ELSEVIER

Neuroscience Letters 358 (2004) 79–82

Neuroscience Letters

www.elsevier.com/locate/neulet

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).

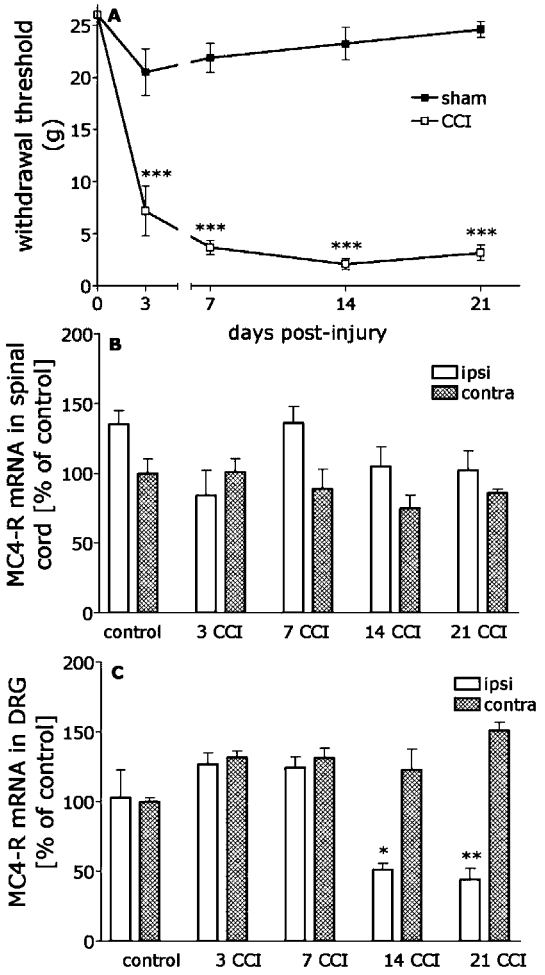

Fig. 1. Development of tactile allodynia (A); quantitative real-time RT-PCR analysis of MC4 receptor (MC4-R) level in spinal cord (B); and dorsal root ganglia (DRG) (C). Tactile allodynia was assessed using von Frey filaments (1.0–26.0 g). The MC4-R mRNA level is expressed as % of MC4-R expression in 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-

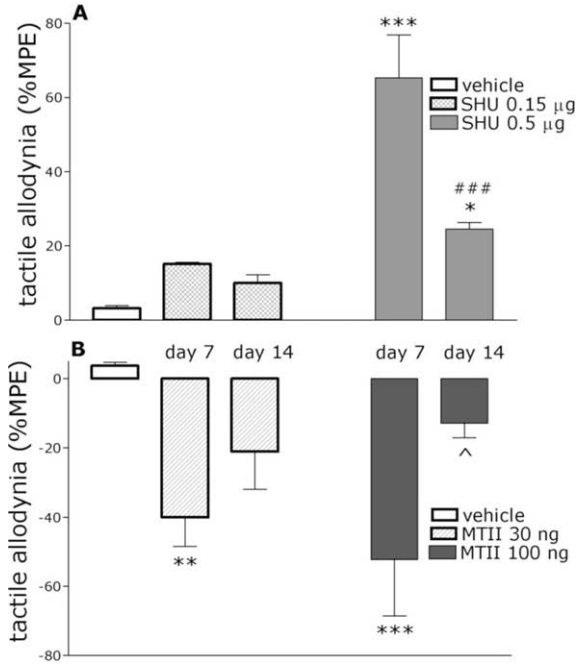

Fig. 2. The effects of intrathecal (i.th.) administration of MC4-R antagonist (A) SHU9119 (0.15 and 0.5 μg) and MC4-R agonist (B) MTII (30 and 100 ng) on tactile allodynia 7 and 14 days after CCI in rats. Data are expressed as a percentage of maximal possible effect (% MPE), mean ± SEM ($n = 8$). Bonferroni 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.

References

[1] G.J. Bennett, Y.K. Xie, A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man, Pain 33 (1988) 87–107.
[2] A. Bertolini, R. Poggioli, W. Ferrari, ACTH-induced hyperalgesia in rats, Experientia 35 (1979) 1216–1217.
[3] S.R. Chaplan, F.W. Bach, J.W. Pogrel, J.M. Chung, T.L. Yaksh, Quantitative assessment of tactile allodynia in the rat paw, J. Neurosci. Methods 53 (1994) 55–63.
[4] P. Chomczynski, N. Sacchi, Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction, Anal. Biochem. 162 (1987) 156–159.
[5] T. Kishi, C.J. Aschkenasi, C.E. Lee, K.G. Mountjoy, C.B. Saper, J.K. Elmquist, Expression of melanocortin 4 receptor mRNA in the central nervous system of the rat, J. Comp. Neurol. 457 (2003) 213–235.
[6] V. Kistler-Heer, M.E. Lauber, W. Lichtensteiger, Different developmental patterns of melanocortin MC3 and MC4 receptor mRNA: predominance of MC4 in fetal rat nervous system, J. Neuroendocrinol. 10 (1998) 133–146.
[7] H.A. Lekan, S.M. Carlton, R.E. Coggeshall, Sprouting of A beta fibers into lamina II of the rat dorsal horn in peripheral neuropathy, Neurosci. Lett. 208 (1996) 147–150.
[8] K.G. Mountjoy, M.T. Mortrud, M.J. Low, R.B. Simerly, R.D. Cone, Localization of the melanocortin-4 receptor (MC4-R) in neuroendocrine and autonomic control circuits in the brain, Mol. Endocrinol. 8 (1994) 1298–1308.
[9] K.G. Mountjoy, J.M. Wild, Melanocortin-4 receptor mRNA expression in the developing autonomic and central nervous systems, Brain Res. Dev. Brain Res. 107 (1998) 309–314.
[10] C.A. Sandman, A.J. Kastin, Intraventricular administration of MSH induces hyperalgesia in rats, Peptides 2 (1981) 231–233.
[11] K. Starowicz, B. Przewlocka, The role of melanocortins and their receptors in inflammatory processes, nerve regeneration and nociception, Life Sci. 73 (2003) 823–847.
[12] K. Starowicz, R. Przewlocki, W.H. Gispen, B. Przewlocka, Modulation of melanocortin-induced changes in spinal nociception by mu-opioid receptor agonist and antagonist in neuropathic rats, NeuroReport 13 (2002) 2447–2452.
[13] R. van der Neut, E.M. Hol, W.H. Gispen, P.R. Bar, Stimulation by melanocortins of neurite outgrowth from spinal and sensory neurons in vitro, Peptides 13 (1992) 1109–1115.
[14] D.H. Vrinten, R.A. Adan, G.J. Groen, W.H. Gispen, Chronic blockade of melanocortin receptors alleviates allodynia in rats with neuropathic pain, Anesth. Analg. 93 (2001) 1572–1577.
[15] D.H. Vrinten, W.H. Gispen, G.J. Groen, R.A. Adan, Antagonism of the melanocortin system reduces cold and mechanical allodynia in mononeuropathic rats, J. Neurosci. 20 (2000) 8131–8137.
[16] J.E. Wikberg, Melanocortin receptors: perspectives for novel drugs, Eur. J. Pharmacol. 375 (1999) 295–310.
[17] D.W. Williams Jr, J.M. Lipton, A.H. Giesecke Jr, Influence of centrally administered peptides on ear withdrawal from heat in the rabbit, Peptides 7 (1986) 1095–1100.
[18] C.J. Woolf, P. Shortland, R.E. Coggeshall, Peripheral nerve injury triggers central sprouting of myelinated afferents, Nature 355 (1992) 75–78.
[19] T.L. Yaksh, T.A. Rudy, Analgesia mediated by a direct spinal action of narcotics, Science 192 (1976) 1357–1358.
[20] M. Zimmermann, Ethical guidelines for investigations of experimental pain in conscious animals, Pain 16 (1983) 109–110.