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European Journal of Pharmacology 495 (2004) 145–152

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Accelerating sensory recovery after sciatic nerve crush: non-selective versus melanocortin MC_4 receptor-selective peptides

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Received 17 March 2004; received in revised form 14 May 2004; accepted 20 May 2004

Abstract

Melanocortin receptor ligands accelerate functional recovery after peripheral nerve crush. It is not known which mechanism is involved or via which melanocortin receptor this effect occurs, albeit indirect evidence favours the melanocortin MC_4 receptor. To test whether the melanocortin MC_4 receptor is involved in the effects of melanocortins on functional recovery, we used melanocortin compounds that distinguish the melanocortin MC_4 receptor from the melanocortin MC_1, MC_3 and MC_5 receptors on basis of selectivity and agonist/antagonist profile. Activation and binding studies indicated that the previously described peptides JK1 (Ac-Nle-Gly-Lys-*D*-Phe-Arg-Trp-Gly-NH_2) and [*D*-Tyr^4]melanotan-II ([*D*-Tyr^4]MTII. Ac-Nle-c[Asp-His-*D*-Tyr-Arg-Trp-Lys]NH_2) are selective for the rat melanocortin MC_4 receptor as compared to the rat melanocortin MC_3 and MC_5 receptors, but are also potent on the melanocortin MC_1 receptor. Both peptides did not accelerate sensory recovery in rats with a sciatic nerve crush, whereas the non-selective melanocortin agonist melanotan-II (MTII, Ac-Nle-c[Asp-His-*D*-Phe-Arg-Trp-Lys]NH_2) was effective. The melanocortin MC_3/MC_4 receptor antagonist SHU9119 (Ac-Nle-c[Asp-His-*D*-Nal(2)-Arg-Trp-Lys]NH_2) also enhanced sensory recovery. This effect was probably not due to interaction with the melanocortin MC_4 receptor, since JK46 (Ac-Gly-Lys-His-*D*-Nal(2)-Arg-Trp-Gly-NH_2), a selective melanocortin MC_4 receptor antagonist, was ineffective. Taken together, these data suggest that melanocortins do not accelerate sensory recovery via interaction with the melanocortin MC_4 receptor. From the known melanocortin receptors, only the involvement of the melanocortin MC_5 receptor in acceleration of recovery could not be excluded.
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Keywords: Melanocortin; Sensory recovery; Peripheral nerve injury; Sciatic nerve

1. Introduction

Peripheral nerves have limited intrinsic capacity to regenerate after axonal injury. It is well known that melanocortins, for example adrenocorticotropic hormone (ACTH) and α-melanocyte stimulating hormone (α-MSH) can stimulate this process. In 1980, Strand and Kung (1980) described that ACTH enhances sensorimotor recovery after sciatic nerve crush. Further studies showed that this neurotrophic property of α-MSH and other compounds is contained in the melanotropic moiety (ACTH-(4–10)) (Bijlsma et al., 1981, 1983). The enhanced functional recovery is accompanied by increased numbers of motor endplates (Strand and Kung, 1980) and myelin-positive sprouts (Verhaagen et al., 1986). Both place and timing of peptide administration are important: the effects were augmented when peptides were applied soon after injury or near the lesion site (Edwards et al., 1984, 1986).

The exact mechanisms via which the melanocortins exert their neurotrophic effects are unknown, although several possibilities have been investigated. First, melanocortins could act directly on the regenerating nerve cells. Indeed, melanocortins increase neurite outgrowth in cultured dorsal-root ganglion neurons (Strand et al., 1993; Van der Neut et al., 1992) and in spinal cord-slices (Van der Neut et al., 1988). Second, the effects could be via the neuro-supporting glial cells. Astrocytes (Zohar and Salomon, 1992) respond to melanocortins and the potent [Nle^4-*D*-Phe^7]α-MSH binds

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doi:10.1016/j.ejphar.2004.05.037

to a protein expressed by Schwann cells (Dyer et al., 1993), indicating that these cells may facilitate the neurotrophic effects of these ligands. Especially Schwann cells play an important role in peripheral nerve regeneration (Fawcett and Keynes, 1990) and are therefore good candidates. Third, since the immune system plays an important role in the first phase after nerve injury, the Wallerian degeneration (Fawcett and Keynes, 1990; Lu and Richardson, 1993), the neurotrophic effects of melanocortins may be mediated by immune cells. In particular macrophages are important in this process. The anti-inflammatory effects of melanocortins on the immune system are well described (Lipton and Catania, 1998; Wikberg et al., 2000), and melanocortin MC_1 and MC_5 receptors are expressed on monocytes and lymphocytes, respectively (Bhardwaj et al., 1997; Buggy, 1998; Star et al., 1995; Wikberg et al., 2000). However, no direct role for immunomodulation by melanocortins in nerve regeneration has been established. A fourth option is that at least for sensory recovery, melanocortins may influence central neuronal networks, for instance in the spinal cord (van der Kraan et al., 1999). The adapted network could become more efficient in conducting signals, resulting in a better performance in recovery tests. Although these putative mechanisms all seem viable options, the mechanism underlying the action of exogenous melanocortins in sensory recovery has not been identified.

Knowing which melanocortin receptor subtype(s) mediate(s) the enhancing effects of melanocortin receptor ligands would certainly advance the elucidation of the mechanism of the stimulating effects of melanocortins on sensory recovery. Until now, five melanocortin receptor subtypes have been cloned, which were named melanocortin MC_1 to MC_5 receptor (Chhajlani et al., 1993; Gantz et al., 1993a, 1993b, 1994; Griffon et al., 1994; Mountjoy et al., 1994, 1992; Roselli-Rehfuss et al., 1993). The melanocortin MC_2 receptor is not a likely candidate, since α-MSH is effective in regeneration, while it does not activate the melanocortin MC_2 receptor (Cammas et al., 1995; Schioth et al., 1996). Conversely, γ-MSH is ineffective in nerve regeneration, while it is a potent melanocortin MC_3 receptor ligand, pleading against a role for the MC_3 receptor. This leaves the melanocortin MC_1, MC_4 and MC_5 receptors as the putative receptors involved in nerve regeneration. The melanocortin MC_4 and MC_5 receptors are both expressed in neuronal tissue, but the melanocortin MC_4 receptor is the only subtype found in neuronal tissue outside the brain. Also, melanocortins stimulate neurite outgrowth in Neuro 2A cells via endogenously expressed melanocortin MC_4 receptors (Adan et al., 1996), providing evidence for a direct role for the MC_4 receptor in enhancing neuronal growth. Therefore, the melanocortin MC_4 receptor is considered the most likely candidate for mediating the neurotrophic effects of melanocortins. However, lack of selective melanocortin compounds has hampered identification of the melanocortin receptor subtypes that play a role. Recently, the potent melanocortin analogue MTII (Ac-Nle^4-c[Asp^5,D-Phe^7,Lys^{10}]α-MSH-(4–10)-NH_2) was shown to be very effective in enhancing sensory recovery after sciatic nerve crush in the rat, and it was protective in cisplatin-induced neuropathy (ter Laak et al., 2003). This underlined the importance of the melanocortin system in nerve regeneration and protection. However, since MTII only possesses moderate selectivity for the melanocortin MC_4 and MC_1 receptors over the melanocortin MC_3 and MC_5 receptors (Wikberg, 1999), no conclusion could be drawn about involvement of the melanocortin MC_4 receptor in these processes. Therefore, in the present study the role of the melanocortin MC_4 receptor in sensory recovery was further investigated with the melanocortin receptor agonist melanotan II (MTII) and the melanocortin MC_4 receptor-selective agonists JK1 (Ac-Nle-Gly-Lys-*D*-Phe-Arg-Trp-Gly-NH_2 (Nijenhuis et al., 2003) and [*D*-Tyr^4]MTII. Also, the non-selective melanocortin MC_4 receptor antagonist SHU9119 (Ac-Nle-c[Asp-His-*D*-Nal(2)-Arg-Trp-Lys]NH_2 (Hruby et al., 1995), and the new melanocortin MC_4 receptor-selective antagonist JK46 (Ac-Gly-Lys-His-*D*-Nal(2)-Arg-Trp-Gly-NH_2) were tested.

2. Materials and methods

2.1. Peptides

NDP-MSH ([Nle^4,*D*-Phe^7]α-MSH), SHU9119 (Ac-Nle^4-c[Asp^5,*D*-Nal(2)^7,Lys^{10}]α-MSH-(4–10)-NH_2) and MTII (Ac-Nle^4-c[Asp^5,*D*-Phe^7,Lys^{10}]α-MSH-(4–10)-NH_2) were purchased from Bachem (Bubendorf, Switzerland). JK46 and JK1 were synthesized on an automatic ABI 433A Peptide Synthesizer using FastMoc 0.25 mmol chemistry as described previously (Nijenhuis et al., 2003). [*D*-Tyr^4]MTII was synthesized using Fmoc solid phase synthesis as reported elsewhere (Schaaper et al., 1998).

2.2. Cell culture and transfection

Human embryonal kidney (HEK) 293 cells were grown in Dulbecco's Modified Eagle Medium DMEM (Gibco, Paisley, Scotland) supplemented with 10% fetal calf serum (Integro, Zaandam, the Netherlands), 2 mM glutamine (Gibco) and non-essential aminoacids (Gibco). DNA was transfected into cells with a standard calcium phosphate precipitation protocol. cDNAs of the rat melanocortin MC_3, MC_4 and MC_5 receptors and the mouse melanocortin MC1 receptor, each cloned in the vector pcDNA3 (Invitrogen. Carlsbad, CA) were used. The CRE-LacZ reporter gene construct as described by Chen et al. (1995) was used to measure receptor activation.

2.3. Binding assay

IC_{50} values were determined by displacement of ^{125}I-NDP-MSH. Iodinated NDP-MSH was produced using bovine lacto-peroxidase (Calbiochem, La Jolla, CA) and ^{125}I-

Na (ICN, Aurora, OH) according to Oosterom et al. (1999) and subsequently high-pressure liquid chromatography-purified on a C18 column (μBondapak 3.9 × 300 mm, Waters, Milford, MA).

Cells growing in 24-well plates were washed with Tris-buffered saline (TBS) (saline 0.14 M, Tris 25 mM, potassium chloride 5 mM, pH 7.4) supplemented with 2.5 mM calcium chloride and incubated for 30 min at room temperature with peptides and tracer diluted in Ham's F10 medium (Gibco) supplemented with 2.5 mM calcium chloride, 0.25% bovine serum albumine (ICN) and 200 KIU/ml aprotinin (Sigma, Steinheim, Germany). After two washes with ice-cold TBS (+2.5 mM calcium chloride) to remove non-bound tracer, the cells were lysed in 1 M sodium hydroxide and samples were counted in a γ-counter.

2.4. Activation assay

HEK293 cells growing in 10-cm dishes were co-transfected with 100–200 ng receptor construct and 7 μg of CRE-LacZ construct (Chen et al., 1995). After transfection, the cells were plated into 96-well plates (BectonDickinson). Two days after plating, the cells were incubated with peptides at the appropriate concentrations in serum-free medium (DMEM containing 0.2% bovine serum albumine (ICN), glutamine (Gibco) and non-essential aminoacids (Gibco) according to manufacturer's instruction). After 5–6 h of incubation, the medium was aspirated and 40 μl of lysis mix (PBS containing 0.1% triton-X-100 (Boehringer, Mannheim, Germany)) was added. The plates were stored at −20 °C and after thawing 80 μl of substrate mix (0.1 M phosphate buffer, pH 7.4 containing 1.6 g/l *o*-nitrophenyl β-D-galactopyranoside (ONPG, Molecular probes, Leiden, The Netherlands), 67.5 mM β-mercaptoethanol (Merck, Darmstadt, Germany) and 1.5 mM magnesium chloride) was added. Absorbance at 405 nm was determined in a Victor^2 microplate reader (PerkinElmer, Brussels, Belgium).

2.5. Animals

Male Wistar rats, 6–7 weeks old and weighing 130–150 g, at the start of the study, were used. Animals were housed in groups of two to three in plastic cages on sawdust bedding and kept at a 12/12-h light/dark cycle, with food and water available ad libitum. All testing procedures in this experiment were approved of by the Ethics Committee on Animal Experiments of the Utrecht University.

2.6. Surgery and sciatic nerve crush

Rats were anaesthetized by subcutaneous injection of 0.8 ml/kg Hypnorm (Janssen Pharmaceuticals, Grove, Oxford). Sciatic nerve crush was performed as described previously (De Koning et al., 1986). Briefly, an incision over the length of the right hip was made and after exposing the sciatic nerve a haemostatic forceps was used for exactly 30 s to induce crush injury of the sciatic nerve.

2.7. Drug administration and functional recovery

In the first experiment, 48 rats were randomly divided into four groups of 12 animals. The animals received every 48 h subcutaneous injections of MTII (20 μg/kg), [*D*-Tyr^4]MTII (20 μg/kg), SHU9119 (20 μg/kg) or saline. In the second experiment, 4 treatment groups of 12 animals each received JK46 (20 μg/kg), JK1 (20 μg/kg), JK1 (100 μg/kg) or saline. Treatment started immediately after nerve crush and was continued throughout the whole experiment. The MTII-treated animals and the saline controls of the first experiment were tested daily for recovery, starting at day 12 post-operation until day 25 post-operation. The remaining groups were tested only on day 19.

Sensory recovery was measured with the foot reflex withdrawal test (De Koning et al., 1986). In this test, a small electrical current ranging from 0.1 to 0.6 mA is applied with steps of 0.1 mA to determine at what level sensory function is regained. Non-lesioned rats will retract the stimulated paw already at 0.1 mA, whereas lesioned rats will not respond even to 0.6 mA shortly after surgery. Thus, if rats responded already at 0.1 mA, functionality was defined as 100%, whereas if the rat did not respond even to 0.6 mA, recovery was scored as 0%. Per rat, the lowest current at which a reaction is observed represents the level of functional sensoric recovery (0.1 mA = 100%, 0.2 mA = 84%, 0.3 mA = 67%, 0.4 mA = 50%, 0.5 mA = 34%, 0.6 mA = 17%, >0.6 mA = 0%).

Table 1
Alignment of the peptides that were used in the sciatic crush model to α-MSH and NDP-MSH

Peptide	Sequence
	1 2 3 4 5 6 7 8 9 10 11 12 13
α-MSH	Ac-Ser-Tyr-Ser-Met- Glu - His - Phe - Arg - Trp- Gly- Lys-Pro-Val-NH_2
NDP-MSH	Ac-Ser-Tyr-Ser-Nle- Glu - His- *D*-Phe - Arg - Trp- Gly- Lys-Pro-Val-NH_2
MTII	Ac-Nle-c[Asp-His- *D* –Phe – Arg - Trp- Lys]NH_2
JK1	Ac-Nle– Gly– Lys- *D*-Phe – Arg – Trp– Gly-NH_2
[D-Tyr^4]MTII	Ac-Nle-c[Asp-His- *D*-Tyr – Arg – Trp- Lys]NH_2
SHU9119	Ac-Nle-c[Asp-His- *D*-Nal(2)-Arg - Trp- Lys]NH_2
JK46	Ac-Gly– Lys–His -*D*-Nal(2)-Arg – Trp- Gly- NH_2

Numbering according to relative position in α-MSH. Note that in [*D*-Tyr^4]MTII, it is the *D*-Phe residue of MTII (which aligns to position 7 of α-MSH) which is replaced by a *D*-Tyr residue.

Table 2
IC_{50} and EC_{50} values of NDP-MSH and the peptides used in the sciatic crush model

	IC_{50} (nM)				EC_{50} (nM)			
	MC1R	MC3R	MC4R	MC5R	MC1R	MC3R	MC4R	MC5R
NDP-MSH	0.79 ± 0.05	1.4 ± 0.14	7.8 ± 1.0	0.62 ± 0.079	ND	ND	ND	ND
MTII	0.55 ± 0.029	2.2 ± 0.14	2.4 ± 0.28	22 ± 3.4	0.020 ± 0.0084	0.23 ± 0.077	0.086 ± 0.0071	12 ± 1.9
[*D*-Tyr^4]MTII	4.0 ± 0.13	146 ± 4.9	9.4 ± 0.46	1915 ± 146	0.31 ± 0.14	7.3 ± 1.6	1.5 ± 0.39	15%
JK1	0.36 ± 0.038	66 ± 0.75	1.4 ± 0.17	542 ± 101	0.053 ± 0.024	6.0 ± 0.79	0.31 ± 0.070	46%
SHU9119	1.3 ± 0.12	0.46 ± 0.048	0.74 ± 0.15	3.4 ± 0.082	0.48 ± 0.080	–	–	3.3 ± 0.69
JK46	153 ± 30	21 ± 0.82	1.2 ± 0.23	423 ± 13	100 ± 35	–	–	37%

IC_{50} values were obtained by displacement of iodinated NDP-MSH. EC_{50} values were determined in a *Lac*Z reporter gene assay. In case no complete dose–response curves were obtained in the activation assay due to low activity of the compound, percentages are used to indicate the activity of 1 μM compound relative to the maximal α-MSH activity. "–", no activity at concentrations up to 1 μM; "ND", not determined. Data represent mean of three to five independent experiments (± S.E.M.).

2.8. Statistical analysis

IC_{50} and EC_{50} values were calculated with non-linear regression using Prism software (GraphPad, San Diego, USA). Averages of multiple (3–5) independent experiments were calculated for each peptide–receptor combination. For recovery, differences between drug treatment groups at day 19 were first analysed using the Kruskal–Wallis test because of the non-parametric nature of the data. Each group was then compared with the saline controls using the Mann–Whitney *U* test. A probability level of 5% was considered significant.

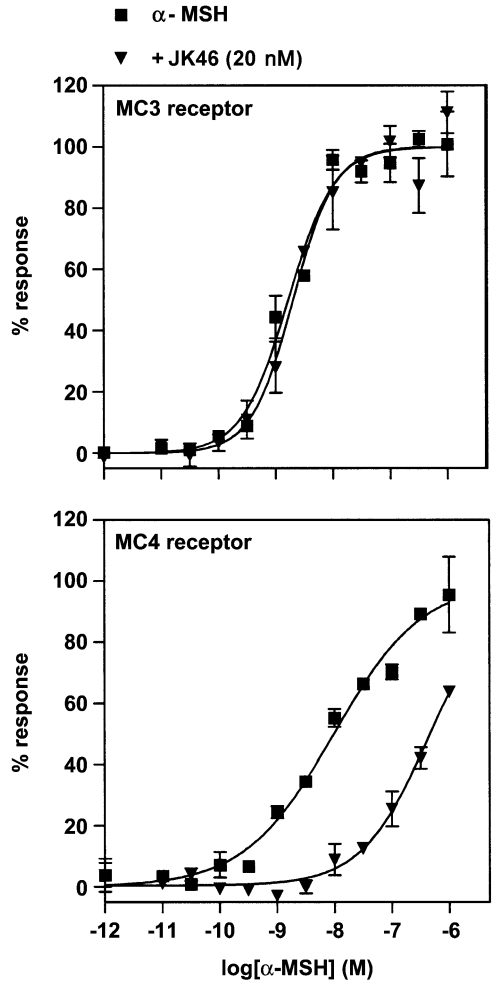

Fig. 1. Displacement of the α-MSH dose–response curves on melanocortin MC_3 and MC_4 receptors by JK46 (20 nM).

3. Results

Five melanocortin analogues were tested for their effects on sensory recovery after sciatic nerve crush. The primary structures of these peptides are shown in Table 1. First, IC_{50} and EC_{50} values of the peptides were determined for the rat melanocortin MC_3, MC_4 and MC_5 receptors and the mouse melanocortin MC_1 receptor (Table 2). All peptides that induced receptor activation as measured in the reporter gene assay behaved as full agonist, i.e. no partial agonism was observed.

MTII activated all receptors and displayed no considerable selectivity for any of the receptor subtypes, although the affinity for the melanocortin MC_5 receptor was lower as

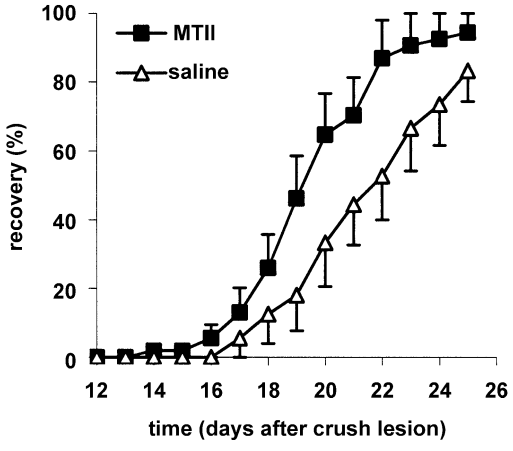

Fig. 2. The effect of subcutaneous injections of MTII on sensory recovery following sciatic nerve crush. Animals received either 20 μg/kg MTII or saline every 48 h. Recovery was measured daily with the foot reflex withdrawal test. Drug treatment groups were analyzed using the Kruskal–Wallis test and subsequently each group was compared with the respective saline controls (Mann–Whitney *U* test). *Statistically significant ($p < 0.05$).

compared to the other receptor subtypes. The agonist [*D*-Tyr^4]MTII was selective for the melanocortin MC_4 receptor as compared to the melanocortin MC_3 and MC_5 receptors (15-fold lower affinity for the melanocortin MC_3 receptor and 200-fold lower affinity for the melanocortin MC_5 receptor), but also possessed high affinity and potency for the melanocortin MC_1 receptor. Like [*D*-Tyr^4]MTII, JK1 is an agonist for all receptor subtypes with selectivity for the melanocortin MC_4 receptor as compared to the melanocortin MC_3 and MC_5 receptors (47-fold and 180-fold selectivity. respectively). JK1 also has high affinity and potency for the melanocortin MC_1 receptor. In agreement with previous studies (Haskell-Luevano et al., 2000; Schioth et al., 1999), SHU9119 was non-selective, with full agonistic activity at the melanocortin MC_1 and MC_5 receptors and an antagonistic profile for the melanocortin MC_3 and MC_4 receptors. The new compound JK46 also is an antagonist for the melanocortin MC_4 receptor, since it had high affinity for, but did not stimulate the melanocortin MC_4 receptor (Table 2). In addition, JK46 induced a rightward shift of the dose–response curve of α-MSH at the melanocortin MC_4 receptor (Fig. 1). The IC_{50} values indicated that JK46 has similar affinity for the melanocortin MC_4 receptor as SHU9119, but in contrast JK46 is selective for the melanocortin MC_4 receptor. This was confirmed by the relatively low potency of JK46 for the melanocortin MC_1 and MC_5 receptors and the fact that it did not induce a shift of the dose–response curve of α-MSH at the melanocortin MC_3 receptor (Fig. 1).

In a previous study, the effective dose of MTII for sensory recovery was found to be 20 μg/kg (ter Laak et al., 2003), and this dose was therefore used in the present study. As expected, application of MTII at this dose accelerated sensory recovery in animals bearing a sciatic nerve crush (Fig. 2). Recovery reached about 50% at day 19 for MTII-treated animals. Therefore, at this day recovery was measured to compare the effects of JK1, [*D*-Tyr^4]MTII, SHU9119 and JK46 with that of MTII. Analogous to Van Der Zee et al. (1991), these data are expressed as the percentage recovery of MTII-treated animals above control (Fig. 3). At the same dose as MTII, both JK1 and [*D*-Tyr^4]MTII did not accelerate sensory recovery compared to saline controls. JK1 was also ineffective at a higher dose (100 μg/kg). Unexpectedly, the melanocortin MC_3/MC_4 receptor antagonist SHU9119 enhanced recovery. JK46 on the other hand, was ineffective.

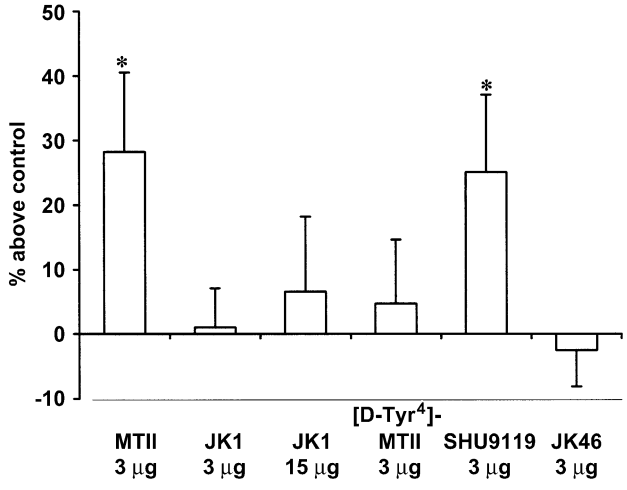

Fig. 3. Effects of peptides on sensory recovery following sciatic nerve crush. Recovery was measured with the foot reflex withdrawal test at day 19 after crush lesion. The mean percentage of recovery (MPR) was calculated for each group and subsequently the MPR of saline-treated animals was subtracted from the MPR of animals that received peptide to calculate percentage recovery above saline controls. Error bars indicate S.E.M. and are given for visualization purposes only. *Significantly different ($p < 0.05$) from saline controls (Kruskal–Wallis test, followed by a Mann–Whitney *U* test).

4. Discussion

Based on the available data, the melanocortin MC_4 receptor was always considered the best candidate to mediate the effects of exogenously applied melanocortins on nerve regeneration. However, the results described in this study suggest that the melanocortin MC_4 receptor is not involved.

The effects of the peptides on functional recovery did not correlate with their pharmacological profile for the melanocortin MC_4 receptor. First, the melanocortin MC_4 receptor-selective agonists JK1 and [*D*-Tyr^4]MTII did not enhance sensory recovery after sciatic nerve crush, while the non-selective agonist MTII was effective at the same dose. JK1 was also ineffective at a higher dose, indicating that the lack of effect of JK1 is not due to lower potency at the melanocortin MC_4 receptor as compared to MTII. It seems unlikely that JK1 and [*D*-Tyr^4]MTII were ineffective due to lack of in vivo efficacy at the melanocortin MC_4 receptor. Previously, we have shown that intracerebroventricular (i.c.v.) applied JK1 and [*D*-Tyr^4]MTII induce grooming, which is a melanocortin MC_4 receptor mediated behavioral response (Adan et al., 1999), and intrathecal injections modulated neuropathic pain (Nijenhuis et al., 2003; Vrinten et al., 2000). Moreover, i.v. applied JK1 was also able to induce grooming behaviour. Although a different delivery route was used in the present study, these data show that JK1 and [*D*-Tyr^4]MTII are effective at the melanocortin MC_4 receptor in vivo. Second, if the neurotrophic effects of melanocortin agonists are mediated by the melanocortin MC_4 receptor, antagonists for this receptor should not enhance recovery. Rather, inhibition of recovery would be expected if endogenous melanocortins influence sensory recovery via the melanocortin MC_4 receptor. Indeed, a MC_4 receptor antagonist inhibited α-MSH induced neurite outgrowth in Neuro 2A cells in vitro (Adan et al., 1996). The stimulatory effect of the melanocortin MC_4 receptor antagonist SHU9119 in the present in vivo study thus questions the putative role of this receptor in sensory recovery. In addition, JK46 did not stimulate recovery at

the dose at which SHU9119 was effective, although JK46 has similar affinity for the melanocortin MC_4 receptor as for SHU9119. Thus, JK46 and SHU9119 are two compounds with a similar pharmacology for the MC_4 receptor, but with different effects on sensory recovery.

Taken together, these data argue against a role for the melanocortin MC_4 receptor in the acceleration of sensory recovery by administered melanocortins. This seems in contradiction with a previous report (Adan et al., 1996), where neurite outgrowth by Neuro 2A cells was stimulated in vitro via endogenously expressed melanocortin MC_4 receptors. Though, it should be noted that those in vitro findings might not represent a mechanism that is actually involved in functional recovery in vivo. One other argument that has been put forward to implicate the melanocortin MC_4 receptor in functional recovery is the presence of melanocortin MC_4 receptors in the spinal cord (van der Kraan et al., 1999). Based on our results, these spinal cord melanocortin MC_4 receptors thus seem not to enhance sensory recovery after sciatic nerve crush. In the present study however, only sensory recovery was measured, whereas melanocortin receptor ligands show neurotrophic effects in other processes as well. For instance, there may still be a role for (spinal) melanocortin MC_4 receptors in neural development (Alves et al., 1997; King et al., 1991; Rose et al., 1988; Smith and Strand, 1981) and motoric recovery (for references see (Strand et al., 1993). Also, it was already noted before that the exact localization of the melanocortin MC_4 receptor within the spinal cord favored a role in nociception rather than in recovery. In fact, these receptors have been shown to modulate neuropathic pain (Vrinten et al., 2000), which may represent their main function.

Of the other three receptor subtypes that were tested in this study, the melanocortin MC_1 and MC_3 receptors are also not likely candidates. As for the melanocortin MC_4 receptor, JK1 is a potent agonist for the melanocortin MC_1 receptor and the lack of effect of JK1 at the two tested doses also argues against involvement of the melanocortin MC_1 receptor in accelerating sensory recovery. SHU9119 is an antagonist for the melanocortin MC_3 receptor, and would be expected not to have effect if the melanocortin MC_3 receptor is the mediator of the effects of melanocortin agonists. Thus, the positive effect of SHU9119 on sensory recovery pleads against a role for the melanocortin MC_3 receptor.

With the pharmacological profiles of the peptides used in this study we cannot exclude a role for the melanocortin MC_5 receptor. The enhancing effect of SHU9119 could be explained by its agonistic profile and high affinity for the melanocortin MC_5 receptor. Conversely, the low potency of JK1 and [*D*-Tyr^4]MTII and the low affinity of JK46 for the melanocortin MC_5 receptor could explain the lack of effects of these peptides. If the melanocortin MC_5 receptor were involved, what would be the mechanism? The important role of the immune system in nerve regeneration and the expression of the melatonin MC_5 receptor in the spleen and by lymphocytes (Buggy, 1998; Griffon et al., 1994; van der Kraan et al., 1998) tempts to speculate a role for the immune system. In line with this, melanocortins seem to act at the crush site (Edwards et al., 1986), which is also the site of action of immune cells. The anti-inflammatory effects of melanocortins are well known (Lipton and Catania, 1998), and therefore melanocortins could well diminish the inflammatory responses following nerve injury. The immune system can induce secondary damage beyond the lesion itself (Hirschberg et al., 1994) and reducing this secondary damage would advance recovery. However, more proof is needed to conclude that the melanocortin MC_5 receptor is the subtype that mediates the enhancing effects of melanocortins on functional recovery. In addition, it should be noted that involvement of another, yet undefined receptor subtype has been suggested. This comes from the observation that the melanocortin analogue ORG2766 clearly has neurotrophic effects (Bijlsma et al., 1981; Van Der Zee et al., 1988), but it does not bind to melanocortin receptors (Adan et al., 1994; Roselli-Rehfuss et al., 1993). Thus, interaction of MTII and SHU9119 with this putative receptor also may facilitate the enhancing properties of these compounds.

In conclusion, we tested whether the melanocortin MC_4 receptor plays a role in acceleration of sensory recovery after sciatic nerve crush using several melanocortin receptor ligands. It is suggested that the melanocortin MC_4 receptor is not involved in the stimulating effects of melanocortins on sensory recovery, since the effects of the ligands do not correlate with their melanocortin MC_4 receptor pharmacology. It remains to be investigated which receptor subtype is involved, but based on this study the melanocortin MC_5 receptor is an interesting candidate.

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