificity of the ^{125}I-NDP-MSH binding is indicated by its inhibition in the presence of 3 μM NDP-MSH, which reduced binding to background level (figs. 1C and D).

As demonstrated previously[19], specific ^{125}I-NDP-MSH binding was highest in the superficial dorsal horn (corresponding to lamina I-II) and lamina X. In CCI animals binding in lamina I-II on both the ipsi- and contralateral side was significantly increased as compared to sham animals (125.5 % and 118.7% of sham values, respectively). In contrast, binding to lamina X did not differ between groups (fig. 2).

Baseline values for temperature and mechanical stimulation

In control animals, the mean baseline mechanical withdrawal threshold for all 4 groups at all time points was 21.09 ± 0 g (mean ± S.E.M.) In CCI animals the overall mean baseline was significantly lower (5.32 ± 0.21 g, rang-

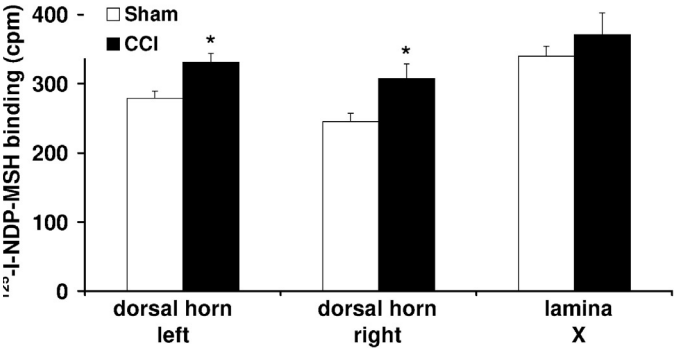

Figure 2.

^{125}I-NDP-MSH binding levels in different anatomic regions in rat lumbar spinal cord (L4-L6) cryostat sections.

Spinal cords were collected 4 weeks after CCI or sham surgery. Regions were analysed by sampling the corresponding region of an X-ray film autoradiogram, using a template as depicted in fig. 1A. Values were converted to cpm using a linear calibration curve. Specific binding within each region was determined by substracting the mean background value obtained from the dorsal white matter from the same section. For each region the overall mean of levels L4-L6 from 1 or 2 sections was calculated per rat.

Data are represented as mean ± S.E.M. of 4 (sham) or 3 (CCI) rats (* $p<0.05$ vs. sham).

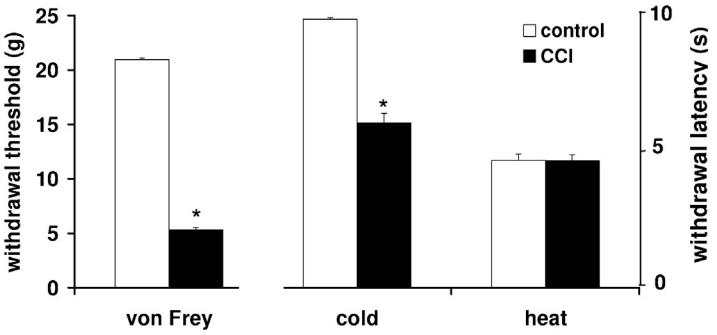

Figure 3.
Baseline withdrawal thresholds to von Frey stimulation and baseline withdrawal latencies to cold stimulation (4.5 °C) and heat stimulation (47.5 °C) in control and neuropathic rats. Data are presented as mean ± S.E.M. of 13 groups of 10 rats (CCI) or 4 groups of 11 rats each (control). (* $p<0.05$)

ing from 4.83 ± 0.50 to 6.67 ± 1.06 g for the 13 different randomized groups), thus indicating a mechanical allodynia.
At 4.5°C overall mean baseline withdrawal latency in control animals was 9.86 ± 0.06 s (ranging from 9.75 ± 0.19 to 10 ± 0 s). In CCI animals, mean baseline withdrawal latency was significantly lower (6.05 ± 0.35 s, ranging from 3.47 ± 0.41 to 7.36 ± 1.07 s), thus demonstrating a cold allodynia.
At 47.5 °C overall mean baseline withdrawal latency in control animals was 4.52 ± 0.22 s (ranging from 3.57 ± 0.26 to 5.47 ± 0.45 s). This value was not significantly different from that in CCI animals (4.66 ± 0.22 s, ranging from 3.47 ± 0.41 to 6.98 ± 1.26 s). These data are shown in fig. 3.
Although baseline values could differ between randomized groups there was no correlation between these values and administration of either an agonist or antagonist, nor were baseline values consistently changed by previous administration of either an agonist or antagonist (data not shown).

Vehicle injection

An injection of 10 µl of saline through the cisterna magna cannula had no effect on the responses to any of the tests performed, neither in CCI rats, nor in control rats.

Administration of SHU9119

In CCI rats, treatment with SHU9119 (0.15, 0.5 and 1.5 µg) produced a tac-

tile anti-allodynic effect, as shown by a dose-dependent increase in withdrawal thresholds to von Frey stimulation (fig. 4A), compared to vehicle treatment. Peak effects were reached 15 min after injection, resulting in a withdrawal threshold of up to 170.5 ± 7.25 % of baseline value (mean ± S.E.M.) with 1.5 μg SHU9119 (fig. 5A).

As for the mechanical withdrawal thresholds, withdrawal latencies to cold

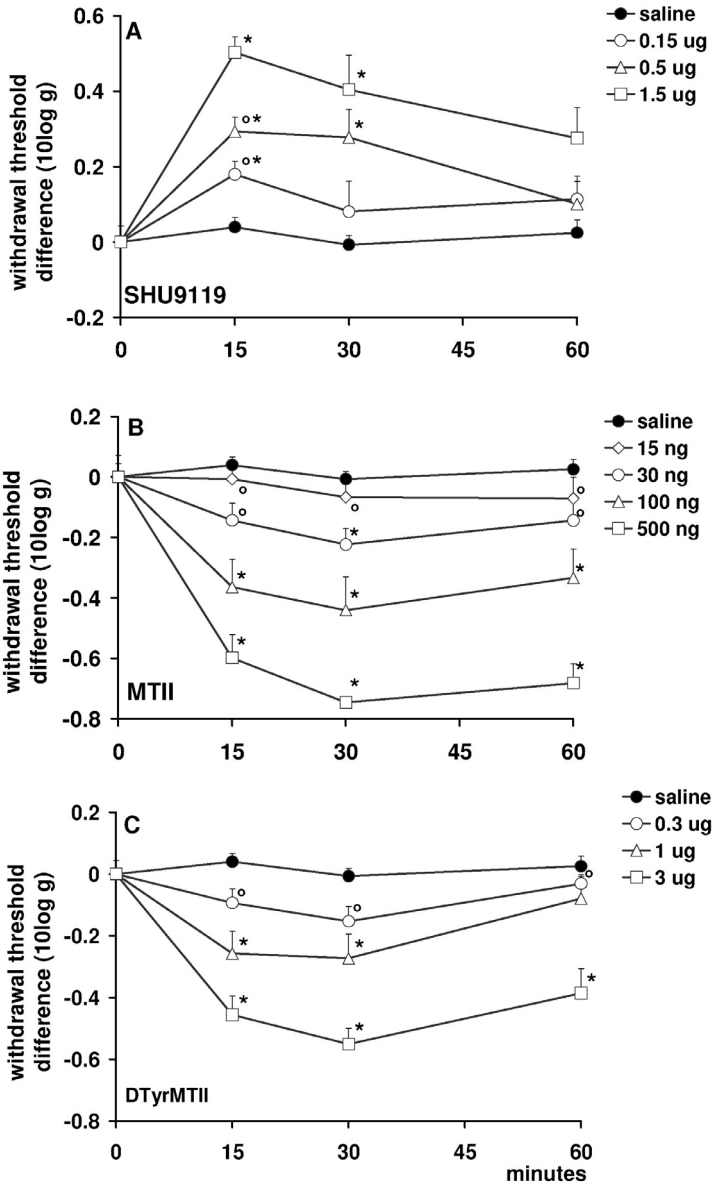

Figure 4.
The effect of i.t. SHU9119 (A), MTII (B) and D-Tyr-MTII (C) on withdrawal thresholds to von Frey stimulation in neuropathic rats. Thresholds are transformed to the logarithm of the applied force. Differences between post-injection and pre-injection (baseline) value are plotted.
Data are presented as mean ± S.E.M. of 10 rats each, except 1.5 mg SHU9119 (N=4) (* $p<0.05$ vs. vehicle; °$p< 0.05$ vs. highest dose of MTII, D-Tyr-MTII or SHU9119).

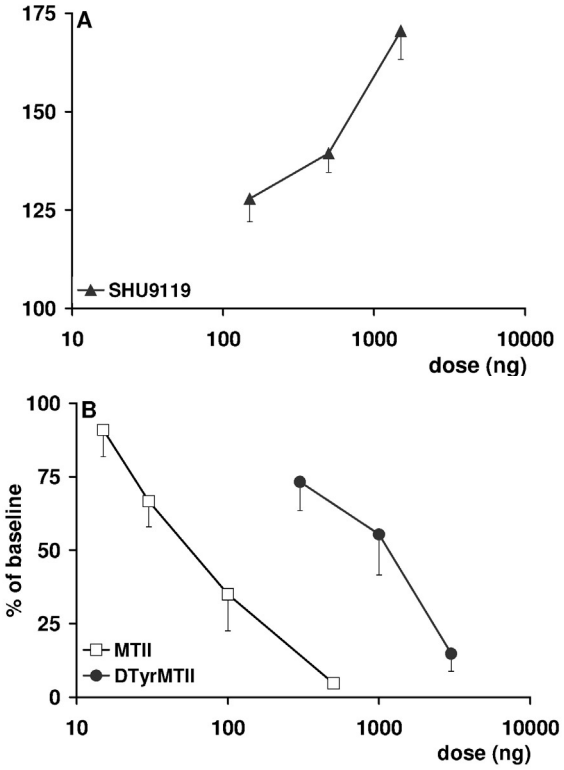

Figure 5.
Dose-response curves of the effect of i.t. SHU9119 (A), MTII and D-Tyr-MTII (B) on von Frey withdrawal thresholds in neuropathic rats. Values represent threshold at 30 min post-injection as a percentage of baseline threshold.
Data are presented as mean ± S.E.M. of 10 rats each, except 1.5 mg SHU9119 (N=4).

stimulation also increased upon administration of SHU9119 (fig. 6A). The cold anti-allodynic effect of the 2 lowest doses of SHU9119 showed a dose-dependency as observed for the tactile anti-allodynic effect. However, the highest dose tested (1.5 μg) only produced a small increase in withdrawal latencies. This group consisted of only 4 animals and baseline withdrawal latencies of 2 of these 4 animals were already at cut off value, leaving no room for a further increase in latency. In the remaining 2 animals latencies, however, did increase to cut off value in one case and to 174% of baseline in the other case. Treatment with SHU9119 did not cause any changes in withdrawal latencies at 47.5°C (data not shown).
In control rats, administration of 1.5 μg SHU9119 had no effect on responses to mechanical, cold or heat stimulation (data not shown).

Administration of MTII and D-Tyr-MTII

In CCI rats, administration of the MC receptor agonist MTII (15, 30, 100 and 500 ng) produced a dose-dependent decrease in withdrawal thresholds

to mechanical stimulation (fig. 4B). 30 min after injection withdrawal thresholds were reduced to 4.64 ± 0.52% of baseline (mean ± S.E.M) with the highest dose tested (fig. 5B).

Similarly as for tactile thresholds, MTII dose-dependently decreased withdrawal latencies at 4.5°C (fig. 6B). The most potent effect was observed with

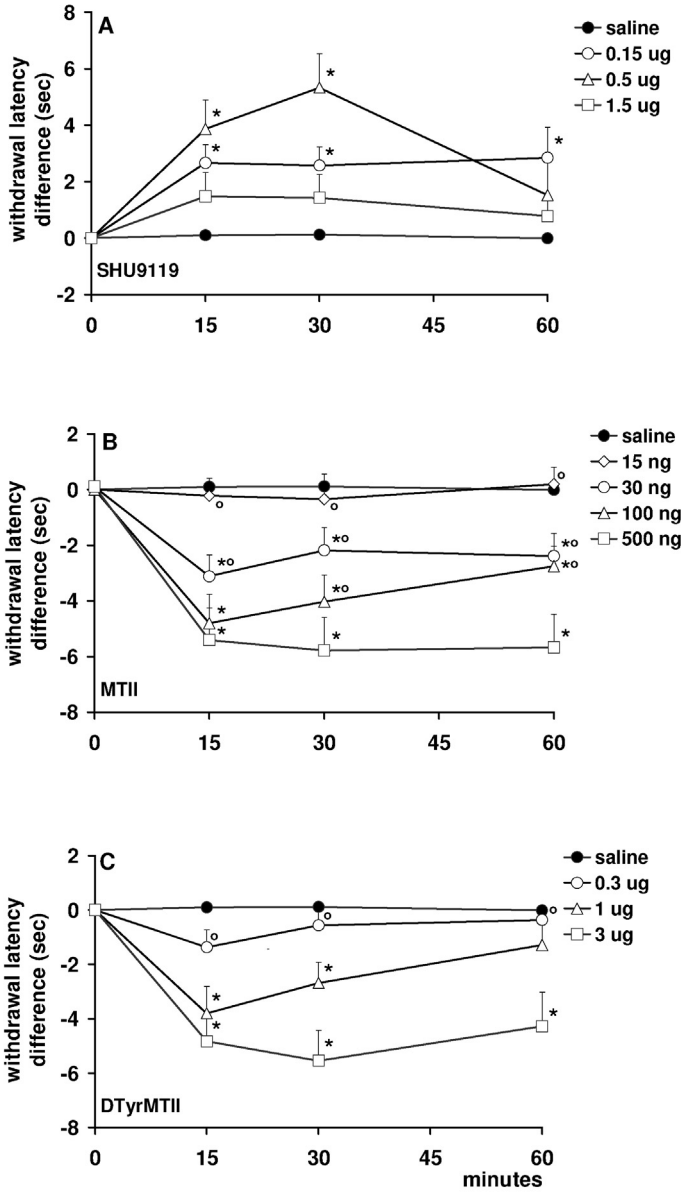

Figure 6.
The effect of i.t. SHU9119 (A), MTII (B) and D-Tyr-MTII (C) on withdrawal latencies to cold stimulation (4.5 °C) in neuropathic rats. Differences between post-injection and pre-injection (baseline) value are plotted.
Data are presented as mean ± S.E.M. of 10 rats each, except 1.5 mg SHU9119 (N=4) (* $p<0.05$ vs. vehicle; ° $p< 0.05$ vs. highest dose of MTII, D-Tyr-MTII or SHU9119).

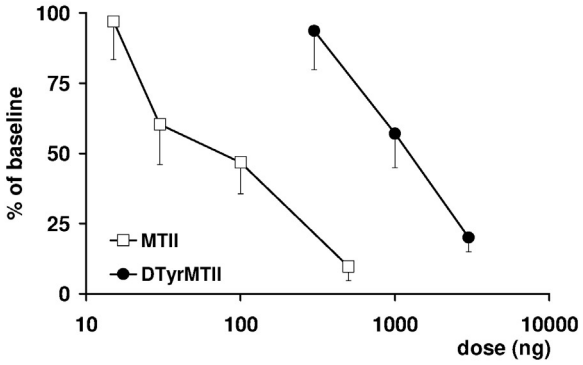

Figure 7.
Dose-response curves of the effect of i.t. MTII and D-Tyr-MTII on withdrawal latencies to cold stimulation (4.5 °C) in neuropathic rats. Values represent threshold at 30 min post-injection as a percentage of baseline threshold.
Data are presented as mean ± S.E.M. of 10 rats each.

the highest dose tested, which reduced latencies to 9.68 ± 5.07 % (mean ± S.E.M.) of baseline value (fig. 7). As for SHU9119, treatment with MTII caused no significant changes in withdrawal latency to a heat stimulus.
Administration of the more selective MC4 receptor agonist D-Tyr-MTII produced similar results as those observed with MTII, with approximately 10 times higher doses (0.3, 1 and 3 μg) resulting in a dose-dependent decrease in withdrawal thresholds to von Frey stimulation (fig. 4C) and in withdraw-

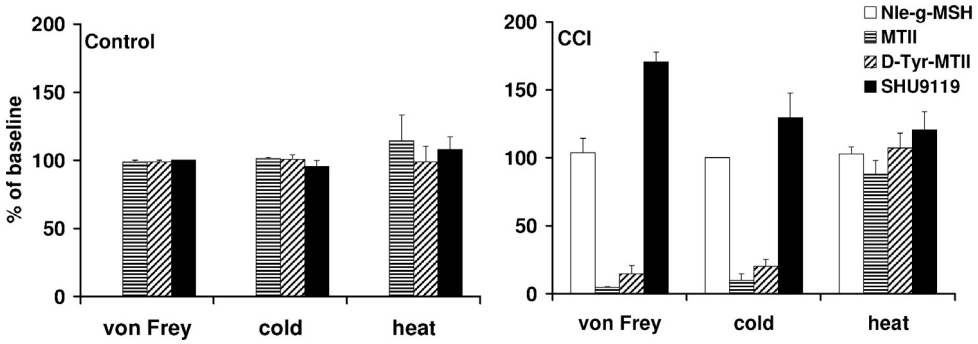

Figure 8.
Summary of the effects of i.t. administration of the MC3-R selective ligand Nle-g-MSH (5 μg), the MC4-R selective ligands MTII (500 ng), D-Tyr-MTII (3 μg) and the MC-R antagonist SHU9119 (1.5 μg) on the responses of CCI and control rats to different stimuli (indicated on X-axis). Values represent thresholds at 30 min (MTII, D-Tyr-MTII and Nle-g-MSH) or 15 min (SHU9119) post-injection as a percentage of baseline threshold. Data are presented as mean ± S.E.M. of 11 (control) or 10 (CCI) rats each, except 1.5 mg SHU9119 (N=4 CCI rats).

al latencies to cold stimulation (fig. 6C). Values were decreased to 14.73 ± 6.04 % and 20.04 ± 5.14 % (mean ± S.E.M.) of baseline values, respectively (figs. 5B, 7). As for MTII, administration of D-Tyr-MTII had no effect on withdrawal latencies at 47.5 °C.
In control rats, the highest dose of both ligands (500 ng MTII or 3 μg D-Tyr-MTII) did not cause any changes in responses to mechanical, cold or heat stimulation (data not shown).

Co-administration of MTII and SHU9119

Co-administration of 15 ng MTII, a dose which by itself had no effect on sensory thresholds (figs. 4B, 6B), and 0.5 μg SHU9119 in CCI rats, resulted in a complete inhibition of the cold and mechanical anti-allodynic effect of SHU9119 (data not shown).

Administration of Nle-γ-MSH

In CCI rats, a single, high dose (5 μg) of the selective MC3 agonist Nle-γ-MSH was tested. No decreased or increased response was observed to either mechanical or thermal stimulation (data not shown).

In figure 8 a summary of the described effects of the different MC receptor ligands is presented.

Grooming behavior

Total grooming scores after injection of saline or 500 ng MTII into the cisterna magna were 50.67 ± 6.39 and 54.5 ± 14.5, respectively (mean ± S.E.M.). These values were not significantly different (data not shown).

Discussion

In the spinal cord the expression of the MC4 receptor overlaps with that of the POMC derived peptides α-MSH and ACTH in nociception-associated areas[19,21]. Therefore we hypothesized that at the spinal level, the MC-system is involved in the processing of nociceptive information. Here we show for the first time that changes in the spinal cord MC-system occur following a

chronic constriction injury (CCI) to the rat sciatic nerve, a lesion that causes neuropathic pain. As shown in fig. 2, *in situ* binding of the synthetic MC receptor ligand ^{125}I-NDP-MSH is increased in lumbar (L4-L6, corresponding with sciatic nerve input) spinal cord sections of CCI rats as compared to sham operated animals, suggesting an upregulation of spinal cord MC receptors. It is not likely that these changes are caused by the profound deafferentation associated with a CCI lesion *per se*[29,30], since van der Kraan et al.[19] have shown that crushing the sciatic nerve, another lesion producing extensive nerve fibre loss, did not lead to significant differences in ^{125}I-NDP-MSH binding levels compared to sham surgery.

Of the anatomic regions we investigated, which were the regions with the highest intensity of binding, the superficial dorsal horn both ipsi- and contralateral to the lesion showed this increased binding. Bilateral changes associated with CCI have been described for other systems as well, including opioid binding sites[31], calcitonin-gene related peptide and substance P immunoreactivity[32], metabolic and nitric oxide synthase activity[33,34] and transsynaptic degeneration[35]. The contralateral changes might be explained by changes in primary afferents that cross the midline, commissural connections between intrinsic spinal neurons[36] or descending control systems affecting both sides of the spinal cord[37].

The superficial dorsal horn, the area that displayed an increased ^{125}I-NDP-MSH binding in our experiments, corresponds with the predominant entry zone of cutaneous fine diameter primary afferents of the sciatic nerve. In contrast, the gray matter surrounding the central canal, an area that receives mostly visceral input, showed no differences in binding. These findings suggest that changes in the endogenous MC-system in the spinal cord might be involved in the increased pain state associated with the CCI lesion, and prompted us to investigate whether tonic activity of the MC-system contributed to this increased sensitivity. As shown in figs. 4-6, administration of SHU9119, an antagonist at the MC4-receptor, induced a significant anti-allodynic effect in both the cold- and mechanical stimulation tests, indicated by an increased withdrawal latency upon immersion in a 4.5 °C water-bath and a higher threshold to von Frey stimulation, respectively. The observation that administration of an MC receptor antagonist produced hypoalgesia by itself indeed suggests a tonic influence of the MC-system on noci-

ceptive transmission.

The increase in MC receptor level in the superficial dorsal horn in CCI rats suggests an increased sensitivity for MC receptor agonists in neurons in this area. Treatment with the MC receptor agonist MTII resulted in an opposite effect compared to SHU9119, producing an increased sensitivity to both cold and mechanical stimulation. Similar results were obtained with D-Tyr-MTII, a MC receptor agonist that displays a higher affinity for the MC4 receptor as compared to the MC3 receptor. Co-administration of MTII and SHU9119 demonstrated the specificity of the anti-allodynic effect of SHU9119. Injection of 15 ng of MTII, a dose which by itself caused no significant changes in nociceptive thresholds, completely blocked the anti-allodynic effect of 0.5 μg of SHU9119, when administered simultaneously, thereby demonstrating that the effects of these compounds are indeed mediated through the same receptor. Administration of the selective MC3 receptor agonist Nle-γ-MSH had no effect on sensitivity. Since both MTII and D-Tyr-MTII altered the responses to cold and von Frey stimulation whereas Nle-γ-MSH had no effect, we suggest that the observed changes in nociception are mediated through the MC4 receptor.

In the present study we administered MC receptor ligands through a cannula placed in the cisterna magna , directly into the fluid surrounding the spinal cord. Adan et al.[38] have demonstrated that a dose of 4.5 pmol MTII is already sufficient to induce grooming, when administered intracerebroventricularly. In contrast, we demonstrate that a more than 100-fold higher dose (500 ng, approximately 488 pmol) failed to induce grooming when injected into the cisterna magna. We cannot exclude the possibility that a small portion of the drugs administered into the cisterna magna will retrogradely reach the ventricular system and surrounding structures, and that these structures play a role in the (anti-) nociceptive effects described here. However, since no grooming was observed after injection of a high dose of agonist, we suggest that the effects we observed in the present study are predominantly excerted at the spinal level.

In the dorsal horn, immunoreactivity has been demonstrated for the MC receptor agonists α-MSH and ACTH as well as for the opioid peptide β-endorphin[21], all of which are derived from the POMC peptide. Furthermore, the μ and δ-opioid receptor subtypes, for which β-endorphin displays a high

affinity, has also been demonstrated in the same area by immunocytochemistry[27,39]. Therefore we hypothesize that the observed anti-allodynic effects of SHU9119 might be caused by blockade of a tonic influence of endogenous α-MSH on nociception, through the MC4 receptor in the spinal cord. This could tip the balance in favor of the anti-nociceptive actions of β-endorphin, co-released with α-MSH in the same POMC projection areas, thus producing analgesia.

The exact source of spinal POMC expression is not known: it might be intrinsic to the spinal cord[19,20], but may also originate from a supraspinal source, namely the nucleus tractus solitarius[21] or the hypothalamus[40,41]. Elias et al.[40] have demonstrated that hypothalamic POMC expressing neurons innervate the interomedial lateral cell column (IML) at a thoracic level, where sympathetic preganglionic cells are located. In this region MC4-R mRNA is also expressed[42].

The melanocortin system is suggested to play a role in the regulation of autonomic function, since centrally administered melanocortins can increase sympathetic nerve activity[43], possibly through activation of the MC4 receptor[18,42,44]. Although its exact role is still a matter of debate, there are several lines of research indicating that the sympathetic nervous system is involved in neuropathic pain[45-48]. One of the potential mechanisms by which sympathetic activity influences nociception is through an increased norepinephrin responsiveness in C-fibers, the primary afferents activated by noxious stimuli (for review, see Janig et al.[49,50], Bennett[51]).

In this present study we only demonstrated changes in the MC system in areas of the spinal cord that correspond to sciatic nerve input. We can however not exclude the possibility that changes also occur at other spinal levels, or that the ligands we used act through MC receptors located more rostrally. Thus, an alternative explanation for the observed effects of MC receptor ligands on neuropathic pain might be a change in sympathetic activity, mediated at the level of the IML.

As shown in figure 3, baseline values for von Frey and cold stimulation were significantly lower in CCI rats compared to control rats, confirming the development of mechanical and cold allodynia associated with the CCI lesion[22,52]. However, in contrast to other groups[22,52,53], we observe no differences in sensitivity to noxious heat between control rats and CCI rats. The

reason for this discrepancy is not clear but may result from genetic variability between various rat strains. As previously suggested, this may lead to differences in predisposition for the development of neuropathic conditions[54] or in sensitivity to noxious stimuli, due to variations in endogenous opiate systems or adrenergic sensitivity[55,56].
Interestingly, we only observed effects of the MC receptor ligands in CCI rats, and only on responses to cold and mechanical stimulation. From a clinical point of view this is promising, since in this study the effects of melanocortins appear to be specific for the allodynia associated with a neuropathic pain state, without altering normal pain sensation by inducing a more general analgesia. This specificity for the hyperalgesia underlying neuropathic pain without affecting baseline pain detection has also been reported for the α2-adrenergic agonist tizanidine[57].

In summary, in this present study we show that intrathecally administered MC receptor ligands alter the sensitivity to cold and mechanical stimulation in a rat model for neuropathic pain, the CCI. Our data suggest that these effects are mediated through the MC4 receptor located in the spinal cord. SHU9119 produces profound anti-allodynia, whereas MTII and D-Tyr-MTII increase sensitivity to cold and mechanical sensitivity. We therefore suggest that selective MC4 receptor antagonists may be of value in the treatment of neuropathic pain, and that further research into the mechanisms through which the effects of these ligands are excerted is needed.

Acknowledgements

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5

Chronic blockade of melanocortin receptors alleviates allodynia in rats with neuropathic pain

Dorien H. Vrinten
Roger A.H. Adan
Gerbrand J. Groen
Willem Hendrik Gispen

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Abstract

In the present study we investigated the involvement of the spinal cord melanocortin (MC) system in neuropathic pain. Since we recently demonstrated that MC receptor ligands acutely alter nociception in an animal model of neuropathic pain we here tested whether chronic administration was also efficacious. We hypothesise that chronic blockade of the spinal MC system might decrease sensory abnormalities associated with this condition. The effects of the MC receptor antagonist SHU9119 (0.5 μg/day) and agonist MTII (0.1 μg/day) were evaluated in rats with a chronic constriction injury of the sciatic nerve. Drugs were continuously infused into the cisterna magna. Antinociceptive effects were measured using tests involving temperature (10°C or 47.5°C) or mechanical (von Frey) stimulation. Administration of MTII increased mechanical allodynia whereas SHU9119 produced a profound cold and mechanical anti-allodynia, altering responses to control levels. The anti-allodynic effects of SHU9119 were very similar to those produced by the α_2–adrenergic agonist tizanidine (50 μg/day). The effects of SHU9119 and MTII are most likely mediated through the MC4 receptor, as this is the only MC receptor subtype present in the spinal cord. We conclude that chronic administration of MC4 receptor antagonists might provide a promising tool in the treatment of neuropathic pain.

Introduction

Neuropathic pain (pain after a lesion to the central or peripheral nervous system) remains one of the most difficult forms of pain to treat. Conventional treatment with the two major classes of analgesics, non-steroidal anti-inflammatory drugs and opioids, is seldom effective. Moreover, the wide variety of drugs currently used in the treatment of neuropathic pain, including tricyclic antidepressants, anticonvulsants, systemic administration of local anesthetics, and NMDA receptor antagonists do not often provide adequate pain relief[1].

Extensive research using experimental animal models has led to the discovery of an array of potential new drug targets[2]. A possible target in the control of neuropathic pain that has received very little attention is the melanocortin (MC) system. Several lines of research have suggested an involvement of the MC system in nociception. Previous studies have demonstrated hyperalgesia in different tests of acute nociception after intracerebroventricular administration of the melanocortins α-MSH (α-melanocyte stimulating hormone) and ACTH (adrenocorticotropic hormone)[3,4]. In addition, the analgesic effects of morphine and β-endorphin are antagonized by these peptides[5].

At the spinal cord level, the presence of a functional MC system is suggested by the presence of the pro-opio-melanocortin (POMC) derived peptides ACTH and α-MSH, and the MC-4 receptor[6,7]. Interestingly, these are all co-localized in the superficial dorsal horn, an area that is important in nociception. Taken together, these findings suggest an involvement of the spinal cord MC system in nociceptive transmission.

Recently we investigated the spinal cord MC system as a new drug target for the control of neuropathic pain. We have shown that a chronic constriction injury (CCI) of the sciatic nerve[8], a condition that causes a syndrome similar to human neuropathic pain, induces an increase in ^{125}I-NDP-MSH binding to the spinal cord, suggesting an increase in MC receptors. Furthermore, acute intrathecal administration of the MC receptor antagonist SHU9119 (directly into the cisterna magna) reduced cold and mechanical allodynia in CCI rats, whereas MC-4 selective agonists had the opposite effect[9].

Most drugs used in animal models of neuropathic pain are tested in an acute administration paradigm. Since it is a chronic form of pain, drugs suitable for chronic administration, providing long-lasting pain relief, are interesting from a clinical point of view. In light of the anti-allodynic effect of acutely administered SHU9119, in the present study we have investigated the effects of this compound, as well as the MC receptor agonist MTII, upon chronic administration.
The effects of these MC receptor ligands were compared with those of the α_2-adrenergic agonist tizanidine, of which the anti-nociceptive and anti-allodynic actions in experimental animals have been well documented[10,11].

Materials and Methods

All procedures in this study were performed according to the Ethical Guidelines of the International Association for the Study of Pain[12] and approved of by the Ethics Committee on Animal Experiments of the Utrecht University.

Animals and drugs

Forty-eight male Wistar rats weighing 350-400 g at the start of the study were used. They were socially housed in groups of 2-3 on a sawdust bedding. The animals were kept at a 12/12h light/dark cycle, with food and water available *ad libitum*.
Animals were randomly assigned to different treatment groups. CCI rats were treated with either vehicle (N=10), SHU9119, 0.5 μg/day (N=11), tizanidine hydrochloride, 50 μg/day (N=7) or MTII, 0.1 μg/day (N=10). Sham rats (N=10) were treated with vehicle.
SHU9119 (cyclo-[Nle^4, Asp^5, D-Nal(2)^7, Lys^{10}]α-MSH-(4-10)) was synthesized using 9-fluorenyl-methoxycarbonyl (fmoc)-based solid phase synthesis as reported elsewhere[13] and purified using reversed phase preparative high-pressure liquid chromatography (HPLC) to a purity of ± 90%, estimated after analysis by analytical HPLC at 215 nm. Molecular weight was confirmed by mass spectrometry performed on a Micromass Quattro sq.

Tizanidine hydrochloride was purchased from Novartis Pharma AG (Basel, Switzerland). MTII (Melanotan-II or cyclo-[Nle^4, Asp^5, D-Phe^7, Lys^{10}]α-MSH-(4-10)) was purchased from Bachem Feinchemicalien (Bubendorf, Switzerland).

Drugs were dissolved in saline and continuously administered into the cisterna magna via an Alzet osmotic minipump (Charles River, Someren, the Netherlands, type 2002, pump speed 0.5 µl/hr for 14 days).

Surgery

Prior to surgery, all animals were anesthetized with a single subcutaneous injection of Hypnorm (Janssen Pharmaceutical LTD., Grove, Oxford) containing 0.315 mg/mL fentanyl citrate and 10 mg/mL fluanisone (a butyrophenone), at a dose of 0.3 mL/kg bodyweight.

Chronic constriction injury: a chronic constriction injury was produced by placing four loose ligatures of 4-0 chromic catgut (Ethicon INC., Norderstedt) around the nerve as previously described by Bennet and Xie[8]. Subsequently the incision was closed with silk sutures and the animals were allowed a 2-3 day recovery period. For the sham condition the same procedure was performed except placement of the ligatures.

Cisterna Magna cannulation: two weeks after the initial surgery a steel cisterna magna cannula (20 * 0.4 mm) was placed as described by Lankhorst et al[14]. An Alzet osmotic minipump (type 2002, pumping rate 0.5 µL/h, duration 14 days), filled with the appropriate solution, was implanted in the right flank and connected to the cannula by subcutaneously tunnelled polyethylene tubing (PP 25). The incision in the flank was closed with silk sutures. Since the tube connecting the minipump to the cannula was filled with saline and as a consequence of the length of the tube and pump speed, the drugs were delivered into the cerebrospinal fluid (CSF) starting three days after implantation of the pumps. This allowed the animals a three day recovery period before testing was initiated.

The day on which the drugs were delivered into the CSF is referred to as 'treatment day 1'.

Sensory testing

1 Temperature stimulation test

Withdrawal latency to a temperature stimulus was measured by immersing the hind paws on each side into a 10°C or 47.5°C water bath. Upon immersion of the paw an electronical circuit including a timer was closed. Withdrawal of the paw resulted in a discontinuation of the circuit which stopped the timer, thus allowing a precise registration of the withdrawal latency time. Cut-off time was set at 10 s to avoid skin damage. Interval time between consecutive tests was at least 10 min to allow restoration of original foot temperature.

2 Mechanical stimulation test

Prior to testing, each rat was placed in a plastic testing box with a metal mesh floor, and allowed to acclimatize to this environment. Mechanical allodynia was determined by measuring the paw withdrawal threshold following probing of the foot plantar surface with a series of calibrated von Frey filaments (Stoelting, Wood Dale, IL), ranging from 1.08 to 21.09 g. Probing was only performed if the hind paw was in contact with the mesh floor, to correct for paw lifting in response to spontaneous pain. Filaments were applied to the mid-plantar surface of both feet through the mesh floor until the filament bent, and kept in this position for 6-8 s[15]. The smallest force that elicited a foot withdrawal response was considered the threshold stimulus.

Temperature and mechanical stimulation tests were performed the day before the beginning of pharmacological treatment ('baseline value') and at 2-3 day intervals thereafter ('treatment days 1-10'). Throughout the experiment bodyweight was monitored at regular intervals.

Statistical analysis

Data are plotted starting from the day before the onset of treatment ('baseline') through treatment day 10. Withdrawal latencies are expressed as mean ± standard error of the mean (SEM). Withdrawal thresholds to von Frey stimulation are expressed as median and 25^{th} to 75^{th} percentile range, with values plotted on a logarithmic scale.

Differences in bodyweight between treatment groups were compared using

a repeated measures analysis of variance (ANOVA). All other data were analyzed using non-parametric tests. To obtain a linear scale of perceived force in the mechanical stimulation test, withdrawal thresholds were converted to the log of the actual bending force of the filament. Statistical analysis was performed on the transformed data. Differences in baseline values were analyzed using a Mann-Whitney U test. Overall group differences in mechanical and temperature stimulation tests were analyzed by a Kruskall-Wallis test. The effects of the different drugs were compared to the vehicle-treated CCI-group and, in case of a significant difference, to the sham group using a Mann-Whitney U test with Bonferroni-correction. In the mechanical stimulation test, for the CCI group treated with MTII, thresholds on treatment days 1 and 3 were compared to baseline values using a paired T-test. Results were considered significant when $p<0.05$. For the cold and mechanical stimulation tests, the effect of SHU9119 or tizanidine was quantified as the percentage of maximum possible effect (%MPE), using the following formula:

% MPE = 100 x (postdrug value – baseline value) / (cutoff value – baseline value)

Results

At the end of the experiments, proper placement of cannulas and connection to the minipumps was checked. Pump reservoirs were checked to control accurate drug delivery. All reservoirs were empty. One animal was excluded because of improper connection of the pump and one animal died during anesthesia. Both were CCI animals. There were no significant differences in bodyweight between treatment groups at any time point (data not shown).

All CCI groups developed a cold allodynia of the ligated hindpaw as indicated by a significant reduction in withdrawal latency upon immersion in a 10°C waterbath (mean pre-drug value ± SEM for all CCI groups: 5.5 ± 0.7 sec.). In the sham group none of the animals showed signs of cold allodynia (mean withdrawal latency ± SEM: 9.5 ± 0.3 sec). The cut-off latency for the test was 10 s. No signs of cold allodynia developed in the contralateral hind-

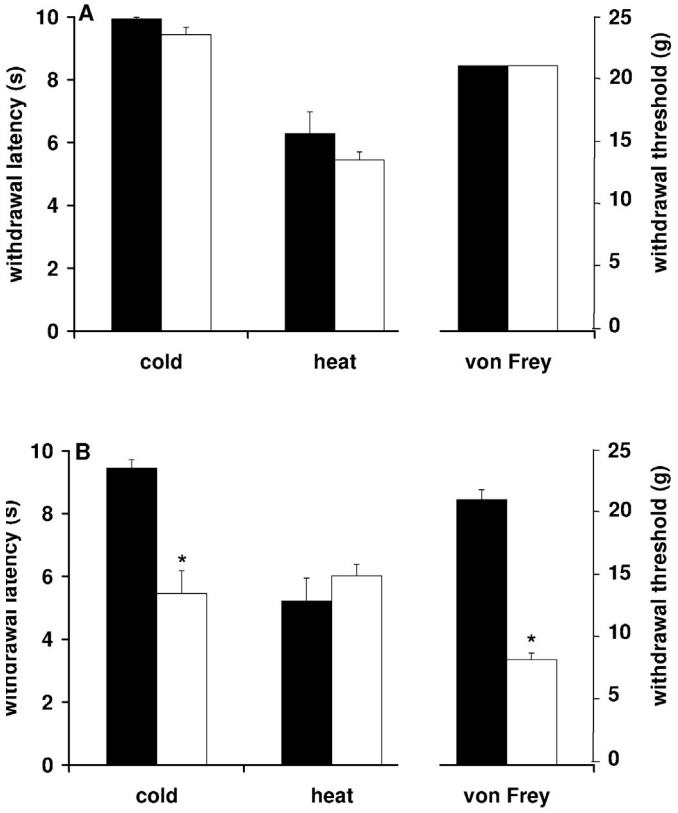

Figure 1.
Baseline withdrawal latencies to cold (10 °C) and heat (47.5 °C) stimulation and baseline withdrawal thresholds to von Frey stimulation in sham (black bars) and CCI (open bars) rats. Measurements were taken on the unoperated (A) and experimental (B) hind paw. Withdrawal latencies are presented as mean ± SEM and withdrawal thresholds as median and 25th-75th percentile range (logarithmic scale) of 10 (sham) or 36 rats (CCI). (* $p<0.05$ vs. sham)

paw of either sham or CCI animals.
There were no significant differences in baseline responses to heat (47.5 °C) stimulation between sham and CCI animals on either side.
CCI animals displayed a tactile allodynia on the ligated side as shown by a significant decrease in withdrawal threshold to von Frey stimulation (pre-drug value ± SEM for all groups of 8.5 [6.2-8.5] g) (median and [25^{th} – 75^{th} percentile]) . Sham animals failed to respond to any filament up to the maximum of 21.09 g, as was the case for the contralateral hindpaw of CCI animals.
These data are summarized in figure 1.

Drug effects

1 Temperature stimulation test

As shown in fig. 2A, treatment with SHU9119 (500 ng/day) significantly prolonged withdrawal latency of the ligated hindpaw to a cold stimulus as

compared to the vehicle CCI-group ($p<0.05$ on treatment days 1 and 3), restoring latencies to near cut-off values (non-significant vs. sham). %MPE was 86.9 ± 8.1 and 90.7 ± 9.3 respectively.

This effect of SHU9119 was similar to that of 50 μg tizanidine/day ($p<0.05$ vs. vehicle CCI-group on treatment days 1 and 3) (fig. 2A); %MPE was 97.3 ± 2.7 and 100 ± 0%, respectively. On treatment day 5 withdrawal latencies decreased again in both SHU9119 and tizanidine treated groups and did not significantly differ from vehicle CCI-group throughout the rest of the testing period.

Treatment with MTII (100 ng/day) resulted in a transient, non-significant decrease in withdrawal latencies of the ligated hindpaw (fig. 2A).

All treatments were ineffective in causing any significant changes in withdrawal latencies to the cold stimulus on the contralateral side. Also, there were no significant differences in withdrawal latency to a heat stimulus between the vehicle treated CCI group and any of the other treatment groups on eihter side (data not shown).

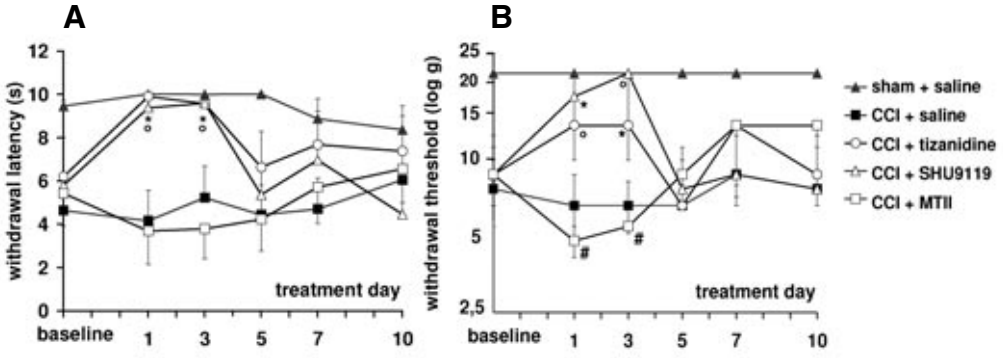

Figure 2.

The effect of SHU9119 (500 ng/day), MTII (100 ng/day) and tizanidine (50 μg/day) on withdrawal latencies to cold (10 °C) stimulation (A) and withdrawal thresholds to mechanical stimulation (B) in rats with a chronic constriction injury. Drugs were continuously administered in the cisterna magna by an osmotic minipump. Data are plotted from the day before beginning of treatment ('baseline') to treatment day 10.

Withdrawal latencies are presented as mean ± SEM and withdrawal thresholds as median and 25th-75th percentile range (logarithmic scale) of 10 (sham, CCI+vehicle and SHU9119), 9 (MTII) or 7 (tizanidine) rats each. (* $p<0.05$, SHU9119 vs. vehicle; ° $p< 0.05$, tizanidine vs. vehicle; # $p<0.05$ vs. baseline)

2 Mechanical stimulation test

As shown in fig. 2B, treatment with SHU9119 (500 ng/day) increased the withdrawal threshold on the ligated side up to 17.0 [13.2-20.9] and 20.9 [13.2-20.9] g (median and [25th – 75th percentile]) on treatment days 1 and 3; % MPE was 73.0 [26.4-100] and 100 [46.6-100] respectively. These thresholds were significantly higher than those in the vehicle CCI group ($p<0.05$) and did not significantly differ from the maximum threshold as observed in the sham operated group.

Administration of 50 μg tizanidine/day resulted in a similar increase in threshold. On treatment days 1 and 3, thresholds were 13.2 [13.2-13.2] and 13.2 [13.2-20.9] respectively ($p<0.05$ vs vehicle CCI group), with corresponding % MPE of 46.6 [24.3-52.2] and 46.6 [37.7-100] (median and [25th – 75th percentile]) Only on treatment day 3 this threshold did not significantly differ from the threshold of the sham-operated group.

On treatment day 5 thresholds in both the SHU9119 and tizanidine treated groups decreased again and were not significantly different from the vehicle CCI group for the remainder of the testing period.

Treatment with 100 ng MTII/day produced a decrease in withdrawal thresholds on treatment days 1 and 3 (median and [25th – 75th percentile] of 4.7 [4.1-5.4] and 5.4 [5.0-5.4]) (fig. 2B). When compared to the vehicle CCI group, this decrease was not significant, due to a slightly higher baseline value in the MTII treated group. However, within the MTII treated group, withdrawal thresholds on treatment days 1 and 3 were significantly lower than baseline value.

None of the treatments caused any significant changes in withdrawal threshold on the contralateral side (data not shown).

Discussion

Here we demonstrate that the MC receptor antagonist SHU9119, when infused chronically into the cisterna magna, reduces cold and mechanical allodynia in CCI rats. Infusion of MTII, a MC receptor agonist, into the cisterna magna induces an increased sensitivity to mechanical stimulation. Since the MC4 receptor is the only MC receptor subtype known to be present in

the spinal cord[16], it is most likely that the observed effects of MTII and SHU9119 are mediated through this receptor, as we have previously suggested[9].

The mechanism through which SHU9119 alleviates allodynia remains to be elucidated. Possibly the endogenous MC receptor agonist αMSH, released in the dorsal horn[6] exerts a tonic influence on nociception. Consistent with this view is the induction of hyperalgesia upon intracerebroventricular administration of MC receptor agonists[3,4] and the observed upregulation of spinal cord MC receptors in CCI rats[9], which could contribute to the increased sensitivity associated with the lesion. We hypothesize that the anti-allodynic effects of SHU9119 are caused by a blockade of the endogenous αMSH tonus.

Melanocortins modulate a variety of body functions, including fever, immunity and body weight homeostasis[17]. The latter has received much attention, since the MC4 receptor has been shown to play a role in disorders of energy balance, such as obesity[18]. In rats, MTII and SHU9119 respectively inhibit and stimulate food intake, when administered intracerebroventricularly[19]. With the use of melanocortins as possible analgesic drugs, changes in bodyweight are unwanted side effects. Therefore, in the present study, drugs were continuously infused into the cisterna magna, downstream of the cerebroventricular system. The osmotic minipumps we used have a very low infusion speed (0.5 μL/h), thus allowing a very gradual release of drugs without creating pressure in the direction of the ventricular system. The continuity and speed of drug delivery by the minipums have been verified earlier in our laboratory (unpublished results). Since at the end of the study pump reservoirs were empty, it is very unlikely that there was backflow from CSF into the pumps. Thus in this way drugs were delivered directly in the CSF surrounding the spinal cord, where their proposed site of action, the spinal MC4 receptor, is located. The doses of SHU9119 and MTII we used here (0.5 and 0.1 μg/day, respectively) have been shown in rats to readily affect bodyweight when administered intracerebroventricularly[19]. Here we show that the anti-allodynia induced by chronic administration of the MC receptor antagonist SHU9119 into the cisterna magna is not accompagnied by any changes in bodyweight, further confirming a spinal site of action.

The magnitude of the anti-allodynic effects we find with SHU9119 are

comparable to those of tizanidine, and were so large that responses were normalized to control (sham) levels. Moreover, the cold anti-allodynia corresponds well with that described by Leiphart et al.[10] (over 80% decrease in paw withdrawal after 50 μg tizanidine intrathecally). In contrast, they describe an MPE of only 19% in the paw pinch test whereas we observed a MPE of up to 46% in the von Frey test with 50 μg tizanidine/day. This might be explained by the fact that the paw pinch test measures pressure hyperalgesia, whereas the von Frey stimulation we used measures mechanical allodynia. It is however difficult to make full comparisons between tizanidine and SHU9119, since here only single doses are tested and the mechanisms of action (involving the α-adrenoreceptors for tizanidine and the melanocortin receptors for SHU9119) are clearly distinct.

In contrast to other groups[8,20] we found no differences in heat sensitivity between CCI and control rats. This discrepancy might result from genetic differences between various rat strains, leading to variations in the predispostion for the development of neuropathic conditions[21] or in sensitivity to noxious stimuli[22]. In the present study, none of the drugs tested induced changes in the CCI animals' responses to heat. Also we have previously demonstrated that injecting a high dose of MTII (500 ng) or SHU9119 (1.5 μg) into the cisterna magna does not affect pain perception in control rats[9]. Taken together, these data suggest that both tizanidine and the melanocortins specifically affect the sensory abnormalities associated with the neuropathic pain state, without affecting normal pain perception. Our data confirm previous reports of this selectivity of tizanidine effects in neuropathic pain[10,11].

In spite of its potent antinociceptive actions in experimental animals, clinical trials performed with tizanidine had less promising results. In patients with trigeminal neuralgia, the efficacy of tizanidine was inferior to that of carbamazepine[23], and there was a rapid recurrence of painful attacks during tizanidine treatment[24].

Levy et al.[11] have reported that upon chronic intrathecal infusion of tizanidine, after several days rats became tolerant to its analgesic effects. In the present study, we observed a similar time-course of the effects of tizanidine. Surprisingly, we found that also MTII and SHU9119 had only temporarily effects. Possibly the organism quickly adapts to both a lack of tonic α-MSH

as well as a continous (over-)stimulation of MC receptors, as occur with chronic infusion of SHU9119 and MTII, respectively. These adaptations or development of tolerance might be suppressed by using other dosages of drugs or different drug administration regimens such as repeated injections at various intervals. Future experiments, employing these strategies, will be helpful in further addressing this question.

However, this rapid decline of effects of the melanocortins should not exclude melanocortin antagonists from further consideration in the treatment of neuropathic pain in humans, since differences in the speed of the development of tolerance between rats and humans do occur. For instance, tolerance to intrathecal morphine develops quickly in rats in several tests of acute nociception[25], whereas in humans morphine tolerance can take several months to develop and can provide long-lasting adequate pain relief in cancer pain[26] and non-malignant pain[27].

In summary, we demonstrate that chronic intrathecal infusion of the MC receptor antagonist SHU9119 has profound anti-allodynic effects in rats with a chronic constriction injury of the sciatic nerve. We suggest that these effects are mediated through the spinal cord MC4 receptor. SHU9119 appears to be specifically effective in altering the sensory abnormalities associated with the neuropathic pain state, without affecting normal pain perception. Therefore we suggest that selective MC4 antagonists might be of value in the treatment of neuropathic pain.

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6

Interaction between the spinal melanocortin and opioid systems in a rat model of neuropathic pain

Dorien H. Vrinten
Willem Hendrik Gispen
Cor J. Kalkman
Roger A.H. Adan

Anesthesiology, accepted

Abstract

We recently demonstrated that administration of the melanocortin-4-receptor (MC_4R) antagonist SHU9119 decreased neuropathic pain symptoms in rats with a sciatic chronic constriction injury (CCI). We hypothesised that there is a balance between tonic pro-nociceptive effects of the spinal MC system and tonic anti-nociceptive effects of the spinal opioid system. We therefore investigated a possible interaction between these two systems, and tested whether opioid effectiveness could be increased through modulation of the spinal MC system activity.

In CCI rats, MC and opioid receptor ligands were administrered through a lumbar spinal catheter and their effect on mechanical allodynia was assessed by von Frey probing. Naloxone (10-100 μg) dose-dependently increased allodynia (% of maximum possible effect (%MPE) of 67 ± 9%), which is in agreement with a tonic anti-nociceptive effect of the opioid system. SHU9119 decreased allodynia (%MPE of 60 ± 13%) and this effect could be blocked by a low dose of naloxone (0.1 μg), which by itself had no effect on withdrawal thresholds. Morphine (1–10 μg) dose-dependently decreased allodynia (%MPE of 73 ± 14% with the highest dose tested). When 0.5 μg of SHU9119 (%MPE of 47 ± 14%) was given 15 minutes prior to morphine there was an additive anti-allodynic effect of both compounds. Together, these data confirm that there is an interaction between the spinal MC and opioid systems and that combined treatment with MC_4-R antagonists and opioids might possibly contribute to the treatment of neuropathic pain.

Introduction

Neuropathic pain is characterised by allodynia (pain due to a normally non-painful stimulus) and hyperalgesia (increased pain in response to a normally painful stimulus). It has become clear that numerous pathophysiological changes in response to neuronal or axonal damage contribute to neuropathic pain[1,2]. Not only neuroatomical changes occur, such as loss of axotomised primary afferent fibers[3], apoptotic cell loss[4] and reorganization of dorsal horn circuitry[5], but also the expression of different neurotransmitters and their receptors in sensory neurons is altered. Such plasticity has been described in a number of messenger systems, including substance P, calcitonin-gene related peptide, cholecystokinin and neuropeptide Y[6,7]. We recently demonstrated that such plasticity also occurs in the spinal melanocortin (MC) system, as demonstrated by an upregulation of MC_4-receptors (MC_4-R) in the spinal cord dorsal horn in a rat model for neuropathic pain, the chronic constriction injury (CCI)[8,9]. Since MCs have been shown to induce hyperalgesia[10,11], this increase in spinal MC_4-R might contribute to the increased sensitivity in neuropathic pain, through activation by the endogenous MC-R agonist α-MSH, which is also known to be present in the dorsal horn[12].

An interaction between the central MC and opioid system has been described previously. MCs can reduce morphine induced analgesia[13,14] inhibit the development of opioid tolerance[13,15], counteract opioid addiction[16] and induce morphine withdrawal-like symptoms[17]. However, it is not known if such a functional antagonism between the MC and opioid system also exists at the spinal level.

It is generally accepted that in neuropathic pain, opioids are less effective. To obtain adequate pain relief high doses of opioids are needed[18], thus their use is often limited by unwanted side effects. Possible explanations for this right-shift in the dose response curve of opioids include a loss of opioid receptors on primary afferent terminals after axotomy[19,20] and an increased activity of endogenous anti-opioids such as dynorphin[21] or cholecystokinin[22,23] in the spinal cord. Considering the functional antagonism between the MC and opioid system it is possible that the aforementioned changes in the spinal MC system in neuropathic pain[9] might also contribute to the reduced analgesic effect of morphine in this condition.

We therefore investigated a possible interaction between MC and opioid systems at the spinal level, by administering different combinations of the opioid receptor antagonist naloxone and agonist morphine, and the MC_4 receptor antagonist SHU9119 and agonist MTII. We hypothesized that through modulation of the activity of the spinal MC system it is possible to increase the effectiveness of opioids in neuropathic pain.

Materials and methods

Animals

30 Male Wistar rats weighing 250-300 g at the start of the study were used. Animals were housed in groups of 2-3 in plastic cages on sawdust bedding. They were kept at a 12/12hr light/dark cycle, with food and water available *ad libitum*. All testing procedures in this study were performed according to the Ethical Guidelines of the International Association for the Study of Pain[24] and approved of by the Ethics Committee on Animal Experiments of Utrecht University.

Surgery

Animals were anesthetized with a single intramuscular injection of Hypnorm (Janssen Pharmaceutical LTD., Grove, Oxford) containing 0.315 mg/ml fentanyl citrate and 10 mg/ml fluanisone, at a dose of 1 ml/kg bodyweight.

4 Ligatures were placed around the right sciatic nerve, as previously described by Bennett and Xie[8], and the incision was closed with silk sutures. Two weeks after the CCI lesion rats were anesthetized with a mixture of O_2/N_2O (1:2) and 2.5% halothane. A lumbar spinal catheter was placed, as described by Storkson et al.[25], and subcutaneously tunneled to the neck region. After recovery from anesthesia, and also at the end of all experiments, proper placement of the catheters was checked by injecting 15 µl of 2% lidocaine, which gives an immediate and short-lasting motor paralysis of the hindlimbs upon intraspinal injection. Due to incorrect placement of the spinal catheter 3 rats were excluded from this study, thus leaving a total of 27 animals. They were allowed to recover before testing was initiated.

Drugs

For *in vivo* administration MTII (Melanotan-II or cyclo-[Nle^4, Asp^5, D-Phe^7, Lys^{10}]α-MSH-(4-10)), SHU9119 (cyclo-[Nle^4, Asp^5, D-Nal(2)^7, Lys^{10}]α-MSH-(4-10)), naloxone (naloxone-hydrochloride) and morphine (morphine-hydrochloride) were used. MTII was purchased from Bachem Feinchemicalien (Buberdorf, Switzerland); SHU9119 was synthesized using Fmoc solid phase synthesis as reported elsewhere[26]. Naloxone and morphine were obtained from the Utrecht University Medical Center Pharmacy.
Drugs were dissolved in 5 μl of saline and injected through the spinal catheter by means of a 25 μl Hamilton syringe, followed by a saline flush (12 μl).

Treatment paradigms

The following 17 different treatments were given: saline, naloxone (0.1, 10, 30 and 100 μg), SHU9119 (0.5 and 1.5 μg), morphine (1, 3 , 10 and 30 μg), MTII (0.5 μg), or the following combinations: SHU9119 (1.5 μg) with naloxone (0.1 μg) pretreatment, morphine (1, 3 or 10 μg) with SHU9119 (0.5 μg) pretreatment, or MTII (1.5 μg) and morphine (100 μg) simultaneously. In experiments where a "pre-treatment" was given, the first drug was given 15 min prior to the second drug.
On a typical testing day all 27 animals were randomly divided into 3 groups (n= 8-9 each, except for simultaneous administration of MTII and morphine, where n=4). Each group randomly received one treatment, with the experimenter blinded to the allocation. Hereafter animals were given at least two days rest, to mimimize any possibility of drug interactions or development of tolerance. On the next testing day all animals were again randomly divided into 3 groups, and 3 other treatments were given. This design was continued until all treatments had been given to one group of animals. Thus in total 17 groups were tested, and therefore each animal was used multiple times on consecutive testing days.

Mechanical stimulation test

Foot withdrawal threshold in response to a mechanical stimulus was determined using a series of von Frey filaments (Stoelting, Wood Dale, IL), ranging from 1.08 to 21.09 g. Animals were placed in a plastic cage with a metal mesh floor, allowing them to move freely. They were allowed to acclimatize

to this environment prior to the experiment. The filaments were presented to the midplantar surface as described by Chaplan et al[27], and the smallest filament eliciting a foot withdrawal response was considered the threshold stimulus.
Baseline values were determined and measurements were repeated 60 min after drug or vehicle administration (in case of a pre-treatment, measurements were taken 60 min after injection of the second drug).

Data analysis and statistics

All data are expressed as mean ± standard error of the mean (S.E.M) for visualization purposes only. Effects of treatments resulting in an increase or decrease in mechanical allodynia are plotted on a negative or positive axis, respectively. For the mechanical stimulation test the effect of different drug treatments was quantified as the percentage of maximum possible effect (%MPE), using the following formula:
% MPE = 100 x (postdrug value – baseline value) / (cutoff value – baseline value).
Non-parametric tests were performed to analyze the data: overall group differences were analyzed by a Kruskal-Wallis test followed by a 2-tailed MannWhitney U test to analyze differences between groups. A probability level of <5% was considered significant.

Results

Baseline mechanical withdrawal thresholds

Two weeks after the CCI lesion, mean baseline withdrawal threshold to von Frey stimulation was reduced from 20.3 ± 0.4 g to 5.3 ± 0.6 g, confirming the presence of mechanical allodynia. Baseline withdrawal thresholds were not altered by placement of the spinal catheters, and remained stable throughout the testing period.

Administration of naloxone, and SHU9119 with low-dose naloxone pre-treatment.

With increasing doses of naloxone (10, 30 and 100 μg), a significant decrease

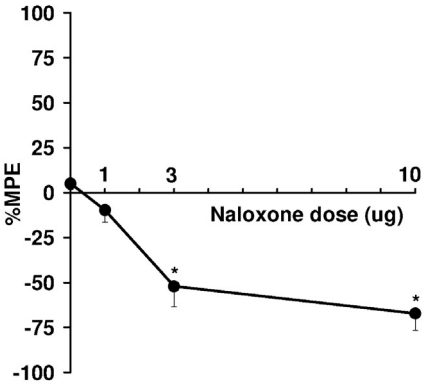

Figure 1. Effects of naloxone on mechanical allodynia
Effects of spinal administration of naloxone on mechanical allodynia, as measured by withdrawal thresholds to von Frey stimulation. Doses ranged from 0 (saline) to 100 μg naloxone. Data are presented as % MPE (mean ± S.E.M) of 8-9 rats each, 60 min after injection of naloxone. (* $p<0.01$ vs. saline)

in withdrawal thresholds was observed, with a %MPE of -67.2 ± 9.3 at the highest dose tested (fig. 1).
1.5 μg SHU9119 increased withdrawal thresholds, with an %MPE of 60.0 ± 13.3. When 0.1 μg naloxone, a dose which by itself had no effect on mechanical withdrawal thresholds, was administered 15 min prior to SHU9119 (1.5 μg), this anti-allodynic effect of SHU9119 was largely decreased (%MPE was reduced to 14.8 ± 10.9, which was not significantly different from saline, fig. 2).

Co-administration of high dose morphine and high dose MTII

Administration of 0.5 μg MTII significantly decreased withdrawal thresholds, with a %MPE of -93.8 ± 6.2, whereas 30 μg of morphine increased thresholds, with a %MPE of 89.6 ± 10.4. A combination of approximately 3

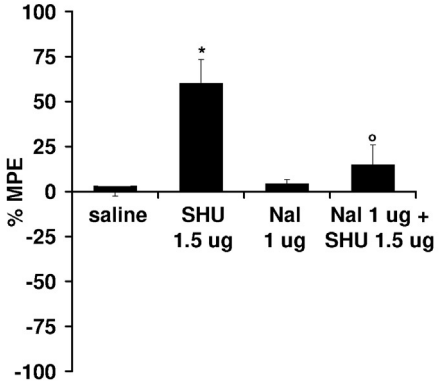

Figure 2. Modification of the anti-allodynic effect of SHU9119 by naloxone
Effect of spinal administration of 1.5 μg of SHU9119 (SHU 1.5), 1 μg of naloxone alone (Nal 1) or 1 μg naloxone 15 min prior to SHU9119 (Nal 1, SHU 1.5), on mechanical allodynia. Mechanical withdrawal thresholds were assessed by von Frey probing. Data are presented as % MPE (mean ± S.E.M) of 8-9 rats each, 60 min after the last injection.
(* $p<0.01$ vs. saline; o $p<0.01$ vs. SHU9119 alone)

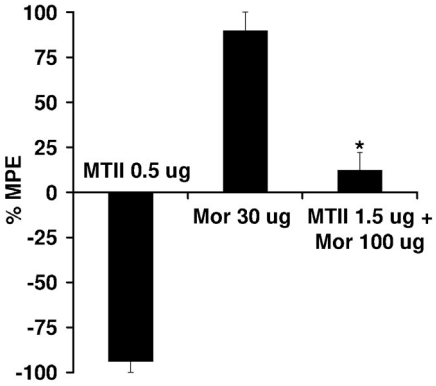

Figure 3. Neutralisation of the pro- and anti-allodynic effects of MTII and morphine by co-administration of both drugs
Effect of spinal administration of 0.5 μg of MTII (MTII 0.5) or 30 μg of morphine (Mor 30) alone, or co-administration of 1.5 μg MTII and 100 μg of morphine (MTII 1.5, Mor 100), on mechanical allodynia. Mechanical withdrawal thresholds were assessed by von Frey probing. Data are presented as % MPE (mean ± S.E.M) of 8-9 rats each (MTII and morphine alone) or 4 rats (combination of both drugs). (* $p<0.05$ vs. MTII or morphine alone)

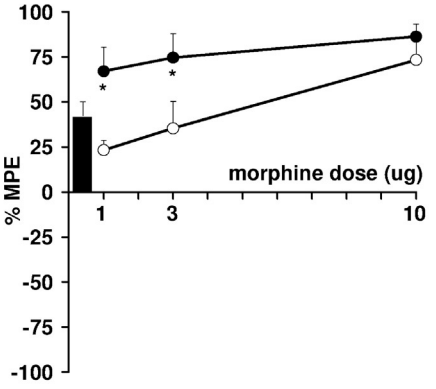

Figure 4. Additive anti-allodynic effects of SHU9119 and morphine
Effects of spinally administered morphine (1, 3 or 10 μg) (open circles) alone, or 15 min after 0.5 μg SHU9119 (black circles), on mechanical allodynia. Mechanical withdrawal thresholds were assessed by von Frey probing. Black bar indicates the anti-allodynic effect of 0.5 mg SHU9119. Data are presented as % MPE (mean ± S.E.M) of 8-9 rats each, 60 min after the last injection.
Data are presented as % MPE (mean ± S.E.M) of 8-9 rats each. (* $p<0.05$ vs. morphine alone)

times higher doses of both drugs (1.5 μg MTII and 100 μg morphine) resulted in an intermediate %MPE of 12.2 ± 9.8, which was not significantly different from saline (fig. 3).

Administration of morphine with SHU9119 pre-treatment

Spinal administration of morphine (1, 3 and 10 μg) produced a significant anti-allodynic effect, as shown by a dose-dependent increase in withdrawal thresholds to von Frey stimulation (fig. 4), compared to saline treatment. A single dose of SHU9119 (0.5 μg) also increased withdrawal thresholds, with an %MPE of 47.3 ± 13.9. When given 15 min prior to morphine, 0.5 μg of SHU9119 resulted in an additive and significant increase in withdrawal thresholds, with a maximum %MPE of 86.2 ± 6.9 (0.5 μg SHU9119 and 10 μg morphine) (fig. 4).

Discussion

In the present study we were able to demonstrate an interaction between the spinal MC and spinal opioid system in a rat model for neuropathic pain. In the spinal cord, the MC_4-R[28,29], as well as α-MSH, an endogenous ligand for the MC-R, and its precursor molecule pro-opiomelanocortin (POMC)[12,29,30] are shown to be expressed in the dorsal horn, an area involved in the processing of nociceptive information. Proteolytic cleavage from POMC yields not only α-MSH but also the endogenous opioid β-endorphin (βEP), which is also present in the dorsal horn[12]. Moreover, both the μ- and δ-opioid-receptors (μOR and δOR) for which βEP displays a high affinity[31], are expressed in the same area[32]. Thus both a functional MC and opioid system appear to be present at the same anatomical site in the spinal cord. Possibly there is a natural balance between these two systems, each with opposite activities on nociceptive processing (see also Adan and Gispen[33]). In neuropathic pain, this balance might be out of equilibrium, by increased activity of the MC system[34] as well as decreased activity of the opioid system[19-22].
Considering this balance between the spinal MC and opioid systems, we proposed that by blocking the pro-nociceptive activity of the spinal MC system with SHU9119[34], tonic analgesic effects of βEP, through activation of OR in the dorsal horn, will predominate (see also Vrinten et al.[35]). As shown in figure 1, administration of naloxone increased mechanical allodynia, consistent with blockade of a tonically active endogenous opioid agonist at the spinal level. This confirms previous work, in which high doses of naloxone increased pain-related behavior in mononeuropathic and spinally injured rats[36,37]. When a low dose of naloxone, which by itself had no effect on allodynia, was administered 15 min prior to SHU9119, the anti-allodynic effect of SHU9119 was almost completely blocked, as can be seen in figure 2.
These data suggest that the MC and opioid systems act at the same common pathway. Other evidence for the close interactions between the MC and opioid system has been provided previously. For instance, chronic morphine treatment is shown to induce a downregulation of MC_4-R in several brain areas[38], whereas ablation of pituitary POMC neurons induces both hypothalamic POMC overexpression and a downregulation of μOR in several brain areas[39]. Our present experiments suggest that an interaction is also pres-

ent at the spinal level. However, from these data it is not possible to establish the neuro-anatomical origin of this interaction. The MC and opioid systems might be organised in a linear pathway, with the MC system either up- or downstream of the opioid system. Another possibility is that the two systems are organised in a parallel fashion. The MC_4-R and OR could be located on different neurons, with pathways converging further downstream, or they could be co-localised on the same neuron. The latter has been demonstrated in locus coeruleus derived cells, where α-MSH and β-EP respectively increase and decrease the level of the second messenger cAMP (via adenylate cyclase) through MC-R and δOR that are expressed in the same cell[40]. Adenylate cyclase might thus function as an integrator of MC and opioid mediated signalling, thereby regulating the output of the cell *in vivo.* A similar mechanism has been proposed to be involved in the effect of MCs on opioid addiction[16].

In order to further investigate the nature of the neuroanatomical substrate underlying the interaction between the spinal MC and opioid system, we tested the effect of co-administration of a high dose of the MC-R agonist MTII and morphine. As seen in figure 3, each of this drugs alone already had an almost maximal effect on allodynia with a 3 times lower dose than we used for the co-administration. When the two systems are organized in a linear fashion it would be expected that the resultant effect of the combined doses on allodynia resembles that of activation of the downstream receptor. Thus when the OR is downstream of the MC-R, this combination of drugs would produce anti-allodynia, and *vice versa*, whereas with parallel pathways the resulting effect would expected to be in between. The net effect of the combined drugs was indeed between those of the separate drugs (see figure 3), suggesting a parallel organization of the MC and opioid system. Since a dose of naloxone which was in itself inactive, blocked the anti-allodynic effect of the MC-R antagonist SHU9119, we conclude that the MC and opioid system converge on the same pathway, possibly the same neurons, involved in nociceptive processing. In order to address the question on which neurons the two systems converge, it will be necessary to conduct neuro-anatomical or electrophysiological studies.

From a clinical point of view an integration of the MC and opioid system is interesting, since by co-administration of MC receptor antagonists it might be possible to modulate opioid effectiveness. We tested this hypothesis by administering SHU9119 15 min prior to morphine. With this treatment paradigm, SHU9119 and morphine had an additive anti-allodynic effect, as shown in figure 4. Although this does not imply a modulation of opioid effect by MC, it raises the possibility that combined treatment with MC_4-R antagonists and opioids might have a place in the management of neuropathic pain. With concomitant administration of MC_4-R antagonists, the total dose of opioid needed to obtain adequate analgesia might be reduced. This might diminish the incidence and severity of opioid side effects, development of tolerance, and possible tolerance-associated hyperalgesia[41,42]. Also, by changing the inter-injection interval of SHU9119 and opioids, it could be that the combined anti-allodynic action is enhanced, as has been demonstrated for the combination of NMDA receptor antagonists and morphine[43].

In conclusion, we were able to demonstrate an interaction between the spinal MC and opioid systems in a rat model for neuropathic pain. The anti-allodynic actions of the MC_4-R antagonist SHU9119 were largely blocked by a low dose of naloxone, whereas SHU9119 and morphine had an additive anti-allodynic effect. It is conceivable that co-administration of MC_4-R antagonists and opioids might contribute to a better treatment of human neuropathic pain.

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7

General discussion

Chronic neuropathic pain is a major clinical problem, since in many cases treatment is inadequate and unsatisfactory. In the past decades our understanding of the pathophysiological mechanisms involved in this condition has increased tremendously. Although a large number of clinical investigations have been performed, much of our insight into the mechanisms underlying neuropathic pain has come from the study of different animal models. In the following paragraphs we discuss various aspects of these animal models, including differences between human and experimental animal situations, and the methods that are used to assess neuropathic pain in animals.
The main focus of this thesis is the role of the melanocortin system in neuropathic pain. We studied this in the sciatic nerve chronic constriction injury model of neuropathic pain. The main findings described in the preceding chapters include plastic changes in the spinal cord MC system in these animals, and an anti-allodynic effect of blockade of the spinal MC_4 receptor, with both acute and chronic administration. Moreover, we demonstrated an interaction between the spinal MC and opioid systems in these rats. These results and their implications are discussed below, and suggestions for future research are given.

1 Experimental animal models of human neuropathic pain

It is unclear to what extent animal models of neuropathic pain can be extrapolated to the human clinical situation. A major difference between patients and pre-clinical animal models is the extremely high incidence of symptoms in the latter. This might be explained by the fact that experimental nerve injuries are tightly controlled in order to be reproducible whereas in humans there is extreme variability in the extent and etiology of nervous system injury. Genetic factors also appear to play a role, since the development of a neuropathic pain syndrome with the same type of lesion can be different between animal strains[1-4]. There are also genetic differences in baseline susceptibilty to pain in animals[5,6]. Moreover, animals selected for various phenotypes in open field activity or autotomy in a neuroma model, differ in their likelihood to develop neuropathic pain symptoms and hyperexcitability of sensory neurons[7-9]. Other factors that influence the development and magnitude of a neuropathic pain syndrome are age, gender and even diet[2,10-13]. Together, these data underscore the importance of genetic and environ-

mental factors in neuropathic pain. Since inbred laboratory animal strains are genetically almost identical, and experimental groups used in studies of neuropathic pain are usually very homogeneous with respect to age, gender and environment this may explain part of the discrepancies between human and animal neuropathic pain.

In chapter 2 we describe the development of a neuropathic pain syndrome with two types of sciatic nerve lesion, the chronic constriction injury and the sciatic nerve crush. Both lesions were performed in young adult male Wistar rats of the same age (8-9 weeks). Irrespective of the difference in time course of the neuropathic syndrome between lesions, which appears to be regeneration-related, the magnitude of sensory abnormalities was similar in both groups. Moreover, we did not observe signs of heat hyperalgesia with either lesion type, in contrast to other groups[14-17]. This further suggests that homogeneity of experimental groups is an important factor in the manifestation of symptoms.

Notwithstanding the many differences between the human and animal situation, several treatment regimens derived from basic animal research have proven to be effective in treating clinical neuropathic pain. For example, blockade of the NMDA receptor, which plays an important role in the development and maintenance of central sensitization, as assessed in animal studies[18,19] was reported to have a positive effect in clinical studies[20]. Also intrathecal administration of adenosine, a drug shown to be effective in preclinical studies, has been reported to be efficacious in human neuropathic pain[21,22]. Moreover, increased understanding of the various mechanisms involved in neuropathic pain has yielded various drug targets, that are currently in clinical and preclinical development[23]. Thus, although they are not perfect, the use of experimental animal models is essential, not only for development and testing of new analgesics for neuropathic pain, but also for increasing our understanding of the neural mechanisms involved.

2 Outcome parameters in the assessment of neuropathic pain

2.1 Spontaneous pain

Hind paw guarding, reluctance to bear weight on the affected limb and paw lifts unrelated to any apparent external stimulus are interpreted as signs of spontaneous pain[24-26]. Also autotomy, the major outcome parameter in animal

models involving complete nerve transsection, such as Wall's neuroma model[27], has been used as an index of spontaneous pain[28-30]. It can be difficult to quantify these manifestations of spontaneous pain and to exclude that these responses are evoked by indistinct external stimuli such as floor contact. Moreover, the question whether autotomy is an expression of spontaneous pain or rather reflects an attempt of the animal to get rid of an denervated body part is still a matter of debate[31,32]. Therefore, most studies focus on stimulus-evoked pain, by investigating the animal's response to various mechanical, temperature or chemical stimuli.

2.2 Stimulus-evoked pain

2.2.1 Thermal stimulation

Increased sensitivity to various thermal stimuli is often employed to study neuropathic pain in animals. Measurements of cold allodynia include the number of foot withdrawal responses after application of acetone[25] or ethyl chloride[33] to the plantar surface of the paw. Heat hyperalgesia can be quantified by determining withdrawal latencies to a radiant heat source, or a CO_2 laser[34,35]. This is however a costly method. Another frequently used and easy method is determining the withdrawal latency or total duration of paw lifting or upon placement on a cold or hot plate[35-37]. A potential drawback of this approach is a paw withdrawal in response to the mechanical stimulus of touching the floor rather than to the temperature stimulus, since heat hyperalgesia and cold allodynia are often accompagnied by mechanical allodynia. Therefore we chose to investigate the effect of temperature stimulation by immersion of the animals paw into a water bath, to minimize any influence of mechanical hypersensitivity. This method has also been used by others[16,17]. In our experimental setup we included an electronical timer with was automatically started upon immersion of the paw and stopped upon withdrawal. This allowed for a rapid and semi-automated quantification of withdrawal latencies upon thermal stimulation.

A possible disadvantage of paw immersion in a water bath is that it is not possible to limit the skin area to which the stimuli are applied to the sciatic nerve's territory, since the whole paw is immersed. Therefore it cannot be excluded that changes in sensitivity are caused by alterations in adjacent intact nerves, such as the saphenous nerve, innervating the medial dorsal part

of the hind paw, as has been suggested by Kingery and colleagues[38]. However, with careful registration of the time course of responses to thermal stimuli and comparison with responses to other stimuli that can be limited to the sciatic nerve's territory, such as von Frey probing, these problems can be circumvented (see also chapter 2).
The waterbath immersion test as we described here can thus be used as a rapid and semi-automated method to quantify responses to various thermal stimuli in animals.

2.2.2 Mechanical stimulation

Hypersensitivity to mechanical stimuli is most commonly assessed by means of probing with von Frey filaments. Although the bending forces of these filaments are very reproducible and inter- or intra-experimenter variability is low[39], there are some problems with this method. Apart from possible effects of temperature, ambient humidity and extensive use on bending forces[40,41], the many ways in which filaments are applied and withdrawal thresholds are determined makes it very difficult to compare results obtained from different studies.
In chapter 3 we compared mechanical withdrawal thresholds determined by von Frey filaments with different parameters of locomotion obtained by CatWalk analysis. With the CatWalk method data acquisition is performed by computer, thus making it a fast and objective method. We found a very high degree of correlation and parallel time courses between von Frey thresholds and pressure applied by the experimental hind paw during locomotion, suggesting that both parameters might reflect mechanical allodynia. Indeed, a decrease in applied pressure has also been described in inflammatory pain, a condition associated with mechanical allodynia[42], and decreased weight bearing of the limb has been shown to be present in neuropathic conditions[26]. We also observed a significant decrease in the duration of the stance phase, probably reflecting a reluctance of the animal to place the affected paw on the floor. This parameter also had a similar time course and a high correlation with von Frey thresholds. It is not likely that these high correlations and parallel time courses between von Frey thresholds, stance phase duration and pressure merely reflect a recovery from locomotor deficits. Nervous system injuries associated with locomotor deficits exhibit

an increase in pressure and stance phase rather than a decrease[43-45]. Moreover, as described in in chapter 2, we found no correlation between locomotor recovery and resolution of mechanical allodynia in CCI rats. Therefore we hypothesise that the pressure and stance phase parameters obtained by the CatWalk method indeed reflect the magnitude of mechanical allodynia. It would be useful to conduct an experiment with anti-allodynic drugs, to investigate whether these CatWalk parameters are indeed altered, to further confirm the usefulness of CatWalk analysis as a measure of mechanical allodynia.

3 Melanocortins in neuropathic pain

3.1 Plasticity of the spinal MC system

Plastic changes in spinal cord and DRG receptors following nerve lesions have been described for many receptors, including opioid (see below), vanilloid[46], metabotropic glutamate (DRG)[47], galanin[48] and GABA receptors[49]. In chapter 4 we demonstrated that spinal MC receptors also undergo plastic changes in CCI rats, as demonstrated by a 25% increase in in situ ^{125}I-NDP-MSH binding to spinal cord dorsal horn as compared to sham operated animals. Since the only known MC-R subtype present in the spinal cord is the MC4-R, this increased binding most likely represents an upregulation of the spinal MC4-R This increase is not robust, but its magnitude is comparable to the changes observed in for instance opiate receptor levels upon nerve injury. Quantitative autoradiography demonstrated an initial increase in dorsal horn μOR of 29%, 2 days after a chronic constriction injury[50], followed by a long-lasting decrease of 28% [51]. De Groot et al found a 17-49% decrease in μOR immunoreactivity two weeks after axotomy[52]. Similarly, Zhang and colleagues found a 30 –50 % decrease in μOR immunoreactivity in the rat dorsal horn in the same model[53]. In the monkey however, this decrease was much larger, indicating that there are species differences in μOR plasticity upon nerve damage[53]. Similarly, differences between monkey and rat have also been described for plastic changes in galanin in the dorsal horn after axotomy, this upregulation being far greater in the monkey[48]. This suggests that, after nerve injury, some plastic changes in the spinal cord are more pronounced in primates (including humans?) than in the rat. At present it is not known whether this also holds true for upregulation of spinal MC receptors.

3.2 The spinal MC4 receptor in neuropathic pain

In spite of the relatively small increase in spinal MC-R, we showed that blockade of these receptors by SHU9119 reduces symptoms of neuropathic pain in CCI rats, whereas activation by MTII and D-Tyr-MTII had the opposite effect (chapters 4 and 5). Also the more selective MC_4-R agonist JK1 increased neuropathic pain symptoms[54], further confirming that these effects are mediated through the MC_4 receptor.

In these experiments ligands were administered through a cisterna magna cannula, directly into the cerebrospinal fluid surrounding the spinal cord. Chronic administration of SHU9119 and MTII had no effect on body-weight, and a single high dose of MTII induced no grooming behaviour, both centrally mediated MC effects (for review, see Adan and Gispen[55]). This suggests that the observed effects of MCs in neuropathic pain are mediated at the spinal level. From these experiments the possibility that these effects are (in part) mediated at the level of the brainstem, an area where MC_4 receptors are also located[56], cannot be excluded. However, the only somatosensory area in the brainstem containing MC_4 positive cells is the caudal part of the spinal nucleus of the trigeminal nerve[56]. Moreover, by using a lumbar spinal catheter as the route of administration, thus limiting the spread of ligands to the lumbar spinal cord[57], similar results are obtained (see chapter 6), further pointing at a spinal site of action.

The early reports describing melanocortin-induced hyperalgesia demonstrated decreased withdrawal latencies to a noxious heat stimulus upon administration of α-MSH or ACTH[58-60]. In contrast, we found no effects of MC_4-R agonists on withdrawal latencies to a heat stimulus in control rats. This might be explained by the fact that in these aforementioned studies melanocortins were administered intracerebroventricularly, whereas we administered ligands at the spinal level, indicating that brain and spinal melanocortin systems might have different effects on nociception.

In summary, our data suggest that the effects of melanocortins on neuropathic pain symptoms are mediated through the spinal MC_4 receptor.

3.3 Modulation of sympathetic activity

The mechanism underlying the effects of melanocortins on nociception is not known. As already mentioned in chapter 4, one possibility is that these

effects are exerted through a modulation of sympathetic activity. It has been demonstrated that central administration of α-MSH or ACTH increased sympathetic outflow and lumbar sympathetic nerve activity[61,62]. I.c.v. administration of the MC-R agonist MTII also increased sympathetic activity, and this effect could be blocked by SHU9119[63]. These effects are likely to be mediated through the MC_4-R[56,61].

In the experiments descibed in chapter 4 we demonstrated an upregulation of MC_4-R at the lumbar spinal level, but we cannot exclude the possibility that upregulation also occurs at other levels of the spinal cord, e.g.more rostrally. Here the intermediolateral nucleus, containing cell bodies of the preganglionic sympathetic fibers, is located. MC_4-R positive cells have been demonstrated in this nucleus, colocalised with choline acetyltransferase, indicating that these neurons are cholinergic in nature (J. Elmquist, personal communication). By using a cisterna magna cannula or a lumbar spinal catheter, drugs are allowed to spread over several spinal segments, thus it is possible that in our experiments sympathetic outflow is modulated.

It has been long known that sympathetic activity can contribute to certain types of neuropathic pain, and sometimes patients benefit from sympathetic blockade (for review, see Janig and Baron[64]). Various studies in animal models have demonstrated adaptations in the sympathetic nervous system in neuropathic pain, and accumulated evidence for the development of a functional sensory-sympathetic coupling[64,65]. After partial nerve injury, primary afferent fibers may begin to express α-adrenoreceptors, thus rendering them sensitive to sympathetic stimulation and norepinephrin[23,35]. Nerve injury also induces sprouting of sympathetic axons into the dorsal root ganglion, where they form baskets around the cell bodies of sensory neurons[23,66,67]. Its relevance in neuropathic pain is however not clear, since Kim and colleagues found no correlation between the magnitude of neuropathic pain symptoms and the extent of sympathetic sprouting[68] (see also Michaelis[65]). Moreover, the effect of sympathicolysis in animal models of neuropathic pain is highly variable[69-71], and has very little effect in the CCI model[35,72,73]. It has been suggested that mechanisms other than sympathetic changes are more important in the CCI model[74].

Therefore we hypothesize that the effects of MCs on allodynia are not mediated through modulation of sympathetic activity, but that other mechanisms

are involved. In view of the presence of both α-MSH and β-endorphin, both derived from POMC, in the dorsal horn, and the abundant evidence pointing at an interaction between the melanocortin and opiate system (see chapter 6), an interaction with the spinal opiate system is a possible candidate mechanism.

4 Melanocortins and the opiate system

Opioids are the cornerstone in the treatment of severe pain, but their effectiveness in neuropathic pain has been a matter of debate for many years. Several clinical studies have demonstrated a lack of potent analgesic efficacy of opioids in neuropathic pain[75,76]. On the other hand, various studies demonstrated pain relief with opioid treatment (for review, see Rowbotham[77]), and it is now generally believed that neuropathic pain is not opioid-resistant but there merely is a reduced sensitivity to these drugs. Thus in order to obtain adequate pain relief higher doses of opioids are needed[78,79], thus their use is often limited by unwanted side-effects[80].

There are several possible explanations for this rightward shift in the opioid dose-response curve, such as a loss of opioid receptors after nerve injury (see above, paragraph 3), or an upregulation of the endogenous opioid functional antagonist cholecystokinin[81,82]. In view of the known functional antagonism between the MC and opioid system (see chapter 1), we hypothesized that the plasticity in the spinal MC system in neuropathic pain we observed[83] might also contribute to the decreased opioid efficacy.

In parallel with the clinical situation, the analgesic effect of opioids is also decreased in animal models of neuropathic pain. Several experimental studies have demonstrated only a weak or absent analgesic effect of intrathecally administered morphine in these models[84-87]. In our experiments intrathecally administered morphine was effective, but the doses we used (approximately 30-90 μg/kg bodyweight) were very high when compared to the human situation, also pointing at a rightward shift of the dose-response curve.

As described in chapter 6, we investigated whether it is possible to increase opioid sensitivity through modulation of the spinal MC system activity. We suggest that there is a functional interaction between the spinal MC and opiate system, as demonstrated by a blockade of the anti-allodynic effect of

SHU9119 by pretreatment with a low dose of naloxone, which by itself had no effect on allodynia.

In our experiments we found no indications for synergistic anti-allodynic effects of SHU9119 and morphine, since when SHU9119 was given 15 min prior to morphine, the effects of both drugs were additive. When morphine and SHU9119 were given simultaneously, this additive effect of SHU9119 was however not observed. Different treatment strategies have been described in other studies demonstrating synergism between morphine and other drugs, in either neuropathic or non-neuropathic conditions. For instance, synergistic anti-allodynic effects have been described with coadministration of ketorolac or neostigmine and morphine[87,88], paracetamol 10 min before or diltiazem 30 min before morphine[89,90] or with gabapentin two hours after morphine[91]. Apparently, the relative timing of administration of both drugs is important. This has also been suggested by Belozertseva and colleagues[92], who demonstrated that the ability of NMDA receptor antagonists to affect morphine antinociception depends on the inter-injection interval. In this regard it is possible that other treatment regimens, e.g. a longer interval between SHU9119 and morphine, or repeated administration of SHU9119 prior to morphine, will give different, possibly synergistic, results.

As described in chapter 4, we propose that the anti-allodynic effects of SHU9119 are caused by blockade of a tonic MC_4-R activation by endogenous α-MSH, thus disclosing the tonic analgesic effects of βEP which is co-released with α-MSH in the spinal cord. Possibly, the rapid decline of the anti-allodynic effects upon chronic administration of SHU9119, as seen in chapter 5, reflects the development of tolerance for the endogenous opioid βEP, instead of tolerance for SHU9119.

This rapid development of tolerance does however not preclude MC_4 receptor antagonists for possible clinical use. As is the case with differences in the susceptibility to neuropathic pain syndromes (see above, paragraph 1), there are also differences in sensitivity to morphine and the development of tolerance between animals[93]. Moreover, there are large differences in the development of tolerance between animals and humans. In animal studies, morphine tolerance can develop already within a few days, or even after a single dose[94-97], whereas in humans long-term opioid therapy can be success-

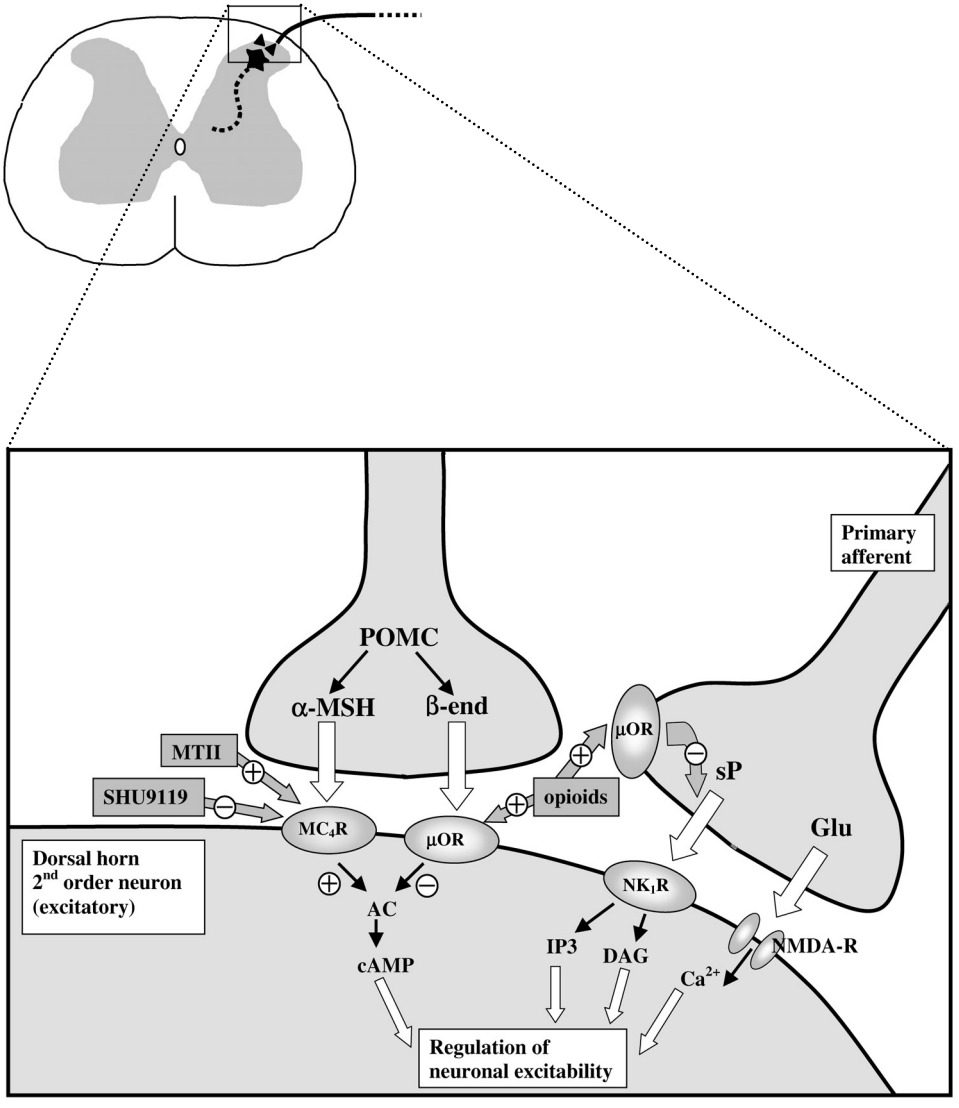
ful[98-100] (see also Rowbotham[77] and chapter 5). .Mayer and colleagues suggested that repeated administration of morphine could not only lead to the development of tolerance but contribute to hyperalgesia as well[101,102]. This might initiate a vicious cycle, leading to progressively higher opiate doses, and subsequently more tolerance and hyperalgesia. With polypharmacy regiments, combining two analgesic drugs with additive effects such as SHU9119 and morphine, it might be feasible to obtain adequate analgesia with a lower total dose of opioids. This would reduce opioid-therapy related problems such as adverse effects, development of tolerance and tolerance-associated hyperalgesia. Moreover, treatment with MC_4-R antagonists might be a useful "add-on" therapy, considering the reduced opioid responsiveness present in neuropathic pain states.

5 Potential avenues for future research.

From the work presented in chapter 4 and 5, we conclude that both acute and chronic intrathecal administration of the MC-R antagonist SHU9119 alleviates symptoms of neuropathic pain in the rat chronic constriction injury model. We hypothesise that these effects are mediated through the spinal MC_4-R. Since the endogenous MC-R agonist α-MSH is co-released with the endogenous opioid β-EP in the spinal cord, we hypothesise that the anti-allodynic effects of MC-R blockade are caused by disturbance of a balance between the MC and opioid system, allowing the anti-allodynic effects of β-EP to predominate. Indeed, an interesting finding in this thesis was an interaction between the spinal MC and opioid system, as demonstrated by a blockade of the antiallodynic effects of SHU9119 by a low dose of naloxone, which by itself had no effect on allodynia (see chapter 6). We could however not provide an anatomical basis for this interaction. Therefore this finding would be greatly supported by neuroanatomical studies, directed at the neural circuitry underlying these effects.

Since no MC_4-R mRNA could be demonstrated in DRG neurons, its presence in the dorsal horn is not likely to be from primary afferent origin[103]. An in situ hybridisation study has demonstrated MC4-R positive neurons in the spinal cord dorsal horn (Elmquist, J., personal communication). Both the μ- and δOR, for which β-endorphin has high affinity, are known to be expressed in the dorsal horn[104-106]. Although the δOR is suggested to play a

Figure 1.
Schematic summary of the possible anatomical organisation of the spinal melanocortin and opioid systems, relative to sP-containing primary afferents in the dorsal horn. A dorsal horn second-order neuron is depicted, which is activated by substance P (sP) and glutamate (glu) released from primary afferents upon noxious stimulation. These postsynaptic responses could be modulated through activation of the melanocortin-4-receptor (MC4R) and m-opioid receptor (μOR) which are oppositely coupled to the adenylate cyclase pathway (AC). In addition, sP release is also inhibited through activation of presynaptic mOR. Sites of action of various ligands affecting neuropathic pain symptoms are also indicated (i.e. MTII, SHU9119 and opioids). For further details, see text.
DAG: diacylglycerol, IP3: inositol triphosphate, NK1-R: neurokinin-1-receptor, β-end: β-endorphin, POMC: pro-opiomelanocortin.



role in antinociception at the spinal level (see Przewéocki[107]), the analgetic effects of opioids, including βEP and morphine, are mostly mediated through the μOR[108-110] and almost all clinically available opioids are μ-selective ligands[111]. Therefore, in future studies into the neuroanatomical basis for the interaction between the spinal MC and opioid system, it appears logical to focus on the μOR.

In the dorsal horn, the μOR is localised both pre- and postsynaptically[104,112,113]. Several lines of evidence have suggested that opioid analgesia is mediated through presynaptic inhibition of stimulation-induced substance P (sP) release from primary afferents[114-117]. However, only few primary afferents exhibit opioid receptor and sP co-localisation[113,118]. Moreover, noxiously evoked sP release from primary afferent origin in the dorsal horn of the cat was unaffected by superfusion with high concentrations of morphine[119]. Many of the μOR positive dendrites in the dorsal horn co-localise with the sP receptor NK1[120], and μOR internalisation in dorsal horn neurons correlates with the level of analgesia produced by the μ-OR agonist DAMGO[121]. These observations have led to the suggestion that μOR ligands modulate sP mediated nociceptive responses predominantly through inhibition of postsynaptic excitatory neurons[120-122]. Indeed, electrophysiological studies have revealed a hyperpolarisation of substantia gelatinosa neurons by DAMGO[123-125]. More specifically, Cheunsuang and colleagues have reported similar results in NK1-positive neurons[126], further confirming that opioid analgesia involves inhibitory mechanisms postsynaptic of sP input. Possibly the neurons that are inhibited by μOR agonists also contain sP, considering the reported co-localisation of the μOR and sP in dorsal horn neurons[118]. Thus, since postsynaptic μORs appear to play a role in antinociception through inhibition of excitatory interneurons and both the μOR and the MC_4-R are localised on local dorsal horn neurons, it is conceivable that they are co-localised on the same cells, which could be containing sP. Hypothetically, the output of these excitatory neurons, involved in nociceptive transmission, might be increased by MC_4-R activation and decreased by μOR activation. At present there are no good MC_4-R antibodies available; therefore one possible neuroanatomical approach to solve this question might be to combine MC_4-R in situ hybridisation with μOR (and sP) immunohistochemistry to demonstrate a possible co-localisation of both

receptor types. Doublestaining for the sP receptor NK1 might demonstrate whether the MC_4 positive neurons are localised post-synaptically of the sP containing afferents, as is the case for the μOR[120]. Since upon noxious stimulation, c-Fos protein is induced in postsynaptic spinal cord neurons[17,127-129], including NK1-positive neurons[130], c-Fos staining combined with labelling of the MC_4 (and NK1) will provide further assessment of the role of MC_4-positive neurons in the nociceptive pathways in the spinal cord. Electrophysiological studies, investigating the effects of combinatined administration of MC_4 receptor and μOR ligands on excitatory transmission in dorsal horn neurons could allow further elucidation of the underlying mechanism of the MC and opioid system interaction (see also figure 1). In our experiments we demonstrated additive anti-allodynic effects of SHU9119 and morphine. However, as discussed above, it is possible that other treatment strategies could further enhance the anti-allodynic effects of this combined treatment. Therefore it will be interesting to test different administration paradigms. Also, since intrathecal catheters are not first choice options in clinical practice, it will be worthwhile to investigate other routes of administration. Moreover, considering the known functional antagonism between the MC and opioid system, it will be interesting to investigate whether modulation of the activity of the spinal MC system could influence the development of opioid tolerance.

In conclusion, the studies described in this thesis, concerning the role of the spinal MC system in neuropathic pain and its interaction with the spinal opioid system, are clearly just the beginning of a new lead in the putative pharmacotherapy of neuropathic pain. From a clinical perspective these results are promising and merit further study, since MC_4-R antagonists and possibly combined therapy with opioids might aid to the relief of human neuropathic pain.

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Nederlandse samenvatting

Neuropathische pijn is gedefinieerd als "pijn veroorzaakt door een lesie of dysfunctie van het zenuwstelsel". Kenmerkende symptomen zijn allodynie (pijn als reactie op een normaliter niet-pijnlijke prikkel) en hyperalgesie (toegenomen pijn als reactie op een pijnlijke prikkel). Dit pijnsyndroom is vaak moeilijk te behandelen en reageert over het algemeen onvoldoende op pijnstillers als NSAIDs en opiaten.
In hoofdstuk 1, de inleiding van dit proefschrift, wordt een overzicht gegeven van neuropathische pijn, waarbij met name wordt ingegaan op de classificatie, symptomatologie, verschillende experimentele diermodellen en tot nu toe bekende pathofysiologische mechanismen.Verder wordt er dieper ingegaan op de mogelijke betrokkenheid van het melanocortine-systeem bij dit type pijn.
Melanocortines zijn peptiden zoals het α-melanocyt-stimulerend hormoon (α-MSH) en adrenocorticotroop hormoon (ACTH), en daaraan gerelateerde peptiden. Ze zijn betrokken bij tal van fysiologische processen zoals pigmentatie, inflammatie, zenuwregeneratie en energiemetabolisme. Er zijn ook aanwijzingen dat melanocortines functionele antagonisten van het opiaat systeem zijn, en dat ze invloed hebben op pijngedrag in proefdieren. De precieze basis van deze effecten is echter onbekend.
In dit proefschrift wordt de mogelijke rol van het melanocortine-systeem in neuropathische pijn onderzocht. Hiertoe werd gebruikt gemaakt van een diermodel: het "chronic constriction injury (CCI)" model in de rat.

In hoofdstuk 2 worden het CCI model en de verschillende uitleesparameters van dit model gevalideerd. In dit model worden een viertal losse ligaturen rond de nervus ischiadicus gelegd. Hierdoor ontstaan zwelling en verhoogde druk in de zenuw en sterft een groot deel van de zenuwvezels af. Dieren met een CCI letsel ontwikkelen na enkele dagen symptomen die sterk lijken op de klinische uitingen van neuropathische pijn, zoals een versterkte reactie op verschillende normaal niet-pijnlijke of pijnlijke prikkels (allodynie en hyperalgesie). Deze symptomen werden gekwantificeerd door de reacties van de dieren op applicatie van kou, hitte, mosterdolie en von Frey filamenten op de achterpoten te meten.

We hebben ratten met een CCI letsel vergeleken met het crush-letsel van de nervus ischiadicus, een veelgebruikt model voor zenuw-regeneratie onderzoek. Beide typen letsels veroorzaakten, naast een verstoring van de locomotor-functie, tekenen van allodynie en hyperalgesie. In het CCI model onstonden deze symptomen al binnen enkele dagen na het aanbrengen van het letsel, terwijl ze in het crush-model pas optraden na regeneratie van de zenuw en herstel van locomotie. Deze resultaten suggereren dat een crush-letsel ook kan dienen als een additioneel diermodel voor neuropathische pijn, met een ander tijdsverloop en onderliggend zenuwletsel dan in het CCI model.

Een veelgebruikte methode om mechanische allodynie te kwantificeren is de bovengenoemde applicatie van von Frey filamenten. Hoewel dit een reproduceerbare methode is, zijn er toch enkele nadelen aan verbonden; testprocedures en definities van drempelwaarden komen niet altijd overeen. Daarnaast zijn de filamenten zelf gevoelig voor diverse invloeden, zoals temperatuur, vochtigheidsgraad en slijtage door veelvuldig gebruik. In hoofdstuk 3 hebben we een alternatieve methode voor het meten van mechanische allodynie onderzocht: de CatWalk. Dit is een systeem, recent ontwikkeld op ons laboratorium, waarbij locomotor-parameters automatisch gekwantificeerd kunnen worden. We hebben gekeken naar de duur van de verschillende fasen van de stap-cyclus en de hoeveelheid druk die door de achterpoten uitgeoefend wordt tijdens het lopen. Het bleek dat dieren met een CCI letsel minder druk uitoefenen met de aangedane achterpoot en dat de duur van de stand-fase van deze poot significant verkort is. Er was een sterke correlatie tussen deze parameters en de mechanische drempelwaarden gemeten met de conventionele von Frey methode. Dit wijst erop dat analyse van relevante CatWalk parameters mogelijk gebruikt zou kunnen worden in het onderzoek naar mechanische allodynie.

In hoofdstuk 4 en 5 is de betrokkenheid van het melanocortine-systeem in neuropathische pijn bestudeerd. Tot dusver zijn er vijf verschillende subtypen melanocortine-receptoren bekend. Hiervan is de melanocortine-4-receptor (MC4-R) de enige die voorkomt in het ruggenmerg, en met name in gebieden die betrokken zijn bij het verwerken van pijnsignalen (nocicepsis),

waaronder de dorsale hoorn.
We hebben aangetoond dat de hoeveelheid binding van ^{125}I-NDP-MSH, een radioactief gelabeld α-MSH derivaat, in de dorsale hoorn is toegenomen in CCI dieren. Dit wijst op plastische veranderingen in het spinale melanocortine-systeem en waarschijnlijk een toename van het aantal spinale MC4 receptoren in neuropathische pijn. Door middel van spinaalcatheters hebben we verschillende melanocortines rechtstreeks in de liquor rondom het ruggenmerg toegediend, zowel acuut als chronisch (m.b.v. een subcutaan geïmplanteerde osmotische minipomp), en de effecten hiervan op de symptomen van neuropathische pijn onderzocht in het CCI model. Onze resultaten laten zien dat activatie van de MC4 receptor, door middel van verschillende agonisten, de symptomen versterkt, terwijl blokkade van deze receptor, door middel van de antagonist SHU9119, de symptomen vermindert. Dit bevestigt dat het melanocortine-systeem inderdaad betrokken is bij nocicepsis, en meer specifiek bij neuropathische pijn.
Het feit dat toediening van de MC receptor antagonist SHU9119 op zichzelf een effect heeft suggereert dat het melanocortine-systeem tonisch pro-nociceptief actief is, waarschijnlijk via de endogene MC receptor agonist α-MSH die aanwezig is in de dorsale hoorn. Blokkade van deze activiteit, door SHU9119, onthult mogelijk een tonisch anti-nociceptief systeem wat zorgt voor de vermindering van symptomen. We veronderstellen dat dit het spinale opiaat systeem betreft. Er zijn een tweetal redenen voor deze veronderstelling: 1) er is reeds bekend dat het melanocortine- en opiaat-systeem functioneel antagonisme vertonen en 2) β-endorfine, een endogene agonist voor de opiaat receptor, is afkomstig van hetzelfde voorloper-molecuul als α-MSH en is ook aanwezig in de dorsale hoorn.
Mogelijk bestaat er in het ruggenmerg dus een balans tussen de pro-nociceptieve activiteit van het melanocortine-systeem en de anti-nociceptive activiteit van het opiaat systeem, en beïnvloeden deze systemen elkaar. Deze hypothese wordt in hoofdstuk 6 onderzocht. Hier tonen we aan dat spinale toediening van naloxon, een opiaat receptor antagonist, de symptomen van neuropathische pijn verergert, wat suggereert dat het opiaat systeem inderdaad tonisch anti-nociceptief actief is. Een zeer lage dosis naloxon, die op zichzelf geen effect had, was in staat om het anti-nociceptive effect van SHU9119 volledig te blokkeren. Dit bevestigt onze hypothese dat er een

interactie bestaat tussen het melanocortine en opiaat systeem in het ruggenmerg. De precieze neuro-anatomische basis voor deze interactie dient nog verder onderzocht te worden.
Daarnaast hebben we gekeken naar het effect van gecombineerde behandeling met SHU9119 en morfine, een opiaat receptor agonist. We hebben aangetoond dat de effecten van deze twee stoffen additief zijn.
Al deze resultaten tezamen worden tenslotte in hoofdstuk 7 bediscussieerd en suggesties voor verder onderzoek worden gegeven. Op basis van de experimenten beschreven in dit proefschrift menen wij dat behandeling met MC4 receptor antagonisten in de toekomst eventueel een plaats verdient in de behandeling van neuropathische pijn. De interactie met het opiaat systeem is interessant, gezien de verminderde effectiviteit van opiaten in neuropathische pijn. Een toegenomen activiteit van het melanocortine-systeem zou mede ten grondslag kunnen liggen aan deze verminderde effectiviteit, en toediening van een combinatie van opiaten en MC4 -receptor antagonisten zou mogelijk bij kunnen dragen aan een adequate behandeling van dit pijnsyndroom.

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Curriculum Vitae

De auteur van dit proefschrift werd geboren op 5 maart 1970 te 's Hertogenbosch. In 1988 behaalde ze het VWO diploma aan het Titus Brandsma College te Vught. Tijdens de laatste twee jaar van het VWO begon ze haar studie aan het Brabants Conservatorium te Tilburg, met als hoofdvakken piano (J. de Tiège en T. Demmers) en kerkorgel (B. Beekman). In 1990 ving ze aan met de studie Medische Biologie aan de Universiteit Utrecht, waar ze in 1991 haar propaedeuse (cum laude) behaalde. In 1994 sloot zij deze studie af (cum laude), met als hoofdvak Moleculaire Neurobiologie bij het Rudolf Magnus Instituut te Utrecht (Dr. P.N.E. de Graan) en als bijvak Biologische Psychiatrie bij de vakgroep Psychiatrie van het Academisch Ziekenhuis Utrecht (Dr. H.G.M. Westenberg). Hierna ving zij aan met de studie Geneeskunde aan de Universiteit Utrecht, waar ze in 1998 haar artsexamen behaalde. In datzelfde jaar begon ze met de opleiding Anesthesiologie bij de Divisie Peri-operatieve zorg, Anesthesie en Pijnbestrijding van het Universitair Medisch Centrum Utrecht (Prof. dr. J.T.A. Knape), gecombineerd met wetenschappelijk onderzoek aan het Rudolf Magnus Instituut te Utrecht (Prof. dr. W.H. Gispen, prof.dr. R.A.H. Adan en prof. dr. C.J. Kalkman). De resultaten van dit onderzoek staan beschreven in dit proefschrift.

Publications

Vrinten DH, Gispen WH, Groen GJ, Adan RA: Antagonism of the melanocortin system reduces cold and mechanical allodynia in mononeuropathic rats. *J Neurosci 2000; 20: 8131-7*

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