Evidence for the involvement of MC4 receptors in the central mechanisms of opioid antinociception

De mogelijke rol van MC4 receptoren
in de centrale mechanismen van opioïde antinociceptie
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de Rector Magnificus, Prof. Dr. W.H. Gispen ingevolge het besluit van het College voor Promoties in het openbaar te verdedigen op maandag 4 april 2005 des middags te 10:30 uur

door

Katarzyna Starowicz

Geboren op 12 september 1976 te Kraków, Polen

Promotores: **Prof. Dr. W. H. Gispen**
Prof. Dr. R. Przewłocki
Co-promotor: **Prof. Dr. B. Przewłocka**

The research described in this thesis was supported by the Institute of Pharmacology statutory funds, and by grant No. K062/P05/2003 from the State Committee for Scientific Research, Warsaw, Poland and by a grant from Utrecht University, The Netherlands as part of the collaborative research program, between the International Institute of Molecular and Cell Biology, Warsaw and the Academical Biomedical Center at Utrecht University. Research was performed at the Department of Molecular Neuropharmacology, Institute of Pharmacology Polish Academy of Sciences, Cracow, Poland and at the Rudolf Magnus Institute for Neuroscience, Utrecht University.

ISBN: 90–393–3933–3

Print: Technet, Kraków

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ABBREVIATIONS

ACTH	adrenocorticotropic hormone
α-MSH	alpha-melanocyte stimulating hormone
Arc	arcuate nucleus of the hypothalamus
AUC	area under the curve
CCI	chronic constriction injury
CCK	cholecystokinin
CFA	complete Freund`s adjuvant
CNS	central nervous system
CP	cyprodime
DAMGO	Tyr-D-Ala-Gly-MePhe-Gly-ol, μ-opioid receptor specific agonist
DRG	dorsal root ganglia
i.c.v.	intracerebroventricular
i.p.	intraperitoneal
i.t.	intrathecal
IR	immunoreactivity
JKC–363	Mpr-Glu-His-(D-Nal)-Arg-Trp-Gly-Cys-Pro-Pro-Lys-Asp-NH_2, a potent and selective antagonist for MC4-R
MC	melanocortin
MCR	melanocortin receptor
MC4R	melanocortin type 4 receptor
MTII	Ac-Nle-Asp-His-D-Phe-Arg-Trp-Lys-NH_2, Melanotan-II, agonist of the melanocortin receptors
NMDA	N-methyl-D-aspartate
NRM	nucleus raphe magnus
PAG	periaqueductal gray
POMC	proopiomelanocortin
ROD	relative optical density
RVM	rostral ventromedial medulla
SHU9119	Ac-cyclo-(Nle^4-Asp^5-D-Nal(2)^7-Lys^{10})α-MSH_{4-10} -NH_2), antagonist of the melanocortin receptors
δOR	delta-opioid receptor
κOR	kappa-opioid receptor
μOR	mu-opioid receptor

Chapter I

General introduction

Contents

1. PROOPIOMELANOCORTIN: A COMMON PRECURSOR OF MELANOCORTINS AND OPIOIDS

The melanocortins and opioid peptides are products of posttranslational modifications of the proopiomelanocortin (POMC) prohormone. These modifications lead to the production of different POMC-derived peptides by different cell types, what, therefore, provides an opportunity for the control of multiple physiological functions by the same prohormone. POMC gene encodes 31 kDa (241 amino acids) precursor protein that is cleaved into a number of biologically active peptides, including adrenocorticotropic hormone (ACTH), α-melanocyte stimulating hormone (MSH), β- and γ-MSH as well as the opioid peptide β-endorphin. POMC is processed by the prohormone convertases PC1 and PC2, which recognize the lysine-arginine pairs and cleave the bond between them (Korner et al., 1991; Zhou et al., 1994). Proconvertase 1 (PC1) generates ACTH and β-LPH, while PC2 generates α-MSH by the cleavage of ACTH. The entire 13 amino-acid sequence of α-MSH is contained within the N-terminal region of ACTH, which is a 39 amino-acid peptide. Schematic representation of POMC processing is shown in Fig 1. Furthermore, carboxypeptidases and aminopeptidases subsequently remove dibasic residues, and enzymatic modifications such as *N*-α-acetylation and COOH-terminal amidation may occur (Gantz and Fong 2003). These processes further regulate final biological activity of the POMC-derived peptides, with α-MSH and β-endorphin being regulated in an opposite manner: N-acetylated MSH displays increased potency (O`Donohuue et al., 1982), whereas N-acetylated β-endorphin is essentially inactive (Akil et al., 1981). Depending on the stimulus and site of production, POMC can be cleaved into an amalgamate of melanocortins and opioids at different proportions. N-acetylation, an enzymatic function controlled by neurotransmitters and tightly associated with the rate of secretion, appears to be a critical point in modulation of this composite signal. Indeed, it has been shown that under the conditions of stimulated exocytosis, the ratio of diacetyl- to desacetyl-MSH released from rat hypothalamic slices increases dramatically (Jegou et al., 1989; Bunel et al., 1990). Thus, the biological signal generated by the co-stored POMC-derived peptides, melanocortins and opioids varies according to the physiological status of the secreting cell. Furthermore, α-MSH and related molecules can stimulate the adenylate cyclase pathway through five distinct receptor types (Low et al., 1994; Siegrist and Eberle 1995), whereas the opioids, decrease cAMP formation through binding to μ, δ and κ receptors (Kieffer 1995; Rene et al., 1998) Hence, recent data suggest that the biological signal(s) generated by POMC may

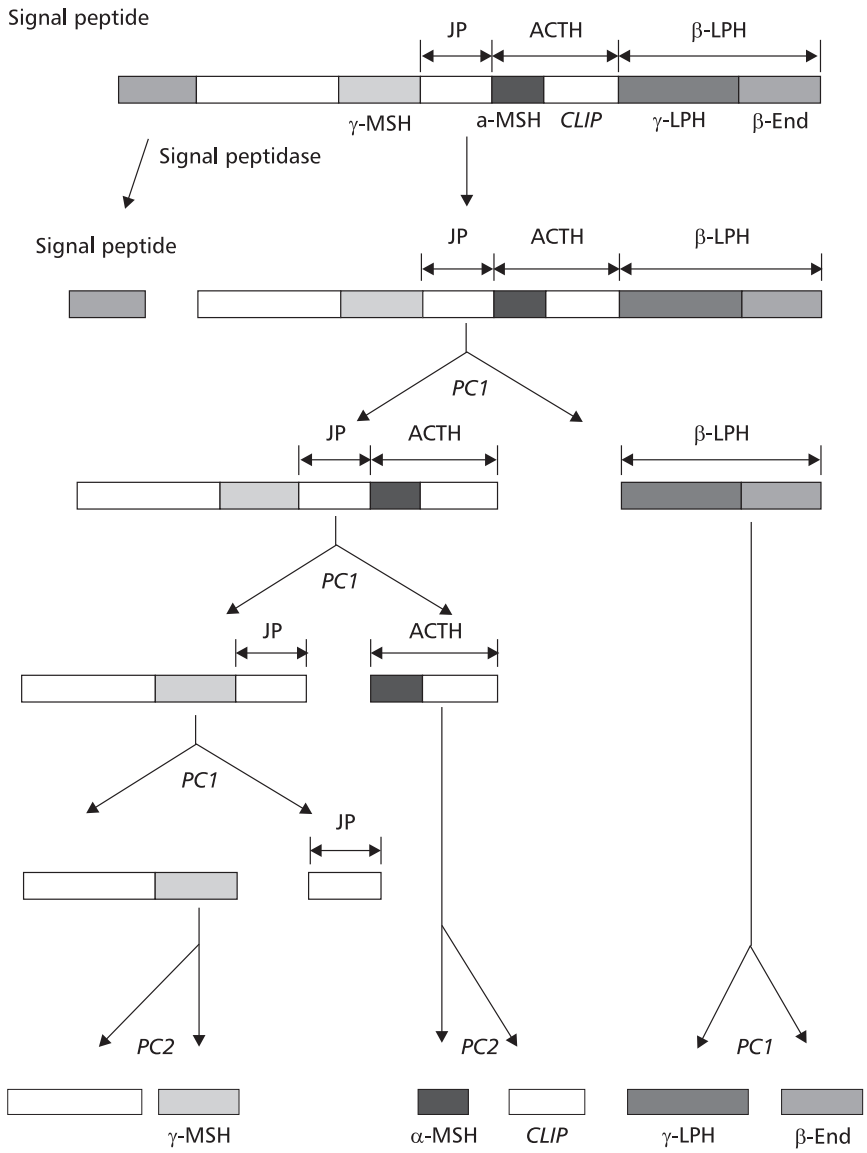

Fig. 1. Proteolytic processing of POMC. After the signal peptide is removed form the pre-POMC, the remaining peptide undergoes an ordered process of endoproteolysis by proconvertase 1 and 2 (PC1 and PC2) at dibasic residues between adjoining domains. PC1 is involved in the early steps of POMC processing and liberates the bioactive peptides adrenocorticotropic homone (ACTH), β-endorphin and γ-lipotropic hormone (γ-LPH). PC2 cleaves ACTH into corticotrophin-like intermediate lobe peptide (CLIP) and α-melanocyte stimulating hormone (α-MSH) and also releases γ-MSH from the N-terminal portion of the propeptide. The joining peptide (JP) is the region of the precursor between ACTH and γ-MSH. *Adapted form: Molecular neuropharmacology, by E. Nestrel, S. Hyman and R. Malenka; 2001.*

ultimately be mediated by a family of peptides which are in complex functional interaction with each other. The presence of POMC-derived peptides in supraspinal and spinal central nervous system pathways as well as in the pituitary gland, involved in stress-related phenomena, suggests that POMC may serve as a link between stress response systems in the body and endogenous pain control (Roberts et al., 1993; Przewłocki and Przewłocka 2001; Przewłocki 2002, van Meeteren et al., 1997; Akil et al., 1986). It is likely that both melanocortin and opioid receptors that bind POMC-derived peptides may mediate opposite biological functions.

2. OPIOID SYSTEM

2.1. Endogenous opioid peptides

The first indication of existence of endogenous opioids came from studies showing that brain extracts contained opioid-like activity (Terenius and Wahlström 1974; Kosterlitz and Waterfield, 1975). Further investigations led to the isolation and characterization of the enkephalins, the first discovered endogenous opioids (Hughes et al., 1975). There appeared to be two pentapeptides, Met- and Leu-enkephalin. The structure of Met-enkephalin was also present as the N-terminal part of the C fragment, a part of the earlier isolated fat-mobilizing pituitary hormone β-lipotropin (Bradbury et al., 1976). The C fragment, was termed β-endorphin (after endogenous morphine). Enkephalins and β-endorphin were shown to induce similar actions as morphine in a number of *in vitro* and *in vivo* test procedures, in particular they showed analgesic activity. Both, β-endorphin and the enkephalins were self-administered by laboratory animals, indicating the rewarding properties and addictive liability of these substances (Belluzzi and Stein 1977; Van Ree et al., 1979; Goeders et al., 1984). Furthermore, repeated administration of the peptide led to tolerance to its analgesic action and to morphine-like withdrawal symptoms upon a challenge with naloxone (Van Ree et al., 1976; Wei and Loh 1976). Thus, the endogenous opioids share all typical opioid-like actions with morphine, both after acute and chronic administration. A few years later, another class of endogenous opioids, the dynorphins were discovered (Goldstein et al., 1979; 1981). Endogenous opioids are generated by enzymatic processing from three precursor molecules: pro-opiomelanocortin (POMC), proenkephalin (PENK), and prodynorphin (PDYN) (Nakanishi et al., 1979; Kakidani et al., 1982; Noda et al., 1982). These precursor molecules are

translation products of separate genes. Their structures have been determined using recombinant DNA techniques (e.g. Numa 1984). The proteolytic processing of the precursor proteins and the receptor selectivity of the opioid peptides has been reviewed in detail (e.g. Höllt 1986). Each of these precursors has a unique anatomical distribution throughout the central nervous system (CNS) and in peripheral organs (Akil et al., 1984; Khachaturian et al., 1985). The anterior and neurointermediate lobes of the pituitary gland are major sites of POMC biosynthesis. In the brain, there are two distinct nuclei that contain POMC mRNA: the arcuate nucleus of the hypothalamus and the nucleus tractus solitarius. These nuclei send their widespread projections throughout the brain. PENK-containing neurons are widely distributed throughout the brain and comprise neurons both forming local circuits and extending long projections to remote areas. PENK is the source of Leu- and Met-enkephalin and several longer forms of these pentapeptides. PDYN-containing cell bodies have a characteristic widespread distribution throughout the CNS. PDYN-containing neurons have both short- and long-projection pathways and can generate several opioid peptides, including α- and β-neoendorphin, dynorphin A, and dynorphin B (van Ree et al., 1999). Recently, a novel group of peptides has been discovered in the brain and named endomorphins: endomorphin–1 and endomorphin–2. They are unique in comparison with other endogenous opioid peptides, having characteristic structure and exhibiting high selectivity for μ-opioid receptors (μORs) (Zadina et al., 1997). Their analgesic properties are subject of extensive studies (Przewłocka et al., 1999, Przewłocki et al., 1999; Soignier et al., 2000, 2004).

2.2. Opioid receptors

Martin et al. (1976) were the first who postulated the existence of multiple types of opioid receptors. Based on behavioral and neurophysiological findings in the chronic spinal dogs, they distinguished between the μ-type (for morphine), the κ-type (for ketocyclazocine), and σ-type (for SKF10,047 or *N*-allylnormetazocine) receptors. Later, the fourth type of opioid receptor, named δ (from vas deferens) was identified (Lord et al., 1977). Additional research revealed that the σ-type receptor was nonopioid in nature (Manallack et al., 1986; Roman et al., 1988), leaving three main type of opioid receptors, μ, δ, and κ. Almost 20 years after the existence of opioid receptors had been suggested, a complementary DNA (cDNA) encoding the mouse δOR was isolated simultaneously by 2 independent laboratories using expression cloning in mammalian cells (Evans et al., 1992; Kieffer et al., 1992). Cloning by homology was then accomplished using mouse δOR as a probe in polymerase chain reaction or low-stringency screen-

ing procedures, leading to the identification of the human homologue (hDOR) as well as cDNAs encoding μ- and κORs in rodents and humans (Kieffer 1995; Satoh and Minami 1995). Sequence analysis confirmed that these genes belong to the G-protein-coupled receptor family. The cloned receptors are highly homologous, differing only at the N- and C-termini and at the extracelluar loops that confirms different binding specificity (Reisine and Bell 1993).

Interestingly, there seems to be some preference of different endogenous opioid ligands for the different receptors, viz. β-endorphin preferentially binds to μ receptor, enkephalins to δ receptor, and dynorphins to κ receptors. Classification of these receptors into subtypes has been proposed (μ_1, μ_2; δ_1, δ_2; κ_1, κ_2, κ_3) on the basis of pharmacological studies (e.g. Dhawan et al., 1996) and some evidence is available that some other receptor types may exist [e.g., the ε-receptor which was labeled as β-endorphin specific (Wüster et al., 1979; Narita and Tseng 1998). The International Union of Pharmacology subcommittee on opioid receptors has proposed another terminology to distinguish the opioid receptors: OP1, OP2, and OP3 for the δ, κ, and μ receptor, respectively (Dhawan et al., 1996) (Table 1).

An interesting consequence of opioid receptor cloning was the isolation of the fourth, highly homologous gene. Another opioid-like receptor has been cloned, termed ORL–1. i.e. opioid receptor-like recoptor–1 (Fukuda et al., 1994; Mollereau et al., 1994; Lachowitz et al., 1995). This receptor has nearly 70% sequence homology with the opioid receptors. The ORL–1 receptor does not bind opioids with high affinity. However, an endogenous peptide was later isolated and characterized as a high-affinity ligand of this receptor and referred to as nociceptin or orphanin FQ (Meunier et al., 1995; Reinscheid et al., 1995). The functional role of this novel neurotransmitter system is the subject of intensive investigations (Butour et al., 1997; Darland et al., 1998).

Table 1. Nomenclatures of opioid receptors, International Union of Pharmacology (IUPHAR) recommendations, for details see: Dhawan et al. (1996).

Preferential Endogenous Opioid Ligands	Opioid Receptors		
	IUPHAR Recommendation	Pharmacology Nomenclature	Molecular Biology Nomenclature
Enkephalins	OP1	δ	DOR
Dynorphins	OP2	κ	KOR
β-endorphin	OP3	μ	MOR

As already mentioned, endogenous opioid ligands display preference for different opioid receptors. The μOR binds morphine but endomorphins may be its endogenous ligands although this receptor is the main target for β-endorphin or longer peptides derived from PENK. β-endorphin also binds to δORs. The enkephalins bind to the δOR with great affinity but they also interact with μOR, though to a lesser extent. Dynorphins bind primarily to κORs (Chavkin et al., 1982; James et al., 1982) but they also might interact with μOR. The majority of opioid peptides do not bind exclusively to one specific opioid receptor type, but have some affinity and may interact with other opioid receptors as well.
The discovery of a multiplicity of opioid receptors and the endogenous ligands thereof raised a question of what is the role of individual receptor types and their potential therapeutic importance in response to pain. Several research groups addressed this issue by evaluating agonists acting at multiple opioid receptor sites, and their utility as antinociceptive agents has been demonstrated. However, in elucidating the role of multiple opioid receptors in response to pain, the μOR has been the focus of much attention.

2.3. Opioid analgesia

The endogenous opioid system regulates nociception. In the past years, the opioid peptide precursor genes PENK, PDYN and POMC and genes encoding μ, δ and κ receptors have been inactivated in mice by homologous recombination (Kieffer and Gaveriaux-Ruff 2002). The analysis of behavior in mutant mice has demonstrated significant roles of each gene in modulating locomotion, pain perception and emotional behaviors (Kieffer and Gaveriaux-Ruff 2002) and allowed for an evaluation of the specific contribution of each receptor. Most relevant are the studies of nociceptive responses of mutant mice in acute pain models. Mice lacking μOR exhibited lower pain thresholds in nociceptive responses to thermal stimuli, both in the tail-flick and the hot plate tests. In these tests, κOR-deficient mice showed no alteration but otherwise displayed a dramatically enhanced response to acetic acid injections (writhing test) compared with wild type mice (Simonin et al., 1998). These data highlight a specific contribution of μOR and κOR receptors to regulation of responses to different noxious stimuli, namely thermal and visceral pain. No change in pain perception was described in δOR-deficient mice (Zhu et al., 1999). Moreover, using distinct targeting strategies, several research groups have obtained mice lacking each gene encoding opioid peptides. Mice lacking POMC and other opioid peptide precursor genes have been produced (Rubinstein et al., 1996; Yaswen et al., 1999). Two strains of PENK gene-knockout mice have been described (König et al.,

1996; Ragnauth et al., 2001). Mice lacking the PDYN gene were obtained in two laboratories (Sharifi et al., 2001; Zimmer et al., 2001). No change in baseline sensitivity to thermal stimuli was reported in mice devoid of β-endorphin, but PENK- and PDYN-knockout mutant mice showed increased pain response in the hot plate and tail-flick tests, respectively (König et al., 1996; Wang et al., 2001). Up until now, only PDYN-knockout mice were analyzed in a model of persistent neuropathic pain (Wang et al., 2001). After spinal nerve ligation, wild-type mice showed sustained increased response to noxious thermal, as well as innocuous mechanical stimulation. The knockout mice showed first similar responses, but both thermal and mechanical sensitivities were close to baseline values within a few days. Together with indications of dynorphin up-regulation, these data highlight the pronociceptive activity of dynorphin in the maintenance of neuropathic pain. The analysis of responses of mutant mice to exogenous opiates has definitely clarified the essential role of μOR in both morphine analgesia and addiction, and have also demonstrated that δOR and κOR remain promising targets for pain treatment (Kieffer and Gaveriaux-Ruff 2002).

2.3.1. Supraspinal analgesia

Numerous supraspinal sites of μOR-mediated analgesia have been established (Besson and Chaouch 1987). They have been localized to areas in the medial brain stem around the nucleus raphe magnus and extending rostrally to periaqueductal and periventricular grey and other areas with the monoamines appearing to be critical transmitters in these pathways (Yaksh et al., 1988). The roles of these areas in morphine analgesia have been evaluated in microinjection studies and confirmed by the ability of naloxone applied locally into these areas to reduce the effects of systemic morphine treatment (Dickenson 1997). Microinjection studies into the rat brain have identified a number of regions sensitive to morphine, including the periaqueductal gray, locus coeruleus, nucleus raphe magnus, nucleus gigantocellularis and amygdala (Jacquet and Lajtha 1973; Mattia et al., 1991; Pert and Yaksh 1974; Mansour et al., 1987; Pickel and Colago 1999). Additional studies allowed for classification of these actions via μ_1-OR based on the activity of selected agonists and their sensitivity to naloxonazine (Bodnar et al., 1988). Supraspinal analgesic mechanisms mediated by μORs have also been demonstrated in mice (Heyman et al., 1988; Ling and Pasternak 1983; Paul et al., 1989; Pick et al., 1992). Agonists of μOR acting at supraspinal site have been shown to be involved in antinociceptive (and antihyperalgesic) effects against different forms of noxious stimuli (Goodchild et al., 2004; Pham et al., 2003; Zurek et al., 2001; Harte et al., 2000).

The δOR ligands are far less active supraspinally than spinally, leading some investigators to speculation that supraspinal δ systems are not very important in pain modulation (Porreca et al., 1984, 1987).
The involvement of PDYN-derived peptides on nociception is not as clear as those originating form POMC or PENK. For example dynorphin, agonist of κOR does not influence the nociceptive threshold to thermal stimulation after i.c.v. injection (Walker et al., 1982). Interestingly a synthetic κOR agonist [D-Pro10]-dynorphin(1–11) administered i.c.v., did not show any activity against thermal stimulus but, in contrast, produced a dose-related effect against chemical pain (Gairin et al., 1988).

2.3.2. Spinal analgesia

The spinal cord is an important site of action of opioids. Early observations have indicated that opiates can produce a significant reduction in the magnitude of the reflex activity evoked by the activation of high-threshold cutaneous afferents in animals with transsected spinal cord as measured by motor (Bodo and Brooks 1937) or spinal electrophysiological (Fujita et al., 1953, 1954) responses. These observations provided the evidence that opiates could exert a direct and powerful influence upon spinal nociceptive reflexes. Studies in chronically catheterized animals revealed that in spite of the powerful effect upon motor cells in the ventral horn, intrathecal opioids could selectively block the response of the animal to high-, but not low-intensity stimulation i.e. could produce analgesia (Yaksh and Rudy 1976) with no effect upon motor activity.
In terms of relative density of binding sites, the density of all three classes of opioid binding sites are higher in the dorsal horn than in the ventral horn, while in terms of relative levels of total spinal binding, μOR represents the highest level, and κOR binding is greater than δORs. One of the major sites through which opioid receptor agonists produce antinociception are the superficial layers of the dorsal horn in the spinal cord (lamina I and II), where the main terminals of thin somatosensory afferent fibers are localized (Dickenson et al., 1987, Light and Perl 1979; Sugiura et al., 1986). Behavioral and pharmacological studies have shown that spinal opioid antinociception likely involves all types of opioid receptors (Besse et al., 1990., Schmauss and Yaksh 1984, Stevens and Yaksh 1986). All opioid receptor types have been localized by receptor autoradiography using radiolabeled ligands in the superficial layers of the dorsal horn of the rat (Waksman et al., 1986), primate (Wamsley et al., 1982) and human (Faull and Villiger 1987). Estimates of the relative densities of the μ-, δ- and κ-binding sites in lamina I and II of the spinal cord vary, but at least in rodents,

μORs are the most abundant. It has been estimated that μORs represent 70%, δ- 24% and κ- 6% of opioid binding sites in the rat spinal cord (Besse et al., 1990). Studies on opiate receptor binding sites in human spinal cord revealed that κ-receptors were the predominant type (approximately 50%), followed by μ-receptors (approximately 40%), and only small numbers of δ-receptors were detectable (Czlonkowski et al., 1983).
Within the spinal cord opiates can act either presynaptically to inhibit the synaptic transmission (Macdonald and Nelson 1978) or postynaptically to increase potassium conductance thus resulting in hyperpolarization. A presynaptic mechanism of opioid interference with the perception of pain is supported by biochemical studies suggesting that opiate drugs, including morphine, and opiate peptides, such as enkephalin, can reduce the potassium-evoked release of glutamate (Grudt and Williams 1994) and/or substance P (Go and Yaksh 1987) from nociceptive afferents in the dorsal horn. The reduced release of these excitatory transmitters from primary afferents would markedly diminish the response of neurons in the dorsal horn to sensory input. A postsynaptic mechanism of opioid action is supported by previous studies showing that endogenous opioid peptides, Met^{4}- and Leu^{5}-enkephalin hyperpolarize neurons within the spinal cord by increasing potassium conductance (Williams et al., 1982).

2.3.3. μ-opioid receptor in nociception

The μORs are widely distributed throughout the CNS with particularly dense binding observed in the basal ganglia, limbic structures, thalamic nuclei and regions important to nociceptive and visceral regulation (Mansour and Watson 1993). In the rat, the frontal, parietal and temporal corticies show dense μ-receptor binding in layers I and IV of the cerebral cortex, with light and moderate densities in the remaining cortical layers. The amygdaloid nuclear complex is an area of dense μ-receptor binding. In the rat hippocampal formation, μ-receptor binding is dense in the stratum lacunosum-moleculare, the pyramidal cell layer and the molecular and granular cell layers of the ventral dentate gyrus. In the caudate-putamen of the rat, μ-receptor binding sites are the densest in patches and in the subcallosal streak. The rat hypothalamic nuclei display little or no μ-binding. More rostally, μORs are densely distributed in the thalamus. In the mesencephalon, μ-binding sites are seen in the periaqueductal gray (PAG), superior and inferior colliculi, interpeduncular nucleus, substantia nigra and raphe nuclei. More caudally in the brainstem, dense levels of μ-binding sites are seen in the nucleus tractus solitarius and the spinal trigeminal and parabrachial nuclei (Mansour and Watson 1993). Studies on the location of binding have shown it to

be heterogeneous within the dorsal horn: μOR binding is relatively the highest in the substantia gelatinosa and lamina III, IV, V and VIII, δOR binding tends to be the highest in the marginal layer (lamina I) whereas κOR binding has been shown to be the highest in the substantia gelatinosa (Yaksh 1993). Estimates of the relative densities of the μ-, δ-, and κ-binding sites in lamina I and II of the spinal cord vary, but, at least in rodents, μORs are the most abundant and predominately located on primary afferents (Besse et al., 1990).

Clearly the role of opiates as analgesics is supported by their localization in the dorsal horn of the spinal cord, PAG and thalamus. The μOR appears to be critical for the opioid analgesia. The ability of the supraspinally and spinally administered μ-opioids to produce analgesia is well documented. Agonists at μORs act on brain structures to produce analgesia by activating the descending pain modulatory pathways (Light et al., 1986; Renno et al., 1992). Evidence is accumulating for the role of central POMC system in nociception. The μOR agonists, β-endorphin, morphine or Tyr-D-Ala-Gly-MePhe-Gly-ol (DAMGO) administered into lateral ventricles produce strong analgesia both in animals and in humans (Yaksh 1983; Pavlovic et al., 1996; Onofrio and Yaksh 1983). The μORs are found in the PAG region (Mansour et al., 1995), which is a brain region that participates in descending antinociceptive control. Fields (1983) demonstrated that electrical stimulation of PAG or medullary nucleus raphe magnus (NRM) neurons stimulated opioid peptide secretion. Focal electric stimulation in the PAG produces a profound antinociception, and this effect was antagonized by naloxone (Akil et al., 1976; Yaksh et al., 1976). Furthermore, microinjection of morphine into the ventrolateral PAG inhibits pain behavior in animals (Yaksh et al., 1988; Behbehhani 1995). However, morphine is believed to increase activity of PAG output neurons projecting to the medullary nucleus raphe magnus. This results in activation of NRM neurons that project to the spinal dorsal horn. They inhibit nociceptive transmission in the spinal cord. This idea is based largely on the observation that many PAG neurons project to the NRM but few of them project directly into the spinal cord (Kuypers and Maisky 1975; Castiglioni et al., 1978; Mantyh and Peschanski 1982). To explain the excitatory effect of opioids, a mechanism of disinhibition has been proposed (Yaksh et al., 1976; Basbaum and Fields 1984; Reichling et al., 1988; Fields et al., 1991). It has been suggested that PAG-NRM projection neurons are tonically inhibited by GABAergic interneurons in the PAG. The μORs are often located on GABAergic PAG neurons (Kalyuzhny and Wessendorf 1998). Opioids block the activity of interneurons, thus disinhibiting PAG-NRM neurons (Reichling and Basbaum 1990; Williams and Beitz 1990).

The dorsal horn of the spinal cord is an important site of the analgesic action of μOR agonists. A powerful analgesia is produced following intrathecal administration of opioids in rats (Yaksh and Rudy 1976) and in humans (Onofrio and Yaksh 1990). As already reported by Dickenson et al. (1987) μORs could modulate the transmission of nociceptive information at the spinal cord level. The intrathecal administration of μOR agonist, DAMGO produced dose-dependent inhibition of C-fibre-evoked neuronal activity (noxious stimuli) whilst A-fiber activity (provoked by natural innocuous stimuli) was relatively unchanged. DAMGO-produced inhibition was completely reversed by naloxone.

There is much evidence for a presynaptic action of opioids from studies of opioid-induced inhibition of C-fiber evoked release of transmitters (substance P and glutamate) and from *in vitro* and *in vivo* electrophysiological studies (Yaksh and Noueihed 1985). Their pre-synaptic actions on transmitter release result from an opening of potassium channels (μ- and δ-receptors) or a closing of calcium channels (κ) both of which lead to a reduction in calcium influx into C-fiber terminals thereby diminishing transmitter release (North 1989). Consequently, the presynaptic opioid action consisting in reduction of the release of many transmitters will be a highly effective route to induce analgesia since it will equivalently block multiple postsynaptic receptors. Evidence of functional opioid actions at postsynaptic receptors is based on electrophysiological and behavioral approaches (Duggan and North 1984; Lombard and Besson 1989). Postsynaptic hyperpolarization again results from the opening of K^+ channels or the closing of calcium channels (North 1989). These receptors could hyperpolarize the dendrites of projection neurons, interneurons (both would be selective for noxious transmission) or the cell body of projection cells which may not be selective for nociceptive inputs, since many but not all neurons in the dorsal horn receive both nociceptive and tactile inputs. The disinhibitory effect of opioids mediated via GABAergic and enkephalinergic neurons in the substantia gelatinosa is their important indirect postsynaptic action which leads to an inhibition of output neurons. Hence, some neurons in the substantia gelatinosa can be stimulated by opioids, which is an action that requires GABA-A receptor function. There is both morphological and electrophysiological evidence to support this action (see: Magnuson and Dickenson 1991).

On the other hand, there is evidence that in the spinal cord μORs can be activated endogenously by pain-producing stimuli. For example, neurochemical studies demonstrated that endogenous peptides derived form PENK were released into cerebrospinal fluid after repeated noxious stimulation, which could target and activate spinal μORs (Le Bars et al., 1987; Bourgoin et al., 1990; Cesselin et al., 1989). Furthermore, the newly discovered endogenous μOR pep-

tide agonists, endomorphin–1 and endomorphin–2, are localized in the spinal cord dorsal horn, within small diameter neurons containing substance P and calcitonin gene-related peptide (CGRP) (Martin-Schild et al., 1998; Pierce et al., 1998). It has also been demonstrated that β-endorphin administered peripherally displays analgesic effects in the hyperalgesia associated with the local inflammation (Stein et al., 1989). The μORs localized on the nerve terminals of primary afferents or on immune cells are presumably most likely candidates for mediating these effects. Endomorphin–1 and endomorphin–2 administered i.c.v. or i.t. have been reported to produce analgesia in acute, inflammatory and neuropathic pain (Zadina et al., 1997; Stone et al., 1997; Grass et al., 2000; Przewłocka et al., 1999).

3. MELANOCORTINS

3.1. Melanocortin system

The melanocortin system consists of the melanocortin endogenous peptides α-, β- and γ-melanocyte-stimulating hormone (α-, β-, γ-MSH) and adrenocorticotropic hormone (ACTH); a family of melanocortin receptors, and the endogenous melanocortin antagonists, agouti and agouti-related protein (AGRP). Knowledge of melanocortins (MC) and their receptors has increased tremendously over the last few years. The cloning of five different melanocortin receptors between 1992–1994 started a new era in the research on the melanocortin functions. Before this time, the receptors for MSH and ACTH were known mainly from the physiological effects of these hormones, i.e. skin pigmentation elicited by MSH, and ACTH-induced secretion of corticosteroids.

On the basis of homology with other members of a large family of G protein-coupled receptors, the mouse and human MSH (MC1) and ACTH (MC2) receptors were cloned from cDNA isolated from melanoma cells containing a high level of specific [^{125}I]NDP-α-MSH binding (Mountjoy et al., 1992; Chhajlani and Wikberg 1992). To date, five melanocortin receptor (MCR) types have been cloned (Mountjoy et al., 1992; Chhajlani and Wikberg 1992; Gantz et al., 1993a, b; Chhajlani et al., 1993). Each receptor is the product of a separate gene (Abdel-Malek 2001). These discoveries facilitated research into the physiological roles of the five melanocortin receptors, and the compounds exhibiting selective actions at some of them became available. Melanocortin receptors are proteins with seven transmembrane domains positively coupled to G_s proteins

and belonging to the G protein-coupled receptor (GPCR) family. When compared with other GPCRs, they have a short second extracellular loop, an intracellular carboxy-terminal domains and a short extracellular amino-terminal domain. The melanocortin receptors exhibit sequence homologies ranging from 40% to 60% and have several N-glycosylation sites in their N-terminal domains. They also have conserved cysteine residues in their C-termini (a potential site of acetylation with fatty acid). The melanocortin receptors are functionally coupled to adenylate cyclase (AC) and mediate their effects primarily by activating the cAMP-dependent signaling pathway. The melanocortin receptors have recognition sites for protein kinase C (PKC), and some also for protein kinase A (PKA) (Mountjoy et al., 1992), indicating that they may be regulated by phosphorylation. Melanocortin receptor signaling has also been associated with increases in intracellular Ca^{2+} concentration secondary to activation of inositol trisphosphate (Konda et al., 1994), influx of extracellular Ca^{2+} (Kojima et al., 1985) and activation of the MAP kinase (Englaro et al., 1995), janus kinase (Buggy et al., 1998) and with the PKC pathways (Kapas et al., 1995).
ACTH, α-MSH and β-MSH are potent, high-affinity agonists of all melanocortin receptors except for the MC2 receptor. The melanocortin MC2 receptor is the most selective one, recognizing only ACTH sequence. Selectivity of melanocortin receptors is summarized in Table 2.

3.2. Melanocortin receptors

MC1 receptor was the first member of the melanocortin receptor gene family which was cloned. It was cloned from a mouse melanoma cell line and from a primary culture of normal human melanocytes by Mountjoy et al. (1992) and from human melanoma cell by Chhajlani and Wikberg (1992). MC1R is the "classic" melanocyte α-MSH receptor. It is expressed by cutaneous melanocytes, where it plays a key role in determining skin and hair pigmentation. However, other cell types in the skin also express MC1R, including keratinocytes, fibroblasts, endothelial cells, and antigen-presenting cells (Luger et al., 1999). Other tissues and cell types have also been found to express MC1R (Chhajlani 1996). In relation to this, it is notable that MC1R is expressed by leukocytes, where it mediates the anti-inflammatory and immunomodulatory properties of melanocortins. *In situ* hybridization studies and immunohistochemical methods demonstrated the presence of MC1 receptor on a few scattered neurons of the periaqueductal grey matter in both rat and human brains (Wikberg 1999). Moreover Chhajlani (1996) reported that the MC1R was present in the pituitary. Interestingly, recent studies of Mogil et al. (2003) have identified MC1R as a novel modulator of nociception. In addition to the well-known localization of MC1R

Table 2. Selectivity of melanocortin receptors

Receptor	Agonists in increasing order of affinity	mRNA expression	Function
MC1	α-MSH = ACTH > β-MSH > γ-MSH	Melanocytes, Leydig cells, testis, corpus luteum, placenta, macrophages/ monocytes, neutrophils, endothelial cells, fibroblasts, glioma cells and astrocytes. Also in: pituitary and periaqueductal gray (PAG)	Hair and skin pigmentation, immunomodulation and anti-inflammatory effects
MC2	ACTH	Adrenal cortex	Lipolytic activity
MC3	α-MSH = β-MSH = γ-MSH = ACTH	Brain: hypothalamus, medial habenula nucleus, lateral septal nucleus, ventral tegmental area. Peripheral tissues: placenta, duodenum, pancreas, stomach	Cardiovascular functions, thermoregulation, control of feeding behavior
MC4	α-MSH = ACTH > β-MSH > γ-MSH	Brain: hypothalamus, olfactory cortex, septal region, hippocampus, superior colliculus, nucleus of the optic tract, brainstem and spinal cord. During ontogeny: brain, spinal cord, autonomic nervous system and adrenal medulla	Involvement in autonomic and neuroendocrine functions, regulation of food intake, weight homeostasis, hyperalgesia, pain, grooming behavior, stress
MC5	α-MSH > ACTH = β-MSH > γ-MSH	Peripheral tissues: skin, adrenal gland, skeletal muscle, bone marrow, spleen, thymus, testis and ovary, uterus, lung, liver, thyroid, thymus, stomach, kidney, exorcine glands. Brain: cortex, cerebellum	Function not very well understood, and mostly speculative: neuro/ myotropic, gastric and inflammatory effects, regulation of aldosterone secretion, weak lipolytic activity of α-MSH on adipocytes, regulation of hair lipid production, water repulsion, thermal regulation, exorcine gland function

in the periphery, MC1Rs are expressed in the brain glial cells (Wikberg 1999) and neurons of the ventral periaqueductal gray (Xia et al., 1995), i.e. the brain area of critical relevance to the modulation of nociception.

MC2 receptor was cloned from the adrenal gland shortly after cloning of MC1 receptor (Mountjoy et al., 1992, Chhajlani et al., 1993). ACTH preferentially binds to MC2 receptor, hence, it is considered to be the ACTH receptor. MC2 receptor is mainly expressed in the adrenal cortex (Mountjoy et al., 1992), where it mediates the effects of ACTH on steroid secretion (Xia and Wikberg 1996). The ACTH receptor (MC2) mRNA and mRNAs for three enzymes obligatory for steroid synthesis, including cytochromes P450scc, P450c17 and P450c21, were shown using RT-PCR method to be expressed in normal and pathologic human skin (Slominski et al., 1996). MC2R is also expressed in the white adipose tissue of rodents (Boston and Cone 1996) but, in contrast, human adipocytes do not express melanocortin MC2 receptor (Xia and Wikberg 1996). MC2 receptor, expressed in adipocytes of various mammals mediates mostly lipolytic activity of ACTH (Boston 1998).

MC3 receptor is expressed in many areas of the CNS and in several peripheral tissues, including the gastrointestinal tract and placenta (Chhajlani 1996). All of the melanocortins are roughly equipotent at MC3R. Notably, among the MCR subtypes, γ-MSH has the greatest affinity for MC3R, that is assumed to be of physiological significance, namely it indicates MC3R involvement in energy homeostasis (Gantz and Fong 2003). Wide expression of MC3R within the CNS: in the hypothalamus, thalamus, hippocampus, anterior amygdala, and in the cortex suggests its role in the regulation of cardiovascular functions and thermoregulation, as well as in the control of feeding behavior (Low et al., 1994). High densities of MC3 receptor are also present in the ventromedial nucleus of the hypothalamus (including the arcuate nucleus) and posterior hypothalamus. ^{125}I-NDP-MSH binding studies (Lindblom et al., 1998) revealed high densities of the MC3 receptor in the ventromedial nucleus of the hypothalamus and in the nucleus accumbens, and also in the medial preoptic area and central gray. The presence of MC3 receptor in the nucleus accumbens may suggest possible involvement of the melanocortin system in food intake and mechanisms of reward (Fan et al., 1997, Olszewski et al., 2003).

MC4 receptor is expressed predominantly in the CNS. The MC4 receptor expression is quite widespread in the brain, as it was detected in the thalamus, hypothalamus and brainstem. MC4R involvement in autonomic and neurocrine functions (the regulation of energy homeostasis) has been suggested. The distribution of MC4 receptor in the CNS is much wider than the expression of MC3

receptor. The MC4 receptor is abundant in the paraventricular nucleus, which suggests its role in the central control of hypothalamic and pituitary functions. MC4 receptor is also expressed in the spinal cord, superficial dorsal horn; lamina I, II and the gray matter surrounding the central canal, i.e. lamina X (Mountjoy and Wong 1997), that are regions implicated in pain transmission.

MC5 receptor gene was the last cloned melanocortin receptor gene (Labbe et al., 1994). Its expression has been shown in the adrenal glands, fat cells, kidneys, liver, lung, bone marrow, thymus, mammary glands, testis, ovary, uterus, pituitary, stomach, skin, and skeletal muscles (Chhajlani 1996; van der Kraan et al., 1998, Gantz et al., 1994). Although *in situ* hybridization in the brain proved to be unsuccessful, RT-PCR studies indicate that MC5 receptor transcript is present in several brain regions, including the olfactory bulb, substantia nigra and striatum (Griffon et al., 1994). Expression of MC5 receptor mRNA has been detected in mouse adipocytes but at a lower level than MC2 receptor mRNA. MC5 receptor might mediate the weak lipolytic activity of α-MSH on the adipocytes of several rodent species (Boston and Cone 1996) and regulate hair lipid production, water repulsion and thermal regulation (Chen et al., 1997, Chen 2000). MC5 receptor is a prime candidate for mediating the secretion of stress-induced alarm substances, or stress pheromones (Chen 2000).

3.3. Role of MC4 receptor in nociception

An understanding of the physiological role of the MC4 receptor have now started to emerge, and some of the new findings indicate the important role of melanocortin receptors in the CNS physiology. Of all five cloned melanocortin receptors, the MC4 receptor attracts particular attention in the aspect of its role in nociception. MC4 receptor is widely distributed within the brain and spinal cord of rodents and mammals (Mountjoy et al., 1994, 1998). The expression of the MC4 receptor in areas involved in nociception, like the thalamus, and also in the superficial dorsal horn of the spinal cord (lamina I, II) and the gray matter surrounding the central canal, lamina X (Plantinga et al., 1995) strongly implicates its role in pain. Direct effects of melanocortins on nociception have also been described. Sandman and Kastin (1981) reported hyperalgesia in the tail-flick test upon i.c.v. injection of α-MSH; a similar effect was shown for i.c.v. injected ACTH (Bertolini et al., 1979). Analgesic effects produced by central injection of α-MSH have been described by some early studies (Ohkubo et al., 1985, Walker et al., 1980), but most recent studies suggest that melanocortins increase sensitivity to painful stimuli, which is in line with their functional antagonistic influence on opioid-induced analgesia. Present data indicate that activation

of certain MCRs, primarily MC4R, could be a novel strategy to control chronic pain (Vrinten et al., 2000; Starowicz et al., 2002; Bellasio et al., 2003).

4. CHRONIC PAIN

Normally, pain occurs in response to the activation of a specific subset of high-threshold peripheral sensory neurons, the nociceptors. Acute pain is a normal sensation triggered in the nervous system to alert an individual to possible injury. Thus, nociception has a protective role, allowing an individual to react appropriately to the stimulus and to minimize its effects. On the other hand, chronic pain which can arise either from damage to the nervous system (neuropathic pain) or chronic inflammatory states such as arthritis (Costigan and Woolf 2000) persists. Pain signals keep firing in the nervous system for weeks, months, even years. Accordingly to the current International Association for the Study of Pain (IASP), neuropathic pain is initiated or caused by a primary lesion or dysfunction in the nervous system. Peripheral neuropathic pain occurs when the lesion or dysfunction affects the peripheral nervous system. Central pain may be retained as the term when the lesion or dysfunction affects the central nervous system. Usually neuropathic pain is accompanied by allodynia (pain due to a stimulus which does not normally provoke pain) and hyperalgesia (an increased response to a stimulus which is normally painful). Mechanisms, symptoms and signs of neuropathic pain are subject of extensive studies and have been reviewed in detailed by Jensen and Baron (2003), Smith et al. (2002), Baron (2000) and Devor et al. (2002). Drug effectiveness in neuropathic pain states is related to the exact etiology of the disease or injury (Nicholson 2000). There is no single, well-tolerated drug that is effective in all types of neuropathic pain (Smith et al., 2002). It is clear, therefore, that new drugs and therapeutic approaches are required.

4.1. Opioids and chronic pain

4.1.1. Inflammatory pain

The effects of opioids in animal models of inflammatory pain have been studied in great detail. Inflammation in the periphery influences the central sites and changes the opioid action. Interestingly, inflammation results in an increase in μOR density in primary afferents (Kayser and Guilbaud 1983; Stein et al., 1988) that increases spinal potency of various opioid receptor agonists (Millan and Colpaert 1991; Ossipov et al., 1995). In general, the antinociceptive potency

of opioids against various noxious stimuli is greater in animals with peripheral inflammation than in control animals. Inflammation-induced enhancement of opioid antinociceptive potency is dependent predominantly on μORs, since there is a greater increase in spinal potency of morphine than of agonists of δ- and κOR. Enhancement of the potency of μOR agonists during inflammation could arise from the changes occurring in opioid receptors, predominantly in affinity or number of the μORs (for review see: Przewłocki and Przewłocka 2001).
The involvement of the μOR has been demonstrated in various models of chronic inflammatory pain (complete Freund`s adjuvant (CFA), carrageenan or formalin injection into the animals' paw). The role of endogenous β-endorphin in the arcuate nucleus of the hypothalamus (Arc) in nociception in rats with carrageenan-induced inflammation has also been reported (Sun et al., 2003). The antiserum against β-endorphin, administered to the Arc, dose-dependently reduced the hindpaw withdrawal latency in rats with inflammation, further supporting that role of endogenous β-endorphin in the endogenous antinociceptive system in rats with inflammation. Zangen et al. (1998) demonstrated that the extracellular levels of β-endorphin in the Arc increased markedly in response to intraplantar injection of formalin. These results indicate that central β-endorphin is activated and modulates the formalin-induced nocifensive response. The study of Hurley and Hammond (2000) demonstrated antihyperalgesic and antinociceptive effects of μOR agonists microinjected into the rostral ventromedial medulla (RVM) after the induction of an inflammatory injury by injection of CFA in one hindpaw. Finally, the significant role of μORs in the formalin-induced chronic pain has also been indicated. An increase in β-endorphin immunoreactivity at a supraspinal site resulting from formalin-induced inflammation has been observed and i.c.v. pretreatment with anti-β-endorphin antiserum markedly increased the behavioral response to formalin in the rat and mouse (Porro et al., 1991; Wu et al., 2001). Additional results providing a strong evidence for involvement of μOR in peripheral analgesia, particularly in inflammatory pain, were reported by Jin et al. (1999) who showed that intraplantar injection of endomorphin–1 produced dose-dependent reduction of carrageenan-induced c-Fos expression and peripheral edema, which were completely blocked by i.p. co-administration of naloxone. Furthermore, Łabuz et al. (2003) demonstrated that endomorphin–2 and long-acting analog of the peptide potently suppressed formalin-induced expression of c-Fos in the dorsal horn of the spinal cord.

4.1.2. Neuropathic pain

Several clinical studies have shown that opioids, particularly morphine, lack potent analgesic efficacy in neuropathic pain in humans (Arner and Meyerson 1988). A vast body of evidence suggests that the reduced sensitivity to systemic opioids is observed in neuropathic pain, and an increase in their dose is necessary in order to obtain adequate analgesia. Reduction of morphine antinociceptive potency may be a result of nerve injury-induced reduction of the activity of spinal opioid receptors or opioid signal transduction. On the other hand, a few studies showed that morphine could be effective in some patients suffering from neuropathic pain (Rowbotham et al., 1991; Portenoy et al., 1990). These authors suggested that neuropathic pain was associated with the reduced sensitivity to systemic opioids, and an increase in their dose was necessary in order to obtain adequate analgesia (Portenoy and Hagen 1990). Moreover, prolonged and repeated exposure to opioid agonists in chronic pain reduces the responsiveness of opioid receptors thus this was hypothesized to contribute to opioid tolerance (Ueda et al., 2003). Beside dependence, tolerance is a major limitation of the long-term clinical use of opioids. Mechanisms involved in tolerance development after chronic administration of opiates are extremely complex, involving interactions between opioidergic and non-opioidergic systems (Vaccarino and Kastin 2000).

The limitations of existing treatments of neuropathic pain and the inability to provide relief to many patients has stimulated ongoing studies that examine different approaches to preventing neuropathic pain and to counteracting tolerance to morphine analgesic effect. Identification of the involved mechanisms may be of importance to understanding of the molecular mechanism of opioid action in neuropathic pain, as well as to development of better and more effective drugs for the treatment of neuropathic pain in humans.

4.2. Anti-opioid systems

The analgesic function of opioids is subject to modulation by a range of non-opioid systems. These systems may modulate antinociception induced by exogenously administered opioids, and may be involved in the development of opioid tolerance, dependence and opioid insensitivity in some pain states. Literature describes several types of such an interaction namely: synergistic antinociception occurs between opioids and other systems that have antinociceptive properties, e.g. interaction between spinally administered opioids and α_2-adrenoreceptor agonists, causing enhanced nociception (Fleetwood-Walker et al., 1985; Jensen

and Yaksh 1986; Reddy et al., 1980). Interestingly, some opioid targets may be components of homeostatic systems tending to reduce the effects of opioids. "Anti-opioid" properties have been attributed to various neuropeptides, especially cholecystokinin (CCK) and nociceptin. The analgesic action of β-endorphin was effectively suppressed by i.c.v. injection of CCK (Itoh et al., 1982). Cellular basis of the interaction between μORs and cholecystokinin at the spinal level was discovered by Zhang and his colleagues (Zhang et al., 2000) who demonstrated coexistence of these two molecules in local interneurons. Nociceptin has also been reported to be an anti-opioid peptide (Tian et al., 1997; Pan et al., 2000; Mika et al., 2004). Data of Ueda et al. (2000) suggest that the spinal nociceptin system develops anti-opioid plasticity observed in morphine tolerance and dependence. Antagonistic action of dynorphin on the effects of opioids has also been demonstrated (Friedman et al., 1981). Dynorphin-(1–13) has been shown to have significant effects on β-endorphin-induced analgesia despite lacking any analgesic activity itself (Friedman et al., 1981). The physiological implication of the nonopioid actions of dynorphin is particularly relevant to animal models of peripheral neuropathy caused by inflammation or nerve injury. These conditions give rise to abnormal pain states, including hyperalgesia and allodynia, and are associated with an elevated level of spinal dynorphin (Drasci et al., 1991; Kajander et al., 1990). Przewłocki et al. (1986, 1987) revealed that chronic stress as well as chronic pain induced dynorphin immunoreactivity in the lumbar enlargement of the rat spinal cord. Data of Nichols proved that anti-dynorphin A antiserum blocked thermal hyperalgesia and restored the efficacy of morphine against allodynia in spinal nerve ligation injury model (Nichols et al., 1997). The further evidence of the dynorphin A contribution to the hyperesthetic states, possibly through modulating the NMDA receptor function via a nonopioid mechanism when the endogenous level of dynorphin is abnormally elevated has also been reported by Obara et al. (2003). Rothman (1992) presented the hypothesis that the brain synthesizes and secretes neuropeptides which act as parts of a homeostatic system to attenuate the effects of morphine and endogenous opioid peptides. A deeper understanding of interactions between opioid and anti-opioid systems in the central nervous system may lead to new treatment strategies of chronic pain, substance abuse, and psychiatric disorders. Among neuropeptides, melanocortins can be considered as one group of endogenous peptides with putative anti-opioid function.

4.3. Opioid-melanocortin interaction

The melanocortin and opioid systems have opposite activities in many tests, and, therefore, been considered as functional antagonists. Melanocortin agonists have been reported to antagonize opiate self-administration as well as opiate analgesia (Contreras and Takemori 1984; Szekely et al., 1979). Melanocortin–opioid interaction was also reported by van Ree and his colleagues (1983) who showed that POMC may serve as a precursor molecule for peptides, which differentially modulate the acquisition of heroin self-administration in rats. Moreover, Alvaro et al. (1996) suggested the involvement of MC4R in opioid addiction and hypothesized that decreased melanocortin function, via down-regulation of MC4R expression, might contribute to the development of these opiate-induced behaviors. The opioid peptide β-endorphin is co-synthesized and released with melanocortins from POMC neuronal terminals. The release of α-MSH is always accompanied by the release of β-endorphin at the same anatomical site. Interestingly, as already mentioned, opioid receptors are negatively coupled to adenylate cyclase whereas melanocortin receptors are positively coupled to this enzyme (Adan and Gispen 2000). This set of data raises the question about the physiological significance of antagonistic signals generated from the same gene. It would be interesting to answer the question what is the physiological significance of this functional unit in which two peptides are co-synthesized, co-stored and co-released, reach a common postsynaptic target and exert opposing effect. Nociception, which is one of the main functions of opioids, and opioid tolerance are the fields of possible interactions between melanocortins and opioids which may provide cues to elucidate nature of this phenomenon.

5. AIMS AND OUTLINE OF THE THESIS

As already mentioned in previous sections, interplay between the melanocortin and opioid systems has recently received more attention in relation to their involvement in nociceptive processes and data have been accumulated which open a new avenue for understanding of their role in mechanisms of chronic pain, particularly in neuropathic pain. However, further basic research aimed to further understanding of the mechanisms underlying these interactions are needed. Thus, the main objectives of the studies presented herein were to investigate whether the melanocortin system is involved in spinal antinociception, and whether and if so how it can modulate spinal and supraspinal antinocicep-

tion induced by opioids in pathological states such as chronic pain or tolerance to morphine analgesic effect.

In order to better understand a role of MC4R in nociception, we studied whether this receptor is expressed in the regions important to pain transmission, in DRG and spinal cords of rats. In chapter 3 and Addendum #2, we described the MC4R mRNA expression as well as the MC4R immunoreactivity in control animals. To our knowledge this is the first report demonstrating the presence of MC4R expression in peripheral sensory ganglia, since it is generally accepted that the expression of MC4R is restricted to the CNS. The possible involvement of MC4R ligands in nociception was the subject of our previous unpublished study; MTII an agonist of MC4R was shown to produce hyperalgesia, whereas receptor antagonists did not influence the baseline thresholds of physiological pain.

In the experiments on neuropathic pain described in this thesis, the chronic constriction injury (CCI) model was used. In chapter 2, the effects of i.t. administered MC4 receptor ago- and antagonist, MTII and SHU9119, on the mechanical and thermal allodynia in CCI rats were presented. It was demonstrated that MC4R ligands when administered i.t. to the lumbar spinal cord influenced nociception in neuropathic rats, namely, MTII enhanced hyperalgesia and allodynia which accompanied the neuropathic pain while and SHU9119 alleviated it. Moreover, the modulation of action of MC4R ligands in CCI animals was demonstrated by pretreatment of animals with opioid ago- and antagonist, DAMGO and cyprodime, respectively. The interaction of melanocortins and opioids was also addressed in Addendum #1. It was shown that blockade of the MC4R prior the morphine injection resulted in potentiation of the morphine analgesic effect in neuropathic rats. To further elucidate the role of MC4R, chapter 3 presents a study describing the changes in MC4R mRNA in the lumbar spinal cord and dorsal root ganglia of neuropathic rats. It was demonstrated that the neuropathic pain does not alter the MC4R mRNA levels in the spinal cord as measured by quantitative Real-Time PCR, whereas a down-regulation of these receptors one week after the nerve injury was observed in the DRG.

Various studies on animal models of neuropathic pain revealed changes in spinal μOR. Ample evidence suggests that following tissue injury there are multiple changes in the CNS function that contribute to the development and maintenance of chronic pain. It has been suggested that lower potency of systemic morphine in neuropathic pain could be, at least partly, caused by the decreased μOR expression in DRG. However, other possible explanations for the lower potency of opioids might rely on the enhanced activity of the melanocortin system. Using light microscope immunohistochemistry, we investigated the

relationship between the opioid and melanocortin receptors on primary afferent dorsal root ganglion neurons and at the lumbar level of the spinal cord. In Addendum #2, we demonstrated that neuropathic pain was accompanied by a decrease in μOR immunoreactivity (IR), both in the DRG and at the spinal level, whereas the MC4R-IR gradually increased with the development of neuropathic pain in rats. Up-regulation of the MC4R and alteration of balance between MC4 and μOR, may indeed contribute to the decreased efficacy of morphine in neuropathic pain.

Finally, to further elucidate the role of melanocortins in opioid-mediated nociception, we studied the mechanisms of opioid tolerance. Morphine tolerance and its mechanisms are addressed in chapter 4, where the involvement of an interaction between β-arrestin and spinal opioid receptors in tolerance and neuropathy was delineated. The delay of the development of these two phenomena related to the repeated μOR activation, i.e. tolerance to antinociceptive effect of morphine and nerve injury-induced hyperalgesia and allodynia after knockdown of β-arrestin by antisense oligonucleotides, was observed. In chapter 5 and 6, investigations on the involvement of the supraspinal and spinal melanocortin system in tolerance to morphine analgesic effect are detailed. We administered MC4R antagonists to morphine-tolerant rats in a paradigm that produces tolerance to its analgesic action. Based upon the results of these studies we conclude that both supraspinal and spinal melanocortin system undergoes plastic changes during development of tolerance, and that antagonistic effect of the MC4R is potent in restoring morphine analgesic effect and is able to significantly delay the development of morphine tolerance.

The main findings and implications of these studies are summarized and discussed in chapter 7.

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Chapter II

Modulation of melanocortin-induced changes in spinal nociception by μ-opioid receptor agonist and antagonist in neuropathic rats

Katarzyna Starowicz, Ryszard Przewłocki[1],
Willem Hendrik Gispen[2], Barbara Przewłocka[1]

[1] Institute of Pharmacology, Polish Academy of Sciences, Cracow, Poland
[2] Department of Medical Pharmacology and Anestesiology, Rudolf Magnus Institute for Neurosciences, University Medical Centre Utrecht, the Netherlands

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SYNAPTIC TRANSMISSION NEUROREPORT

Modulation of melanocortin-induced changes in spinal nociception by μ-opioid receptor agonist and antagonist in neuropathic rats

Katarzyna Starowicz, Ryszard Przewlocki,[1] Willem Hendrik Gispen[2] and Barbara Przewlocka[1,CA]

International Institute of Molecular and Cell Biology, UNESCO/PAN, 4 Ks. Trojdena Str., 02-109 Warsaw; [1]Institute of Pharmacology, Polish Academy of Sciences, 12 Smetna Str., 31-343 Cracow, Poland; [2]Department of Medical Pharmacology and Anesthesiology, Rudolf Magnus Institute for Neurosciences, University Medical Centre Utrecht, Utrecht, the Netherlands

[CA]Corresponding Author: przebar@if-pan.krakow.pl

Received 23 July 2002; accepted 28 October 2002

DOI: 10.1097/01.wnr.0000047683.08940.57

Co-localization of opioid and melanocortin receptor expression, especially at the spinal cord level in the dorsal horn and in the gray matter surrounding the central canal led to the suggestion that melanocortins might play a role in nociceptive processes. In the present studies, we aimed to determine the effects of melanocortins, administered intrathecally, on allodynia, and to ascertain whether there is an interaction between opioid and melanocortin systems at the spinal cord level. Neuropathic pain was induced by chronic constriction injury (CCI) of the right sciatic nerve in rats. Tactile allodynia was assessed using von Frey filaments, while thermal hyperlagesia was evaluated in cold water allodynia test. In the present experiments, melanocortin receptor antagonist, SHU9119 was much more potent than μ-opioid receptor agonist, morphine after their intrathecal (i.th.) administration in neuropathic rats. SHU9119 alleviated allodynia in a comparable manner to DAMGO, a selective and potent μ-opioid receptor agonist. Administration of melanocortin receptor agonist, melanotan-II (MTII) increased the sensitivity to tactile and cold stimulation. Moreover, we demonstrated that the selective blockade of μ-opioid receptor by cyprodime (CP) enhanced antiallodynic effect of SHU9119 as well as pronociceptive action of MTII, whereas the combined administration of μ receptor agonist (DAMGO) and SHU9119 significantly reduced the analgesic effect of those ligands. DAMGO also reversed the proallodynic effect of melanocortin receptor agonist, MTII. In conclusion, it seems that the endogenous opioidergic system acts as a functional antagonist of melanocortinergic system, and μ-opioid receptor activity appears to be involved in the modulation of melanocortin system function. *NeuroReport* 13: 2447–2452 © 2002 Lippincott Williams & Wilkins.

Key words: Allodynia; Alpha-melanocyte stimulating hormone (α-MSH); Antinociception; Chronic constriction injury (CCI); Melanocortin 4 receptor; Melanocortins; Neuropathic pain; μ-Opioid receptor; Opioids; Spinal cord

INTRODUCTION

Peptide hormones and neurotransmitters are synthesized as high mol. wt precursors, which are processed intracellularly into smaller, active fragments. Melanocortins, the peptides that include ACTH, α-MSH, β-MSH and γ-MSH are produced by the proteolytic processing of the proopiomelanocortin (POMC) precursor protein. POMC is most highly expressed in the anterior and intermediate lobes of the pituitary [1]. In the central nervous system, POMC expression has been confirmed in the arcuate nucleus of the hypothalamus and nucleus tractus solitarius (NTS) of the medulla [2]. The melanocortinergic fibers have been discovered throughout the brain, projecting into hypothalamus, thalamus, mesencephalon, amygdala, hippocampus, cortex, medulla and spinal cord [3]. In 1999, expression of the precursor protein of melanocortins, POMC was demonstrated in the spinal cord and dorsal root ganglia (DRG) by van der Kraan *et al.* [4], who confirmed the results of Plantiga *et al.* [5]. Immunoreactivity for α-MSH and another POMC-derived peptides has also been found in the rat spinal cord [6], although this immunoreactivity has been largely ascribed to supraspinal POMC expression. Van der Kraan *et al.* [4] demonstrated that POMC expression was also intrinsic to the spinal cord, and contributed to local synthesis of POMC-derived peptides.

Sandman and Kastin [7] have suggested that α-MSH plays a physiological role of an endogenous antiopiate, causing hyperalgesia. α-MSH modulates the threshold of pain sensitivity and produces a dose-dependent hyperalgesia, which can be blocked by s.c. naloxone. In addition to the melanocortin sequence, POMC contains also one copy of opioid-defining amino acid sequence, β-endorphin.

Melanocortin peptides have been reported to directly affect nociception. When full processing of POMC occurs, β-endorphin and α-MSH are subsequently stored in the same synaptic vesicles, thus two opposed acting peptides may be released at the same anatomical site. Hence, melanocortins and opioids originate from the same precursor molecule, but they have been considered functional antagonists, which was substantiated by many observations. A number of studies have suggested that melanocortins antagonize the action of exogenous opiates. Electrophysiological studies have indicated that melanocortins block morphine-induced depression of evoked potentials in the isolated frog spinal cord [8]. α-MSH is capable of antagonizing the analgesic effect of morphine [9]. It has also been shown that melanocortin peptides attenuate acquisition of heroin self-administration [10]. Co-localization of opioid and melanocortin peptides in the spinal structures suggests their functional interaction in nociceptive processes. α-MSH has opposite actions to those observed after the administration of an endogenous opiate, β-endorphin in acute pain states. However, under pathological conditions, such as neuropathic pain, the interactions between melanocortins and opioids are still an open issue.

We investigated the effect of intrathecally administered MC4 receptor ligands, SHU9119 MC4 receptor antagonist, and MTII MC4 receptor agonist, on nociceptive information processing in rats after the sciatic nerve injury. We have also proposed a possible interaction between the opioidergic and melanocortinergic systems at the spinal cord level in pain perception. In the present study, we also investigated the modulation of melanocortinergic system activity at the spinal level by selective μ-opioid receptors ligands: cyprodime and DAMGO in rats with chronic constriction injury to the sciatic nerve.

MATERIALS AND METHODS

Experimental animals: The study was conducted on male Wistar rats, weighing 250–300 g at the start of the experiment. The animals were housed individually in plastic cages on a sawdust bedding and kept under a 12:12 h light:dark cycle (lights on 08.00–20.00 h) with food and water available *ad lib.* All test procedures in presented study were performed in compliance with the recommendations of the Local Bioethical Committee and were carried out according to the NIH Guide for the Care and Use of Laboratory Animals.

Surgery: Animals were chronically implanted with intrathecal catheters, and surgical procedure was carried out under pentobarbital anesthesia. They were placed on the David Kopf stereotaxic table, and an incision was made in the atlanto-occipital membrane. A catheter (PE 10, Clay Adams, Sparks, MD) was carefully introduced to the subarachnoid space at a rostral level of the spinal cord enlargement according to Yaksh and Rudy [11].

For chronic constriction injury (CCI), rats were anesthetized as described above 1 week after the implantation of cannulas. The right sciatic nerve was exposed and a lesion was produced by placing four loose ligatures at 1 mm intervals around the nerve as described by Bennett and Xie [12]. The same procedure was performed in control animals except that the placement of the ligatures around the nerve was omitted (sham-operated animals).

Peptides and drug administration: Melanotan-II (MTII), SHU9119, [D-Ala^2, N-Me-Phe^4, Gly^5-ol]-enkephalin (DAMGO), morphine and cyprodime (CP) were administered *in vivo*. MTII and SHU9119 were purchased from Phoenix Pharamceuticals, Inc., DAMGO was obtained from Sigma, morphine was from Polfa; and cyprodime (CP) was from H. Schmidhammer, Institute of Organic and Pharmaceutical Chemistry, University of Innsbruck, Innsbruck, Austria.

All tested drugs were administered intrathecally (i.th.) in a volume of 5 μl followed by an injection of 10 μl distilled water to flush the catheter. DAMGO was administered at doses of 0.1, 0.25 and 0.5 μg, and morphine was given at 10, 20 and 30 μg. Melanocortin receptor agonist MTII was administered at doses of 0.03, 0.1 and 0.5 μg and MC receptor antagonist SHU9119 was injected at 0.15, 0.5, and 1.5 μg. Cyprodime, a μ-opioid receptor antagonist, was used at a dose of 30 μg.

Behavioral evaluation: Mechanical allodynia was measured as the foot withdrawal threshold in response to a mechanical stimulus using electronic von Frey filaments, which were used to apply slight pressure to the skin. The animals were placed in a plastic cage with wire net floor, and allowed to habituate for 5 min before the experiment. The von Frey filament was applied from underneath the floor to the midplantar surface of the paw. Each probe was applied three times to the paw, or less if the filament probes elicited withdrawal response of a foot. The cut-off value was set at 80 g.

Withdrawal latency was measured by water cold allodynia test [13]. The animals were placed on a metal stage submerged to a depth of 2.5 cm in an ice-cold water (0°C). The cut-off latency for the test was 30 s. A positive response was observed when an animal lifted the injured paw on the ligated side above the water level.

Baseline values were determined before drug injection, and measurements were repeated 15, 30 and 45 min after drug administration.

Statistical analysis: The antinociceptive effects were estimated by measuring the test latency (TL) after drug administration. Thus, the data were expressed as a percentage of maximal possible antinociceptive effect (% MPE), and calculated using the equation $=\%\text{MPE} = [(\text{TL}-\text{BL})/(\text{CUT-OFF-BL})] \times 100$ where BL = baseline latency, TL = respective test latency.

Statistical analysis of the data was carried out using ANOVA (Bonferroni's multiple comparison test). $p < 0.05$ was considered statistically significant.

RESULTS

Effect of i.th. administration of SHU9119: A significant delay of paw withdrawal after mechanical (von Frey test) stimulation was observed in the animals administered melanocortin receptor antagonist in the rat CCI model. Figure 1a shows reduction of allodynia after all SHU9119 doses (0.15, 0.5 and 1.5 μg i.th.). Maximal effects were

reached 15 min after peptide administration, when withdrawal threshold rose to 70 ± 4.1% of maximal possible effect for 1.5 μg SHU9119.

The increase in withdrawal latency to cold stimulation was even more pronounced upon administration of SHU9119 in the cold allodynia test, and the effect of the highest dose was also observed after 30 min (Fig. 1b).

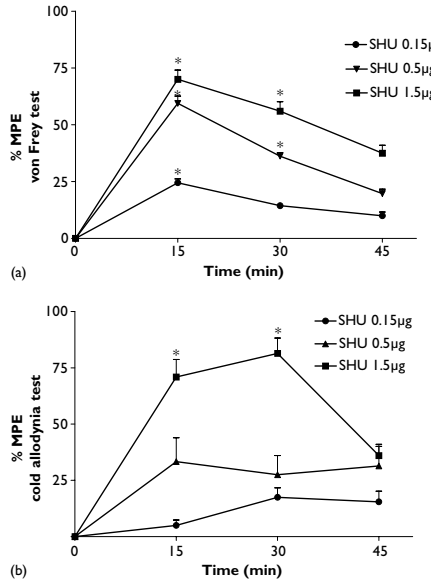

Fig. 1. The effect of intrathecal administration of melanocortin 4 receptor antagonist, SHU9119 (SHU), on withdrawal threshold to von Frey stimulation (**a**) and cold allodynia (**b**) in neuropathic rats 3 weeks after nerve injury. Data are presented as the mean of maximal possible effect (% MPE) ± s.e.m. of 8–10 rats each group. *$p < 0.05$ *vs* vehicle.

Effect of i.th. administration of MTII: The melanocortin receptor agonist MTII enhanced allodynia. Three weeks after CCI, we observed a decrease in withdrawal threshold to mechanical stimulation after administration of MC receptor agonist. MTII (0.03 μg, 0.1 μg, 0.5 μg, i.th.) administration dose-dependently decreased the threshold to mechanical stimulation (Table 1), whereas in the early phase of neuropathic pain development (days 3 and 10 after CCI, data not shown), the effect did not reach statistical significance, becoming significant 3 weeks after nerve injury. In the cold allodynia test, the melanocortin receptor agonist caused an increase in sensitivity to stimulus. However, the effect was much less pronounced than was seen with tactile allodynia (Table 1). MTII also affected the uninjured paw, and allodynia was observed on both ipsilateral and contralateral sides (data not shown).

Comparison of actions of opioid and melanocortin receptor ligands in neuropathic rats: The antiallodynic potency of the μ-opioid receptor agonists DAMGO and morphine were measured in von Frey and cold allodynia tests. DAMGO was much more potent than morphine. The antiallodynic effect of SHU9119 was observed at considerably lower concentrations (0.15–1.5 μg, i.th.) in comparison with μ-opioid agonists. DAMGO and SHU9119 both antagonized allodynia when given in similar dose ranges. In contrast, MTII administration (0.03–0.5 μg, i.th.) enhanced allodynia (Fig. 2).

Modulation of melanocortin antinociceptive effects: The nociceptive effect of melanocortins in CCI rats can be modulated by the changes in the opioidergic system activity. The dose of 0.5 μg SHU9119, i.th., was used in further studies, since it brought satisfactory effect in alleviating neuropathic pain. The administration of selective μ-opioid antagonist (cyprodime; CP, at a dose of 30 μg, i.th.) potently ($p < 0.001$) augmented the effect of SHU9119. When an agonist of MC4 receptor, MTII (0.5 μg, i.th.) was used, we observed weak but discernible proallodynic effect. Interestingly, administration of CP strongly augmented also the effect of MTII. As a result, the administration of an μ-opioid receptor antagonist potentiated the effect of melanocortins in either case, i.e. whether agonists or antagonists of

Table 1. Effect of melanocortin receptor agonist, melanotan II (MTII) on mechanical and cold allodynia in CCI rats 3 weeks after nerve injury.

	0.03 μg	0.1 μg	0.5 μg
Mechanical allodynia (g)			
Vehicle	47.6 ± 3.2	47.6 ± 3.2	47.6 ± 3.2
15 min	38.2 ± 3.9	27.5 ± 2.5**	29.4 ± 3.9*
30 min	32.4 ± 3.4	30.1 ± 1.2**	34.3 ± 3.1*
45 min	33 ± 2.3*	28.7 ± 1.9**	32 ± 2.6*
Cold allodynia (s)			
Vehicle	9 ± 1.14	9 ± 1.14	9 ± 1.14
15 min	7.8 ± 0.9	5.7 ± 1.2	7.1 ± 1.1
30 min	6 ± 1.1	4.8 ± 0.75*	5.3 ± 1.3
45 min	5.2 ± 1.4	4.5 ± 0.8*	6 ± 0.9

Baseline value was determined before drug injection and measurements were repeated 15, 30 and 45 min after drug administration. Data are presented as mean ± s.e.m. for 8–10 animals per group.
*$p < 0.01$ *vs* vehicle.
**$p < 0.001$ *vs* vehicle.

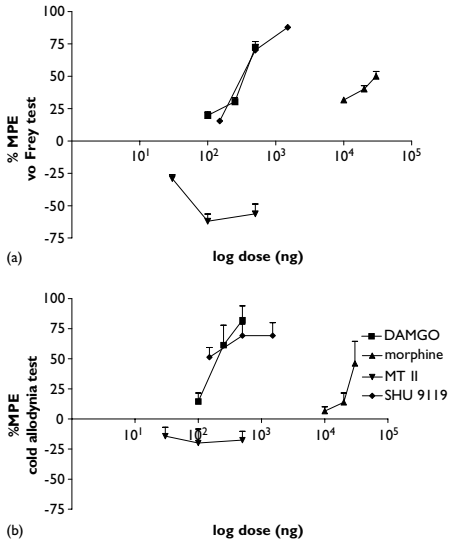

Fig. 2. Comparison of μ-opioid receptor agonists (morphine and DAMGO) and melanocortin receptor ligands (SHU9119 and MTII) antiallodynic action in rat model of neuropathic pain, 3 weeks after nerve injury in von Frey (**a**) and cold allodynia (**b**) tests. Data are presented as the mean of % maximal possible effect (% MPE) ± s.e.m. of 10 rats each group.

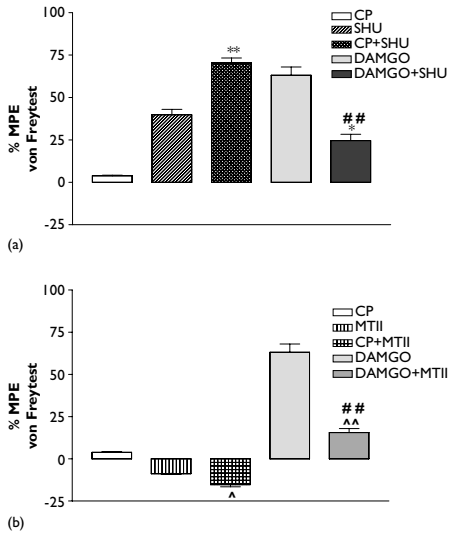

Fig. 3. Modulation of (**a**) SHU9119 (SHU) and (**b**) MTII nociceptive effects by μ-opioid receptor antagonist cyprodime (CP) and by μ-opioid receptor agonist, DAMGO. Data are presented as the mean of % maximal possible effect (% MPE) ± s.e.m. of 8 rats each group. $*p < 0.05$ *vs* SHU9119 given alone and $*p < 0.05$ *vs* DAMGO administered alone, $\hat{}p < 0.05$ *vs* MTII administered alone. Statistical analysis *vs* CP was not considered, since CP administered alone did not caused any behavioral effects.

MC4 receptor were used (Fig. 3). The μ-opioid agonist DAMGO, when administered alone, proved to be even more effective in alleviating neuropathic pain symptoms than SHU9119 did, conversely, when co-administered along with SHU9119, it reduced the antiallodynic effect of that MC4 receptor antagonist. It was a striking observation, as one could expect the additive effect of the two potent analgesics. Co-administration of DAMGO with MC4 receptor agonist reversed the behavioral profile of the response (Fig. 3). DAMGO also reversed the weak but significant proallodynic effect of MC4 receptor agonist MTII, which enhanced allodynia in CCI rats, but displayed an antiallodynic effect when co-administered with DAMGO.

DISCUSSION

We have shown that spinal administration of MC4 receptor antagonist, SHU9119 results in significant reduction of allodynia associated with neuropathic pain, as measured by von Frey and water cold allodynia tests. Moreover, opioids modulate the activity of the melanocortinergic system. Estimates of the relative densities of the μ-, δ-, and κ-binding sites in lamina I and II of the spinal cord vary, but, at least in rodents, μ-opioid receptors (MOR) are the most abundant and predominately located on primary afferents [14]. The analgesic function of opioids is subject to modulation by many non-opioid peptides. Several types of interactions between opioids and non-opioids have been described, including synergistic antinociceptive action of opioids and α_2-adrenoreceptor agonists, causing enhanced antinociception [15] or antagonization of the effects of opioids by anti-opioid endogenous peptides such as α-melanocyte stimulating hormone [9]. Literature data suggest the involvement of melanocortin peptides (ACTH/α-MSH) in hyperalgesia [7,16]. It has also been reported that melanocortins antagonize the analgesic effect of morphine and β-endorphin [17,18], although the mechanisms underlying the above-mentioned effects remain still unclear. Due to this antagonism, melanocortin peptides are called antiopiates. Among five cloned melanocortin receptors, MC4 receptor received more attention, due to its role in pain control. MC4 is the only melanocortin receptor type, whose mRNA has been detected in the spinal cord [19], and it is the most likely candidate to mediate the spinal α-MSH effects. As shown in the present paper, melanocortin receptor antagonist, SHU9119 dose-dependently increased the withdrawal threshold to mechanical stimulation, in contrast, MTII, a cyclic α-MSH analog, enhanced allodynia in the rat model of neuropathic pain.

Tissue injury-induced plasticity involves changes in the inhibitory system [20]. Opioid peptides and their receptors change after nerve injury. Autoradiographic binding studies

indicated that rizotomy induced a reduction in the level of opioid receptors in the dorsal horn of the spinal cord [21]. In contrast to receptors, the level of opioid peptides altered a little in the dorsal horn following peripheral nerve injury. In our study, peripheral nerve injury induced up-regulation of MC4R mRNA in ipsilateral L4-L6 part of the spinal cord (Starowicz *et al.*, in preparation). An increase in α-MSH binding sites and MC4 receptor immunoreactivity in the spinal cord was also observed [22]. This is in agreement with the fact that morphine and other opioid peptides are less effective in alleviating neuropathic pain compared to nociceptive or inflammatory pain [23]. Poor opioid efficacy may be explained by a loss of opioid receptors in the dorsal horn after nerve injury, reduced receptor function or altered function of intracellular messengers, or finally, by higher efficacy of anti-opioid systems. The majority of all opioid receptors are μ-opioid receptors localized in lamina I and II of the spinal cord. Their site of expression overlaps the expression of MC4 receptor. A subpopulation of the cells in lamina I and II of the dorsal horn may be an anatomical basis for an interaction between activation of MOR and activation of MC4 receptor-containing neurons. It has been demonstrated that melanocortin and opioid receptors are also co-localized in the locus coeruleus (LC) cells, and oppositely regulate the activity of adenylate cyclase, melanocortins activating and opioids inhibiting the enzyme activity [24]. Interestingly, MC4 receptor antagonist, SHU9119 attenuated neuropathic pain when administered intrathecally. A similar effect was observed after SHU9119 injection into the cisterna magna [22], but in that case we could not exclude the supraspinal mechanisms of its action. We noticed that antinociceptive effect of SHU9119 was even augmented by μ-opioid antagonist, cyprodime, which suggested inhibitory control of μ-opioid receptor.

Receptor co-localization is likely to underlie the efficacy of the combined drug therapies targeted at MOR and MC4 receptors, which appear to be more effective than single-drug therapies in the treatment of pain. DAMGO, μ-opioid agonist, when administered alone, proved to be even more effective in alleviating neuropathic pain symptoms than SHU9119 did, conversely, when co-administered along with SHU9119, it reduced the antiallodynic effect of MC4 receptor antagonist. Moreover, DAMGO also reversed the weak but significant proallodynic effect of MC4 receptor agonist, MTII. MTII enhanced allodynia in CCI rats, but displayed the antiallodynic effect when coadministered with DAMGO. Antiopiates may not only modulate antinociception induced by exogenously administered opioids, but they may also regulate sensitivity to opioids in some pathological pain states.

Lamina II of the rat spinal cord contains a neurochemically diverse population of neurons, most of which appear to have axons that do not reach supraspinal levels. We postulate that co-localization of both receptor subtypes: μ-opioid and MC4 on GABAergic neurons might be a basis for an interaction between the opioidergic and melanocortinergic system resulting in the modulation of nociceptive response at the spinal cord level, although the MC4 receptor-containing neurons have not been localized yet, due to the lack of specific antibodies. The superficial layer of the dorsal horn contains many GABAergic neurons [25], and GABA inhibits noxious stimulation-produced responses in the spinal cord. It has been suggested that GABA is co-localized with an endogenous opioid, methionine^5-enkephalin, in many neurons of the superficial dorsal horn, and that opioids exert their antinociceptive effects, at last partly, by increasing the sensitivity of postsynaptic $GABA_A$ receptors in the spinal cord. MOR agonists activate receptor on the postsynaptic neurons in lamina I and II thus enhancing the inhibitory effects of GABA on these neurons.

Many potential analgesics have been examined in preclinical studies, to evaluate their effectiveness in alleviating neuropathic pain. Particular attention was focused on endogenous analgesics, opioids, since the role of opioid peptides and their receptors in pain perception is well established. The inability of morphine to treat this kind of pathological pain prompted the conclusion that there is a need for new analgesic compounds. The present investigation showed a significant change in nociceptive information processing after administration of melanocortin receptor ligands into the lumbar enlargement, corresponding to the sciatic nerve input. The endogenous opioidergic system seems to be involved in modulation of melanocortinergic system activity, which influences the level of sensitivity to painful stimuli. The question arises whether opioidergic system controls the action of melanocortins, and what is the neuronal and/or molecular basis of this interaction. Explanation of the interaction between melanocortins and opioids will open new perspectives in the search for effective neuropathic pain treatment strategies.

CONCLUSION

In neuropathic rats, the selective MC4 receptor antagonist, SHU9119 attenuates allodynia, and antagonists of μ-opioid receptor potentiate this effect. The above results suggest that, in CCI rats, melanocortinergic system in the spinal cord remains under the control of the opioid/μ-opioid system. Interestingly, while opioid antagonists augmented the effect melanocortins, the opioid agonists attenuated the effect of melanocortins.

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Acknowledgements: This study was supported by statutory funds from the State Committee for Scientific Research (KBN; Warsaw, Poland).

Addendum #1

Modulation of morphine analgesia by melanocortin receptor antagonist in neuropathic rats

Katarzyna Starowicz[1,2], Ilona Obara[1],
Ryszard Przewłocki[1], Barbara Przewłocka[1]

[1] Dept. of Molecular Neuropharmacology, Institute of Pharmacology, PAS, Cracow Poland
[2] International Institute of Molecular and Cell Biology UNESCO/PAS, Warsaw, Poland

ABSTRACT

Antagonists at MC4 receptor are known to alleviate neuropathic pain symptoms. Furthermore, at the spinal cord level, an interaction between melanocortin and opioid systems has previously been demonstrated. We studied whether the melanocortin-opioid interaction will be altered when different experimental paradigms and/or different μ opioid receptor agonists (DAMGO vs. morphine) will be used. Potentation of the analgesic effect of morphine in neuropathic rats was observed when a MC4R antagonist was given 15 min prior to morphine. Thus, blockade of MC4R would contribute to the enhanced analgesic potency of morphine. These results further indicate an interaction between the spinal melanocortin and opioid systems, as the response to morphine is enhanced in rats that were previously exposed to a MC4R antagonist. An interplay between μ-opioid and MC4 receptor seems to be crucial to the final behavioral effect observed.

INTRODUCTION

Accumulating evidence supports the role of MC4 receptor in neuropathic (Vrinten et al., 2000, 2001; Starowicz et al., 2002, 2004; Starowicz and Przewłocka, 2003) and inflammatory (Bellasio et al., 2003) pain. Interaction between melanocortin and opioid systems has been proven. In chapter 2, we demonstrated that the intrathecally administered DAMGO, a μ-opioid receptor agonist and cyprodime, an antagonist of this receptor, influence melanocortin-induced nociceptive effects by attenuation of the hyperalgesic effect of MTII and anti-allodynic action of SHU9119, MC4R ago- and antagonist, respectively. However, others (Vrinten et al., 2003) reported a potentation of antiallodynic effect of SHU9119 administered prior to morphine. To this end, we studied whether the melanocortin-opioid interaction will indeed be affected when different experimental paradigms and/or different μ-opioid receptor agonists (DAMGO vs. morphine) are used.

METHODS

Animals

Male Wistar rats weighing 200–250 g at the beginning of the study were used. Rats were housed in single cages on a sawdust bedding under standard conditions (12 h light/dark cycle, lights on from 8:00 a.m.) with food and water available *ad libitum.* All experiments had the approval of the Local Bioethics Committee of the Institute of Pharmacology (Cracow, Poland) and were in accordance with "Ethical guidelines for investigations of experimental pain in conscious animals" (Zimmermann, 1983).

Drugs

SHU9119 (Phoenix Pharmaceuticals, USA) was stored as stock solutions at –20°C and was diluted to the appropriate concentrations right before the experiment. SHU9119 was tested at the dose of 0.5μg. Morphine hydrochloride (Polfa Kutno, Poland) was administered at doses of 5, 10, 20 and 30 μg. All drugs were injected i.t.

Surgical preparations and schedule of drug administration

Rats were prepared for intrathecal (i.t.) injection of drugs by implanting a polyethylene (PE–10) tube into the lumbar spinal cord as described by Yaksh and Rudy (Yaksh and Rudy, 1976). After the surgery, animals were allowed a minimum one-week recovery before the experiment. Saline or drugs were delivered slowly (1–2 minutes) through the i.t. catheter followed by 10 μl of saline, which flushed the catheter.

Seven days after catheter implantation, chronic constriction injury (CCI) was inflicted under sodium pentobarbital anesthesia (50mg/kg, i.p.) by tying 4 loose ligatures spaced at 1 mm around the nerve at about l cm from the nerve trifurcation, as described by Bennett and Xie (1988).

Tactile allodynia test. The assessment of tactile allodynia consisted in measuring the withdrawal threshold of the paw ipsilateral to the site of injury in response to mechanical stimulus with von Frey filaments (Stoelting, USA) calibrated to apply a pressure from 1.0 to 26.0 g. Briefly, for testing, animals were individually placed on a wire mesh floor. Cages were mounted in a position that allowed the experimenter access to the bottom of the cage. The tactile stimulus was applied to the middle plantar surface of the right paw by placing the von

Frey filament perpendicular to the surface of the paw, starting with the smallest filament (1.0 g) as described by Chaplan et al. (1994). The von Frey filament was held in this position with enough force to cause a slight bend. 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. Baseline values were determined, and measurements were repeated 15, 30, and 60 min after drug or vehicle administration.

Experiment design

The following treatment was used to establish the possible melanocortin-opioid modulation in neuropathic rats: SHU9119 at a dose of 0.5 μg, morphine (5, 10, 20 and 30 μg), and a combination of SHU9119 (0.5 μg) administered 15 min before respective dose of morphine. Rats were tested for the tactile allodynia in a time-course study (15, 30 and 60 min after the morphine administration). Data in the graph represent the mean ±SEM of values obtained 60 min after the last injection.

Data analysis (MPE) and statistics

The results were quantified as the percentage of maximum possible effect (%MPE), using the following formula: % MPE = (post-drug value – baseline value) / (cutoff value – baseline value) x 100. Differences between the groups were analyzed by a one way ANOVA test followed by Tuckey test. Differences with $p < 0.05$ were considered significant.

RESULTS

MC4R antagonist modulates morphine analgesia in neuropathic rats

Spinal administration of morphine (5–30 μg, i.t.) produced a weak anti-allodynic effect in rats after sciatic nerve injury as shown by an increase in withdrawal thresholds to mechanical stimulus (Fig. 1). SHU9119 at a dose 0.5 μg when given 15 min prior to morphine, produced significant increase ($p<0.001$) in withdrawal thresholds to mechanical stimulation with a maximum %MPE of 65.8 ± 7.2, in rats which obtained SHU9119 before 30 μg of morphine (Fig. 1D).

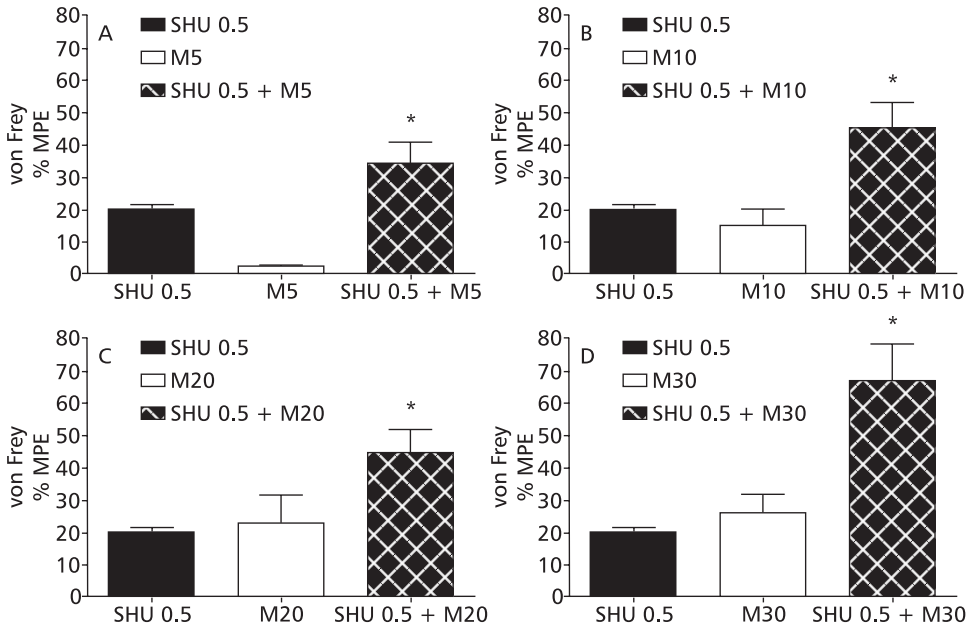

Fig. 1. Additive anti-allodynic action of morphine (M) at dose of 5–30 μg, i.t. and SHU9119 (SHU) at dose of 0.5 μg, i.t. as measured by von Frey test at 60 min after injection. Statistic was performed using the One way ANOVA followed by Tuckey post test, #p< 0.05 vs. respective dose of morphine; *p<0.05 and ***p<0.001 vs. SHU9119 at dose of 0.5 μg

CONCLUSIONS

In a series of experiments with the combined injection of an MC4R antagonist and morphine at different doses (5–30 μg, i.t.), we showed a potentation of morphine analgesic effect in neuropathic rats. Spinal melanocortins have been suggested to interact with opioids. The melanocortins and opioid peptide β-endorphin are products of posttranslational modifications of the proopiomelanocortin (POMC) prohormone. Thus, the possible explanations for spinal interaction between those two neuropeptide systems may be derived form the fact that they have a common precursor molecule, POMC. The signal(s) generated by POMC-derived peptides may ultimately be a resultant of their complex functional interaction with each other. Thus, the biological signal generated by the melanocortins and opioids varies accordingly to the physiological status of the secreting cell; α-MSH and related molecules stimulate the adenylate cyclase pathway (Low et al., 1994), whereas the opioids decrease cAMP formation (Kieffer 1995). The opposite regulation may hint at the possible interpre-

tation of melanocortin-opioid interactions, but the neuroanatomical evidence and reports on the suggested co-expression of MC4 and μ-opioid receptors on the same anatomical sites of dorsal spinal cord are still missing.
The results of our study highlight the importance of drug injection schedule and the interval in-between, what was also demonstrated for morphine and NMDA receptor interaction (Belozertseva et al., 2000). As demonstrated in chapter 2, if a different paradigm was used, namely if the μ-opioid receptor ligands were administered prior to MC4R ligands, an opposite regulation was observed. A combination of μ-opioid receptor agonist, DAMGO, administered i.t. 15 min prior to SHU9119 injection, attenuated its analgesic effect, when compared to effect of SHU9119 given alone to neuropathic animals. Similar was true for the administration of MTII after DAMGO injection, namely, the hyperalgesia produced by the combined drug injection was weaker when compared to the effect of MTII alone. The effect of the combined drug administration varies depending on whether melanocortin or opioid system is first affected by its ligands. Activation of the opioid system would diminish the effects of MC4R, and blockade of MC4R would contribute the enhanced analgesic potency of morphine. Thus an interplay between μ-opioid and MC4 receptor seems to be crucial to the final behavioral effect observed. The hypothesis of the melanocortin and opioid system interaction was also tested in the aspect of morphine tolerance, and the results are described in detail in chapters 5 and 6 of this Thesis.

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Chapter III

Melanocortin 4 receptor is expressed in the dorsal root ganglions and down-regulated in neuropathic rats

Katarzyna Starowicz[1],[2], Wiktor Bilecki[1],
Agnieszka Sieja[1], Barbara Przewłocka[1],
Ryszard Przewłocki[1]

[1] Institute of Pharmacology, Polish Academy of Sciences, Department of Molecular Neuropharmacology, Cracow, Poland
[2] International Institute of Molecular and Cell Biology, Warsaw, Poland

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Melanocortin 4 receptor is expressed in the dorsal root ganglions and down-regulated in neuropathic rats

Katarzyna Starowicz[a,b], Wiktor Bilecki[a], Agnieszka Sieja[a], Barbara Przewlocka[a], Ryszard Przewlocki[a,*]

[a] *Institute of Pharmacology, Polish Academy of Sciences, Department of Molecular Neuropharmacology, 12 Smetna Street, 31-343 Cracow, Poland*
[b] *International Institute of Molecular and Cell Biology, 4 Ks. Trojdena Street, 02-109 Warsaw, Poland*

Received 14 October 2003; received in revised form 16 December 2003; accepted 17 December 2003

Abstract

Recent reports have demonstrated effectiveness of melanocortin antagonists as potent analgesics, and have suggested that the spinal melanocortin 4 receptor (MC4-R) mediates their effects on pain transmission. These findings prompted us to investigate the changes in MC4-R mRNA level in the spinal cord and dorsal root ganglia (DRG) of neuropathic animals at different time points after sciatic nerve injury by quantitative real-time PCR. The spinal MC4-R mRNA level was not affected by sciatic nerve injury. In contrast, down-regulation of MC4-R mRNA in DRG developed 2 weeks after the injury and was parallel with the attenuated effectiveness of MC4-R ligands in neuropathic animals. The MC4-R adaptation in DRG observed in neuropathic rats indicates their important role in presynaptic modulation of activity of the primary afferents in neuropathic pain.

Keywords: Melanocortins (MC); Neuropathic pain; Melanocortin 4 receptor (MC4-R); Dorsal root ganglia (DRG); Chronic constriction injury (CCI); Real-time RT-PCR

Melanocortins (MC) are a family of bioactive peptides derived from proopiomelanocortin. The involvement of melanocortins and their receptors in many important functions of the organism [11,16] including nociception [2,10,17] had been reported. More recently, the melanocortin system has received attention as a potential target for treatment of chronic pain [12,14,15]. It was found that intracisternal [15] and spinal [12] administration of MC3/MC4 receptor antagonist alleviated the neuropathic pain symptoms in the rat. Interestingly, among cloned MC receptors, the only type whose expression has been demonstrated in the spinal cord is the melanocortin 4 receptor (MC4-R) [8]. Both dorsal root ganglia (DRG) and spinal cord are involved in sensory information processing, but the expression of the MC4-R in DRG has only been found during development of the nervous system [9] but not in adult animals [5,8]. Up until now there is limited information about changes in expression of gene coding for melanocortin receptors in neuropathy.

In order to better understand a role of MC4-R in chronic pain, we studied whether this receptor is expressed in the DRG of mononeuropathic rats, and whether it undergoes any adaptational changes, and if so, how the changes are correlated to behavioural effects of MC4-R ligands. To address this issue, we investigated the MC4-R mRNA level in spinal cord sections and in DRG at the lumbar level which corresponds to the sciatic nerve input, in relation to the development of tactile allodynia. We also tested the effects of MC4-R ligands in neuropathic rats at various times after nerve injury.

Male Wistar rats (Rembertow, Poland) 220–250 g were housed in single cages, under standard 12/12 h light/dark cycle (lights on at 08:00 h), food and water available ad libitum. All the handling and testing of the animals was performed in accordance with recommendations of the International Association for the Study of Pain [20] and received approval from the Local Bioethics Committee of the Institute of Pharmacology.

Rats for behavioural tests ($n = 40$) were chronically implanted with intrathecal catheters under sodium pentobarbital anaesthesia (50 mg/kg, i.p.) according to Yaksh and

* Corresponding author. Tel.: +48-12-662-3218; fax: +48-12-637-4500.
E-mail address: nfprzewl@cyf-kr.edu.pl (R. Przewlocki).

0304-3940/03/$ - see front matter © 2003 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.neulet.2003.12.096

Rudy [19]. Seven days after catheter implantation, chronic constriction injury (CCI) was inflicted under sodium pentobarbital anaesthesia (50 mg/kg, i.p.) by tying four loose ligatures spaced at 1 mm around the nerve at about 1 cm from the nerve trifurcation, as described by Bennett and Xie [1]. On day 7 after CCI, rats were randomly divided into five groups: vehicle treatment, SHU 9119 at 0.15 and 0.5 μg and MT II at 30 and 100 ng. Fourteen days after sciatic nerve injury rats were again randomly divided into treatment groups and tested as previously described.

SHU9119 [cyclo-[Nle^4, Asp^5, D-Nal(2)^7, Lys^{10}]-MSH-(4–10)] and MTII [Melanotan-II or cyclo-[Nle^4, Asp^5, D-Phe^7, Lys^{10}]-MSH-(4–10)], (Phoenix Pharmaceuticals, USA) were administered intrathecally (i.th.) in a volume of 5 μl through the lumbar catheter by a Hamilton syringe.

The assessment of tactile allodynia consisted in measuring the withdrawal threshold of the paw ipsilateral to the site of injury in response to probing with von Frey filaments (Stoelting, USA) calibrated to apply a pressure from 1.0 to 26.0 g. The midplantar surface of the paw was touched (trough a metal mesh floor) starting with the smallest filament (1.0 g) as described by Chaplan et al [3]. 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. Baseline values were determined, and measurements were repeated 15, 30, and 60 min after drug or vehicle administration.

The effects of SHU9119 and MTII on allodynia were expressed as a percentage of maximal possible effect (% MPE), and calculated using the equation: %MPE = [(TT-BT)/(CUT OFF-BL)] × 100 where BT = baseline threshold, TT = threshold in von Frey test. The results were analyzed using the one-way ANOVA followed by Bonferroni test.

For evaluation of MC4-R mRNA in spinal cord and dorsal root ganglia, a total of 48 rats were used. Each of six tested group consisted of eight rats (intact rats, rats implanted with i.th. catheters, and neuropathic animals: 3, 7, 14 and 21 days after CCI). Rats were tested for allodynia on the indicated days and sacrificed. Spinal cord (L4–L6) was divided into one ipsi- and one contralateral spinal cord section per sample. The L4–L6 DRG from the right (ipsilateral) and left (contralateral, control) side of two animals were pooled into one experimental sample. All tissues were rapidly frozen on dry ice and stored in −70 °C until the extraction of mRNA.

Tissue samples were homogenized and mRNA was isolated according to Chomczynski and Sacchi [4]. The iCycler iQ Real Time PCR Detection System (BIORAD, USA) for quantitative real-time detection of PCR products was used. For calculating relative amounts of PCR products, we used standard curve of template dilutions. All data concerning the MC4-R were normalized using the glycerol-3-phosphate dehydrogenase (GAPDH) and the relative amount was calculated using control contralateral side as a reference value. Data in the graph are the mean of two to three independent real time RT-PCR runs, which were performed in tetraplicate. Primer concentration was 5 μM and probe concentration was 100 ng per reaction. The following sets of primers were used: rat MC4-R: 5′-GTA ATT GCG CCC TTC ATG TT; 5′-TCG GGC GTT CTT TTT ATC AT. Rat GAPDH: 5′-TGT ACC GAT CGA TGT CTG GA; 5′-CCT GCC CAA GAT TGT TGA GT.

The sciatic nerve ligation decreased paw withdrawal threshold to mechanical stimulation with von Frey filaments. Allodynia developed on day 3 post-injury, when a 65% decrease in withdrawal threshold compared to sham operated animals (7.1 ± 2.4 vs. 20.5 ± 2.2, respectively) was observed ($F_{7,47} = 41.6$; $P < 0.001$). On day 7 and 14 the CCI rats displayed a 83 and 91% decrease ($F_{7,47} = 41.6$; $P < 0.001$), respectively, in withdrawal threshold, as compared to sham-operated animals (2.1 ± 0.5 vs. 23.2 ± 1.6 g, respectively). Strong allodynia (3.2 ± 0.74 g in CCI rats) was still observed 3 weeks after CCI. No significant differences were observed in allodynia between 3 and 21 days after CCI. In sham operated animals no significant changes appeared (Fig. 1A).

There were no statistically significant differences in MC4-R mRNA level between naive and sham-operated animals (data not shown). The intrathecal catheter implantation did not significantly change the MC4-R mRNA level in control rats, neither on contra- nor on ipsilateral side in DRG (0.94 ± 0.3 contra-; 0.86 ± 0.07 ipsilateral) and spinal cord (1.02 ± 0.27 contra-; 1.06 ± 0.3 ipsilateral).

In the spinal cord, MC4-R transcript level was not significantly modified by the CCI procedure, neither on ipsi-nor on contralateral side to the nerve injury (Fig. 1B).

A statistically significant decrease in ipsilateral MC4-R mRNA level in CCI rats' DRG was observed on day 14 (0.51 ± 0.09, $P < 0.05$, $F_{4,19} = 12.9$) and on day 21 (0.44 ± 0.16, $P < 0.01$; $F_{4,19} = 12.8$) after injury compared to control animals (Fig. 1C). The MC4-R mRNA on the contralateral side remained unaffected throughout the testing period.

Based on the biochemical observation that differences in MC4-R mRNA level in DRG of neuropathic rats occurred between day 7 (no change) and day 14 (decrease) after the nerve injury (see Fig. 1C), the behavioural experiments were performed on days 7 and 14 after sciatic nerve injury.

SHU9119 (0.15, 0.5 μg, i.th.) administered to CCI rats 7 days after nerve injury, 30 min after administration showed a dose-dependent effect, 5.1 and 65.3% ($P < 0.001$ vs. vehicle; $F_{5,31} = 26.8$), respectively, in the tactile allodynia test (Fig. 2A). The antiallodynic effect observed 60 min after SHU9119 injection was decreased (data not shown), but those values did not differ significantly from values obtained 30 min after administration (shown in Fig. 2A). One week after nerve injury, intrathecal administration of MC4-R agonist MTII (30 and 100 ng, i.th.) produced 30 min after the injection a decrease in withdrawal thresholds for mechanical stimulation (after 100 ng to −52.3% MPE) (Fig. 2B).

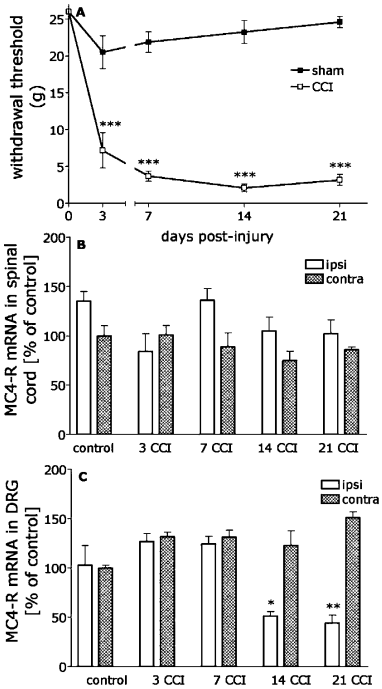

Fig. 1. Development of tactile allodynia (A); quantitative real-time RT-PCR analysis of MC4 receptor (MC4-R) level in spinal cord (B); and dorsal root ganglia (DRG) (C). Tactile allodynia was assessed using von Frey filaments (1.0–26.0 g). The MC4-R mRNA level is expressed as % of MC4-R expression in control sample. All biochemical data were normalized using GAPDH and the relative amount calculated using the control contralateral side as a reference value (= 100%). Results are expressed as the mean ± SEM $^{*}P < 0.05$ versus respective controls (one-way ANOVA, followed by Bonferroni comparison test).

Fourteen days after nerve injury, effects of MC4-R ligands in CCI rats were more markedly attenuated (Figs. 2A,B), than at 7 days after CCI. Though a dose-dependent antinociceptive effect of SHU9119 (Fig. 2A) was still observed (10.0% MPE; 24.5% MPE; $P < 0.05$; for respective tested dose, $F_{5,35} = 23.9$) 14 days after nerve injury (Fig. 2A). Similar effect was observed for MTII (Fig. 2B) at a dose of 100 ng i.th., a significant increase ($F_{5,35} = 6.2$, $P < 0.01$) in withdrawal threshold by 39.4% was observed on day 14 as compared to day 7.

The major finding of the present study is the demonstration that the MC4-R is expressed in the DRG and is regulated after sciatic nerve injury in adult rats. It is generally accepted that in adult animals expression of MC4-R is limited to the CNS [5]. On the other hand, some studies [6,9] show that during development of the rat foetus, MC4-

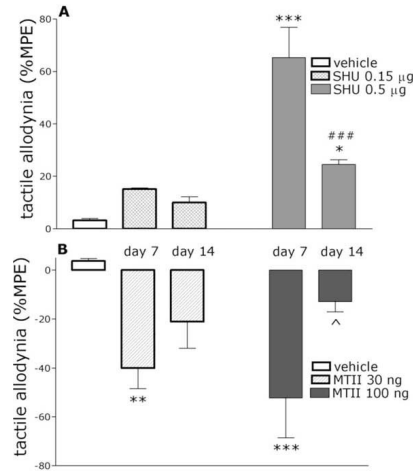

Fig. 2. The effects of intrathecal (i.th.) administration of MC4-R antagonist (A) SHU9119 (0.15 and 0.5 μg) and MC4-R agonist (B) MTII (30 and 100 ng) on tactile allodynia 7 and 14 days after CCI in rats. Data are expressed as a percentage of maximal possible effect (% MPE), mean ± SEM ($n = 8$). Bonferroni post-test was employed to evaluate the statistical differences between treated groups. $^{*}P < 0.05$ versus vehicle; $^{\#}P < 0.05$ SHU9119 on day 14 versus respective SHU9119 dose on day 7; $^{\wedge}P < 0.05$ MTII on day 14 versus analogous MTII dose on day 7.

R mRNA is expressed transiently in the peripheral nervous system (cranial nerve ganglia and sympathetic ganglia) where the appearance of MC4-R mRNA is temporarily correlated with periods of neural network formation. In the present study, the MC4-R mRNA in DRG of adult rats has been demonstrated. Our finding may be of great importance since it might suggest location of this receptor on terminals of primary afferents, which project from DRG to the superficial laminae of the dorsal horn. Furthermore, the presence of MC4-R on primary afferent terminals within the spinal cord could suggest their involvement in presynaptic regulation. Interestingly, in addition to the demonstration of MC4-R in DRG, we found a marked reduction in the DRG MC4-R mRNA levels of CCI animals 2 weeks (but not 1 week) after sciatic nerve injury. This coincided with a decrease in effectiveness of MC4-R ligands in affecting mechanical allodynia. Thus, the down-regulation of MC4-R mRNA in DRG of neuropathic rats seems to be of functional importance. In addition to their presumed role in regulation of nociceptive input to the spinal cord, the altered MC4-R mRNA expression in CCI animals may be involved in a new network formation [13], which occurs after nerve injury when C-fiber terminals undergo atrophy and A-fiber terminals sprout into the superficial laminae of the dorsal horn [7,18].

In the present study, we showed that MC4 receptor transcript remained unchanged in the spinal cord of CCI

animals as revealed by real-time PCR. However, Vrinten et al. [15] found that in CCI animals, MC4 receptor level in laminae I–II on ipsi and contralateral side to the injured nerve at the L4–L6 level of the spinal cord was significantly higher by about 20% in comparison with sham-operated animals as shown by in situ ^{125}I-NDP-MSH binding.

The difference between results of the present paper and that by Vrinten et al. [15] may be due to the fact that these authors measured MC4-R in discrete regions of the spinal cord while our measurements were preformed in the whole spinal lumbar homogenates in which the presumed increase in MC4 receptor expression in superficial layers could have been diminished.

In conclusion, we have demonstrated here that MC4 receptor is expressed both in the spinal cord and also in the DRG, which suggests its involvement in presynaptic regulation of nociceptive input. In a chronic constriction injury model of neuropathic pain, MC4-R mRNA level in DRG undergoes adaptive changes, which parallel changes in anti-allodynic or proallodynic efficacy of melanocortin receptor ligands. This suggests a role of DRG MC4-Rs in neuropathic pain.

Acknowledgements

This study was supported by the grant No. K062/P05/2003 from the State Committee for Scientific Research, Warsaw, Poland.

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Addendum #2

Melanocortin 4 and μ-opioid receptors in the rat dorsal root ganglia and spinal cord after peripheral nerve injury: immunohistochemical studies

Katarzyna Starowicz[1,2], Shaaban Mousa[3], Agnieszka Chocyk[4], Ryszard Przewłocki[1], Krzysztof Wędzony[4], Halina Machelska[3], Barbara Przewłocka[1]

[1]Dept. of Molecular Neuropharmacology, Institute of Pharmacology PAS, Cracow, Poland
[2] International Institute of Molecular and Cell Biology UNESCO/PAS, Warsaw, Poland
[3] Klinik fur Anaesthesiologie und Operative Intensivmedizin, Charite-Universitatsmedizin Berlin, Campus Benjamin Franklin, Berlin, Germany
[4] Dept. of Pharmacology and Brain Biostructure, Institute of Pharmacology PAS, Cracow, Poland

ABSTRACT

The effect of sciatic nerve injury (CCI) on the expression of melanocortin (MC4R) and μ-opioid (μOR) receptors in the rat L4-L5 dorsal root ganglia (DRG) and dorsal horn of the spinal cord was investigated using immunohistochemical techniques. We observed an increase in MC4R immunoreactivity (IR) in the DRG. The degree of MC4R up-regulation parallels the time after nerve injury, viz. a relative increase by 21.1 % and 40.5 % in comparison to control animals was noted 3 and 14 days after the injury, respectively. Spinal cord MC4R-IR ipsilateral to the nerve injury was also increased. In contrast, a decrease in μOR-IR was observed in investigated tissues. Consistently with the development of neuropathic pain, μOR-IR in the rat DRG decreased by 30 and 43% 3 and 14 days after injury, respectively. Examination of the spinal cord revealed a statistically significant reduction of μOR-positively stained cells, ipsi- and contralateral to the injury, though the effect on the ligated sitde was more pronounced. In summary, the higher level of MC4R-IR in the spinal cord and DRG together with lower level of μOR-IR may contribute to the reduced efficacy of morphine in neuropathic pain.

INTRODUCTION

Neuropathic pain develops as a consequence of disease and/or injury of the peripheral nervous system. Its pathomechanism appears to be complex and remains inadequately understood. One of major limitation in neuropathic pain therapy is a reduced analgesic potency of morphine, which may be due to the changes in spinal μ-opioid receptor (μOR) expression. However, changes in μOR expression alone fail to account for the attenuation of opioid activity. The possible role of POMC-derived peptides, melanocortins and melanocortin 4 receptor (MC4R) has recently attracted attention. Recent reports have demonstrated effectiveness of MC4R antagonists as potent analgesics in chronic neuropathic pain (Vrinten et al., 2000; Starowicz et al., 2002). The melanocortin and opioid systems appear to have opposite activities in many pharmacological tests, therefore, melanocortins have been considered to be endogenous functional antagonists of opioids (Contreras and Takemori, 1984; Szekely et al., 1979). On the basis of these findings, a hypothesis about the higher activity of melanocortin system in neuropathic pain was suggested. The study of Beltramo et al. (2003)

demonstrated up-regulation of POMC and MC4R expression, paralleling the presence of neuropathic pain symptoms, namely tactile allodynia and thermal hyperalgesia. In addition, a complex interaction between the melanocortins and opioids in a rat model of neuropathic pain have been reported (Starowicz et al., 2002; Vrinten et al., 2003). Furthermore, our previous study demonstrated the presence of MC4R in the dorsal root ganglia (DRG) and its expression in this tissue in neuropathic rats (Starowicz et al., 2004) indicating their important role in presynaptic modulation of activity of the primary afferents. However, the level of mRNA is not always parallel to the level of its end-products, therefore, to elucidate the physiological significance of the changes in MC4R and μOR expression we used light microscopic immunohistochemical methods to examine the immunoreactivity of both MC4R and μOR receptors in the lumbar DRG and spinal cord of neuropathic rats.

MATERIALS AND METHODS

Animals

Experiments were performed in male Wistar rats (License Breeding House, Rembertow, Poland) weighing 220–250 g at the time of surgery. Animals were individually housed in cages lined with sawdust. Standard laboratory rodent chow and water were available *ad libitum*. The rats were maintained under 12-h:12-h light/dark cycle (lights on at 08:00 h). All the experiments received approval from the Local Bioethics Committee of the Institute of Pharmacology PAS in Cracow and were performed in accordance with recommendations of the International Association for the Study of Pain (Zimmermann, 1983)

Chronic constriction injury to the sciatic nerve (CCI)

CCI was performed according to the method described by Bennet and Xie (Bennett and Xie, 1988). Briefly, under sodium pentobarbital anesthesia (50mg/kg, i.p.), the common right sciatic nerve was exposed and 4 loose ligatures spaced 1 mm were tied around the nerve at about 1 cm from the nerve trifurcation. The tissue was collected for immunohistochemical analysis 3 and 14 days after the injury.

Immunohistochemistry

The rats were deeply anesthetized with an overdose of pentobarbital (100 mg/kg, i.p.) and transcardially perfused with 60 ml of warm saline (0.9% NaCl), followed by 300 ml of 4% (w/v) paraformaldehyde with 0.2% (v/v) picric acid in 0.16 M phosphate-buffered solution, pH 6.9. The spinal cord, and ipsilateral and contralateral L4/L5 DRG were removed, postfixed in the same fixatives for 90 min, and then placed in 15% (w/v) sucrose solution at 4°C for 12 hours. The tissue was embedded in Tissue Tek compound (OCT; Miles), frozen and cut into 14-μm sections (lumbar spinal cord) or 10-μm sections (DRG) on the Microm cryostat, (Zeiss Gruppe, Microm Laborgeräte GmbH, Walldorf, Germany). For light microscopic immunohistochemistry, the free floating spinal cord sections or gelatin mounted DRG sections were incubated overnight with anti-μOR (1:1000; kindly provided by Drs. Stefan Schulz and Volker Höllt, Department of Pharmacology and Toxicology, Otto-von-Guericke University, Magdeburg, Germany, for the specificity of antibodies see Schulz et al., 1998) or anti-MC4R (1:50, Abcam, Cambridge, UK) antibody. Immunohistochemical staining of sections was performed as described previously (Mousa et al., 2001). In brief, the Vectastain avidin-biotin peroxidase complex (ABC; Vectastain Elite Kit, Vector Laboratories, Inc.) was used. The sections were incubated for 90 min with the appropriate biotinylated secondary antibody and with avidin-biotin-conjugated peroxidase. Finally, the sections were washed and stained with 3',3'-diaminobenzidine tetrahydrochloride containing 0.01% H_2O_2 in 0.05 M Tris-buffered saline, pH 7.6, for 3 to 5 min. Then, the sections were washed in tap water, dehydrated in alcohol, cleared in xylene, and mounted in dibutylpthalate polystyrene xylene. To demonstrate specificity of staining, the following controls were included: 1) preabsorption of diluted anti-μOR antibody with a synthetic peptide agonist of μOR (Gramsch Laboratories, Schwabhausen, Germany) for 24 h at 4°C and 2) omission of the primary antisera, the secondary antibodies, or the avidin-biotin complex. These control experiments proved the specificity of the obtained stainings.

Quantification of immunoreactivity

The method of quantification for DRG staining has been described previously (Ji et al., 1995). Briefly, we stained every fourth section of DRG that was serially cut at 10 μm. The total number of MC4R- or μOR-containing neurons was counted by an observer unaware of the experimental protocol. Five squares (38.4 mm^2 each) per section were analyzed using a Zeiss microscope (objective, x40

x10; Carl Zeiss, Oberkochen, Germany). This number was divided by the total number of neurons in each DRG section, and the percentage of MC4R and μOR immunoreactive (IR) neurons was calculated. Percentages from four sections of each DRG were averaged. Eight rats per group were used for analysis. Values are the mean ± SEM. For further details see: Zöllner et al. (2003).

Image Analysis and Quantification (MCID™ Elite, Canada) system was employed to measure the relative optical density (ROD) in the spinal cord. Staining signal of MC4R or MOR was measured across the dorsal horn of the rat spinal cord. Data from lamina I and II were collected from at least four sections per animal, bilaterally. ROD for each section background was also measured, and subtracted from the immunoreactive signal in the regions of interest. Values are ROD means ± SEM.

Statistical analysis

Differences in the percentage of μOR-IR and MC4R–IR in the L4-L5 DRG between control and neuropathic rats at each time point were tested using repeated measurements ANOVA, followed by the Tuckey post hoc test, when the former gave a significant result.

Comparison of spinal cord-IR for different groups at each time point was performed using two-way ANOVA (time and side: ipsi- vs. contralateral, as a source of variation). The level of significance in all cases is presented as follows: *$P<0.05$, **$P<0.01$, ***$P<0.001$.

RESULTS

Effect of sciatic nerve injury on MC4R immunoreactivity in the rat L4-L5 DRG

Microscopic analysis (Fig. 1A-C) and subsequent quantification (Fig. 1D) of MC4R-IR positive neurons showed extensive changes in the receptor expression in a time after lesion dependent degree in different populations of DRG neurons. All animals subjected to CCI exhibited an ipsilateral increase in the number of MC4R-IR neurons paralleling time elapsing after nerve injury, mostly in small-sized and medium neurons. The signal varied from lightly stained cells with granular staining to cytoplasm completely filled with MC4R-IR (Fig. 1A-C). The 44.1 ± 2.6 % of all neurons were MC4R-IR (Fig. 1A). Three days after

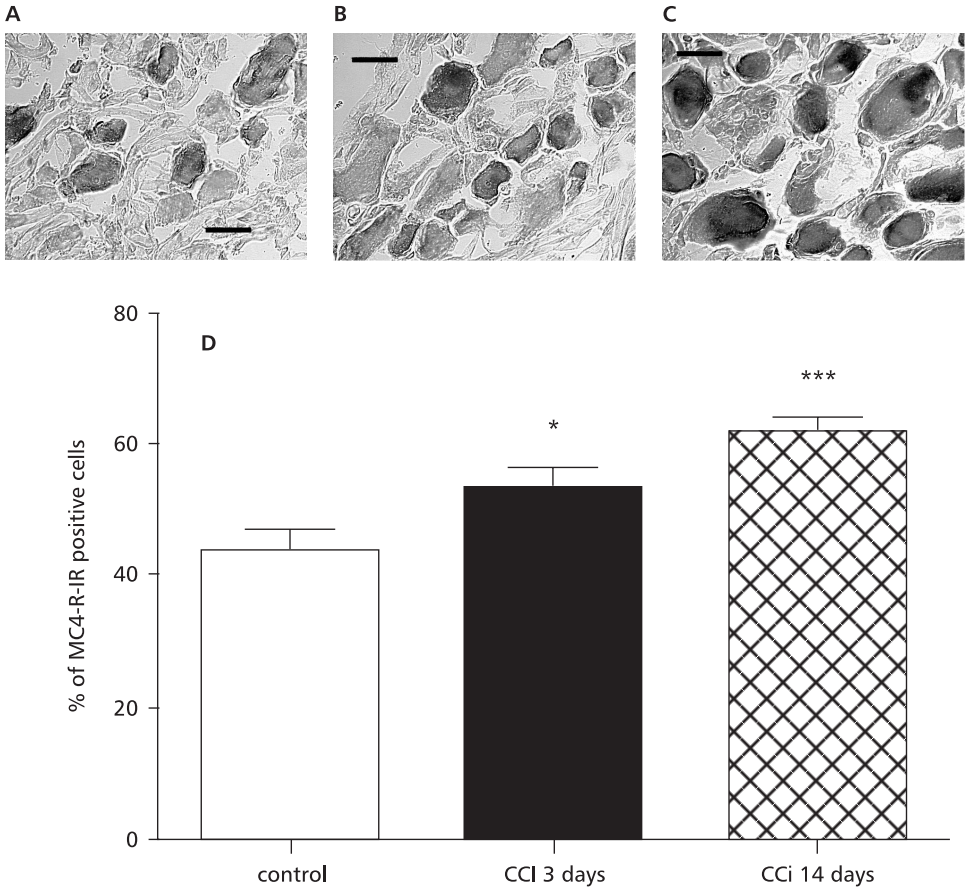

Fig. 1. Effect of unilateral sciatic nerve ligation on the MC4R immunoreactivity (IR) in the ipsilateral L4-L5 rat DRG. Micrographs show MC4R positive neurons in L4 and L5 DRG of control rats (A) and after 3 (B) and 14 (C) days of neuropathic pain, and subsequent analysis (D) of MC4-R positively stained cells. MC4R-IR is mainly seen in small and medium size DRG neurons. Scale bar corresponds to 20 μm. Percent (%) of the immunostained MC4R-IR-positive neurons increases gradually following the injury. Values are the mean ± SEM, n=8 for each group. *$p< 0.05$ and ***$p< 0.001$ vs. control animals.

CCI, there was a noticeable increase in the number of MC4R-positive DRG neurons on the injured side (Fig. 1B), and 53.4 ± 3.0 % of all DRG neurons were MC4R-positive, which represents a relative increase by 21.1% ($p< 0.05$). Development of neuropathic pain (14 days after injury) was followed by the increase in the number of MC4R-positive DRG neurons (Fig. 1C), of which 62.0 ± 2.0 % were MC4-R positive, which corresponds to a relative increase by 40.5 % when compared to control animals ($p<0.001$). F value for the test $F_{2,21} = 12.2$.

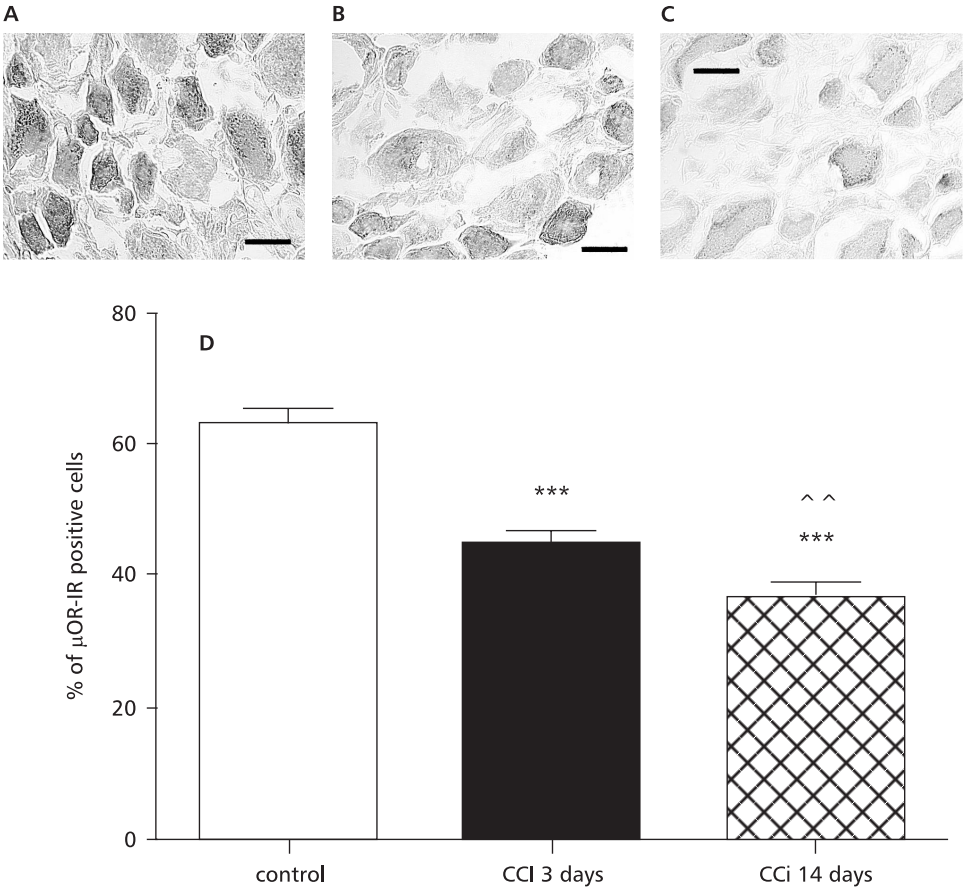

Fig. 2. Effect of unilateral sciatic nerve ligation on the μOR immunoreactivity (IR) in the ipsilateral L4-L5 rat DRG. Micrographs show μOR positive neurons in L4 and L5 DRGs of control rats (A) and after 3 (B) and 14 (C) days of neuropathic pain, and subsequent analysis (D) of μOR positively stained cells. μOR-IR is mainly seen in small and medium size DRG neurons. Scale bar corresponds to 20 μm. Percent (%) of the immunostained μOR-IR positive neurons decreases gradually following the injury. Values are the mean ± SEM, n=8 for each group. *p< 0.05 and ***p< 0.001 vs. control animals.

Effect of sciatic nerve injury on μOR immunoreactivity in the rat L4-L5 DRG

Examination of μOR immunoreactivity in the rat DRG revealed distinct dense areas of μOR-IR within the L4-L5 DRG of control animals as demonstrated by micrographs (Fig. 2A-C) and subsequent quantification (Fig. 2D). In control rats, 63.2 ± 2.0 % of the cells per section were μOR-IR positive (Fig. 2 A).

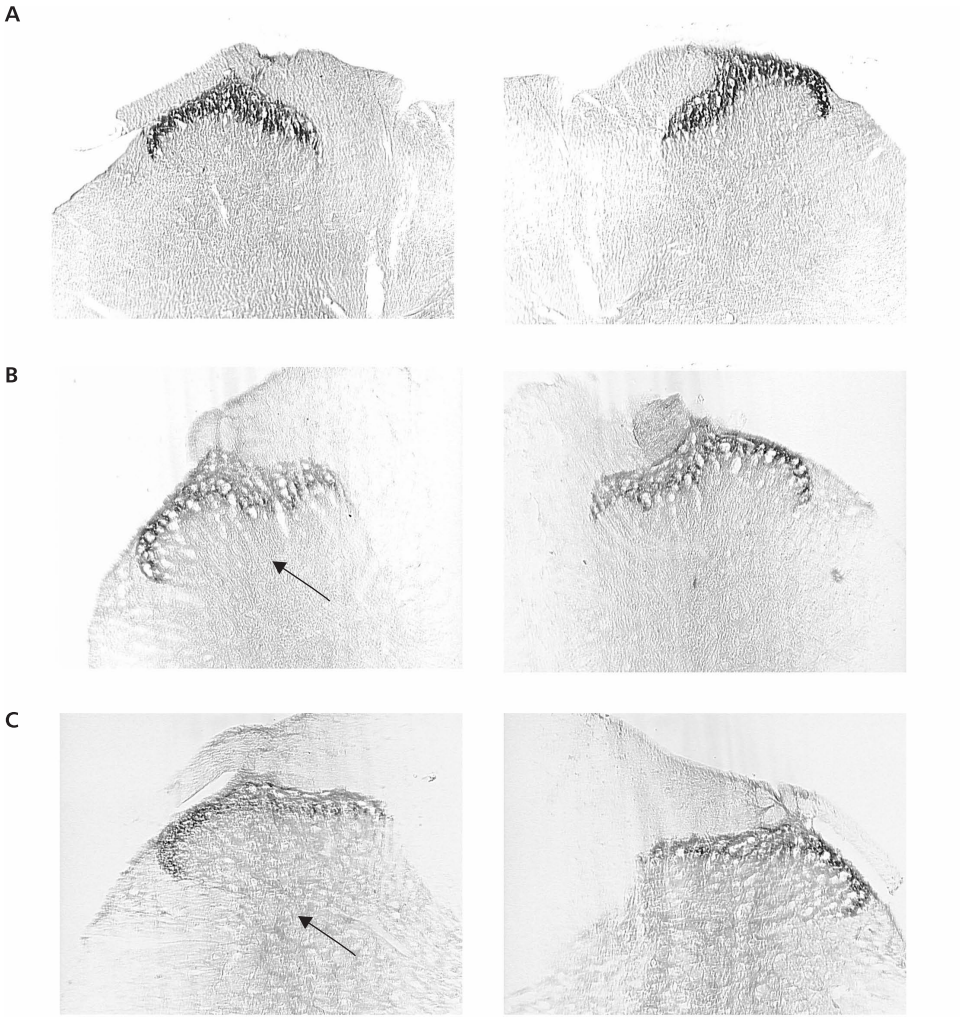

Fig. 3. Representative images of μOR-IR in sham-operated animals (A) and in rats subjected to peripheral nerve ligation, 3 (B) and 14 (C) days after the injury. CCI resulted in a decrease in μOR-IR ipsilateral to the injury (B, C arrow) that was not seen following following sham operation (A).

Peripheral injury to the sciatic nerve caused a decrease in the number of μOR-positive cells by 44.7 ± 2.0 % ($p < 0.001$) and 36.6 ± 2.4 % ($p < 0.001$), 3 and 14 days post injury, respectively (Fig. 2 B, C). Thus, the sciatic nerve ligation caused a 30 and 43 % decrease in μOR positively stained cells in comparison with control individuals. F value for the test $F_{2,21} = 37.1$.

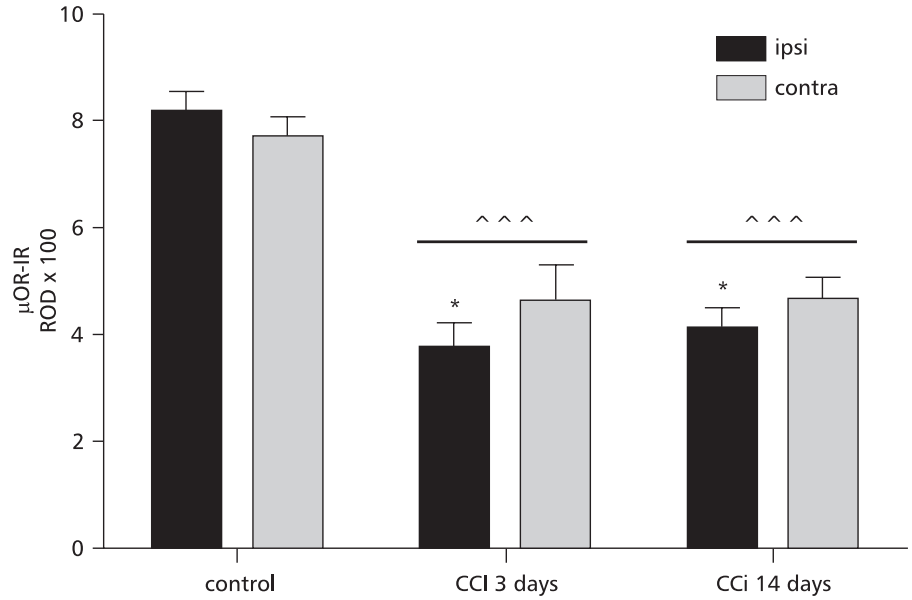

Fig. 4. Effect of unilateral sciatic nerve ligation on and μOR immunoreactivity (IR) in the rat spinal cord. Chronic constriction injury (CCI) induced down-regulation of μOR. Relative optical density (ROD) measurement revealed a significant (***$p<0.001$) changes when time was employed as critical parameter and #$p<0.05$ when the analysis was performed according to group treatment (injured vs. non-injured).

μOR immunoreactivity in the spinal cord of neuropathic rats

Immunohistochemical studies conducted 3 and 14 days after CCI in rats revealed a decrease in μOR-IR on the nerve-injured side when compared with both the contralateral side and control animals (Fig. 3; Fig. 4). The observed changes in the lumbar segments were restricted to the dorsal horn innervated by the sciatic nerve. Quantification of the density of μOR-IR 3 days after the CCI revealed a statistically significant decrease by 52% on the ligated side and by 24% on the contralateral side (Fig. 3 B), when compared to the control animals ($p< 0.001$), the above decrease in the μOR-IR within the studied group was statistically significant ($p< 0.05$). Examination of the spinal cords 14 days following CCI revealed a consistent reduction in spinal μOR-IR. At this time (Fig. 3 C), reduction of the spinal μOR by 47% and 32% was observed ipsi- and contralaterally to the lesion, respectively ($p<0.001$). Chronic injury to the sciatic nerve caused a 23 % decrease in μOR-IR on the ipsilateral side when compared to the contralateral side ($p< 0.05$).

DISCUSSION

In the present study, we used light microscopic immunohistochemical methods to examine the immunoreactivity of both MC4R and μOR in the DRG and lumbar spinal cord of neuropathic rats. Peripheral nerve injury results in a time dependent increase in MC4R immunohistochemical staining, whereas μOR-immunoreactivity decreased gradually following the injury. Furthermore, similar changes were observed at the spinal cord level. Herein we report an opposite regulation of MC4R and μOR in L4-L5 DRG and spinal cord during development of neuropathic pain after constriction injury to the sciatic nerve. Our previous study demonstrated the MC4R mRNA in the DRG and confirmed its expression in the spinal cord. The demonstration of a functional MC4R as indicated by protein detection in tissues implicated in nociception further confirms the influence of melanocortins on pain perception. Furthermore, the MC4R plastic changes concomitant with the neuropathic pain and the demonstrated opposite regulation of MC4R and μOR in CCI model emphasized the important role of melanocortins.

Inhibition of nociceptive transmission at the spinal level by opioids is primarily attributed to an action of μOR in the dorsal horn. Changes in spinal μOR expression have been observed in animal models of neuropathic pain (Stevens et al., 1991; Zhang et al., 1998). It was demonstrated that peripheral axotomy caused a reduction in the number and μOR-positive neurons and intensity of their staining in the rat and monkey DRG and of μOR-IR in the dorsal horn of the spinal cord (Zhang et al., 1998). Furthermore, a decrease in μOR expression in the DRG of nerve-injured mice was also observed (Rashid et al., 2004). Therefore, our results on μOR-IR both in DRG and dorsal horns of spinal cord are in agreement with previous studies. These data suggest that the lower potency of systemic morphine in neuropathic pain could be at least partly caused by the decreased μOR expression in the DRG. However, the changes in μOR-IR alone fail to fully explain the attenuation of opioid activity. It was previously suggested that melanocortins (α-MSH/ACTH) antagonized the antinociceptive effect of opioids (Bertolini et al., 1979; Sandman and Kastin, 1981) and they could represent a group of endogenous neuropeptides which might be activated upon nerve injury. They may interact with MC4 receptors which are believed to play a role in the modulation of the nociceptive transmission at the spinal level. An *in situ* hybridization studies demonstrated MC4R expression in a number of regions important to spinal and supraspinal nociception (Mountjoy and Wild, 1998; Kishi et al., 2003). In the previous study of Beltramo et al. (2003), MC4

transcript was up-regulated in the spinal cord of neuropathic rats. Moreover, data of Vrinten et al. (2000) reported an increased binding of (125)I-NDP-MSH to the dorsal horn. Our studies further elucidate the plastic changes of the spinal MC4 receptors to support its role in development of chronic neuropathic pain. We recently demonstrated the presence of MC4R transcript in the DRG of adult rats (Starowicz et al., 2004). Present histochemical studies further confirm and add new data suggesting the presence of the MC4 receptor in the DRG and alteration of its expression after nerve injury. This may suggest a role for MC4R in the modulation of chronic neuropathic pain and could further support an opposite role of melanocortins to the opioid systems in morphine analgesia. The supposed presynaptic localization of MC4R may indicate that MC4R modulates release of glutamate from primary afferent terminals, thereby influencing sensory transmission from peripheral receptors to spinal dorsal horn neurons. Since glutamate coexists with CGRP in primary afferents (Merighi et al., 1991), presynaptic MC4R may modulate the release of glutamate and CGRP from the same afferents terminals. The present finding that DRG neurons are MC4R positive may support this presynaptic regulatory role and suggests that this receptor may participate in enhancement of primary afferent transmission during chronic neuropathic pain.

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Chapter IV

Knockdown of spinal opioid receptors by antisense targeting beta-arrestin reduces morphine tolerance and allodynia in rat

Barbara Przewłocka, Agnieszka Sieja,
Katarzyna Starowicz, Marcin Maj, Wiktor Bielicki,
Ryszard Przewłocki

Department of Molecular Neuropharmacology, Institute of Pharmacology, Cracow, Poland

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Knockdown of spinal opioid receptors by antisense targeting β-arrestin reduces morphine tolerance and allodynia in rat

Barbara Przewlocka*, Agnieszka Sieja, Katarzyna Starowicz, Marcin Maj, Wiktor Bilecki, Ryszard Przewlocki

Department of Molecular Neuropharmacology, Institute of Pharmacology, 12 Smetna Street, 31-343 Kraków, Poland

Received 31 December 2001; received in revised form 7 March 2002; accepted 8 March 2002

Abstract

The development of morphine tolerance and sciatic nerve injury-induced allodynia after functional knockdown of spinal opioid receptors using antisense oligonucleotides targeting β-arrestin was investigated. Ineffectiveness of morphine in neuropathic pain suggests an implication of the same mechanism in these two processes. The development of morphine tolerance (10 μg intrathecally (i.th.), every 12 h) was significantly inhibited in rats, which received i.th. β-arrestin antisenses (2 nM). Acute and chronic (6 days) i.th. administration of antisenses antagonized the allodynia in the rat model of neuropathic pain. Our results demonstrated that i.th. administration of β-arrestin antisenses delayed development of tolerance to morphine and nerve injury-induced cold allodynia, which suggest that both of the investigated phenomena may be mediated by a similar mechanism, e.g. receptor desensitization. Moreover, the antisense oligonucleotides targeting β-arrestin may constitute a new approach to the therapy of neuropathic pain.

Keywords: Antinociception; Tolerance; Allodynia; Antisense; Neuropathic pain; β-Arrestin

The binding to the opioid receptor has been shown to correlate with the desensitization of G-protein-coupled receptors. In vitro studies have indicated that this process involves phosphorylation of G-protein-coupled receptors and subsequent binding of regulatory proteins called β-arrestins. Functional deletion of the β-arrestin-2 gene in mice resulted in remarkable potentiation and prolongation of the analgesic effect of morphine, suggesting that μ-opioid receptor desensitization may be impaired [4]. The same authors demonstrated that desensitization of the μ-opioid receptor did not occur after chronic morphine treatment in knockout mice lacking β-arrestin-2 (β $arr2^{-/-}$), and that these animals failed to develop tolerance to antinociceptive effect [5]. The vast body of evidence showed that responsiveness to morphine is strongly decreased in rats after sciatic nerve injury (animal model of neuropathic pain). Neuropathic pain is associated with severe chronic sensory disturbances characterized not only by increased responsiveness to painful stimuli but also to normally innocuous stimuli and ineffectiveness of opioids creates a serious problem with its therapy.

The question arises about the contribution of desensitization of the μ-opioid receptor to the mechanisms of morphine tolerance as well as to the neuropathic pain. Therefore, in our study, we investigated the development of morphine tolerance and development of sciatic nerve injury-induced allodynia after knockdown of β-arrestin-2 using the antisense approach.

Male Wistar rats, weighing 250–300 g, were used. The animals were housed in a single plastic cages under a 12/12 h light/dark cycle with food and water available ad libitum. All testing procedures in the present study were performed according to the Institute's Animal Research Committee and were carried out according to the NIH Guide for the Care and Use of Laboratory Animals. In vivo treatment involved the administration of bidistilled water, morphine, antisense and mismatch oligonucleotides for β-arrestin-2. Morphine was obtained from Polfa Kutno (Poland). Antisense (AS-1: TTG TTG GCT CCC TCC TTC A; AS-2: GAG GTT GGT ATC CAC AGG GAT GT) and mismatch (MM-1: TTG TGT GCT CCC TCT CTC A; MM-2: GAG TGT GGT ATC CAC AGG AGT GT) probes were obtained from Sigma. Effect of β-arrestin antisenses was studied on C6 glioma cells in vitro

* Corresponding author. Tel.: +48-12-6623398; fax: +48-12-6374500.

E-mail address: przebar@if-pan.krakow.pl (B. Przewlocka).

0304-3940/02/$ - see front matter
PII: S0304-3940(02)00246-X

using reverse transcription-polymerase chain reaction. The result showed a substantial (about 60%) decrease of β-arrestin mRNA after 5 μM of antisense A1 and A2 when compared with mismatch controls.

All tested substances were administered intrathecally (i.th.) in a volume of 5 μl. In all experiments, 2 nM of the mixture (1 nM of each) of AS-1 and AS-2, as well as MM-1 and MM-2, was injected.

Under pentobarbital anesthesia a catheter (PE 10, Clay Adams) was carefully introduced to the rats lumbar enlargement of the spinal cord, according to Yaksh and Rudy [12]. Animals were allowed to recover for one week after the cannulas implantation.

The experiment was divided into two parts.

Part I: Development of morphine tolerance: In the first part, we study the effect of β-arrestin on morphine tolerance. Rats were divided into three treatment groups. Animals in groups 1, 2 and 3 were given bidistilled water, antisense and mismatch, respectively. All animals were injected with morphine at a dose of 10 μg at 12 h intervals. The tail-flick test was performed 15 min after the morning morphine administration. The 2 nM of the mixture (1 nM of each) of AS-1 and AS-2, as well as MM-1 and MM-2 or bidistilled water, was given 4 h later.

Part II: Development of nerve injury-induced allodynia: The experiment was designed to investigate the influence of β-arrestin on the development of allodynia. The sciatic nerve injury was performed according to Benett and Xie [2].

Acute treatment: For 6 days after nerve injury, the rats were repeatedly injected with bidistilled water, which on the 7th day was replaced with an acute injection of 2 nM of antisense to β-arrestin (AS-1 + AS-2) or with a mismatch (MM-1 + MM-2) to β-arrestin. Oligonucleotides were injected 12 h before the testing procedures (von Frey and cold allodynia tests).

Chronic treatment: All experiments involved repeated injection of 5 μl of bidistilled water, or a mixture of 2 nM of antisense to β-arrestin (AS-1 + AS-2), or 2 nM of mismatch to β-arrestin (MM-1 + MM-2). The oligonucleotides were administered i.th., and the first injection was made 24 h before injury of the sciatic nerve and then injections were given every day for 6–8 days. Oligonucleotides were injected 12 h before the testing procedures (von Frey and cold allodynia tests).

Tolerance development was evaluated using a tail-flick test. Baseline values (3–4 s) were determined before the first morphine injection. The cut-off value was 9 s.

Cold allodynia was measured according to the method described by Hunter et al. [8]. Animals were placed on a metal stage submerged to a depth of 2.5 cm. The positive response was recorded when an animal held the injured paw above the ice-cold water (0 °C) level. The cut-off latency was 30 s.

Tactile allodynia was measured by electronic von Frey test. The von Frey filament was applied to the midplantar surface of the paw. The cut-off value was set at 80 g.

The latency measured in seconds or grams, was converted to the percentage maximum possible effect (%MPE) using the formula: (postdrug latency − predrug latency)/(cut-off time − predrug latency) × 100. The data were statistically evaluated using one-way analysis of variance and presented as means ± SEM from 6–8 animals per group.

Until day 4 of morphine administration, the pattern of morphine tolerance development was the same in rats which received i.th. morphine, mismatch + morphine and β-arrestin antisense + morphine. On the 5th day, the tail-flick latency in the group of rats which received β-arrestin antisense + morphine was significantly higher than in the control groups (saline + morphine, mismatch + morphine). The significant difference was observed until the 8th day (Fig. 1).

Acute i.th. administration of β-arrestin antisense and mismatch oligonucleotides on the 6th day after sciatic nerve ligation antagonized the cold allodynia (Fig. 2, upper panel) and showed such tendency in von Frey test, however, the effect did not reach the level of statistical significance (Fig. 3, upper panel).

Chronic i.th. administration of β-arrestin antisense 1 day before and every day after sciatic nerve ligation significantly antagonized the development of cold allodynia in the antisense-treated group in comparison with the group injected mismatch oligonucleotides, and the effect persisted until day 5 (Fig. 2, lower panel). The same effect was observed in the von Frey test, however, the same response was also observed on days 3–5 after mismatch oligonucleotide administration. Starting from day 6, the antagonism was observed only after β-arrestin antisense administration (Fig. 3, lower panel).

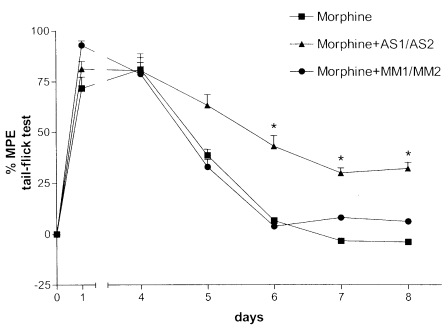

Fig. 1. The development of tolerance in rats after i.th. injection of morphine. Groups of animals were given i.th. water, β-arrestin antisense (AS1/AS2) or mismatch (MM1/MM2) oligonucleotides. All animals were injected with morphine at a dose of 10 μg at 12 h intervals. Tail-flick test was performed 15 min after the morning morphine administration. Tail-flick latency, measured in seconds, was converted to %MPE and data are presented as means ± SEM of six animals per group. *$P < 0.05$ in comparison with the bidistilled water-injected group of animals.

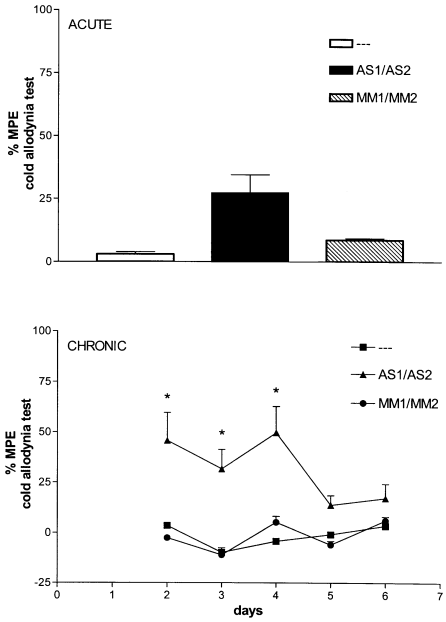

Fig. 2. Effect of acute (A) and chronic (B) i.th. administration of β-arrestin antisense (AS1/AS2) and mismatch (MM1/MM2) oligonucleotides on thermal allodynia (cold allodynia test) which developed after sciatic nerve ligation in rats. The latency, measured in seconds, was converted to %MPE and data are presented as means ± SEM of five animals per group. *$P < 0.05$ in comparison with the bidistilled water-injected group of animals.

The main achievement of the present study was to demonstrate that treatment with antisense oligonucleotides targeting β-arrestin-2 slowed down not only the development of tolerance to morphine but also the development of allodynia which was a consequence of nerve injury. Tolerance is probably the most fully recognized phenomenon that contributes to the modulation of opioid action and depends on a combination of many factors. Tolerance-related changes have been observed at the level of receptor, cell and neuronal circuits. At the receptor level, the homologous desensitization specific to the activated receptor is described as a loss of responsiveness and it negates involvement of the altered G-protein expression or effector activity [6,11,13]. The cytoplasmic protein arrestin, shows a high affinity for the phosphorylated receptor and binds to it, yielding a receptor–arrestin complex that uncouples G-protein and renders the receptor non-functional [7]. Recently, Bohn et al. [4,5] demonstrated the enhanced analgesic effectiveness of morphine in β-arrestin-2 knockout mice, and failure to develop antinociceptive tolerance to morphine in that model. Our study has confirmed the results obtained with β-arrestin-2 knockout mice using a different methodological approach. In our study, we used the antisense oligonucleotides targeting β-arrestin, a technique which is not only a suitable model for studying these effects, but can also be used for therapeutic purposes. In our present experiment, not only morphine tolerance, but also the development of cold allodynia was significantly delayed. These findings suggest that both of the investigated phenomena, i.e. tolerance and neuropathic pain, may be mediated by a similar mechanisms, e.g. receptor desensitization. Lower effectiveness of morphine was also observed in neuropathic pain [1,3,10]. Interestingly, a common cellular mechanism of neuropathic pain and morphine tolerance involving the *N*-methyl-D-aspartate receptor, protein kinase C and nitric oxide activation at the level of the dorsal horn of the spinal cord has been recently suggested [9]. Besides the above hypothesis, the results of our study suggest another possible mechanism stemming from the μ-opioid receptor activation and subsequent intracellular processes in which β-arrestin-2 plays a key role.

Summing up, our results indicate a similar delay in the development of two phenomena related to repeated μ-

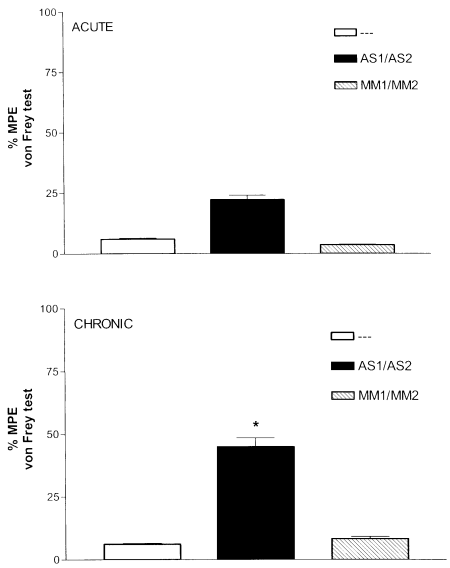

Fig. 3. Effect of acute (A) and chronic (B) i.th. administration of β-arrestin antisense (AS1/AS2) and mismatch (MM1/MM2) oligonucleotides on tactile allodynia (von Frey test) which developed after sciatic nerve ligation in rats. The reaction, measured in grams, was converted to %MPE and data are presented as means ± SEM of five animals per group. *$P < 0.05$ in comparison with the bidistilled water-injected group of animals.

opioid receptor activation, i.e. tolerance to the antinociceptive effect of morphine and nerve injury-induced cold allodynia after the knockdown of β-arrestin by antisense oligonucleotides. Moreover, the antisense oligonucleotides targeting β-arrestin may constitute a new approach to the therapy of neuropathic pain.

This study was supported by a grant (number 4 P05A 093 15) from the State Committee for Scientific Research (KBN, Warsaw, Poland), and partly by a grant for statutory activity.

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Chapter V

The effect of morphine on MC4 and CRF receptor mRNAs in the rat amygdala and attenuation of tolerance after their blockade

Katarzyna Starowicz[1,2], Agnieszka Sieja[1],
Wiktor Bilecki[1], Ilona Obara[1], Barbara Przewłocka[1]

[1] *Department of Molecular Neuropharmacology, Institute of Pharmacology, Cracow, Poland*
[2] *International Institute of Molecular and Cell Biology, Warsaw, Poland*

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Research report

The effect of morphine on MC4 and CRF receptor mRNAs in the rat amygdala and attenuation of tolerance after their blockade

Katarzyna Starowicz[a,b], Agnieszka Sieja[a], Wiktor Bilecki[a], Ilona Obara[a], Barbara Przewlocka[a,*]

[a] *Department of Molecular Neuropharmacology, Institute of Pharmacology, 12 Smetna Street, 31-343 Cracow, Poland*
[b] *International Institute of Molecular and Cell Biology, 4 Ks. Trojdena Street, 02-109 Warsaw, Poland*

Accepted 30 July 2003

Abstract

The relationships between the CRF, which enhances the proopiomelanocortin (POMC) biosynthesis, and POMC-derived peptides (opioids and melanocortins) might be a new target for rational treatment of morphine tolerance. In the present study, we investigated the effect of acute and chronic morphine administration on the level of CRF1 and melanocortin 4 receptor (MC4-R) mRNAs in the rat amygdala by quantitative real-time PCR method. Moreover, we investigated the effect of antagonists of melanocortin and CRF receptors, SHU9119 and α-helical CRF (αh-CRF), respectively, administered bilaterally into the central nucleus of the amygdala, on morphine tolerance using tail-flick and paw withdrawal tests. Our study demonstrated that acute morphine administration decreased the level of MC4-R mRNA in the rat amygdala. This decrease was attenuated following chronic morphine administration, and mRNA level of MC4 receptors was gradually increased and, on 9th day of morphine administration, i.e. in the period when morphine tolerance already developed, the level was significantly increased in comparison with control and with the effect after single morphine dose. In contrast, morphine did not affect the CRF receptor. In behavioral study, we demonstrated that SHU9119 and αh-CRF significantly increased the antinociceptive effect of morphine, when they were injected into the amygdala prior to morphine administration in tolerant rats. We have shown for the first time the contribution of amygdalar melanocortin receptors to morphine tolerance, and we conclude that the altered melanocortin receptor function may play an important role in the development of morphine-induced tolerance. CRF and melanocortin peptides can modulate the phenomena in the same direction, in opposition to opioids. Therefore, antagonists of melanocortin receptors may be regarded as possible therapeutic modulators of morphine tolerance.

Theme: Neurotransmitters, modulators, transporters and receptors
Topic: Receptor modulation, up- and down-regulation

Keywords: Alpha-helical CRF; Melanocortin receptor antagonist: SHU9119; Receptor for corticotropin releasing factor; Melanocortin 4 receptor; Morphine tolerance; Central nucleus of amygdala

1. Introduction

The effectiveness of morphine in the treatment of chronic pain is limited due to the development of tolerance to the analgesic effect of this drug. Many studies have been undertaken to investigate the mechanisms underlying this phenomenon. A new target for investigations into the morphine tolerance and its rational treatment might be the relationships between the CRF, which induces the proopiomelanocortin (POMC) synthesis, and POMC-derived peptides, opioids and melanocortins [9,24]. CRF has been reported to contribute to some symptoms of morphine withdrawal and relapse to its dependence [12,17,20]. CRF and melanocortin peptides (α-MSH/ACTH) share a number of common central effects. The effects of α-MSH may be secondary, due to CRF effects on melanocortin peptide release. CRF receptor agonists are potent stimulators of the synthesis of anterior pituitary POMC-derived peptides, primarily β-endorphin and α-MSH [9,24], and can act on human pituitary tissue in vitro to promote the release of POMC-related peptides, and similar effect was also observed in vivo [6]. CRF may influence melanocortin and

* Corresponding author. Tel.: +48-12-6623398; fax: +48-12-6374500.
E-mail address: przebar@if-pan.krakow.pl (B. Przewlocka).

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doi:10.1016/S0006-8993(03)03444-9

opioid release and in this way modulate central mechanisms of analgesia [16].

CRF-containing cell bodies and fibers have been localized to the central nucleus of the amygdala, while their projections stretch to the hypothalamus and brainstem nuclei. Areas of the brain previously thought to be unrelated to pain processing, such as the limbic system have been shown to play a major role in the experience of pain in animals and humans. CRF may act on a large number of brain structures involved in pain processing. In the rat, the amygdala is a forebrain structure known to be important for the mediation of fear-like and avoidance behavior [10]. It is also critical for the activation of endogenous antinociceptive systems during exposure to certain environmental stressors. The central nucleus of the amygdala sends monosynaptic projections to the population of opioid-sensitive cells in the ventrolateral periaqueductal gray, that are known to be involved in nociception [11]. The central nucleus of the amygdala contains a relatively high concentration of CRF and also considerable density of μ-opioid and melanocortin (MC) receptors [4,14,18,23].

The question arises if the melanocortins, which are known as highly active peptides involved in many functions in the brain, similarly to CRF, influence the effect of chronic morphine administration, since one of their suggested functions is the modulation of nociceptive transmission. Two receptor subtypes for melanocortins, MC3 and MC4 receptors, are expressed within the brain [21]. Highly selective MC4 receptor agonist, administered centrally to rats, increased Fos-like immunoreactivity in the paraventricular nucleus, central nucleus of the amygdala and nucleus of the solitary tract [5].

In the present study, we investigated whether acute and chronic morphine administration influences the level of mRNA coding for MC4 and CRF1 receptors in the amygdala using quantitative real time-PCR method, which could be of a great benefit to elucidation of regulatory mechanisms involved in the behavioral response to opioid treatment. In the behavioral study, we investigated if the blockade of melanocortin and CRF receptors within the central nucleus of the amygdala modifies the action of morphine on nociceptive transmission. We studied also possible influence of MC4 receptor antagonist SHU9119, in comparison with CRF receptor antagonist αhelical-CRF (αh-CRF), administered bilaterally into the central nucleus of the amygdala, on morphine tolerance in rats.

2. Materials and methods

2.1. Animals and surgery

Male Wistar rats (200–350 g) were housed in single cages lined with sawdust, under standard 12/12-h light/dark cycle (lights on at 08:00 h) with food and water available ad libitum. The experiments had the approval of the Local Bioethical Committee and were carried out according to the NIH Guide for the Care and Use of Laboratory Animals.

The rats were divided into groups and two types of experiments were carried out. Four groups of the separately housed rats ($n=4$ each) were appropriated for biochemical tests, i.e. real-time PCR evaluation of the MC4 and CRF receptor mRNA levels in the amygdala. Animals for behavioral evaluation were anaesthetized with pentobarbital and stereotaxically (David Kopf stereotaxic table) implanted with bilateral 23 gauge, 5 mm steel guide cannulas aimed 4 mm above the central nucleus of the amygdala. The injection cannula (connected by polyethylene tubing with Hamilton syringe) was inserted into the guide cannula immediately before the injection and the injection cannula advanced to protrude 4 mm beyond the guide cannula tip, thus reaching the central nucleus of amygdala. The volume of injection was 5 μl delivered at a constant rate over a period of 2 min, and the injection cannula was removed 20 s after the completion of this procedure. Stereotaxic coordinates were based on the atlas of Paxinos and Watson [22]. With the tooth bar positioned at −3.3 mm, the coordinates were: posterior from bregma 2.5 mm, lateral ±4.2 mm and ventral 5.1 mm from the point of entry at the skull surface. Cannulas were fastened to the skull with dental cement and sealed with a stylet wire. After the surgery, animals were allowed a minimum 1 week of recovery before the experiment.

2.2. Drug administration

SHU9119 was provided by Phoenix Pharmaceuticals, USA. Alpha-helical CRF (αh-CRF) was purchased from Sigma, Germany. SHU9119 and αh-CRF were dissolved in distilled water and were injected by hand-held Hamilton syringe connected by polyethylene tubing with injection cannula. Control animals were injected with distilled water and tested according to the same time schedule as described below for the experimental groups.

After the completion of the experiment, the injections of methylene blue were made and, after 1 h, the animals were sacrificed, their brains were dissected and fixed in formalin for verification of the cannula position. The localization of the cannula was marked on the brain scheme (Paxinos and Watson [22]). The results obtained with animals with incorrect cannula position were excluded from calculations and, therefore, a number of animals in the experiment on development of tolerance was 8–10 and, after intraamygdalar injection of SHU9119 or αh-CRF, 6–8 rats per group were tested.

Morphine was injected at a dose of 10 mg/kg (i.p.) twice daily every 12 h. Morphine antinociceptive effect was estimated by tail-flick and paw withdrawal tests 15 min after the morning morphine administration. The rats were

rendered tolerant to morphine after 8 days of morphine injections (see Fig. 1).

On day 9 of the experiment, the rats were challenged with the same dose of morphine but 15 min earlier, bilateral, intraamygdalar injections of SHU9119 (0.15–1.5 μg) or αh-CRF (0.5–1 μg) were made. Antinociceptive effect was measured by tail-flick and paw withdrawal tests 15 min after morphine administration.

2.3. Apparatus and testing procedures

The tail-flick test was carried out using Analgesia Meter (Ugo Basile). A rat was gently restrained by hand and radiant heat was directed into the animal's tail; 9-s cutoff time was employed to prevent tissue damage. The development of morphine tolerance was determined by measuring the respective test latency after drug administration.

The paw withdrawal test was carried out using Paw Withdrawal Apparatus (model 33, IITC, Landing, NJ). The rats were placed in a plastic cage with glass floor and were allowed to habituate for 5 min before the experiment. The light was focused through the glass floor to the midplantar surface of the hindlimb and latency of the foot withdrawal was measured. The cutoff value was set at 20 s.

2.4. mRNA extraction and real-time PCR

Brains for biochemical evaluation of MC4 and CRF1 receptors were collected from four groups: Group 1 consisted of control rats, which were injected with distilled water for 8 days (i.p.), rats in group 2 were injected with distilled water (twice daily, i.p.) and, on day 8 of the experiment, they received a single morphine injection (10 mg/kg i.p.) and were sacrificed 15 min later. Animals chronically injected with morphine (for 8 days, 10 mg/kg i.p., twice daily) were sacrificed either 3 (Group 3) or 24 h (Group 4) after the last morphine injection. Rats from the above groups ($n=4$ for each group) were killed, their brains were isolated and amygdala was dissected and subjected to further analysis. Total mRNA was extracted according to Chomczynski method [7].

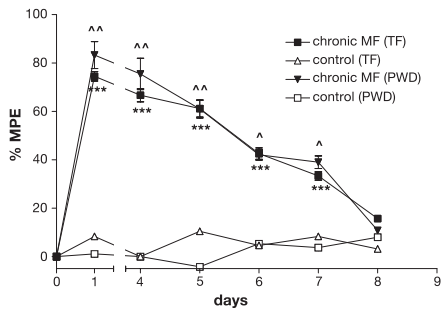

Fig. 1. The development of tolerance after administration of morphine (10 mg/kg i.p.) twice a day for 8 days. The effects of morphine (MF) on nociception were estimated by measuring the tail-flick (TF) and paw withdrawal (PWD) test latency after drug administration. The data were expressed as a percentage of maximal possible effect (%MPE) ± S.E.M. for groups of 8–10 animals each, and calculated using the equation: %MPE=[(TL − BL)/(CUT-OFF − BL)] × 100 where BL = baseline latency and TL = respective test latency. The results were analysed using the one-way ANOVA followed by Bonferroni test; *$p<0.05$ vs. vehicle and ^$p<0.05$ vs. vehicle for TF and PWD test, respectively, was considered to be statistically significant.

The iCycler iQ Real Time PCR Detection System for quantitative real-time detection of PCR products was used. For quantification of RNA targets, QIAGEN QuantiTect SYBR Green RT-PCR Kits were used, containing SYBR Green I as a fluorescent dye. Specificity of RT-PCR product was validated by constructing a melting temperature curve and the result was further confirmed by agarose gel electrophoresis. Threshold value (at least 10 times the mean standard deviation of fluorescence in all wells over the baseline cycles) for each sample was set in the exponential phase of PCR. For analyzing the data, we used standard curve method (series of template dilutions) and changes in fluorescence of discrete samples were transformed into differences in respect to control samples (non-treated animals). As a result, data were presented as percent (%) of control (mean ± S.E.M.). We used β-actin as a housekeeping gene.

The following sets of primers were used: rat β-actin RT-PCR primer sets 5′-GTT GGA TAC AGG CCA GAC TTT GTT G (25-mer), 5′-GAG GGT AGG CTG GCC TAT AGG CT (23-mer); rat MC4-R: 5′-GTA ATT GCG CCC TTC ATG TT (20-mer), 5′-TCG GGC GTT CTT TTT ATC AT (20-mer); and rat CRF-R1: 5′-ATG AGG ATG CGG ACA ATG TT (20-mer), 5′-GTG GAT GTT CGT CTG CAT TG (20-mer). All primers were synthesized by Sigma-ARK, Darmstadt, Germany.

2.5. Data analysis

The results were compared using the one-way ANOVA followed by Bonferroni's test. The data are presented as means ± S.E.M.; $p<0.05$ was considered to be statistically significant, F values resulting from statistical analysis of the test results are also given.

3. Results

3.1. The development of morphine tolerance

Chronic morphine administration twice a day (10 mg/kg i.p.) resulted in development of tolerance to its antinociceptive effect. On the first day of the experiment, significant morphine antinociceptive effect was observed in tail-flick

and paw withdrawal tests as shown in Fig. 1. The latencies of the response in both tests indicate high antinociceptive effect of morphine till day 5 of the experiment, when compared to control group. Tolerance to systemic administration of morphine developed over days 6 and 7, and on day 8, latency of response was close to control values in both behavioral tests.

3.2. The effects of acute or chronic morphine administration on MC4-R and CRF-R1 mRNA level in the rat amygdala

MC4 receptor mRNA level was significantly decreased to 36% after acute morphine treatment as shown in Fig. 2A.

Table 1
Effect of corticotrophin and melanocortin receptor antagonists, αh-CRF and SHU9119 administered alone bilaterally into the amygdala of naive rats measured by tail-flick (TF) and paw withdrawal (PWD) tests 15 min after their injection

αh-CRF (0.5 μg)	SHU9119 (0.15 μg)
TF test	
Control: 4.3 ± 0.3	Control: 3.7 ± 0.5
15 min: 4.3 ± 0.3	15 min: 4.2 ± 0.3
PWD test	
Control: 5.3 ± 0.4	Control: 5.3 ± 0.4
15 min: 5.8 ± 0.5	15 min: 5.4 ± 0.5

Data are expressed as means of the measured test latencies in seconds ± S.E.M. for groups of four animals each.

In contrast, chronic morphine treatment resulted in a time-dependent gradual increase in MC4 receptor mRNA in the amygdala (Fig. 2A), when compared to acute drug administration (99% vs. acute morphine and 35% vs. control group). Nine days of drug treatment significantly increased levels of MC4 receptor mRNA in this brain region, when measured on days 8 (0.86 ± 0.05, $p < 0.05$) and 9

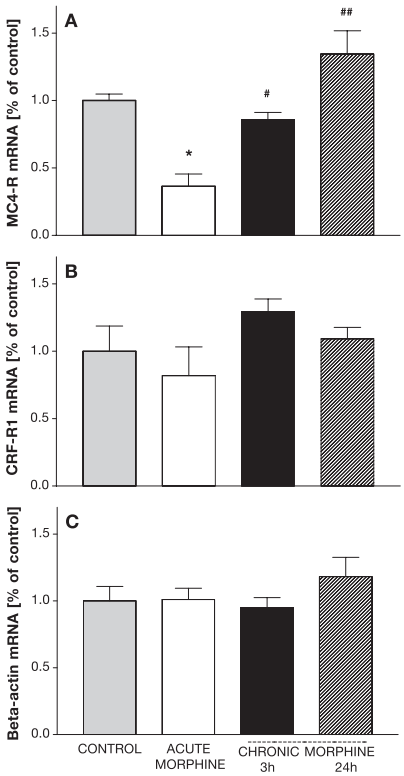

Fig. 2. Changes in the level of mRNAs coding for: (A) MC4-R, (B) CRF-R1 and (C) control gene, β-actin in the amygdala of the rats after acute (15 min after the administration) and chronic (3 and 24 h after the last dose) morphine treatment (10 mg/kg i.p., twice daily at 12-h intervals). Data are expressed as the percent of control mRNA level ± S.E.M. for four animals in each experimental group; $^{*}p < 0.01$ vs. control group, $^{\#}p < 0.05$ and $^{\#\#}p < 0.001$ vs. acute morphine treatment.

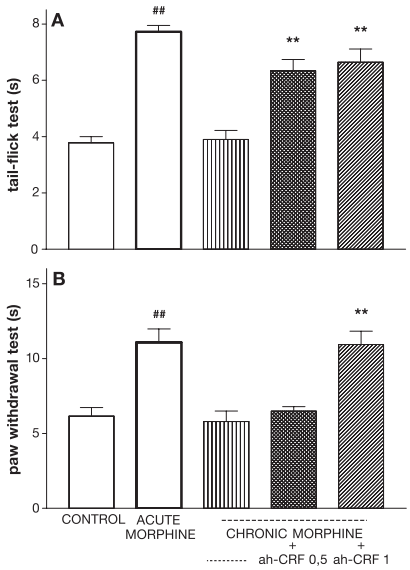

Fig. 3. The effect of bilateral, intraamygdalar injections of αh-CRF (0.5 and 1 μg) on morphine tolerance (10 mg/kg i.p.) as measured by tail-flick (A) and paw withdrawal (B) tests on day 9 of morphine administration. Data are expressed as the reaction latencies in seconds ± S.E.M. for groups of six to eight animals each; $^{\#\#}p < 0.001$ vs. control group and $^{**}p < 0.001$ vs. control group chronically injected with morphine (for 9 days, twice daily, 10 mg/kg i.p.).

(1.35 ± 0.17, $p < 0.001$) after chronic morphine treatment ($F_{3,12} = 15.55$), in comparison with acute morphine administration and also when compared with control. The up-regulation of MC4 receptor mRNA was statistically significant in comparison with acute administration in both cases, although the effect observed on day 9 was more pronounced.

CRF-R1 mRNA was regulated in a significant manner by neither acute nor chronic morphine administration (Fig. 2B). The observed effect did not reach statistical significance at any time point tested, although a decreasing tendency was observed after acute morphine administration when compared to control group.

The mRNA level of control gene, β-actin, remained unaffected (Fig. 2C) throughout the entire testing period.

3.3. Effect of bilateral intraamygdalar αh-CRF microinjection on nociceptive threshold and morphine tolerance

CRF receptor antagonist, αh-CRF administered bilaterally at a dose of 0.5 μg into the central nucleus of the amygdala of naive animals, did not affect the nociceptive threshold (Table 1).

Bilateral intraamygdalar injection of αh-CRF (0.5 and 1 μg) 15 min before the morphine injection on day 9 reversed the morphine tolerance (Fig. 3). In the tail-flick test, the results were as follows, when the data were expressed as a percent of control response: $162\% \pm 2.5$ ($p < 0.001$) and $170\% \pm 3.6$ ($p < 0.001$) for αh-CRF at doses of 0.5 and 1 μg, respectively ($F_{4,33} = 25.6$). In the paw withdrawal test, the results were: $112\% \pm 0.1$ (0.5 μg of αh-CRF) and $189\% \pm 2.9$ (1 μg of αh-CRF). The effect was significant at both tested doses in the tail-flick test (Fig. 3A); however, in paw withdrawal test ($F_{4,33} = 14.5$), it was significant ($p < 0.001$) only for the αh-CRF dose of 1 μg (Fig. 3B).

3.4. Effect of bilateral intraamygdalar SHU9119 microinjection on nociceptive threshold and morphine tolerance

Melanocortin receptor antagonist, SHU9119 (0.15 μg) administered bilaterally into the amygdala of naive animals did not cause any effect itself (Table 1).

In the rats tolerant to morphine, the blockade of brain melanocortin receptors by SHU9119 resulted in reversion of morphine tolerance. The effect was dose-dependent in the tail-flick test, in which intraamygdalar injection of SHU9119 (0.15 and 1.5 μg) significantly potentiated the antinociceptive effect of morphine ($149\% \pm 2.5$, $p < 0.001$ and $181\% \pm 3.6$, $p < 0.001$, respectively; $F_{4,33} = 28$) in rats tolerant to its analgesic effects (Fig. 4A). The morphine antinociception estimated in the paw withdrawal test in rats pretreated with SHU9119 at the doses of 0.15 and 1.5 μg was enhanced ($113\% \pm 0.4$ and $151\% \pm 2.0$, respectively). In the paw withdrawal test ($F_{4,33} = 9.8$), the behavioral results after SHU9119 reached significance ($p < 0.05$) only at its higher dose (Fig. 4B).

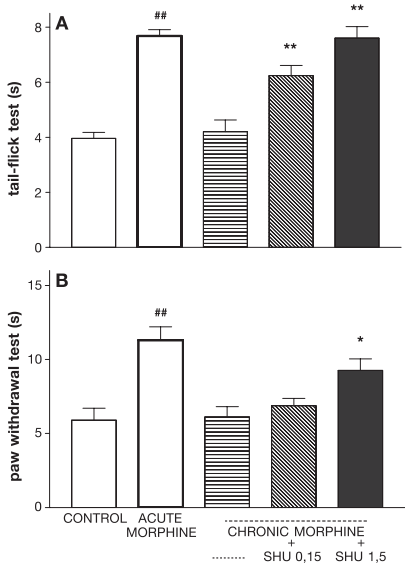

Fig. 4. The effect of bilateral, intraamygdalar injections of melanocortin receptor antagonist: SHU9119 (0.15 and 1.5 μg) on morphine tolerance (10 mg/kg i.p.) as measured by (A) tail-flick and (B) paw withdrawal tests on day 9 of morphine administration. Data are expressed as the reaction latencies in seconds ± S.E.M. for groups of six to eight animals each; $^{\#\#}p < 0.001$ vs. control group, $^{**}p < 0.05$ and $^{**}p < 0.005$ vs. control group chronically injected with morphine (for 9 days, twice daily, 10 mg/kg i.p.).

4. Discussion

The present results show for the first time that an antagonist of MC4 receptor, administered into the rat central nucleus of the amygdala, influences morphine tolerance. Moreover, the results of the current study demonstrated that the level of MC4 receptor mRNA, decreased after single morphine administration, was gradually increased parallel to the development of morphine tolerance. These results indicate that MC4 receptor in the amygdala may be involved in the development of some aspects of morphine tolerance; however, one should bear in mind that changes in mRNA level are not always followed by alterations in the protein level.

The amygdala belongs to the structures involved in major effects of opiates, like rewarding properties. Alvaro et al. [2] demonstrated that chronic morphine administration resulted in a decreased in MC4 receptor mRNA level in the striatum and periaqueductal gray, which was accom-

panied by a concomitant decrease in melanocortin receptor level measured by quantitative radioligand binding and autoradiography [2]. McNally and Akil [19] provide evidence for a role of the amygdalar CRF in opiate dependence. Furthermore, the authors conclude that the increased expression of the CRF gene in the amygdala is a consequence of down-regulation of opiate receptor signaling. In addition to opioid and CRF receptors, the melanocortin receptors are also expressed in the amygdala [21], however, still little is known about the role of amygdalar melanocortin system in the antinociceptive effects of morphine. A role of MC4 receptor in long-term opiate actions is further supported by behavioral studies demonstrating that melanocortins antagonize various functional effects of opiate treatment, e.g. they reduce opiate self-administration [28], decrease opiate physical dependence [8] or there is a correlation between the relative potency of various melanocortin peptides to block tolerance and their ability to activate the MC4 receptor in vitro [13,28]. Moreover, morphine has been shown to increase plasma α-MSH level [15,29], which may be also a consequence of morphine stimulatory effect on CRF secretion. Down-regulation of melanocortin receptors may result from higher release of α-MSH on the first days of morphine administration. Therefore, intraamygdalar administration of SHU9119, may result in attenuation of α-MSH action, which in consequence may restore morphine antinociception. Furthermore, the peripheral multiple i.p. administration of α-MSH, along with repeated morphine administration attenuated the development of tolerance in mice [27], which may suggest the involvement of repeated stress in the effect. Another possibility is that the effect of melanocortins on morphine tolerance could be different at peripheral and central level. The effect of SHU9119 may also be explained by influence on intracellular signal transduction mechanisms. In contrast to morphine, the agonists of MC4 receptors activate adenylyl cyclase (AC) and cAMP formation [1,3]. The antagonist of melanocortin receptor could potentiate the inhibitory effect of morphine on cAMP pattern by antagonizing the stimulatory effect of melanocortins.

The results obtained after intraamygdalar administration of αh-CRF are in line with other animal experimental studies. In our series of experiments, the acutely administered opioid did not modulate the CRF-R1 mRNA in the rat amygdala. In contrast, 24 h after the last morphine injection, at the time when we injected SHU9119 to the amygdala, the level of MC4 receptor mRNA was higher in comparison with single injection of morphine. This finding could correlate with our in vivo experiments, where SHU9119, by blocking the amygdalar MC4 receptors, was effective in restoring morphine analgesic effect when compared to control animals tolerant to morphine. Song and Takemori [25] reported that αh-CRF (9–41), a CRF receptor antagonist, increased the morphine antinociceptive ED50 estimated by tail-flick test, when it was administered intrathecally (i.t.) in tolerant mice. The activation of the CRF receptor is involved in morphine withdrawal signs and relapse to morphine dependence. The morphine withdrawal signs, including jumping, teeth chatter, writhing, shakes, lacrimation, piloerection, irritability and diarrhea, were attenuated by pretreatment with CRF receptor antagonist, αh-CRF (10 μg i.c.v.). Pretreatment with the CRF-R1 antagonist significantly attenuated several behavioral signs of naltrexone-induced morphine withdrawal [17]. In our study, the level of mRNA coding for CRF1 receptor was not changed upon morphine administration. This is in agreement with another study, namely, Zhou et al. [30] demonstrated that neither the activity of the HPA axis nor the β-endorphin and CRF systems in the brain are related by steady-state occupancy of opioid receptors with the long-acting opioid agonist [30]. Chronic methadone, a long-acting opioid agonist, did not alter CRF mRNA in the hypothalamus, POMC and CRF-R1 mRNA in the anterior lobe and neurointermediate/posterior lobe of the pituitary. No change was found in CRF mRNA levels in the frontal cortex, olfactory bulb and amygdala.

Previously, we have demonstrated that at the level of the spinal cord, melanocortin receptor ligands antagonize the action of opioid peptides [26]. The data obtained in the present studies, which were focused on the supraspinal levels, indicate that melanocortin peptides also at this level of CNS display their antagonistic potential toward opioids. The preliminary communication of Zhou et al. [31] indicates that after s.c. administration of another MC4 receptor antagonist, HS-131, several signs of naloxone-precipitated withdrawal were significantly attenuated and that the effect of the MC4 receptor antagonist was comparable to that observed for the neurokinin NK1 receptor antagonist, GR82334, previously shown to reduce the reaction to opioid withdrawal [31].

In conclusion, we hypothesize that gradual increase in MC4 receptor expression, which follows the chronic morphine administration, may be an important adaptation contributing to chronic opiate effects in the brain. Moreover, MC4 receptor function in the amygdala has been demonstrated in vivo, since intraamygdalar injection of SHU9119 reversed morphine tolerance, in a similar manner to αh-CRF, which also increased morphine efficacy in morphine tolerant rats. In conclusion, our data demonstrated for the first time the important role of MC4 receptors located in the amygdala, and confirmed the modulatory effect of melanocortin receptor antagonist on morphine tolerance. Our data suggested also that, at least partly, the effect of CRF, which releases α-MSH in several central nervous system regions, might act behaviorally in the same direction as melanocortins. Moreover, antagonists of melanocortin receptors may be regarded as possible therapeutic modulators of morphine tolerance. In summary, these findings indicate that morphine in animal model of tolerance modulates endogenous melanocortin pathways in the amygdala.

Acknowledgements

This study was supported by the grant no. 4P05A 060 16 and 6P05A 107 20 from the State Committee for Scientific Research (KBN), Warszawa, Poland.

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Chapter VI

Inhibition of morphine tolerance by spinal melanocortin receptor blockade

Katarzyna Starowicz[1,2], Ilona Obara[1],
Ryszard Przewłocki[1], Barbara Przewłocka[1]

[1] Institute of Pharmacology, Dept. of Molecular Neuropharmacology, 12 Smetna str, 31–343 Cracow, Poland
[2] International Institute of Molecular and Cell Biology, Laboratory of Neurodegeneration, 4 Ks. Trojdena str, 02–109 Warsaw, Poland

Pain, submitted

ABSTRACT

Chronic use of morphine is accompanied by the development of morphine tolerance, which is one of the major problems associated with opiate treatment. Possible modulation of opioid effects by melanocortin receptor ligands has been recently demonstrated. Therefore, we investigated the influence of repeated intrathecal injection of a melanocortin receptor antagonist (SHU9119, JKC–363) on the development of morphine tolerance as measured by tail-flick test. It was also examined whether a single i.t. SHU9119 and JKC–363 administration could counteract the loss of analgesic potency of morphine in morphine tolerant rats. We examined also the influence of chronic morphine administration on μ-opioid receptor (μOR) and melanocortin 4 receptor (MC4R) mRNAs in the rat spinal cord and dorsal root ganglia (DRG) during morphine tolerance. Morphine treatment (10 mg/kg, i.p. twice daily) over 8 days induced tolerance as reflected by a significant reduction of withdrawal latency from 181% to 25% above baseline in the tail-flick test. Repeated co-administration of morphine and SHU9119 or JKC–363, significantly prevented the development of morphine tolerance. A single administration of an MC4R antagonist restored morphine analgesic potency in morphine tolerant rats. Using RT-PCR we demonstrated no changes in the spinal cord but there was a decrease in μOR and increase in MC4R gene expression in the DRG of rats tolerant to morphine. These results suggest that MC4R may be involved in the mechanisms of opioid tolerance and antagonists of this receptor may be a possible new target in the search for strategies preventing the development of opioid tolerance.

Keywords: antinociception, μ-opioid receptor (μOR), melanocortin 4 receptor (MC4R), morphine tolerance, spinal cord, dorsal root ganglia (DRG)

INTRODUCTION

Morphine is the most widely used analgesic for moderate and severe pain, and its analgesic effects are mediated primarily through the μ-opioid receptor (μOR) (Matthes et al., 1996). It is widely accepted that μOR participates in the modulation of nociception at primary afferent terminals and mRNA coding for this receptor is expressed in dorsal root ganglia (DRG) (Satoh and Minami 1995; Zhang et al., 1995). Prolonged and repeated exposure to opioid agonists re-

duces the responsiveness of G-protein-coupled opioid receptors and this phenomenon has been hypothesized to contribute to opioid tolerance (Ueda et al., 2003). Numerous *in vivo* studies have attempted to elucidate whether changes in opioid binding sites are responsible for functional alterations observed after chronic morphine treatment, but untill now there is no consensus about the direction, magnitude or role of changes in μOR number in tolerant animals (Brady et al., 1989; Bhargava et al., 1990; Nishino et al., 1990). Interestingly, besides receptor-based mechanisms involved in development of tolerance, also interactions between opiate and non-opiate systems have been postulated (Vaccarino and Kastin 2001). Among the peptides with putative antiopioid function like cholecystokinin (CCK), melanocortins have to been mentioned. The melanocortins are a family of bioactive peptides derived from proopiomelanocortin (POMC). Melanocortins have numerous reported functions; and recently they have been ascribed a role in nociception (Starowicz et al., 2002; Vrinten et al., 2003). Functional interaction between melanocortin and opioid systems has already been documented in our previous study (Starowicz et al., 2002). Current investigations indicate that blockade of certain melanocortin receptor type, primarily melanocortin 4 receptor (MC4R), could be a novel strategy to control pain (Starowicz et al., 2002; Vrinten et al., 2000; Bellasio et al., 2003). MC4R is widely distributed within the brain and spinal cord of rodents and other mammals (Mountjoy et al., 1994, 1998). Recent studies in our laboratory (Starowicz et al., 2002, 2004) demonstrated that MC4R antagonists are involved in the modulation of nociception at the spinal cord level. Vrinten et al. (2000) demonstrated anti-allodynic action of an MC4R antagonist when administered into the cisterna magna. Moreover, enhancement of morphine analgesic effects by the melanocortin receptor antagonist, SHU9119 in neuropathic animals has also been demonstrated (Vrinten et al., 2003). Accordingly to Mao's hypothesis (Mao et al., 1995), similar mechanisms are implicated in the development of morphine tolerance and neuropathic pain. We believe that melanocortins play a significant role in both, mechanisms of pathological pain and opioid tolerance.

In the present study, the mixed MC3/MC4 melanocortin receptor antagonist SHU9119 and the MC4R selective antagonist JKC–363 were injected intrathecally (i.t.) to the lumbar spinal cord 15 min prior to i.p. administration of morphine in order to assess the role of the spinal melanocortin system in the development of morphine tolerance. It was also determined whether a single i.t. administration of SHU9119 or JKC–363 can counteract the loss of analgesic potency of morphine in morphine tolerant rats. Moreover, we investigated the changes in MC4R and μOR mRNA level in the rat spinal cord and DRG after chronic morphine treatment.

METHODS

Animals

Male Wistar rats weighing 200–250 g at the beginning of the study were used. Rats were housed in single cages on a sawdust bedding under standard conditions (12 h light/dark cycle, lights on from 8:00 a.m.) with food and water available *ad libitum*. All experiments had the approval of the Local Bioethics Committee of the Institute of Pharmacology (Cracow, Poland) and were in accordance to "Ethical guidelines for investigations of experimental pain in conscious animals" (Zimmermann, 1983).

Drugs

SHU9119 (Ac-Nle-Asp-His-D-Nal(2')-Arg-Trp-Lys-NH2; amide bridge: Asp3-Lys8) and JKC-363 (Mpr-Glu-His-(D-Nal)-Arg-Trp-Gly-Cys-Pro-Pro-Lys-Asp-NH2; disulfide bridge: Mpr1-Cys8) purchased from Phoenix Pharmaceuticals, USA) were stored as stock solutions at –20°C and were diluted with 0.9 % NaCl (which served as vehicle) to the appropriated concentrations right before the experiment. For pharmacological characterization of the MC4R antagonist, SHU9119 see: Schioth et al. (1998,1999) and for ligand receptor selectivities see review: Starowicz and Przewłocka (2003); Wikberg (1999). Accordingly to the product data sheet description JKC–363 represents potent and selective MC4 antagonist, 3 times more potent than HS014 at the MC4 receptor.
SHU9119 and JKC–363 were tested at the concentrations selected on the basis of previous experiments and Vrinten et al. (2000) (see Fig. 5 for dose-response curves of the effect of SHU9119 on von Frey; and Table 1 for potencies and affinities values, therein). The used doses were as follows 0.46 and 1.4 nmol (0.5, 1.5 μg and 0.7, 2 μg, respectively). Morphine hydrochloride (10 mg/kg) for i.p. injection (Polfa Kutno, Poland) was diluted with 0.9 % NaCl.

Surgical preparations and schedule of drug administration

Rats were prepared for intrathecal (i.t.) injection of SHU9119 and JKC–363 by implanting catheters under pentobarbital anesthesia (60 mg/kg, i.p.). The intrathecal catheter consisted of a polyethylene tubing 12 cm long (PE 10, INTRAMEDIC, Clay Adams, Becton Dickinson and Company, Rutherford, NJ, USA; o.d. = 0.4 mm, dead space = 10 μl), sterilized by immersion in 70%

Table 1. Primers used for Polymerase Chain Reaction

Gene	GenBank accession number	Forward primer/ start position	Reverse primer/ stop position	Product lenght (bp)
MOR	NM 011013	ATC CTCTCT TCT GCC ATT GGT/643	TGA AGG CGA AGA TGA AGA CA/771	127
MC4R	NM 013099	TCG GGC GTT CTT TTT ATC AT/616	GTA ATT GCG CCC TTC ATG TT/796	180
HPRT	XM 343829	AAG ACA GCG GCA AGT TGA AT/817	GGC TGC CTA CAG GCT CAT AG/989	172

ethanol, and fully flushed with sterile water prior to insertion. Rats were placed on the stereotaxic table (David Kopf), and an incision was made in the atlanto-occipital membrane. The catheter (7.8 cm of its length) was carefully introduced into the subarachnoid space at the rostral level of the spinal cord lumbar enlargement (L4-L5) according to Yaksh and Rudy (1976). After the implantation, the first injection of the 10 μl saline was slowly made and the catheter was tightened. One day after the catheter implantation, the rats were monitored for their physical impairments, those showing motor deficits were excluded form further study. After the surgery animals were allowed a minimum one week recovery before the experiment. Saline or respective drugs tested in the experiments were delivered slowly (1–2 min) in a volume of 5 μl through the i.t. catheter and followed by 10 μl saline, which flushed the catheter.

Experimental design

Design of experiment 1

To establish the role of the MC4R receptor antagonists in the development of morphine tolerance, rats were divided into the following groups: group 1 (n=8) consisted of animals receiving morphine (10 mg/kg, i.p.) twice daily at 12 h intervals (8 a.m. / 8 p.m.) for 8 consecutive days; group 2 (n=8) received saline under identical conditions and served as control animals. For testing of the influence of melanocortin receptor antagonist on morphine effects, rats were given an MC4R antagonist: 0.5 μg of SHU9119 (group 3, n=10) or 0.7 μg of JKC–363 (group 4, n= 10), i.t. 15 min prior the morning morphine challenge (10 mg/kg, i.p.) on each testing day (day 1–8). For testing of the action of MC4R antago-

nist per se, rats were injected i.t. with 0.5 μg of SHU9119 (group 5, n= 8) or 0.7 μg of JKC–363 (group 6, n= 8) 15 min prior to the morning saline (i.p.) injection for 8 following days.

Design of experiment 2

In the following experiment, we determined whether a single injection of MC4R antagonist could influence the morphine analgesic effect in morphine tolerant rats. A paradigm to induce morphine tolerance was identical as previously described, briefly, morphine at a dose of 10 mg/kg (i.p.) was administered every 12 h. Control rats (n=6) were injected with saline under identical conditions. Animals (n=72) were rendered tolerant to morphine after 8 days. On day 9 of the experiment at the regular morning time, the rats were divided into 9 experimental groups, each consisted of 8 animals. Groups were treated as follows: group 1 received morphine (10 mg/kg, i.p.); group 2: 0.5 μg (i.t.) of SHU 9119; group 3: 1.5 μg (i.t.) of SHU 9119; group 4: 0.7 μg (i.t.) of JKC–363; group 5: 2 μg (i.t.) of JKC–363. Groups 6- 9 were challenged with i.t. administered MC4R antagonist: SHU9119 (0.5 and 1.5 μg) and JKC–363 (0.7 and 2 μg), respectively, followed by (15 min later) an injection of morphine (10 mg/kg, i.p.).

Determination of tail-flick latency

Analgesia was determined using the tail-flick assay (Analgesia Meter, Ugo Basile, Italy) in which a beam of light was focused on the dorsal tail surface approximately 2 cm from the tip of the tail. The intensity of the light was adjusted so that the baseline flick latencies were 2.5–3 s, if a rat failed to flick its tail by 9 s during testing, the test was terminated and the rat was defined as analgesic. An average of three tail-flick trials each separated by a 1-min intertrial interval was designated the mean baseline latency. Rats were tested for analgesia 30 min following morphine administration. Animals were monitored every day before morning injection to check whether repeated tail-flick testing did not cause tissue damage. Therefore, rats were tested for baseline latencies and then accordingly to the injection paradigm tested for post-drug tail-flick latency. Baseline tail-flick measurements performed daily did not vary significantly, indicating no tissue damage that could influence the results.

Evaluation of μOR and MC4R mRNA changes

On respective testing days, the animals were sacrificed 4 h after the last morphine treatment. Bilateral lumbar DRG (L4-L6) were carefully excised, total

RNA from six DRG of each rat was extracted. Moreover, lumbar (L4-L6) dorsal part of the spinal cords was also isolated for evaluation of the changes in μOR and MC4R mRNA. The collected tissues were rapidly frozen on dry ice and stored at –70°C until mRNA extraction procedure. Total mRNA was extracted according to Chomczynski method (Chomczynski and Sacchi 1987). The amount and purity of the total RNA was determined by spectrophotometry (Beckman, DU 7500) at 260 and 280 nm. A nucleic acid preparation with an A260/A280 ratio $\geq$2.0 was considered as pure.

Real-time PCR analysis was performed on iCycler iQ Real Time PCR Detection System (BioRad, CA, USA). Gene-specific primers were designed and were blasted against the GeneBank to confirm their species and gene specificity (Table 1). Primers were obtained from BIONOVO, Poland. For quantification of RNA targets, QIAGEN QuantiTect SYBR Green RT-PCR Kits were used, containing SYBR Green I as a fluorescent dye. The relative standard curves generated by plotting the threshold value (C_T) vs. the log of the amount of total cDNA added to the reaction. Specificity of RT-PCR product was validated by constructing a melting temperature curve and the result was further confirmed by agarose gel electrophoresis. All data were normalized according to HPRT mRNA level in respective samples. Each RT-PCR amplification with a volume 20 μl contained 400 ng of total RNA and 5 mM of each primer. The conditions were as follows: reverse transcription at 50°C for 30 min, denaturation at 95°C for 15 min, then for each cycle denaturation at 94°C for 15 s, annealing at 57°C for 30 s, and extension at 72°C for 45 s. The number of cycles was 40, the program ended with 5 min at 72°C and storage at 4°C.

Statistics

For evaluating the area under the curve (AUC) the Trapezoidal & Simpson`s Rules, Pharmacologic Calculation System, version 4.0–03/11/86 was used (Tallarida and Murray, 1987). Values from tail-flick tests and quantitative RT-PCR method are expressed as the means ± SEM. Comparisons between groups were performed using analysis of variance (ANOVA) for repeated measurements followed by Tukey multiple comparison test using InStat (GraphPad Software, Inc., CA, USA). $P < 0.05$ was considered significant.

RESULTS

Development of morphine tolerance

Baseline tail-flick latency (before morphine administration) was 2.75 ±0.25 s and did not vary significantly throughout the entire testing procedure. This value did not differ significantly from the controls injected with saline (i.p.) under identical conditions on all tested days. On day 1 and 2, morphine administration (10 mg/kg, i.p.) produced significant analgesia ($p<0.001$) compared to saline-injected rats (8.33 ± 0.24 s *vs.* 2.95 ± 0.22 s and 8.17 ± 0.26 s *vs.* 3.17 ± 0.23 s, respectively). The morphine analgesia on days 1–3 did not differ significantly ($p>0.05$) but it decreased on the following days of chronic morphine treatment. Although until day 6 morphine consistently produced significant ($p<0.001$) antinociception when compared to saline-injected rats, morphine effect significantly decreased when compared to its efficacy on day 1 (8.33 ± 0.24 s *vs.* 5.00 ± 0.38 s, $p< 0.001$; $F_{5,30}= 37.9$, Fig. 1A). On day 7 of chronic morphine administration its analgesic efficacy further decreased (4.7 ± 0.39 s), consequently 8-day i.p. morphine administration (twice daily) produced tolerance to its analgesic effect, and tail-flick latency of morphine-injected rats did not differ significantly form latencies of saline-treated animals (3.57 ± 0.2 s *vs.* 2.85 ± 0.15 s, $F_{2,18} = 12.0$).

Effect of repeated SHU9119 i.t. administration on the development of morphine tolerance in tail-flick test

Repeated administration of SHU9119 at dose of 0.5 μg (i.t.), 15 min prior to the morning saline injection, did not change tail-flick latency. The values did not differ significantly from saline-injected animals on any of the tested days (Fig. 1A). SHU9119-induced changes in development of morphine tolerance are shown in Fig. 1A. There was no significant difference ($p>0.05$) in morphine-induced analgesia between morphine alone or in combination with SHU9119 on day 1 and 2 of the experiment (8.33 ± 0.24 s *vs.* 8.00 ± 0.52 s and 8.17 ± 0.26 s *vs.* 8.18 ± 0.29 s, respectively). Day 3 distinguished the two investigated groups, morphine-injected animals showed decreased latency in tail-lick test (6.2 ± 0.43 s), the SHU9119-pretreated rats displayed significantly higher ($p<0.05$) latency (8.62 ± 0.21 s), compared to the effect of morphine administered on day 1 of the experiment. Further reduction in test latency was observed on the next days until day 7. The analgesic response to morphine on day 8 was still

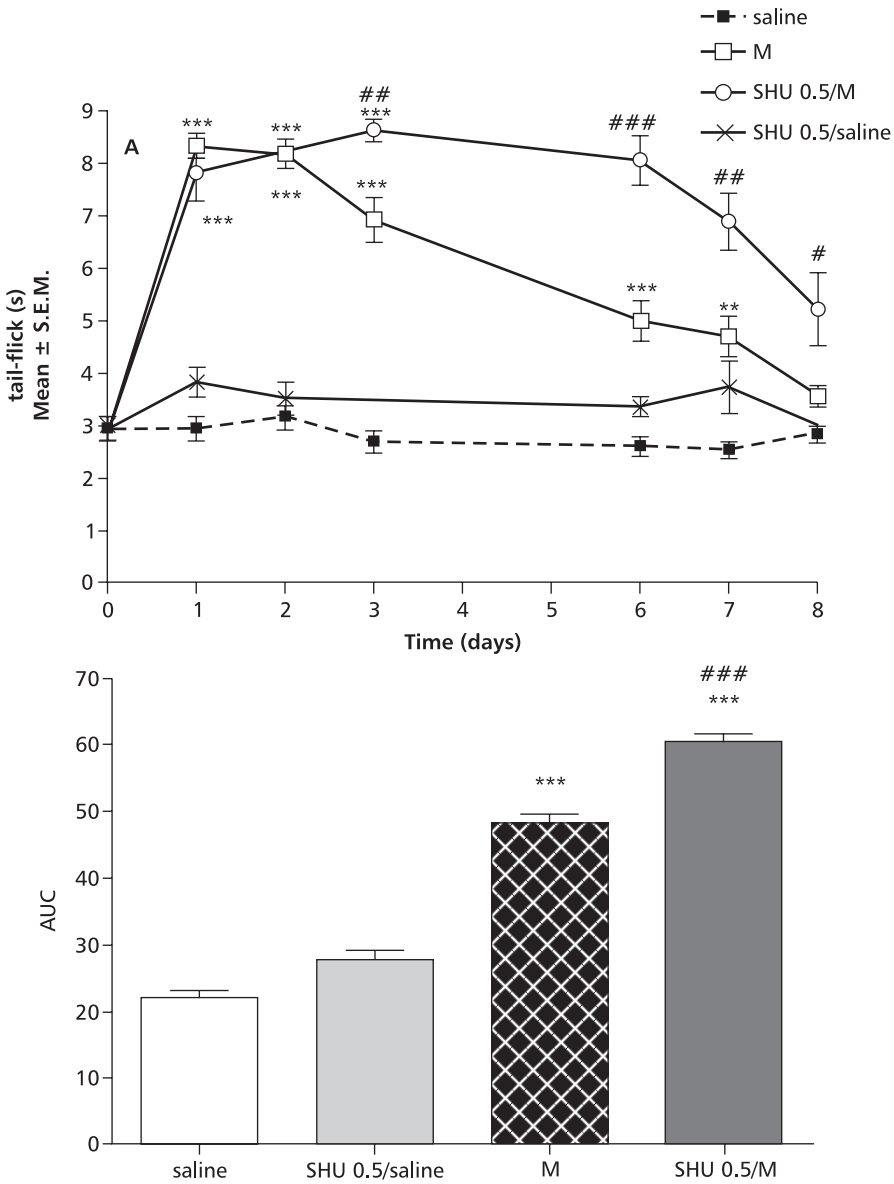

Fig. 1. Effect of SHU9119 (0.5 μg, i.t.) repeated administration on the development of morphine tolerance represented by the tail-flick latency in s (A) and by area under curve (AUC) units (B). Rats received chronic saline (n=8), chronic morphine (M, n=8), SHU9119 before chronic morphine (SHU/M, n=10) and SHU9119 before chronic saline (SHU/saline, n=8). Acute antinociceptive action of the administered drugs was evaluated in tail-flick test on days 1–8 of the experiment (for details see: Materials and Methods). Values are the mean ± S.E.M. *$p<0.05$ in comparison to saline-treated group, #$p<0.05$ in comparison to morphine-administered group.

significantly higher ($p<0.01$) in SHU9119-pretreated animals when compared to the morphine-injected group, which displayed tolerance to its analgesic effect and the values did not vary significantly form saline-receiving group, however the difference was smaller than on day 7 ($F_{3,19} = 19.2$). The above data are also presented as area under the curve (AUC) in Fig. 1B. There was no significant difference ($p>0.05$) between rats treated with SHU9119 prior to saline (27.9 ± 2.4) *vs.* saline alone-injected group (22.2 ± 1.7) as estimated by AUC (0–8 days), whereas pretreatment with SHU9119 prior to morphine significantly increased ($p<0.001$) the AUC (0 – 8 days) values in comparison with the group treated with morphine alone (48.3 ± 1.8 *vs.* 60.5 ± 1.7; $F_{3,28} = 85.8$).

Effect of repeated JKC–363 i.t. administration on the development of morphine tolerance in tail-flick test

The selective MC4R antagonist, JKC–363, also significantly influenced the development of morphine tolerance (Fig. 2A). Rats received i.t. injections of 0.7 μg of JKC–363 prior to each morning morphine administration. There was no significant difference ($p>0.05$) between saline-injected rats when compared to JKC–363 (i.t.) in combination with saline (i.p.), which means that the drug *per se* did not change tail-flick latency. Over a period of 6 testing days, JKC–363 prevented in a significant manner the development of morphine tolerance. The morphine analgesic potency 30 min after the combined administration of JKC–363 (i.t.) and morphine (i.p.) resulted in strong antinociceptive response. The statistically significant ($p<0.05$) difference between morphine-treated animals and rats administered JKC–363 prior to morphine was observed already on day 3 of the experiment. On testing days 6 and 7 i.t. given JKC–363 significantly ($p<0.001$) prevented the development of morphine tolerance (day 6: 8.26 ± 0.31 s *vs.* 5.00 ± 0.35 s; day 7: 7.88 ± 0.43 s *vs.* 4.7 ± 0.39 s) (Fig. 2 B). On day 8 rats receiving morphine alone were tolerant to morphine and the latencies in the tail-flick test did not vary significantly form saline injected animals, whereas chronic treatment with JKC–363 followed by i.p. morphine produced significant ($p<0.001$) analgesia (5.73 ± 0.53 s *vs.* 3.57 ± 0.2 s; $F_{3,19} = 25.6$). The selective MC4R antagonist, JKC–363 significantly increased the tail-flick latency induced by morphine in rats chronically injected with morphine as evaluated by AUC (0 – 8 days). Pretreatment with JKC–363 produced a significant increase (Fig. 2B) in morphine analgesic effect in the AUC (0 – 8 days) in tail-flick test (63.0 ± 2.1) when compared to morphine effect alone (48.3 ± 1.8); whereas JKC–363 *per se* did not influence the baseline values in the tail-flick test (JKC–363/saline 25.0 ± 2.3 and saline 22.2 ± 1.7; $F_{3,28} = 96.8$).

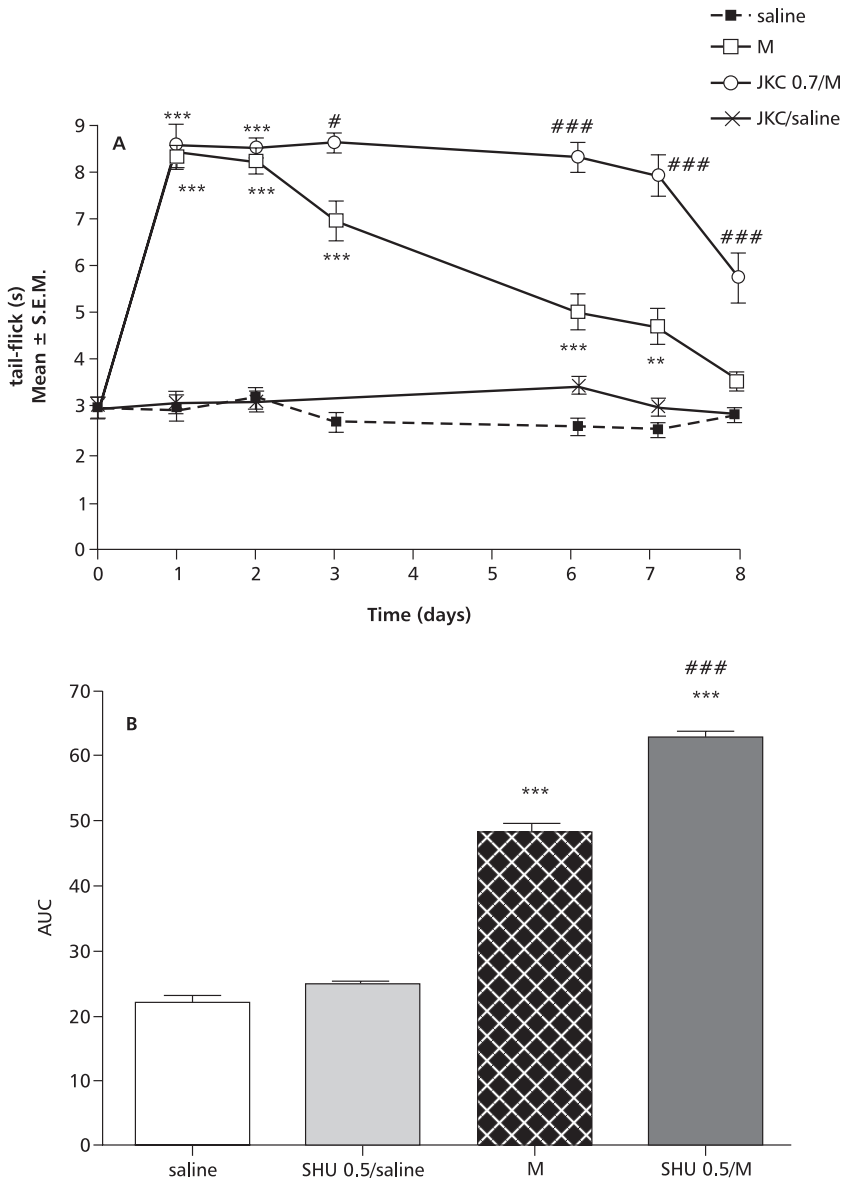

Fig. 2. Effect of JKC–363 (0.7 μg, i.t.) repeated administration on the development of morphine tolerance represented by the tail-flick latency in s (A) and by area under curve (AUC) units (B). Rats received chronic saline (n=8), chronic morphine (M, n=8), JKC–363 before chronic morphine (JKC/M, n=10) and JKC–363 before chronic saline (JKC/saline, n=8). Acute antinociceptive action of the administered drugs was evaluated in tail-flick test on days 1–8 of the experiment (for details see: Materials and Methods). Values are the mean ± S.E.M. *$p<0.05$ in comparison to saline-treated group, #$p<0.05$ in comparison to morphine-administered group.

The effect of a single i.t. SHU9119 or JKC–363 administration on morphine effect in tail-flick test in morphine tolerant rats.

After 8 days of morphine administration (10 mg/kg every 12 h, i.p.), the rats were tolerant to morphine. On day 9 of the experiment the following different treatments were given: morphine (10 mg/kg, i.p.) and SHU9119 (0.5 and 1.5 μg i.t.) or JKC–363 (0.7 and 2 μg i.t.) 15 min prior to morphine. Control group consisted of saline-injected rats. Additional control groups comprised rats tolerant to morphine receiving a single injection of either SHU9119 or JKC–363 in order to establish the influence of MC4R antagonists on analgesia in morphine tolerant rats (for details see Method section).

In rats tolerant to morphine, there was no change in morphine-induced analgesia after repeated drug exposure; latency in tail flick-test after the tested morphine dose was 2.8 ± 0.17 s, which was similar as the test latency in saline-injected rats (2.6 ± 0.15 s). None of the administered MC4R antagonists alone had antinociceptive properties, the tail-flick latency did not vary significantly ($p>0.05$) from the respective values in animals tolerant to morphine or chronically injected with saline. Interestingly, upon spinal administration of an MC4 receptor antagonist, both SHU9119 and JKC–363 produced significant increase in morphine analgesic effect (Fig. 3 A, B). The tail-flick latencies displayed parallel nonsignificant tendency to increase with the increasing dose of the respective MC4 receptor antagonist used. When 0.5 and 1.5 μg of SHU9119 were given 15 min prior to morphine, this resulted in a significant increase in withdrawal threshold by 188 % ($p<0.01$) and 219 % ($p<0.001$; $F_{5,44}= 45.4$) when compared to morphine alone (Fig. 3A) Animals treated with JKC–363 (at different doses) prior to morphine challenge displayed dose-dependent ($F_{5,44}= 17.7$) increase in tail-flick latency to 194% and 221 % vs the effect of morphine alone, for 0.7 and 2 μg of JKC–363, respectively (Fig. 3B).

MC4R and μOR mRNA level in the spinal cord and lumbar DRG in morphine tolerant rats

In these experiments, total RNA was extracted from spinal cord and DRG of rats that had been treated with morphine according to the schedule of behavioral experiments, and used for quantitative (q) RT-PCR. In the series of qRT-PCR experiments, there were no significant differences between intact and saline-treated animals. The control group in the Fig. 4 represents the mRNA level of the respective receptor tested in the saline-treated rats. The continuous mor-

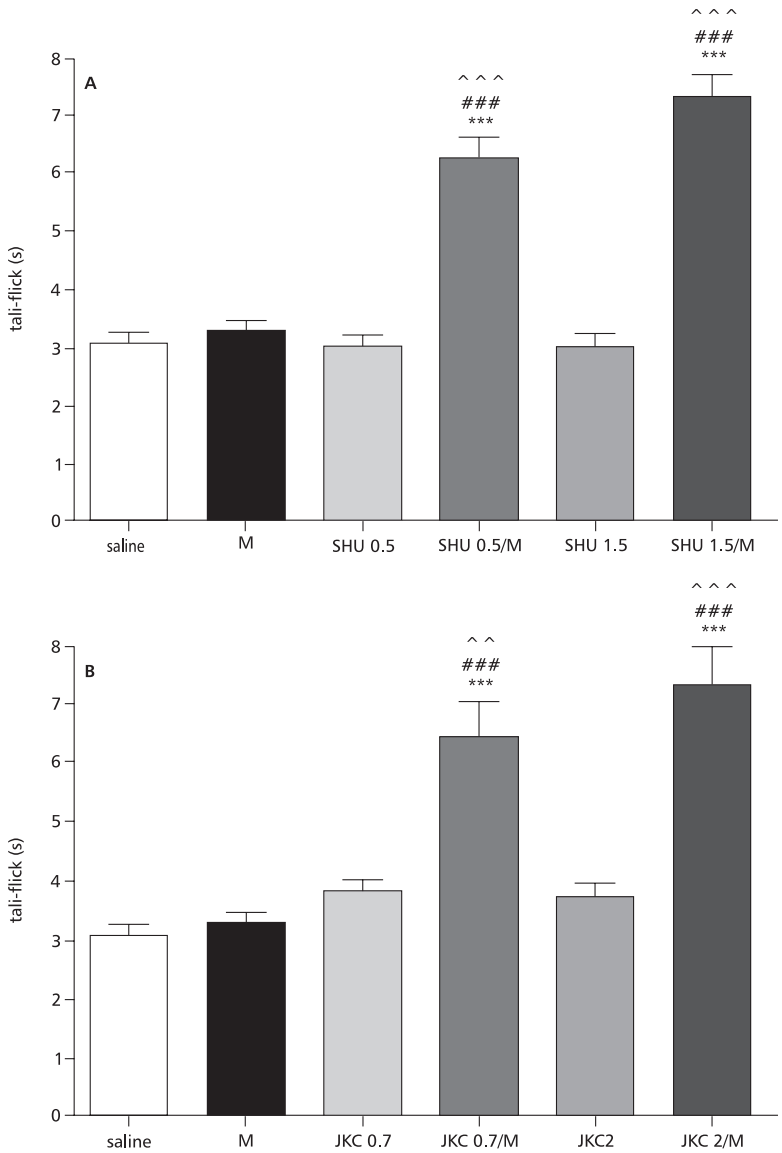

Fig. 3. Effect of a single i.t. injection of MC4-R antagonists, SHU9119 (A) or JKC–363 (B) on analgesic effect of morphine in morphine tolerant rats, measured by the tail-flick latency in s. Rats tolerant to morphine were challenged with saline (n=6), morphine (n=8), MC4-R antagonist alone SHU9119 (0.5 and 1.5 μg, i.t.) or JKC–363 (0.7 and 2 μg, i.t.), n=8 in each group, or MC4-R antagonists (i.t.) in combination with morphine (i.p.), n=8 each group (detailed description in: Materials and Methods). Data are given as the means ± SEM. *$p<0.05$ in comparison to saline-treated group, #$p<0.05$ in comparison to chronic morphine, ^$p<0.05$ the respective MC4-R antagonist alone vs. its administration in combination with morphine.

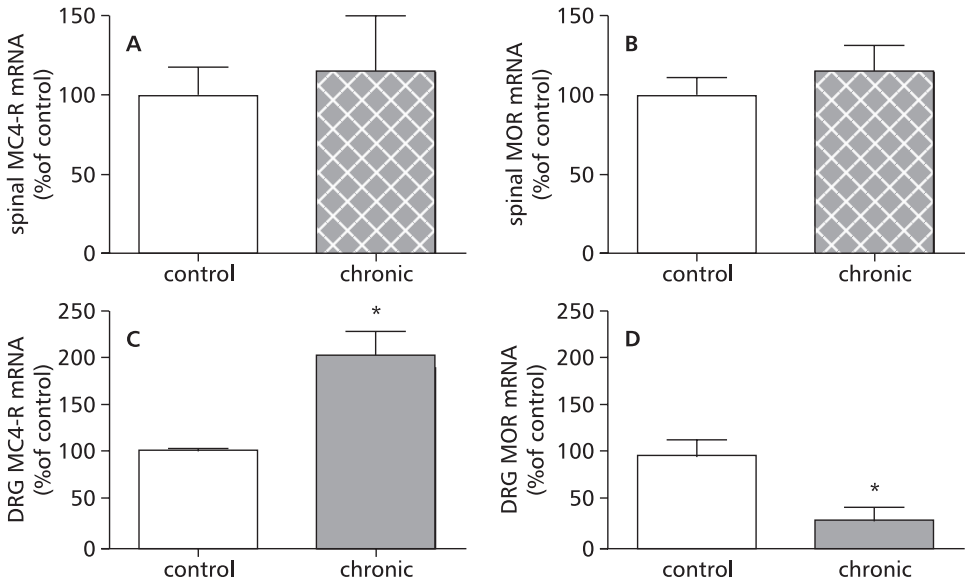

Fig. 4. Changes in MC4-R and MOR mRNA expression of in the lumbar spinal cord (A,B) and dorsal root ganglia (C,D) in rats chronically treated with morphine. Data are expressed as the percent of control mRNA level ±SEM of 6–8 animals in each experimental group. *$p<0.05$ vs. control group was considered statistically significant.

phine exposure that rendered the animals tolerant to the challenge dose of morphine (Fig. 1A open squares) did not alter the levels of MC4R and μOR mRNA measured on day 8 in the dorsal part of the lumbar spinal cord (Fig. 4A, B). However, morphine oppositely regulated the MC4R and μOR gene expression in the DRG of rats tolerant to morphine. Chronic treatment (8 days) with morphine significantly increased the abundance of MC4R in DRG (Fig. 4C), with parallel down-regulation of μOR (Fig. 4D).

DISCUSSION

In the present study, we demonstrated significant delay in development of morphine tolerance in rats daily treated with melanocortin receptor antagonists and ability of their single injection to restore the morphine analgesia in tolerant rats. This is the first report to show reversal of the expression of tolerance to morphine-induced analgesia by a specific MC4R antagonist at the spinal level. We have found that tolerance to analgesic effect of morphine was markedly attenuated

in rats treated with both: mixed MC3/MC4 (SHU9119) and specific (JKC–363) MC4R antagonists. Interestingly, the effect of co-administration of morphine with MC4R antagonists on the first day of the experiment did not differ in comparison with rats treated with morphine alone. On the other hand, the morphine tolerance after its repeated co-administration on the next days, evaluated in tail-flick test, was markedly attenuated in rats treated with SHU9119 or JKC–363. This strongly suggests that the melanocortin system may contribute in part to development of changes which lead to tolerance after chronic morphine treatments. Furthermore, we demonstrated that SHU9119 and JKC–363 could overcome the decrease in analgesic effect that characterizes tolerance to morphine. The MC4R antagonists were not effective *per se*, but influenced development of morphine tolerance. Based on the assumption that the reduction of morphine analgesia during chronic treatment is a consequence of the enhanced activity of anti-opioid systems in the brain, several attempts have been made to characterize morphine analgesic tolerance by the use of noncompetitive NMDA receptor antagonists (Trujillo 1995) or cholecystokinin B antagonist L–365,260 (Mitchell et al., 2000). Therefore, we hypothesized that MC4R antagonists could act similarly to these compounds.

In our previous study, we have demonstrated that SHU9119, microinjected into the central nucleus of the amygdala of rats tolerant to morphine significantly increased the antinociceptive effect of morphine (Starowicz et al., 2003). The abundant distribution of MC4R in the central nervous system may possibly imply that they are involved in both various spinal and supraspinal systems that modulate pain processes. This may further suggests that activity of melanocortin system is increased during morphine treatment and in consequence morphine tolerance develops. As proposed by Ma et al. (2001), the overactivity of pro-nociceptive systems, like the increase in the MC4R mRNA in morphine tolerant rats, observed in the present study, may contribute to opioid tolerance. We currently observed that chronic morphine exposure led to opposite regulation of MC4R and μOR mRNAs in DRG of morphine tolerant rats, increasing the MC4R and decreasing the μOR gene expression. Our data on μOR mRNA in DRG are in accordance with those reported by Meuser et al. (2003). However, we report no change in MC4R or μOR spinal gene expression which are in accordance with the results of Nishino and colleagues (Nishino et al., 1990). Studies on CNS regions have yielded inconsistent results. No change in μOR mRNA levels in brain structures of morphine tolerant rats (Brodsky et al., 1995; Buzas 1996; Castelli et al., 1997) has been reported. On the contrary, a down-regulation of μOR mRNA has been reported in the mediobasal hypothalamus of morphine tolerant guinea pigs with no changes in other brain regions (Ron-

nekleiv and Bosch 1996). In the peripheral nervous system, data of Chen et al. (1997) on isolated rat DRG indicated that *in vitro* exposure to DAMGO, a selective μ-opioid receptor ligand, resulted in a decrease in μ-opioid receptor binding sites that is in agreement with our study which demonstrated lower level of μOR in morphine tolerant rats. The μ-opioid receptors are expressed on primary afferent neurons which terminate in lamina I and II of the dorsal horn and at glutamatergic interneurons in lamina II (Arvidsson et al., 1995; Trafton et al., 2000). Importantly, opioid analgesia besides central nervous system, appears also in the periphery (Stein et al., 1988). The peripheral analgesic effects of opioids are elicited by activation of opioid receptors on nerve endings in the peripheral tissue. The μ-opioid receptors responsible for the local analgesic actions of morphine in the tail presumably are located on sensory nerves with the cell bodies in the DRG (Fields et al., 1980). The results presented herein further support the significant role of μOR synthesized in the DRG in the aspect of morphine tolerance.

There is only limited information about the role of MC4R gene expression both in peripheral and central nervous system in development of morphine tolerance and physical dependence. Although the role of melanocortins has been previously reported in the aspect of both opiate dependence and tolerance, the neural substrates underlying these actions are unknown (Alvaro et al., 1996). The MC4R expression has been demonstrated to be regulated in certain brain structures implicated in the opiate addiction (Alvaro et al., 1996, 1997). The latter authors demonstrated that chronic administration of morphine (over 5 days) resulted in a time-dependent down-regulation of MC4R mRNA expression in the striatum and periaqueductal gray. They also postulated that decreased melanocortin function, via down-regulation of MC4R expression, may contribute to the development of opiate self-administration, tolerance, and physical dependence (Alvaro et al., 1996). More recent study (Alvaro et al., 2003) established a link between cocaine behavioral responses (grooming and locomotor activity) and the central melanocortin system, particularly the MC4R. Our previous study (Starowicz et al., 2003) demonstrated that single morphine administration decreased the level of MC4R mRNA in the rat amygdala. This decrease was, however, attenuated following every next morphine administration, and MC4R mRNA level gradually increased with time, and on 9th day of morphine administration, it was higher than in the control. In our present study, the increase of MC4R mRNA level was observed also in periphery, in the DRG of morphine tolerant rats.

Our present results support earlier suggestions about the involvement of melanocortin system in nociceptive transmission. The MC4R antagonists also produce analgesia in rodent models of neuropathic pain (Vrinten et al., 2000, Starow-

icz et al., 2002), and their analgesic effects in neuropathic pain are exceptionally powerful. Recently, the expression of MC4R in DRG has been reported and modulation of activity of the primary afferents by this receptor has been suggested. Moreover, down-regulation of MC4R mRNA in DRG developed 2 weeks after the nerve injury (Starowicz et al., 2004). The observed opposite regulation of the MC4R mRNA in DRG of tolerant to morphine and neuropathic rats may result from dynamic plastic processes, which accompany those pathological states. The above studies were conducted in various periods after morphine treatment or nerve injury in tolerant and neuropathic rats, respectively. Therefore, further time-course studies are needed in order to clarify the role of MC4R in morphine tolerance and neuropathy, to establish whether common mechanisms are involved and to investigate the subsequent regulation of μOR and MC4R protein density.

In summary, the present results suggest that the melanocortin system is an important component influencing the changes in the spinal neuronal plasticity observed after chronic morphine administration. We demonstrated the reduction of morphine tolerance in SHU9119- or JKC–363-treated rats. Thus, besides other systems, such as the NMDA receptor system (Trujillo 1995), the melanocortin system may also be involved in the changes in spinal neuronal plasticity. It is, however, uncertain what cellular and neurophysiological pathways that opioid and MC4R ligands share in the spinal cord facilitate development of morphine tolerance. The specific MC4R antagonists would be promising compounds to alleviate the side effects of long-term morphine treatment. Their ability to restore morphine analgesia may have clinical benefits. SHU9119 and JKC–363 may widen the options of the use of opioids for treatment of chronic pain by reducing the need for opioid dose escalation, which is often associated with unwanted side effects and by attenuating the development of analgesic tolerance and hyperalgesia following chronic use of morphine.

Acknowledgements

This research was supported by the statutory funds (IO, RP) and by grant No. K062/P05/2003 (KS, BP) from the State Committee for Scientific Research (KBN), Warsaw, Poland.

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Chapter VII

Conclusions and general remarks

Contents

The aim of the thesis was to characterize the role of melanocortin system in chronic pain. Furthermore, the involvement and possible interactions of the melanocortins with the opioid system were also studied. The main findings discussed in the following paragraphs regard the modulation of the MC system activity by opioids, and the plasticity of the MC system at both early and late stage of neuropathic pain and its functional implications. One of the important findings of the study was the identification of the effects of peripheral nerve injury on the expression of MC4R and μ-opioid receptor (μOR) in the rat lumbar 4–5 dorsal root ganglia (DRG) and dorsal horn of the spinal cord using immunohistochemical techniques. It was shown that MC4R immunoreactivity (IR) in the DRG increased, and the degree of the MC4R up-regulation paralleled the time elapsing after nerve injury. MC4R-IR in the spinal cord, ipsilateral to the injury, was also increased. In contrast, the μOR-IR dropped, both in DRG and lumbar spinal cord. Their reciprocal effect of peripheral nerve damage on MC4R and μOR may contribute to the reduced efficacy of opioids in neuropathic pain both in animals and in humans.

Since literature data provide some evidence for functional and molecular similarities between neuropathic pain and tolerance to repeated opioid exposure, we studied the involvement of the supraspinal as well as the spinal melanocortin system in the development of morphine tolerance. We have shown that amygdalar melanocortin receptors may contribute to morphine tolerance, and have concluded that the altered melanocortin receptor function may play an important role in the development of morphine-induced tolerance. We further demonstrated that the MC4R antagonists SHU9119 or JKC–363 prevented the development of morphine tolerance at the spinal cord level. Interestingly, a single administration of an MC4R antagonist restored morphine analgesic potency in morphine-tolerant rats. Further biochemical study demonstrated no changes in MC4R and μOR in the spinal cord while MC4R rose and μOR decreased in the DRG of rats tolerant to morphine. These results suggest that MC4R may be involved in the mechanisms of opioid tolerance, and antagonists of this receptor may be a possible new target in the search for strategies preventing the development of opioid tolerance.

Furthermore, possible interactions between the melanocortins and the opioid system were also studied. The main findings described in the following paragraphs are related to the modulation of the MC system activity by opioids, and to the plasticity of this system at both early and late stage of neuropathic pain and its functional implications. The blockade of melanocortin receptor by the antagonist SHU9119 produced analgesia whereas administration of melanocortin receptor agonist, MTII, increased the sensitivity to pain stimulation in neu-

ropathic rats. Interestingly, the melanocortin-induced effects were a subject of opioid modulation. Blockade of μOR by cyprodime (CP) enhanced the antiallodynic effect of SHU9119 as well as the pronociceptive action of MTII, whereas the combined administration of a μ receptor agonist (DAMGO) and SHU9119 significantly reduced the analgesic effect of those ligands. DAMGO also reversed the proallodynic effect of a melanocortin receptor agonist, MTII. On the other hand, we further demonstrated that the crucial issue for the functional modulation effect is the order of events on opioid or melanocortin receptors. Enhancement of the opioid action depends on the activity of the melancoortin system. Interestingly an additive anit-allodynic action of morphine and SHU9119 after tactile stimulation was observed, when the MC4R blockade preceded the morphine administration.

1. MELANOCORTINS AND NOCICEPTIVE PATHWAYS

Within the CNS, two receptor types for melanocortins are expressed, MC3R and MC4R (Moutjoy et al., 1994). MC4R mRNA was found in multiple sites in virtually every brain region, including the cortex, thalamus, hypothalamus, brainstem, and spinal cord signifying its role in the central control of pain. MC4R is also unique in its expression in numerous cortical and brainstem nuclei (Mountjoy et al., 1994). Its expression has also been demonstrated within the spinal cord (Mountjoy and Wild, 1998). However, MC4R was only shown to be expressed in mammals within the central nervous system (Mountjoy et al., 1994). Therefore, the major finding of this studies, presented in the chapter 3 is the demonstration that the MC4R is also expressed in the DRG of adult rats. Previous studies showed that MC4R mRNA was expressed transiently in the developing peripheral nervous system of the rat fetus (cranial nerve ganglia and sympathetic ganglia) (Kistler-Heer et al., 1998; Mountjoy and Wild, 1998). These studies demonstrated that appearance of MC4R mRNA was temporarily correlated with periods of neural network formation. More recently, Mountjoy et al. (2003) determined MC4R mRNA during ontogeny of the rat using *in situ* hybridization and discovered that MC4R mRNA was expressed in numerous peripheral tissues (heart, lung, kidney and testis) during development. Results presented in the Addendum #2 evidence the peripheral localization of MC4R in the DRG of adult rats. These novel sites of MC4R expression (Staro-

wicz et al., 2004) indicate that the MC4R may not only function within spinal cord neurons but also at the primary afferents. These findings are in line with the findings demonstrating POMC mRNA in rat DRG (van der Kraan et al., 1999), thus suggesting a functional interaction of MC4R with melanocortins in the DRG. The demonstration of the MC4R expression in the DRG of adult rats might indicate location of this receptor on terminals of primary afferents within superficial laminae of the dorsal horn. Furthermore, this observation could suggest involvement of MC4R in presynaptic regulation of primary afferent terminals and indicate their important role in nociception.

2. MELANOCORTINS AND OPIATE TOLERANCE

In chapter 5 and 6, we described a role of supraspinal and spinal MC4Rs in morphine tolerance. We demonstrated that chronic spinal administration of SHU9119 or JKC–363, significantly prevented the development of morphine tolerance. Furthermore, a single administration of an MC4R antagonist was able to restore morphine analgesic potency in morphine tolerant rats. Finally, we demonstrated that SHU9119, when injected into the amygdala prior to morphine administration in tolerant rats, increased the antinociceptive effect of morphine. Thus, we showed for the first time a contribution of spinal and amygdalar melanocortin receptors to morphine tolerance.

The mechanism of this interaction is unknown, however, a contribution of several neurotransmitter systems and their receptors in the development of morphine tolerance has been a subject of previous studies and was reviewed in detail by Vaccario and Kastin (2000). In particular, evidence demonstrating the functional involvement of glutamate receptors (particularly *N*-methyl-D-aspartate (NMDA) receptors) in opiate tolerance has been reported (Bisaga et al., 2000; Fairbanks et al., Trujillo 2000). Tolerance induced by morphine was completely prevented by a competitive NMDA receptor antagonist LY235959 (Allen et al., 2000). Spinal administration of morphine increased NMDA receptor binding activity, and up-regulated neuronal NO synthase (NOS) expression that was partially prevented by MK–801 (Wong et al., 2000). Thus, activation of NMDA receptors can lead to production of the second messenger nitric, oxide (NO) that appears to be involved in morphine tolerance (Fairbanks and Wilcox 2000; Majeed et al., 1994; Kolesnikov et al., 1997; Pasternak et al., 1994) because i.t. infusion of morphine increased NMDA receptor binding activity, and up-regulated neuronal NO synthase (NOS) expression that was partially prevented by

MK 801 (Wong et al., 2000). Cholecystokinin (CCK) is another example of an endogenous neuropeptide and neurotransmitter widely distributed in the brain and spinal cord (Beinfeld et al., 1981). The CNS distribution of CCK parallels that of the endogenous opioids and opioid receptors within pain processing areas, such as periaqueductal gray and the superficial laminae of the spinal cord (Saito et al., 1980). Experimental evidence suggests that CCK modulates opioid effects on pain transmission and the antiopioid effects of this peptide are well documented (O'Neill et al., 1989). In accordance with these findings, behavioral studies revealed that CCK_B-receptor antagonists blocked the development of tolerance to the antinociceptive action of morphine (Kellstein and Mayer, 1991). Furthermore, as proposed by Ma et al. (2001), the overactivity of pro-nociceptive systems in morphine tolerant rats may contribute to opioid tolerance. We demonstrated in chapter 6 that also spinal administration of melanocortin receptor antagonists influenced the development of morphine tolerance. Tolerance to analgesic effect of morphine was markedly attenuated in rats intrathecally treated with both: mixed MC3/MC4 (SHU9119) and specific (JKC–363) MC4R antagonists. Previously it was shown that chronic administration of morphine resulted in a time-dependent down-regulation of MC4R mRNA expression in the striatum and periaqueductal gray of rat (Alvaro et al., 1996). Expression of MC4R mRNA was also found to be decreased in the nucleus accumbens/olfactory tubercle. In the striatum, the reduction of MC4R mRNA was accompanied by a concomitant decrease in melanocortin receptor levels. The authors (Alvaro et al., 1996) hypothesized that the decreased melanocortin function might contribute to the development of opiate tolerance. In contrast, we currently observed that chronic morphine treatment caused an increase in MC4R expression in the DRG but remained without effect within the spinal cord. Furthermore, chronic morphine exposure led to opposite regulation of MC4R and μOR mRNAs in DRG of morphine tolerant rats, increasing the MC4R and decreasing the μOR gene expression. In summary, these results suggest that alteration of primary afferent MC4R function may contribute to induction and expression of opioid tolerance to morphine analgesic effect. Furthermore, μOR/MC4 receptor interaction may play an important role.

Several mechanisms at the cellular level including multiple signaling pathways have been proposed to play a role in opioid tolerance. Shen et al. (Shen et al., 2000) postulated a role of cAMP-dependent protein kinase (PKA) in morphine tolerance in mice. Morphine activates cAMP-PKA pathway *in vivo* (Nestler and Aghajanian 1997) and chronic exposure to an opioid agonist increases the activity of this pathway (Nester and Tallman 1988). On the contrary, opposite cAMP-PKA regulation has been demonstrated for melanocortins (Zhou et al., 2001).

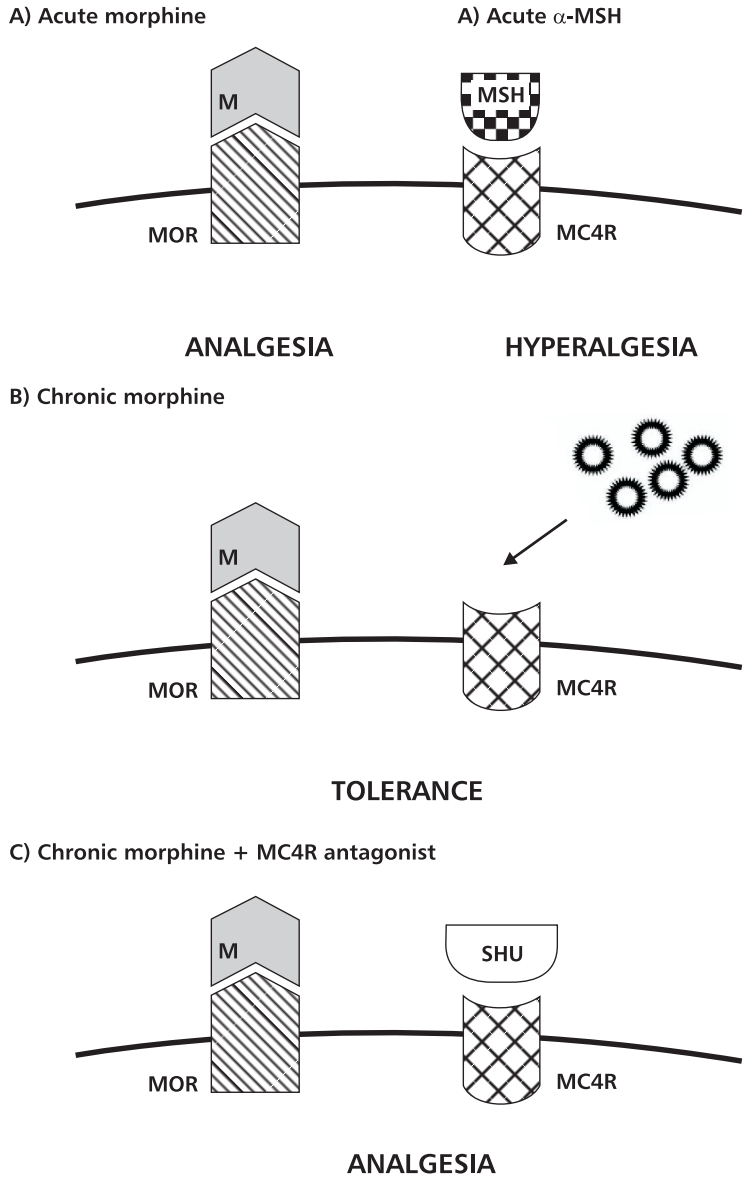

Fig. 1. **A**: Acute administration of the μ opioid receptor (MOR) agonist morphine (M) and MC4 receptor (MC4R) agonist, α-MSH results in opposite regulation: analgesia in case of MOR activation and hyperalgesia after MC4R activation. **B**: Chronic morphine administration is associated with the development of morphine tolerance and a decreased morphine analgesic effect. The above may be further enhanced by the endogenous tonus of α-MSH on MC4R. **C**: A blockade of the MC4R by its antagonist SHU9119 (SHU) may be needed to counteract the increase in PKA activity and thus to restore the analgesic effect of μ-opioid agonists *via* an opioid inhibitory effect on cAMP formation.

In CATH.a cells, activation of MCRs (MC1, MC3- 5) by acetyl MSH stimulates cAMP production and acts at the genomic level by stimulation c-fos and tyrosine hydroxylase (TH) transcription, and the latter effect was mediated by PKA. Exposure to chronic morphine treatment increases activity of the cAMP-PKA pathway, thus the stimulatory effect of the melanocortin system on adelylate cyclase activity is enhanced. To counteract the increase in PKA activity and thus to restore the analgesic effect of μ-opioid agonists *via* an opioid inhibitory effect on cAMP formation, a blockade of another cAMP stimulatory system, namely, the blockade of MC4R by its antagonists, SHU9119 and JKC–363, may be needed (see also Fig. 1). These and other points of regulatory control of the opioid and melanocortin systems remain to be investigated.

As demonstrated in chapter 5, an antagonist of MC4R, administered into the rat central nucleus of the amygdala, influences development of morphine tolerance. The central nucleus of the amygdala sends monosynaptic projections to the population of opioid-sensitive cells in the ventrolateral periaqueductal gray that are known to be involved in nociception (Helmstetter and Landeira-Fernandez, 1990) and contain considerable density of μ-opioid and melanocortin receptors (Bakshi et al., 2002; Mansour et al., 1987; Pickel and Colago, 1999; Kask and Schioth, 2000). Our further study demonstrated that the level of MC4R mRNA, decreased after a single morphine administration but gradually increased parallel to the development of morphine tolerance. These results indicate that MC4R in the amygdala may be involved in the development of some aspects of morphine tolerance.

In summary, recent studies have shown that the development of opioid tolerance may activate melanocortin receptors and possibly melanocortin transmission by primary afferents in spinal cord and amygdala that could counteract the analgesic effects of opioids.

3. SIMILARITIES BETWEEN OPIOID TOLERANCE AND NEUROPATHY

Mao et al. (1995) postulated some similarities between opioid tolerance and neuropathic pain. Neuropathic pain is associated with activation of spinal excitatory amino acid receptors resulting in subsequent intracellular cascades including protein kinase C translocation and activation, nitric oxide (NO) production, and NO-activated poly(ADP ribose) synthetase (PARS) activation. Similar cellular

mechanisms have also been implicated in the development of tolerance to the analgesic effects of morphine (Mayer et al., 1999). The similarity between tolerance and hyperalgesia was demonstrated at the level of excitatory amino acid receptor activation and subsequent intracellular events (Mayer et al., 1999). Interactions between amino acid receptors and opioid receptors can lead to neuroplastic changes in the spinal cord which are associated with opioid tolerance. This alteration may further modify opioid receptor-mediated intracellular signaling. Mice that lack the μOR no longer experience morphine antinociception, indicating that the μOR is primarily responsible for this effect (Sora et al., 1997; Kieffer 1999). Regulation of the μOR is, therefore, an attractive target for affecting the degree of antinociception and tolerance induced by morphine. The overall manifestation of morphine-induced antinociception and tolerance involves complex neuronal interactions and many different signaling components. Morphine induces antinociception by activating μORs in spinal and supraspinal regions of the CNS. β-Arrestin–2, a G-protein-coupled receptor-regulating protein, is one of the proteins putatively involved in regulation of the μ-OR. Furthermore, studies of Shinyama et al. (2003) provided novel insights into the regulation of MC4R signaling and demonstrated that internalization of MC4R by agonist was also β-arrestin dependent.

Bohn et al. (2002) evaluated the contribution of β-arrestin–2 an essential component of the desensitization process, to the antinociceptive actions of morphine and demonstrated that the spinal antinociceptive actions of morphine were regulated by β-arrestin–2. Since regulation of the μOR is an attractive target for affecting the degree of antinociception and tolerance induced by morphine, in chapter 4 we demonstrated the significance of β-arrestin to the phenomenon of morphine tolerance. The binding to the opioid receptor has been shown to be correlated with the desensitization of G-protein-coupled receptors. *In vitro* studies have indicated that this process involves phosphorylation of G-protein-coupled receptors and subsequent binding of β-arrestins. Functional deletion of the β-arrestin 2 gene in mice resulted in remarkable potentiation and prolongation of the analgesic effect of morphine, which suggests that μOR desensitization might be impaired (Bohn et al., 1999). The same authors demonstrated that desensitization of the μOR did not occur after chronic morphine treatment in knockout mice lacking β-arrestin 2 gene (β arr2-/-), and that these animals failed to develop tolerance to the antinociceptive effect (Bohn et al., 2000). As demonstrated in chapter 4, treatments with antisense oligonucleotide targeting β-arrestin–2 delayed not only the development of tolerance to morphine but, more interestingly, also the development of allodynia, resulting from nerve injury.

3.1. Melanocortins in nociception and neuropathic pain

Melanocortins have been implicated in the control of nociception by the CNS. The early reports of Williams et al. (1986) demonstrated that ACTH caused hyperalgesia as indicated by decreases in latency in the rabbit ear-withdrawal test. Sandman and Kastin (1981) demonstrated that centrally administered α-MSH produced enhanced response to painful thermal stimuli in the tail-flick test. Interestingly, α-MSH antagonized the analgesic effect of β-endorphin. The results may suggest that melanocortins when released during stress can influence acute nociception. That study suggests that α-MSH can have opposite actions in relation to the endorphins and postulated that α-MSH and related peptides might be endogenous anti-opiates. POMC mRNA was also demonstrated in the spinal cord (van der Kraan et al., 1999; Beltramo et al., 2003) and immunoreactivity for the POMC-derived peptides β-endorphin, ACTH, and α-MSH has been described in the dorsal horn. MC4R expression has been confirmed in areas important to nociception, like the superficial dorsal horn and in the periaqueductal gray. Thus, the functional melanocortin system is present in the spinal cord and it seems likely that the melanocortin system may modulate pain signal transmission in neuropathic pain. In chapter 2, we described the effects of spinal administration of MC4R ligands on nociceptive information processing in neuropathic rats. In the present experiments, ligands were administered through an intrathecal catheter, directly into the lumbar region of the rat spinal cord. Spinal administration of the MC4 receptor antagonist, SHU9119 results in significant reduction of allodynia associated with neuropathic pain, as measured by von Frey and cold water allodynia tests. The observed anti-allodynic action of SHU9119 was similar to that observed after DAMGO administration. Interestingly, action of both compounds was significantly stronger than the response to morphine. MTII enhanced allodynia, as evidenced by a decrease in the withdrawal threshold to mechanical stimulation after administration of the MC receptor agonist. These observations are in line with investigations of Vrinten et al. (2000). The authors reported that administration of the MC4R antagonist SHU9119 into the cisterna magna had a profound anti-allodynic effect, whereas the administration of the MC4R agonists MTII and d-Tyr-MTII primarily increased the sensitivity to mechanical and cold stimulation, suggesting that the endogenous melanocortin system has a tonic effect on nociception. Interestingly, drugs administered into the cisterna magna may reach the ventricular system and surrounding structures, and thereby MC4R activation or blockade could affect both spinal and supraspinal structures and contribute to the observed pro-

or anti-nociceptive effects described herein (Vrinten et al., 2000). The chronic intrathecal catheter placement employed in our study allows for specific delivery of drugs into the spinal intrathecal space, which limits the direct actions on the spinal cord tissue.

3.2. Melanocortin-opioid interactions in neuropathic pain

As already mentioned, representatives of both systems, β-endorphin and α-MSH have been demonstrated to be present in the spinal cord. They can activate μ-opioid or MC4 receptors therein, thus causing analgesia or hyperalgesia, respectively. Spinal blockade of the MC4 receptors by α-MSH is not produced via MC4R activation. On the other hand, β-endorphin via μOR could act postynaptically and hyperpolarize neurons within the spinal cord by increasing potassium conductance, or enhance the presynaptic inhibition of the synaptic transmission thus causing analgesia.

Experiments described in chapter 2 demonstrated a complex interaction between the opioidergic and melanocortinergic systems at the spinal cord level in pain perception of CCI rats. DAMGO reversed the weak but significant effect of the MC4 receptor agonist, MTII, which potentiated allodynia. Furthermore, the antinociceptive effect of SHU9119 was augmented by the μ-opioid receptor antagonist, cyprodime. However, DAMGO when co-administered with SHU9119 reduced the antiallodynic effect of the MC4R antagonist. Interestingly, as reported in Addendum #1, other melanocortin-opioid interaction may occur when a different treatment paradigm was employed. In difference to the already reported changes in pain perception, if the melanocortin MC4R was first affected by SHU9119 which was followed by morphine administration, we observed potentiation of morphine analgesia. Hence, as reported in Addendum #1 and also by Vrinten et al. (2003), when the injection of morphine was preceded by administration of SHU9119, an additive anit-allodynic effect of both compounds was observed. Consequently, MC4Rs may not only modulate antinociception induced by exogenously administered opioids, but they may also be involved in the regulation of sensitivity to opioids in some pathological pain states.

α-MSH, a natural agonist of melanocortin receptors is generated by POMC cleavage. In subsequent steps of POMC enzymatic cleavage LPH is generated, that is further cleaved into N-terminal peptides: ACTH (4–10) and β-MSH as well as C-terminal peptides: endorphins (Gispen et al., 1977). Two opposite messages are encoded in LPH; in general ACTH (4–10) containing peptides increase the cAMP level and counteract morphine analgesia or inhibition of spinal reflex

activity, whereas opposite effects have been attributed to endorphins. The enzymatic cleavage could release both an excitatory and inhibitory effect on nerve tissue (Gispen et al., 1977). As already suggested by Gispen et al. (1977) such a mechanism should not necessary be "black and white", upon cleavage of LPH a release of information to neurons in a subtle manner should be possible, thus the LPH fragments may influence the same structure, i.e. spinal cord but bind to different receptors. The difference in metabolic activity and/or respective receptor affinity of POMC-derived peptides: α-MSH and β-endorphin, may result in different behavioral effect. Similar could be true for the exogenoulsy derived melanocortin or opioid recepotor ligands. In this aspect the already mentioned drug-injection interval and the receptor activation order may be essential regulation of the final and complex animal`s response to noxius stimuli and could further explain the differences of Chapter 2 and Addendum #1 results.

The different interaction between a melanocortin receptor antagonist and opioid (DAMGO and morphine) could be dependent on the mechanism of μOR internalization. Morphine and opioid peptides differ in their action on μOR internalization. Morphine has been observed to produce little (Keith et al., 1996), whereas DAMGO produced a profound μOR internalization (Keith et al., 1996; Arden et al., 1995). DAMGO-induced μOR internalization correlated with DAMGO-induced behavioral effects (analgesia in hot plate test). Indeed, μOR internalization was observed only when rats were rendered analgesic by intrathecal DAMGO administration (Trafton et al., 2000). As suggested by Vrinten et al. (2001), alleviation of allodynia results from MC4 receptor blockade. Under these conditions, tonic influence of α-MSH on nociception is attenuated, while the analgesic effects of β-endorphin predominates. The exogenously administrated DAMGO has greater affinity for the μOR and may compete with the endogenous β-endorphin. Therefore, the postulated activity of β-endorphin at μOR upon SHU9119 administration may be limited. Thus, one possible explanation would be that DAMGO produces internalization of μOR, β-endorphin remains in the synaptic cleft and does not evoke any of its intracellular events. Exogenous administration of a selective μOR agonist followed by a MC4R antagonist did not result in any additive anti-allodynic effect as evidenced by the response to tactile stimulation. When an opposite treatment paradigm was employed, namely the MC4R was blocked by SHU9119, β-endorphin displayed its analgesic effect *via* μOR and later morphine injection possibly enhanced this effect (see also Fig. 2).

Another possible cue to the elucidation of these distinct behavioral effects=observed after the combined administration of DAMGO or morphine with MC4R antagonist SHU9119, could be the fact that morphine and peptide ligands of

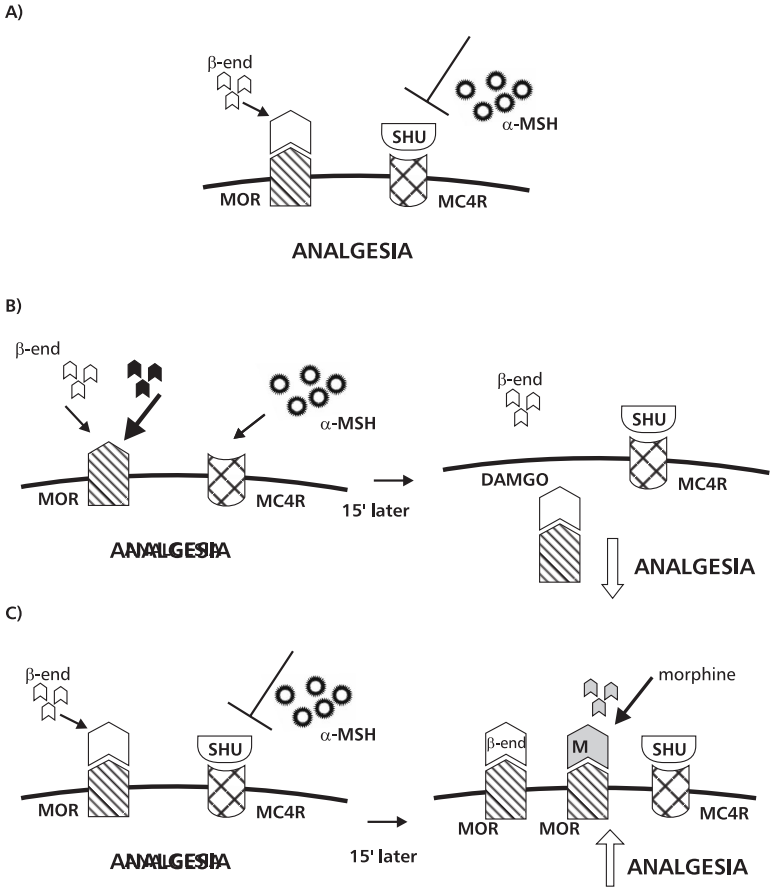

Fig. 2. A: Alleviation of allodynia, as a consequence of melanocortin receptor blockade attenuates a tonic influence of α-MSH on nociception, resulting in a predominant analgesic effect of β-endorphin (β-end). **B**: DAMGO, an exogenously administered agonist of the μ opioid receptor (MOR), competes with the endogenous β-endorphin. DAMGO displays higher efficacy toward the MOR than β-endorphin does, binds to MOR and internalization of the receptor occurs. In the same time the MC4R is a target for endogenous α-MSH. The number of cell surface MOR decreases, β-endorphin remains in the synaptic cleft but is unable to evoke any of its intracellular effects. Exogenous administration of a selective MOR agonist followed by a MC4R antagonist did not result in any additive anti-allodynic effect. **C**: When an opposite treatment paradigm was employed the following picture emerges: the MC4R was blocked by SHU9119, simultaneously β-endorphin displayed its analgesic effect *via* MOR as already suggested (A). Despite the fact that endogenous β-endorphin also evokes MOR internalization, the degree of the process is weaker than in case of DAMGO, efficacy of the ligands is related to desensitization. Some cell surface MOR`s are still accessible for morphine. Analgesia mediated by exogenous morphine (administered 15 min later) may be enhanced by the endogenous β-endorphin effect.

μOR appear to differ in their analgesic activity in experimental neuropathic pain. Opioid action in neuropathic pain depends on the μOR ligand used. Morphine, when applied i.t. at high dose, had little effect on tactile allodynia in rats with sciatic nerve ligation (Bian et al., 1995; Lee et al., 1995), while endogenous ligands of the μOR, endomorphins, and selective μOR peptide agonists seem to be more effective in neuropathic pain than morphine (Przewłocka et al., 1999). Morphine (i.t.) produced a slight anti-allodynic effect; in contrast to endomorphins and DAMGO, which dose-dependently inhibited cold and tactile allodynia. The observed effect was antagonized by cyprodime, clearly indicating the involvement of μORs (Przewłocka et al., 1999). Selective μOR peptide agonists display better anti-allodynic properties in neuropathic pain in rats acting via μORs. Studies of Obara et al. (2004) also demonstrated that opioid peptides, DAMGO and endomorphins were more effective compared with corresponding doses of morphine (opioid alkaloid) in alleviating chronic pain. The reason why μOR ligands exhibit different analgesic effect in neuropathy remains speculative (see: Przewłocki and Przewłocka, 2005), but this can be attributed to their interactions with the μOR. Finally, endogenous β-endorphin that is released by noxious stimuli bind to both presynaptic μORs and δORs, whereas DAMGO is μOR specific, thus differential involvement of μ and δ receptors has to be considered.

Melanocortins may also produce diverse effects on opioid-mediated analgesia, which can depend on some characteristics of the experimental design crucial to the observed behavioral effect, like the drug injection paradigm determining the order of the respective system activation. The recent findings of Bertorelli et al. (2005), indicating the expression of AgRP in spinal cord, could suggest an endogenous tonic inhibitory control on MC system activity. In neuropathic pain conditions, this balance could be insufficient to control the overactive MC system leading to an increase in nociception. These data indicate that targeting MC4R with synthetic antagonists could restore the balance and consequently reduce nociception. Despite these significant advances in research on nociception, still little is known regarding the potential for regulation of MC4R function through ligand-mediated internalization, trafficking, responsiveness, and turnover, leaving an open avenue for studding the underlying molecular mechanisms of melanocortin-opioid interaction.

3.3. Plasticity of melanocortin system in neuropathic pain

Peripheral nerve injury-induced plasticity involves the down-regulation of some excitatory peptides, like substance P and calcitonin gene-related peptide and the up-regulation of the inhibitory neuropeptides, such as galanin, resulting in a reduction of transmission in the dorsal horn (Hökfelt et al., 1994). The observed changes are thought to represent adaptive responses to limit the consequences of peripheral nerve damage to the organism as a whole and to promote survival and recovery of individual neurons. The plastic changes evoked by the neuropathic pain are likely to influence also the melanocortin system.

The disparity between results presented in chapter 3 and those of Vrinten et al. (2000) may be due to the fact that these authors measured MC4R in discrete regions of the spinal cord while our measurements were preformed on homogenates of the whole spinal lumbar segment, in which the presumed increase in MC4 receptor expression in superficial layers could not be detected. Our immunohistochemical analyses within the superficial layers of the spinal cord revealed an increase in the MC4R immunoreactivity following sciatic nerve injury (Starowicz et al., unpublished observation). This observation would further emphasize the subtle changes in MC4 receptor under neuropathic pain conditions within dorsal layers of the spinal cord.

As mentioned above the well-known insensitivity of neuropathic pain to opioid analgesics could be due to a marked reduction in the number of μORs on sensory neurons and interneurons in the dorsal horn of the spinal cord. Addendum #2 provides further evidence of such phenomenon occurring in those tissues. In chapter 3, we also showed a significant decrease in MC4R mRNA level in the ipsilateral DRG of CCI rats, whereas no change in the spinal MC4R transcript level has been reported. However, Vrinten et al. (2000) found that in CCI animals, MC4R level in laminae I-II on ipsi and contralateral side to the injured nerve at the L4-L6 level of the spinal cord was higher by about 20% in comparison with sham-operated animals as shown by *in situ* ^{125}I-NDP-MSH binding. Furthermore, in this thesis we report an opposite regulation of MC4R and μOR in L4-L5 DRG. Thus, neuropathic pain results in a time-dependent activation of the melanocortin system, as demonstrated by the increased MC4R immunohistochemical staining, whereas μOR-immunoreactivity decreased gradually with time after the peripheral nerve injury. Furthermore, we report that a similar change occurred at the spinal cord level. Our results on μOR-IR both in DRG and dorsal horns of the spinal cord are comparable to those obtained after axotomy performed in order to induce neuropathic pain. Zhang et al. (1998)

demonstrated that peripheral axotomy caused a reduction in the number and intensity of μOR-positive neurons in the rat and monkey DRG and of μOR-IR in the dorsal horn of the spinal cord (Zhang et al., 1998). A decrease in μOR expression in the DRG of nerve-injured mice was also observed by others (Rashid et al., 2004). On the other hand, Porreca et al. (1998) examined lumbar spinal cord tissues 7 days after the nerve injury, which is the time when stable allodynia was observed. At this point, no differences were observed in the receptor density or affinity of [^{3}H]DAMGO (μ-selective agonist) or [3H]CTAP (μ-selective antagonist) in the dorsal quadrant of the lumbar spinal cord ipsilateral to nerve injury. Additionally, no change in morphine potency and efficacy in activating G-proteins was observed. In contrast, staining for μOR using μ-selective antibodies revealed a discrete loss of μORs localized ipsilaterally to the nerve injury and specific for sections taken at the L6 level. In these spinal segments, μORs were decreased in laminae I and II. The data indicate that the loss of μORs was confined to these areas and might contribute to the loss of morphine activity due to input from these spinal segments.

However, changes in μOR-IR alone fail to fully explain the attenuation of opioid activity. Smith et al. (2001) provided evidence that reduction of the endogenous opioid in primary afferents was associated with injury-induced chronic pain. The decreased endomorphin–2-IR was observed within 2 days after the injury and was the most pronounced at 2 weeks after injury and was restricted to the medial dorsal horn in the lumbar segments innervated by the sciatic nerve. The decrease in endomorphin–2-IR during the development of chronic pain is consistent with the loss of an inhibitory influence of opioids on pain transmission. The activity of the melanocortin system was subject of studies described in chapter 3 and Addendum #2. The results indicate that the MC4R mRNA and protein levels in the DRG may be oppositely regulated in neuropathy. The increased MC4R-IR does not seem to result from the increased gene transcription; actually, the MC4R mRNA levels are decreased in DRG of neuropathic rats. Thus, it is likely that the accumulation of MC4R in the DRG was due to alterations in post-translational mechanisms regulating protein stability. This regulation may depend on phosphorylation and ubiquitination, which influence the rate of its degradation by the preoteasome and lysosomes. It remains to be established whether neuropathic pain affects these regulation factors. The reported increase in activity of MC4R-IR concomitant with the development of neuropathic pain results in the inhibition mRNA transcription, possibly in order to counteract the enhanced activity of the pronociceptive system.

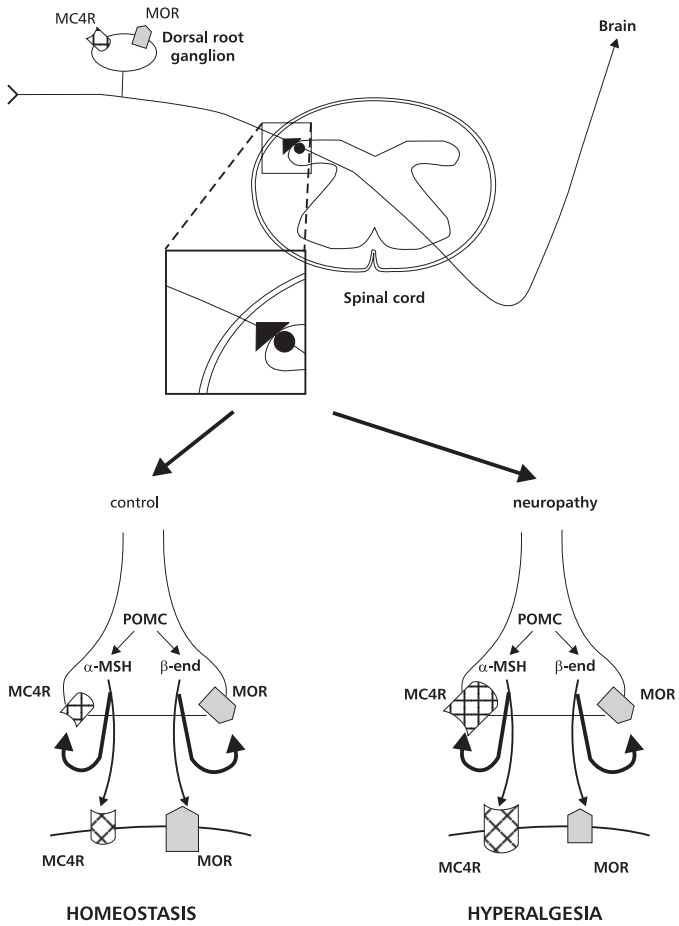

Fig. 3. Schematic representation of the possible anatomical organization of the melanocortin and opioid receptors in primary afferent fibers and dorsal horn of the spinal cord under normal and neuropathic conditions. Development of neuropathic pain results in an increase in the number of melanocortin 4 receptors (MC4R) both in DRG and spinal cord, whereas the μ opioid receptor (MOR) level decreased. Response to a pain stimulus may be subject of modulation by MOR and/or MC4R activation. In control animals opioids acting presynaptically inhibit the release of substance P. Thus, the reduced release of excitatory transmitters from primary afferents would markedly diminish the response of neurons in the dorsal horn to sensory input. Due to the reduced MOR number both in DRG and spinal cord, the pre- and postsynaptic action of opioids is attenuated. The demonstrated presynaptic localization of MC4R may indicate that MC4R modulates release of glutamate and/or substance P from primary afferent terminals, thereby influencing sensory transmission from peripheral receptors to spinal dorsal horn neurons. The novel reported site of MC4R localization, on the primary afferent terminals indicates not only postsynaptic but also presynaptic action of melanocortins

4. CONCLUDING REMARKS

The data described in this thesis extend general knowledge of the involvement of the MC4 receptor in mechanisms of analgesia. The following aspects outlined below constitute novel information. Firstly, the MC4R localization in the DRG is demonstrated. The MC4 receptor was assumed to exist exclusively in the CNS, but our recent data and reports of other authors proved that this assumption needs to be revised. The novel information on the MC4R localization in the peripheral ganglia may facilitate understanding of the role of melanocortins in pain transmission and may open new avenues for future clinical applications (see also Fig. 3). Secondly, the present studies revealed that the modulation of the activity of the spinal and supraspinal melanocortin system delayed the development of morphine tolerance. Thirdly, morphine analgesia was shown to be subject of modulation by melanocortin receptor ligands. The observed effects were very much dependent on the sequence of intercellular events, of the potential value is the interaction observed after the spinal MC4R blockade followed by administration of morphine. Combining the data of our study on the effectiveness of an MC4R antagonist in alleviating neuropathic pain symptoms and in preventing morphine tolerance may prompt preclinical study and future clinical trials on chronic neuropathic pain, where opioid dosing is needed. This may eliminate the need to increase the opioid dose necessary for maintaning a satisfactory pain relief, thus minimizing unwanted side effects of the treatment.

Despite increasing knowledge of the function of the melanocortins, the mode of the function of regulation of the μ-opioid and MC4 receptor remains elusive. Anatomical basis of their interaction needs to be established; therefore co-localization of μ-opioid and MC4Rs both in the lumbar spinal cord and in corresponding DRG would be an interesting issue to address in subsequent studies. Furthermore, it would also be of great interest to localize the MC4R in a subpopulation of primary afferent neurons under neuropathic pain conditions, using specific marker for subpopulations of A- and/or C-fibers. Last but not least, based on the demonstration of the MC4R-IR in the DRG, it would be interesting to investigate MC4R at primary afferents in the dorsal horn of the spinal cord after peripheral injury to the sciatic nerve.

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NEDERLANDSE SAMENVATTING

De belangrijkste vragen die in dit proefschrift experimenteel worden bewerkt, zijn of en hoe het melanocortine systeem de antinoceptische effecten van opioiden kan moduleren.

In **hoofdstuk 1** wordt een overzicht van de relevante literatuur gegeven. Achtereenvolgens worden de opioide- en melanocortinepeptiden en hun receptoren besproken en met name hun rol in nociceptie. Het hoofdstuk eindigt met de beschrijving van de vraagstelling en de opzet van het experimentele gedeelte van het proefschrift.

In **hoofdstuk 2** worden experimenten gepresenteerd waarbij op het niveau van het ruggenmerg de interactie tussen agonisten en antagonisten van aan de ene kant het melanocortine systeem en aan de andere kant het opioide systeem wordt bestudeerd. De co-lokalisatie van zowel opioide als melanocortine receptoren in het ruggenmerg op zich suggereert al een mogelijke functionele interactie van deze receptoren. De agonisten en antagonisten worden intrathecaal toegediend. In het zogenaamde chronische constrictie letsel (CCI) model (nervus ischiadicus) wordt neuropatische pijn bestudeerd aan de hand van tactiele allodynia (von Frey filamenten) en van koud water allodynia. De melanocortine receptor (MCR) antagonist SHU 9119 was veel sterker in het verminderen van allodynia dan de μ-receptor (μOR) agonist morfine, maar ongeveer even sterk als het μ-selectieve opioide DAMGO. Toediening van de MCR agonist MT II verhoogde de gevoeligheid voor zowel de tactiele als de koude stimulatie. Experimenten met gecombineerde toediening van de μOR agonisten/antagonisten met MCR agonisten/antagonisten leverde de gevolgtrekking op dat het opioide systeem als een functionele antagonist van het melanocortine systeem kan worden beschouwd.

Uit **addendum 1** blijkt dat als de MCR antagonist 15 min voorafgaand aan de toediening van morfine wordt gegeven, er eveneens een functionele interactie tussen beide systemen kon worden aangetoond. Maar de relatie was nu omgekeerd. Kennelijk is de volgorde waarin beide systemen worden geactiveerd van belang voor het resultaat van de interactie.

In **hoofdstuk 3** werd met behulp van kwantitatieve RT-PCR de expressie van MC4R mRNA in het ruggenmerg en het dorsale wortel ganglion gemeten op respectievelijk 3,7,14 en 21 dagen na het aanbrengen van het CCI letsel, dat gebruikt werd om de neuropatische pijn op te wekken. Het zenuwletsel bleek geen effect te hebben op de expressie van het MC4R mRNA in het ruggenmerg. Dit in tegenstelling tot het effect op het receptor mRNA in het ipsilaterale dor-

sale wortel ganglion, waar het niveau van expressie 2 en 3 weken na het aanbrengen van het letsel sterk bleek te zijn gereduceerd. Dit zou verband kunnen houden met een MC4R gemedieerde presynaptische modulatie van de activiteit van primaire afferenten in neuropatische pijn.
In **addendum 2** wordt onderzoek met behulp van immuno-histochemische technieken naar de expressie van de MC4R en de μ-OR in de dorsale wortel ganglia (L4-L5) en de dorsale hoorn van het ruggenmerg gepresenteerd. Hoewel verdere karakterisering van het antilichaam gewenst is, werd op 3 en 14 dagen na het CCI letsel in de ganglia een toename van 21.1 respectievelijk 40.5% van de MC4R immuno-reactiviteit waargenomen. Ook in het ipsilaterale gedeelte van het ruggenmerg was de immuno-reactiviteit als gevolg van het letsel verhoogd. De immuno-reactiviteit voor de μ-OR daarentegen bleek zowel in het dorsale wortel ganglion als in het ruggenmerg in de loop der tijd na het plaatsen van het CCI letsel af te nemen. Dit laatste zou mede ten grondslag kunnen liggen aan de afgenomen effectiviteit van morfine in de behandeling van neuropatische pijn.
In **hoofdstuk** 4 worden experimenten beschreven waarin met behulp van intrathecale toediening van anti-sense oligonucleotide gericht tegen β-arrestine, de synthese van opioide receptoren wordt onderdrukt. Het bleek dat de onderdrukking van de synthese van opioide receptoren de ontwikkeling van tolerantie ten opzichte van intrathecaal toegediend morfine significant afremde. Voorts bleek dat de injectie met het oligonucleotide de allodynia,die zich in het CCI model ontwikkelt, onderdrukt.
In **hoofdstuk 5** wordt verder ingegaan op de relatie tussen het opioide en het melanocortine systeem. In dit hoofdstuk wordt onderzocht welk effect morfine heeft op de expressie van de mRNA's van zowel de MC4 receptor en de CRF receptor in de amygdala. Tevens werd onderzocht wat de mogelijke rol is van de activiteit van die receptoren in de amygdala bij de ontwikkeling van tolerantie voor morfine. De rol van de CRF receptoren is in dit verband relevant omdat bekend is dat CRF de synthese van het pro-opiomelanocortine stimuleert. Acute toediening van morfine verhoogde het niveau van MC4R mRNA zoals dat gemeten werd met behulp van kwantitatieve RT-PCR. Deze verlaging nam af bij chronische toediening van morfine en leidde geleidelijk tot een werkelijke toename in het receptor mRNA gedurende de periode dat er tolerantie voor morfine meetbaar was met behulp van de staart-en poot-terugtrek-reflex testen. Deze testen werden gebruikt om de analgetische werking van het morfine te meten. Daartegenover stond dat de toediening van morfine geen effect had op het mRNA van de CRF receptor. De antinoceptieve werking van morfine werd versterkt wanneer SHU 9119 en αh-CRF voorafgaande aan de perifere

morfine toediening, in de amygdala werden geinjecteerd. Deze data vormen een eerste aanwijzing dat mogelijkerwijs het melanocortine systeem in de amygdala een rol kan spelen bij de ontwikkeling van tolerantie voor perifeer toegediend morfine.

In **hoofdstuk 6** werd vervolgens onderzocht wat de mogelijke rol van het spinale melanocortine systeem zou kunnen zijn in de ontwikkeling voor tolerantie die zich tegen perifeer toegediend morfine ontwikkelt. Chronische toediening van morfine resulteerde in tolerantie zoals die tot uitdrukking kwam in een significante verlenging van de reactie tijd van een rat om zijn staart van een hitte prikkel terug te trekken. Herhaalde intrathecale toediening met MC receptor antagonisten in ratten die chronisch met morfine werden behandeld, voorkwam de ontwikkeling van tolerantie. In ratten die reeds tolerant waren voor morfine bleek een enkele intrathecale injectie met een MC receptor antagonist de analgetische werking van morfine weer te herstellen. Met behulp van RT-PCR kon worden aangetoond dat de chronische morfine behandeling geen effect had op de expressie van het mRNA voor zowel de μOR als de MC4R in het ruggenmerg maar wel op de expressie van die mRNA's in het dorsale wortel ganglion. Daar werd een afname van de μOR mRNA gemeten terwijl de MC4R mRNA expressie was toegenomen. Dus ook de data in hoofdstuk 6 suggereren dat de activiteit van MC4 receptoren de ontwikkeling van tolerantie voor morfine kan moduleren.

In **hoofdstuk 7** worden de bevindingen van het proefschrift samengevat en bediscussieerd. Het lijdt geen twijfel dat de in dit proefschrift verzamelde gegevens nieuwe inzichten in de mogelijke rol van de MC4 receptor in mechanismen van analgesie hebben opgeleverd. Interessant is een eerste aanwijzing dat in dorsale wortel ganglia expressie van MC4 receptoren plaats vindt. Ook de observatie dat spinale en supraspinale melanocortine systemen de ontwikkeling van tolerantie voor morfine kunnen beďnvloeden is nieuw. Hoewel nog veel te onderzoeken blijft, wordt gehoopt dat orienterende klinische studies nu zouden kunnen worden uitgevoerd om de betekenis van een melanocortine cotherapie bij pijn bestrijding met behulp van opiaten nader te onderbouwen.

ACKNOWLEDGEMENTS

I wish to express my warmest thanks and appreciation to all who made this thesis possible and especially:

Ryszard Przewłocki for being an excellent supervisor who generously shared his knowledge with me and introduced me into neuroscience. When I joined the group I had no idea on how to work with animals. A bench and pipette were the only "scientists tools" I knew. Whereas now it is a great attitude to compile both biochemical and behavioral methodology. Thank you for giving me an opportunity to participate in numerous conferences and meetings. Finally, for all your criticism and remarks regarding the manuscripts.

Willem Hendrik Gispen my promoter, for your involvement in establishing the collaboration program that offered doctoral positions. It was my pleasure to be one of the PhD students in frames of the Utrecht University PhD Programme. Also, for the exchange of scientific information and ideas, here in Kraków or Warsaw, as well as in Utrecht. For constructive feedback on the "pre-final" version of the thesis and your precious suggestions.

Barbara Przewłocka my co-promotor, for your support and encouragement, enthusiasm and all the ideas you added to my projects. You taught me how to write proper papers and with this knowledge it was much easier to prepare my PhD thesis. Thank you for recommending me as a lecture speaker at the ENC Meeting. I think it was an unforgettable experience for both of us; every next meeting was a less stressful event and another opportunity to improve the data presentation. Additionally, for sharing not only scientific knowledge, but also an interest in Italian cuisine. The dinners you hosted, of course directly associated with various scientific meetings, will forever remain in my memory. I'm more than sure that the delicious taste of mushrooms is an example of a "conditioned place preference" and associates memories with the Neuroscience Course organized by Ryszard and Willem.

Krzysztof Wędzony for your involvement in the MC4-DRG project and your patience in introducing me into the immunohistochemistry. For the discussions on the results and for great quality pictures for the manuscript. Also, for sharing the ski interest and very nice not always scientific talks.

Jacek Kuźnicki, director of the International Institute of Molecular and Cell Biology in Warsaw and the administrative staff at the IIMCB: **Krystyna**

Domańska and **Agnieszka Ziemka** for your assistance, being employed in one institute and work in another one is quite a complicated issue, but thanks to you it was less complicated than it sounds.

Halina Machelska and **Christoph Stein** for giving me the opportunity to be a visiting graduate student at your department in Berlin. Also for making my stay something more than just an official visit of a project-partner.

Shaaban Mousa for your contribution to my project and for the motto "I'll make your life easy". Your improvements of the IHC protocols and the method itself were very helpful.

Dominika Łabuz for your hospitality and kindness during my stay in Berlin.

Past and present lab members at the Department for creating an affable atmosphere. Such a mixture of personalities made every day interesting. **Aga B, Aga L and Aga S, Basia, Dorota, Gosia, Grzesiek, Joasia M and Joasia P, Jasia, Janek, Kuba, Maja, Marcin, Tomek K and Tomek S, Wiola, Wiktor and Wojtek** for your enjoyable company and making the lab much more lively. **Michał**, I really appreciate your help with preparing final figures and drawings for the thesis. Special thanks to **Ilona Obara**, for your assistance with the behavioral experiments and for sharing all, absolutely all moments at the Department. Over the past four years we have spent a few weeks sharing rooms during the meetings and conferences. This was a time of night-long gossips, and hmm… traveling with you was always a challenging event. Most probably you are next to present a PhD Thesis. Please remember deadlines around Christmas time are not the very best solution, choose different ones. Meanwhile, good luck with your experiments and I hope we will keep in touch**.**

Agnieszka Gieryk and **Madelon Pieper,** my paranimfs. Agnieszka, my warmest thanks for all your support and for being a good friend. Although we worked together, we never had enough time to have a chat, that's why the Latino classes and the time thereafter was an excellent alibi. Madelon, thank you for being such a well organized secretary; all the paper work that had to be done before the dissertation was much easier having you on the other site of the computer screen.

Agnieszka Chocyk and **Kasia Markowicz** thank you for teaching me the tricks of IHC and western blot experiments, for all your help, discussions and non-scientific gossips.

My friends from the studies with whom I have been in touch during these four years, for all the meetings in Kraków, Warsaw, Utrecht. Particular thanks to **Monika Mysiak**, for answering all my questions related to the booklet, the dissertation itself, the reception and all other points that I raised in reference to my graduation. You were more than accurate in replaying to my e-mails.

Finally my family. **My dearest parents and my husband Grzegorz** for their support, especially during the last year. You gave me a credit of your patience, trust and a feeling that what I'm doing is right.

Ciao a tutti!

CURRICULUM VITAE

Katarzyna Starowicz was born on the September 12, 1976 in Kraków, Poland. She graduated from the Nowodworski Lyceum in Kraków in June 1995. In October 1995, she commenced her studies at the Faculty of Materials Science of the University of Science and Technology. Later on in October 1996, she continued her studies at the Faculty of Biotechnology (Jagiellonian University). In July 1999, she got an EU ERASMUS/Tempus scholarship and for seven month joined the group of Prof. Erik Fries at the Department of Medical Biochemistry and Microbiology at the Uppsala University in Sweden. The experiments conducted under Profs. Erik Fries and Adam Dubin supervision constituted the basis for her Master's Thesis. The Thesis entitled "Studies on the rapidly sedimenting fraction of the endoplasmic reticulum" was successfully presented to the Committee on the May 10, 2000, and she was granted her M.Sc. degree in Biotechnology and Molecular Biology. In October of the same year, she applied for and was awarded an open position under The Utrecht University International Doctoral Programm based on an agreement between the Polish Network for Cell and Molecular Biology UNESCO/PAS and the Utrecht University, The Netherlands. From November 2000 till January 2005, she has been working as a postgraduate student under the supervision of Prof. Ryszard Przewłocki at the Department of Molecular Neuropharmacology, Institute of Pharmacology, Polish Academy of Sciences in Kraków. Research performed during the Ph.D. training was focused on the involvement of melanocortins, with particular attention given to MC4 receptor, in the mechanisms of opioid antinociception. The results of these studies are the subject of the presented Thesis. In 2005 she will became a postdoctoral fellow in the group of Prof. Vincenzo Di Marzo at the Endocannabonoid Research Group in the Instutute of Biomolecular Chemistry, Consiglio Nazionale delle Ricerche, Pozzuoli, Naples, Italy.

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