ELSEVIER

European Journal of Pharmacology 378 (1999) 249–258

ejp

www.elsevier.nl/locate/ejphar

# Characterization of melanocortin receptor ligands on cloned brain melanocortin receptors and on grooming behavior in the rat

Roger A.H. Adan [a,b,*], Arkadiusz W. Szklarczyk [a,b,1], Julia Oosterom [a,b], Jan H. Brakkee [a,b], Wouter A.J. Nijenhuis [a,b], Wim M.M. Schaaper [a,b], Rob H. Meloen [a,b], Willem Hendrik Gispen [a,b]

[a] *Department of Medical Pharmacology, Rudolf Magnus Institute for Neurosciences, Utrecht University, 3508 TA Utrecht, Netherlands*
[b] *Department of Molecular Recognition, ID-DLO, P.O. Box 65, 8200 AB Lelystad, Netherlands*

Received 15 February 1999; received in revised form 21 June 1999; accepted 25 June 1999

**Abstract**

Since the melanocortin $MC_3$ and melanocortin $MC_4$ receptors are the main melanocortin receptor subtypes expressed in rat brain, we characterized the activity and affinity of nine melanocortin receptor ligands using these receptors in vitro, as well as their activity in a well-defined melanocortin-induced behavior in the rat: grooming behavior. We report here that [D-Tyr$^4$]melanotan-II and RMI-2001 (Ac-cyclo-[Cys$^4$, Gly$^5$, D-Phe$^7$, Cys$^{10}$]α-MSH-$NH_2$) have significantly higher affinity and potency on the rat melanocortin $MC_4$ receptor as compared to the rat melanocortin $MC_3$ receptor. Nle-γ-MSH (melanocyte-stimulating hormone) was the only ligand with higher affinity and potency on the rat melanocortin $MC_3$ receptor. The potency order of melanocortin $MC_4$ receptor agonists, but not that of melanocortin $MC_3$ receptor agonists, fitted with the potency of these ligands to stimulate grooming behavior, when administered intracerebroventricularly. SHU9119 (Ac-cyclo-[Nle$^4$, Asp$^5$, D-Nal(2)$^7$, Lys$^{10}$]α-MSH-(4–10)-$NH_2$) and RMI-2005 (Ac-cyclo-[Cys$^4$, Gly$^5$, D-Nal(2)$^7$, Nal(2)$^9$, Cys$^{10}$]α-MSH-(4–10)-$NH_2$) were able to inhibit α-MSH-induced melanocortin receptor activity in vitro, as well as α-MSH-induced grooming behavior. Melanotan-II, [Nle$^4$-D-Phe$^7$]α-MSH and RMI-2001 were also effective in inducing grooming behavior when administered intravenously. In the absence of purely selective melanocortin $MC_{3/4}$ receptor ligands, we demonstrated that careful comparison of ligand potencies in vitro with ligand potencies in vivo, could identify which melanocortin receptor subtype mediated α-MSH-induced grooming behavior. Furthermore, blockade of novelty-induced grooming behavior by SHU9119 demonstrated that this physiological stress response is mediated via activation of the melanocortin system. 

*Keywords:* Melanocortin receptor; Ligand–receptor interaction; Grooming behavior; MSH (melanocyte-stimulating hormone); ACTH (adrenocorticotrope hormone); Stress

## 1. Introduction

Brain pro-opiomelanocortin (POMC) expression is mainly localised in arcuate neurons of the hypothalamus and in the nucleus of the solitary tract in the brainstem. The arcuate nucleus has projections to many brain areas which suggests the involvement of this system in diverse brain functions. Indeed, it had been documented that melanocortins can modulate neurophysiological and neuropathological phenomena like conditioned avoidance, central control of autonomic systems, different types of behavior (grooming, female sexual behavior, anxiety, aggression), drug addiction and nerve regeneration (De Wied and Jolles, 1982). The demonstration of binding sites for melanocortins in brain (Tatro, 1990) and the cloning of brain specific melanocortin receptors (Chhajlani et al., 1993; Roselli-Rehfuss et al., 1993; Mountjoy et al., 1994) further underscored the significance of the brain melanocortin system. Recently, the deletion of the melanocortin $MC_4$ receptor gene from mice and the identification of Agouti and Agouti-related peptide as endogenous antagonists implicated the brain melanocortin system

* Corresponding author. Department of Medical Pharmacology, Rudolf Magnus Institute for Neurosciences, Medical Faculty, Utrecht University, Universiteitsweg 100, 3584 CG Utrecht, The Netherlands. Tel.: +31-302538810; fax: +31-302539032; E-mail: adan@med.uu.nl

[1] On leave of absence at the Nencki Institute, PL-02-093 Warsaw, Poland.

0014-2999/99/$ - see front matter 
PII: S0014-2999(99)00465-3

in regulation of weight homeostasis (Lu et al., 1994; Fong et al., 1997; Huszar et al., 1997; Ollmann et al., 1997; Shutter et al., 1997). Still, the exact involvement of the melanocortin system in weight homeostasis is not yet understood. Likewise, the mechanism underlying the effects of melanocortins on the diverse brain functions described above are unknown. Selective and potent ligands are essential tools necessary to understand how the melanocortin system modulates these functions. However, the identification and the first application of selective melanocortin receptor antagonists in studies on the brain underscored the involvement of melanocortin receptors in grooming behavior and in the control of feeding and body temperature (Adan et al., 1994b; Fan et al., 1997; Huang et al., 1997; Vergoni et al., 1998).

The stretching and yawning syndrome (Ferrari, 1958) and grooming behavior in the rat (Gispen et al., 1975) were among the first described behaviors which are under control of the melanocortins. Grooming behavior consists of activities directed to the animal body surface like face washing, body grooming, licking, scratching and genital grooming. The display of this behavior is often associated with other factors than the condition of the fur and is also seen following exposure to a novel environment. Although the significance of grooming to animal homeostasis remains to be determined, it was proposed that the display of this behavior may function to reduce arousal, for instance, elicited by mild stress conditions (Gispen and Isaacson, 1986; Spruijt et al., 1992).

Excessive grooming can be induced by intracerebral injection of different neuropeptides (Spruijt et al., 1992). Opioidergic, dopaminergic and serotoninergic brain systems have been demonstrated to stimulate different aspects of grooming behavior. However, the grooming response depends mainly on the melanocortin system, since: (1) the frequency and duration of different grooming elements of melanocortin (but not of other peptides) induced grooming-resembled grooming observed under physiological conditions, i.e., exposure to novelty (Spruijt et al., 1985); (2) ACTH (adrenocorticotrope hormone) and α-MSH (α-melanocyte-stimulating hormone) are potent activators of excessive grooming when delivered into rat brain ventricles (Gispen and Isaacson, 1986); (3) antiserum against ACTH infused into the brain ventricular system blocked novelty-induced grooming in the rat (Dunn et al., 1979); (4) down-regulation of POMC protein using antisense oligonucleotides significantly reduced the grooming response to novelty (Spampinato et al., 1994). Comparison of the activity of melanocortin analogs at melanocortin receptors in vitro with their activity in vivo has led to the suggestion that melanocortin $MC_4$ receptor mediates α-MSH-induced grooming (Adan et al., 1994a), which was supported by demonstrating that melanocortin $MC_4$ receptor antagonists block α-MSH-induced grooming (Adan et al., 1998; Vergoni et al., 1998). Localization of the melanocortin $MC_4$ receptor in brain structures (Mountjoy et al., 1994), which have been shown as critical for the grooming response, i.e., paraventricular nucleus, substantia nigra, periaqueductal gray (Spruijt et al., 1992) further strengthens this suggestion. However, the significance of melanocortin $MC_4$ receptor activation for physiologically evoked grooming has remained unclear.

In the present report, pharmacological effects of earlier identified as well as new MC receptor ligands are described on the main brain melanocortin receptors in the rat, melanocortin $MC_3$ receptor and melanocortin $MC_4$ receptor (Low et al., 1994). Furthermore, we pharmacologically characterize these ligands on melanocortin-induced grooming. We provide evidence that melanocortin $MC_4$ receptor activation mediates melanocortin-induced excessive grooming behavior and that the melanocortin receptor antagonist SHU9119 blocks peptide as well as novelty-induced grooming.

## 2. Materials and methods

### 2.1. Peptides (Fig. 1)

[$Nle^4$-D-$Phe^7$]α-MSH (melanotan-I) and α-MSH were purchased from Bachem (Bubendorf, Switzerland). Melanotan-II (melanotan-II, Ac-cyclo-[$Nle^4$, $Asp^5$, D-$Phe^7$, $Lys^{10}$]α-MSH-(4–10)-$NH_2$) and SHU9119 (Ac-cyclo-[$Nle^4$, $Asp^5$, D-Nal(2)$^7$, $Lys^{10}$]α-MSH-(4–10)-$NH_2$) were gifts from Dr. V.J. Hruby (Department of Chemistry, University of Arizona) and from Dr. R.D. Cone (Vollum Institute, Oregon Health Sciences University). Ac-cyclo-[$Cys^4$, $Gly^5$, D-$Phe^7$, $Cys^{10}$]α-MSH-$NH_2$ (RMI-2001), Ac-[$Nle^3$]$\gamma_2$-MSH-$NH_2$ (Nle-γ-MSH), Ac-cyclo-[$Nle^4$, $Asp^5$, D-$Tyr^7$, $Lys^{10}$]α-MSH-(4–10)-$NH_2$ ([D-$Tyr^4$]melanotan-II), Ac-cyclo-[$Nle^4$, $Asp^5$, D-$Phe^7$, $Lys^{10}$]α-MSH-$NH_2$ (RMI-2004) and Ac-cyclo-[$Cys^4$, $Gly^5$, D-Nal(2)$^7$, Nal(2)$^9$, $Cys^{10}$]α-MSH-(4–10)-$NH_2$ (RMI-2005) were synthesized and using Fmoc solid phase synthesis as reported elsewhere (Schaaper et al., 1998). Peptides were purified using reversed phase preparative HPLC (high pressure liquid chromatography) to a purity of $\pm$90%, estimated after analysis by analytical HPLC at 215 nm.

### 2.2. Binding assay

Approximately $4.10^6$ HEK (human embryonal kidney) 293 cells were transfected with 8 μg of either the rat melanocortin $MC_3$ or rat melanocortin $MC_4$ receptor expressing plasmids and split into poly-L-lysine (Sigma, Brussel, Belgium) coated 24-well Costar plates (specific binding to HEK 293 cells expressing melanocortin $MC_3$ and melanocortin $MC_4$ receptors was approximately 5000 cpm/well and 3000 cpm/well, respectively; non-specific binding was 200 cpm). Two days after transfection, the cells were incubated with 0.2 nM of $^{125}$I-[$Nle^4$-D-$Phe^7$]α-MSH (100.000 cpm/200 μl) and various concentrations

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 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 | $-NH_2$ |
| MT-II | | | | Ac- | Nle | c[Asp | His | D-Phe | Arg | Trp | Lys] | $-NH_2$ | | | |
| D-Tyr-MTII | | | | Ac- | Nle | c[Asp | His | D-Tyr | Arg | Trp | Lys] | $-NH_2$ | | | |
| RMI-2001 | Ac- | Ser | Tyr | Ser | c[Cys | Gly | His | D-Phe | Arg | Trp | Cys] | Lys | Pro | Val | $-NH_2$ |
| RMI-2004 | Ac- | Ser | Tyr | Ser | Nle | c[Asp | His | D-Phe | Arg | Trp | Lys] | Gly | Pro | Val | $-NH_2$ |
| Nle-γ-MSH | | Ac- | Tyr | Val | Nle | Gly | His | Phe | Arg | Trp | Asp | Arg | Phe | Gly | $-NH_2$ |
| SHU9119 | | | | Ac- | Nle | c[Asp | His | D-Nal | Arg | Trp | Lys] | $-NH_2$ | | | |
| RMI-2005 | | | | Ac- | c[Cys | Gly | His | D-Nal | Arg | Trp | Cys] | $-NH_2$ | | | |

Fig. 1. The primary structure of the melanocortin receptor ligands is shown.

of peptides in Ham's F10 medium (Life Technologies, Breda, Netherlands) pH 7.4 containing 2.5 mM $CaCl_2$, 0.25% bovine serum albumin, 10 mM HEPES and 50 μg/ml (150 KIU/ml) aprotinin (Sigma, Brussel, Belgium). After incubation for 30 min at room temperature, the cells were washed twice with ice-cold Tris-buffered saline (TBS) containing 2.5 mM calcium chloride and lysed in 1 M sodium hydroxide. Radioactivity of the lysates was counted in a Packard Cobra γ-counter. Curves were fitted with GraphPad Prism software by non-linear regression and values were calculated with 95% confidence interval. These curves were reproduced at least twice. $K_i$ values were calculated from $IC_{50}$ values using the equation $K_i = IC_{50}/(1 + [L]/K_d)$ ($[L]$ = concentration of $^{125}$I-[Nle$^4$-D-Phe$^7$]MSH in binding assay; $K_d$ = affinity of $^{125}$I-[Nle$^4$-D-Phe$^7$]MSH).

### 2.3. β-Galactosidase activity assay

The assay makes use of a *LacZ* gene which is expressed under the control of a cAMP regulated promotor in a pCRElacZ construct to detect changes in intracellular cAMP as a result of receptor activation (Chen et al., 1995). HEK 293 cells were co-transfected using the calcium phosphate precipitation method with 200 ng of plasmids expressing either the rat melanocortin $MC_3$ receptor (Roselli-Rehfuss et al., 1993) or the rat melanocortin $MC_4$ receptor (Alvaro et al., 1996) and 7 μg pCRElacZ, and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum. Pools of transfected cells were mixed and then split on 96-well plates, and all ligands were tested in one experiment. The data presented in Table 1 are from a representative experiment. Similar data were obtained at least three times. Although the $EC_{50}$ values between experiments varied, the fold difference in $EC_{50}$ values (relative potency) of different ligands in one experiment was always the same. 20 h after transfection the cells were split into 96-well plates. The next day, cells were treated for 6 h with melanocortin receptor ligands in DMEM supplemented with 0.5% bovine serum albumin, 150 KIU/ml aprotinin and 25 mM HEPES (pH 7.4). The agonist activity was measured by stimulating

Table 1
Affinity ($K_i$) and potency ($EC_{50}$) of melanocortin receptor ligands for the rat melanocortin $MC_3$ and $MC_4$ receptors
The affinity (in nM) of nine melanocortin receptor ligands (MT-II = melanotan II; NDP = [Nle$^4$-D-Phe$^7$]α-MSH) was determined on HEK 293 cells expressing either the rat melanocortin $MC_3$ receptor or the rat melanocortin $MC_4$ receptor using $^{125}$I-[Nle$^4$-D-Phe$^7$]α-MSH as radioligand. Data are expressed as mean ± confidence interval.
The potency (in nM) of seven melanocortin receptor ligands was determined on HEK 293 cells expressing either the rat melanocortin $MC_3$ receptor or the rat melanocortin $MC_4$ receptor using *LacZ* as reporter gene for receptor activation. Data are expressed as mean ± confidence interval.

| Ligand | $MC_3$ receptor | | $MC_4$ receptor | |
|---|---|---|---|---|
| | $K_i$ | $EC_{50}$ | $K_i$ | $EC_{50}$ |
| α-MSH | 9.40 ± 3.17 | 11.6 ± 3.2 | 9.17 ± 4.23 | 1.34 ± 0.50 |
| NDP-MSH | 1.19 ± 0.51 | 0.309 ± 0.170 | 3.14 ± 1.18 | 0.0927 ± 0.0471 |
| MT-II | 4.77 ± 2.13 | 0.780 ± 0.173 | 1.74 ± 0.77 | 0.0111 ± 0.0043 |
| D-Tyr-MTII | 204 ± 87.2 | 20.3 ± 7.1 | 3.84 ± 0.83 | 0.466 ± 0.189 |
| RMI-2001 | 4.96 ± 1.06 | 2.22 ± 0.50 | 0.260 ± 0.098 | 0.0300 ± 0.0131 |
| RMI-2004 | 1.23 ± 0.13 | 3.86 ± 0.92 | 4.51 ± 0.99 | 0.326 ± 0.155 |
| Nle-γ-MSH | 1.44 ± 0.26 | 1.26 ± 0.10 | 77.5 ± 37.7 | 11.0 ± 3.92 |
| SHU9119 | 0.879 ± 0.170 | | 0.238 ± 0.060 | |
| RMI-2005 | 4.56 ± 1.30 | | 0.485 ± 0.169 | |

the cells with varying concentration of α-MSH, [Nle$^4$-D-Phe$^7$]α-MSH, melanotan-II and RMI-2001, Nle-γ-MSH, [D-Tyr$^4$]melanotan-II and RMI-2004. Antagonist activity was measured with a fixed concentration of agonist and increasing concentrations of antagonists SHU9119 and RMI-2005. The antagonists were also screened for agonistic activity. After treatment, cells were lysed in PBS with 0.1% Triton X-100, frozen, thawed and assayed for β-galactosidase activity. Data represent means from triplicate data points and curves were fitted with GraphPad Prism software by nonlinear regression sigmoidal dose–response with variable slope.

### 2.4. Animals, implantation of cannulas, intracerebroventricular (i.c.v.) injection, intravenous (i.v.) injections

All experiments were performed with permission of the local ethical committee on animal experiments. Male Wistar rats weighing 120–130 g were used. Rats were housed in single cages in a light–dark cycle of 12 h (lights off 1900–0700 h). Cannulas made from polypropylene tubes were implanted into the foramen intraventriculare under hypnorm anaesthesia (Brakkee et al., 1979). Rats were allowed to recover for 3 days and used for experiments during the next 10 days. In cases where rats were used for more than one grooming experiment, they were allowed to recover for at least 3 days between subsequent experiments. Peptides dissolved in 3 μl of saline (154 mM sodium chloride) were injected i.c.v. by means of a Hamilton syringe. For i.v. injections via the tail, peptides were dissolved in 100 μl saline. In some experiments, SHU9119 was i.c.v.-injected 30 min before the agonist or saline injection (this is indicated by pre-injection) otherwise, the agonist and the antagonist were injected as a single mixture.

### 2.5. Grooming assays

Grooming tests were performed according to Gispen et al. (1975). Briefly, rats were transported from the in-house animal facility, to an observation room at least 1 h before start of the behavioral test. Grooming was induced either by agonist injection or by exposure to a novel environment. In our experiments, the observation cage consisted of a plexiglass box (30 cm × 15 cm × 15 cm) covered with a metal cover in which NAIVE rats were placed immediately after the i.c.v. or i.v. injection. Observation started 15 min after the injection and continued for 50 min. Grooming was scored each 15 s over 50 min, thus, the maximal grooming score for a rat is 200. The grooming elements vibrating, face washing, body and genital grooming, body licking, scratching and paw licking were scored. The grooming tests were performed between 1300 and 1500 h. Each experimental group consisted of at least six rats. Statistical analysis was performed by means of the SPSS for Windows version 6.0 software using independent samples *t*-test or One-way ANOVA (analysis of variance) with

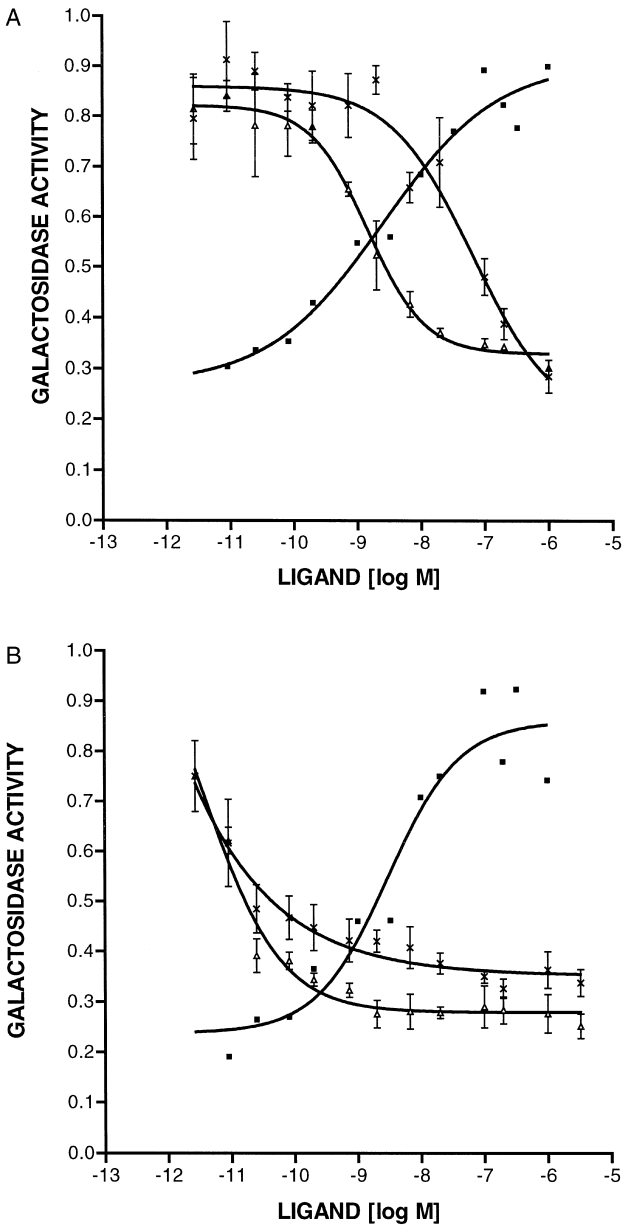


Fig. 2. Inhibition of melanocortin receptor activity by SHU9119 and RMI-2005. (A) HEK 293 cells expressing the rat melanocortin $MC_3$ receptor were stimulated with increasing concentrations of α-MSH (■), a fixed dose of α-MSH (50 nM) with increasing concentrations of SHU9119 (△), or a fixed dose of α-MSH (50 nM) with increasing concentrations of RMI-2005 (x). Activity was determined using *LacZ* as reporter gene (galactosidase activity) for receptor activity. (B) HEK 293 cells expressing the rat melanocortin $MC_4$ receptor were stimulated with increasing concentrations of α-MSH (■), a fixed dose of α-MSH (50 nM) with increasing concentrations of SHU9119 (△), or a fixed dose of α-MSH (50 nM) with increasing concentrations of RMI-2005 (x). Activity was determined using *LacZ* as reporter gene (galactosidase activity) for receptor activity.

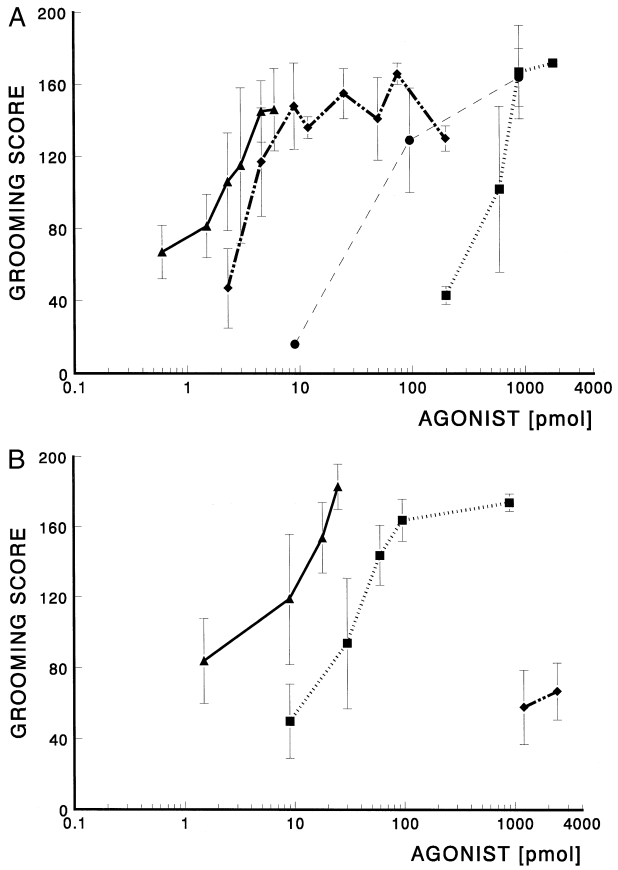


Fig. 3. Potency of melanocortin receptor agonists on the induction of grooming. (A) Rats received i.c.v. injections with different doses (per rat) of α-MSH (■), [Nle$^4$-D-Phe$^7$]α-MSH (♦), melanotan-II (▲) or [D-Tyr$^4$]melanotan-II (●) and 15 min later grooming was scored for 50 min ($n = 6$). (B) Rats received i.c.v. injections with different doses (per rat) of RMI-2004 (■), RMI-2001 (▲) or Nle-γ-MSH (♦) and 15 min later grooming was scored for 50 min ($n = 6$).

Student–Newman–Keuls test as a post-hoc. Data are expressed as means ± S.E.M.

## 3. Results

### 3.1. Binding properties of melanocortin analogues

The affinity ($K_i$) of the nine melanocortin receptor ligands (Fig. 1) was determined in a heterologous binding assay using $^{125}$I-[Nle$^4$, D-Phe$^7$]α-MSH as radiolabel. Table 1 shows the affinity of these ligands on the rat melanocortin $MC_3$ receptor and rat melanocortin $MC_4$ receptor. All these ligands had an affinity for melanocortin $MC_3$ receptor in the low nanomolar range, except for [D-Tyr$^4$]melanotan-II which had an affinity in the submicromolar range. α-MSH had a similar affinity on melanocortin $MC_4$ receptor as compared to melanocortin $MC_3$ receptor. Similar to melanocortin $MC_3$ receptor, most ligands had affinities in the low nanomolar range for melanocortin $MC_4$ receptor, except for RMI-2001, RMI-2005 and SHU9119 which had affinities in the subnanomolar range and Nle-γ-MSH which had submicromolar affinity. In contrast to the melanocortin $MC_3$ receptor, [D-Tyr$^4$]melanotan-II had an affinity in the low nanomolar range on the melanocortin $MC_4$ receptor. Thus, RMI-2001, [D-Tyr$^4$]melanotan-II, RMI-2005 and SHU9119 had significantly higher affinity, being 17-fold, 47-fold, eight-fold and four-fold higher, respectively for the melanocortin $MC_4$ receptor than for the melanocortin $MC_3$ receptor. In contrast, Nle-γ-MSH had 53-fold lower affinity for the melanocortin $MC_4$ receptor than for to the melanocortin $MC_3$ receptor.

### 3.2. Activity of melanocortin analogues on the rat melanocortin $MC_3$ receptor and rat melanocortin $MC_4$ receptor

All agonists displayed full agonistic activity on the melanocortin $MC_3$ and melanocortin $MC_4$ receptors in our experimental system. α-MSH, [D-Tyr$^4$]melanotan-II and RMI-2004 had comparable agonistic activities using the rat melanocortin $MC_4$ receptor which were in the (sub)nanomolar range (Table 1). [Nle$^4$-D-Phe$^7$]α-MSH, melanotan-II and RMI-2001 were more potent ligands, with $EC_{50}$ values of less that 0.1 nM. In contrast, Nle-γ-MSH had very low activity (high $EC_{50}$ value) for melanocortin $MC_4$ receptor. For the melanocortin $MC_3$ receptor, only [Nle$^4$-D-Phe$^7$]α-MSH and melanotan-II had a subnanomolar $EC_{50}$ value. RMI-2001, Nle-γ-MSH and RMI-2004 had $EC_{50}$ values in the low nanomolar range, whereas [D-Tyr$^4$]-melanotan-II had an $EC_{50}$ value of 20 nM. Thus, melanotan-II, RMI-2001 and [D-Tyr$^4$]melanotan-II showed the highest difference between melanocortin $MC_3$ receptor and melanocortin $MC_4$ receptor, the activity being higher for the $MC_4$ receptor. Only Nle-γ-MSH was more potent for the melanocortin $MC_3$ receptor than for the melanocortin $MC_4$ receptor in our test system.

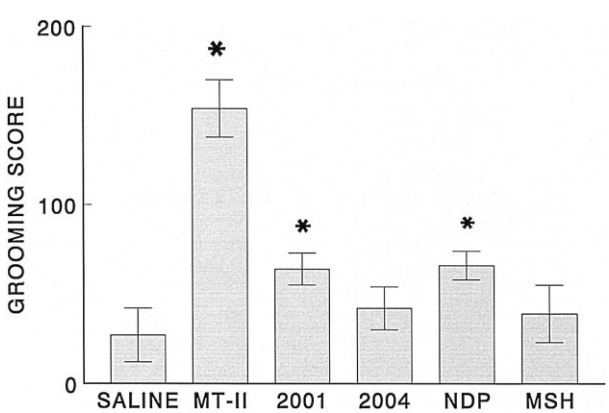


Fig. 4. Induction of grooming behavior by i.v. administration of melanocortin receptor agonists. Rats received i.v. injections with either saline, melanotan-II, RMI-2001 (2001), RMI-2004 (2004), [Nle$^4$-D-Phe$^7$]α-MSH (NDP) or α-MSH (MSH). Each ligand was administered at a dose of 100 μg per rat. Grooming was scored starting 15 min after the injections. An asterisk indicates statistical significant difference as compared to injection with saline ($P < 0.05$; $n = 5$).

### 3.3. Antagonism by SHU9119 and RMI-2005

Fig. 2 demonstrates that SHU9119 and RMI-2005 are able to inhibit melanocortin $MC_3$ receptor and melanocortin $MC_4$ receptor activation by α-MSH. SHU9119 was more potent than RMI-2005 to inhibit the activation of melanocortin $MC_3$ receptor and melanocortin $MC_4$ receptor by 50 nM α-MSH. SHU9119 and RMI-2005 shifted the dose–response curves of α-MSH, [Nle$^4$-D-Phe$^7$]MSH and melanotan-II to the right, without affecting the maximal activity at high doses of agonist (data not shown). Thus, SHU9119 and RMI-2005 can be used as ligands to inhibit receptor activation by melanocortins.

### 3.4. Stimulation of grooming behavior by i.c.v. application of melanocortin analogues

As can be seen in Fig. 3a and b α-MSH, [Nle$^4$-D-Phe$^7$]α-MSH, RMI-2001, [D-Tyr$^4$]-melanotan-II, RMI-2004 and melanotan-II induced excessive grooming after i.c.v. injections, whereas Nle-γ-MSH did not. Also, RMI-2005 and SHU9119 did not induce grooming behavior even at the highest tested dose of 1 nmol. RMI-2001, [Nle$^4$-D-Phe$^7$]α-MSH and melanotan-II had similar potency in the range between 1 and 10 pmol. The lowest doses of these melanocortins that significantly increased grooming as compared to saline-treated rats were 1.5 pmol for [Nle$^4$-D-Phe$^7$]-α-MSH and RMI-2001 and 4.5 pmol for melanotan-II. Thus, the three most potent melanocortin $MC_4$ receptor agonists were also the most potent peptides in inducing excessive grooming behavior. Furthermore, the pharmacological profile of melanocortin-induced grooming fits best with that of the melanocortin $MC_4$ receptor and not with that of the $MC_3$ receptor. For instance, Nle-γ-MSH is more potent and [D-Tyr$^4$]melanotan-II is less potent than α-MSH for the melanocortin $MC_3$ receptor, but [D-Tyr$^4$]melanotan-II is more potent than α-MSH in

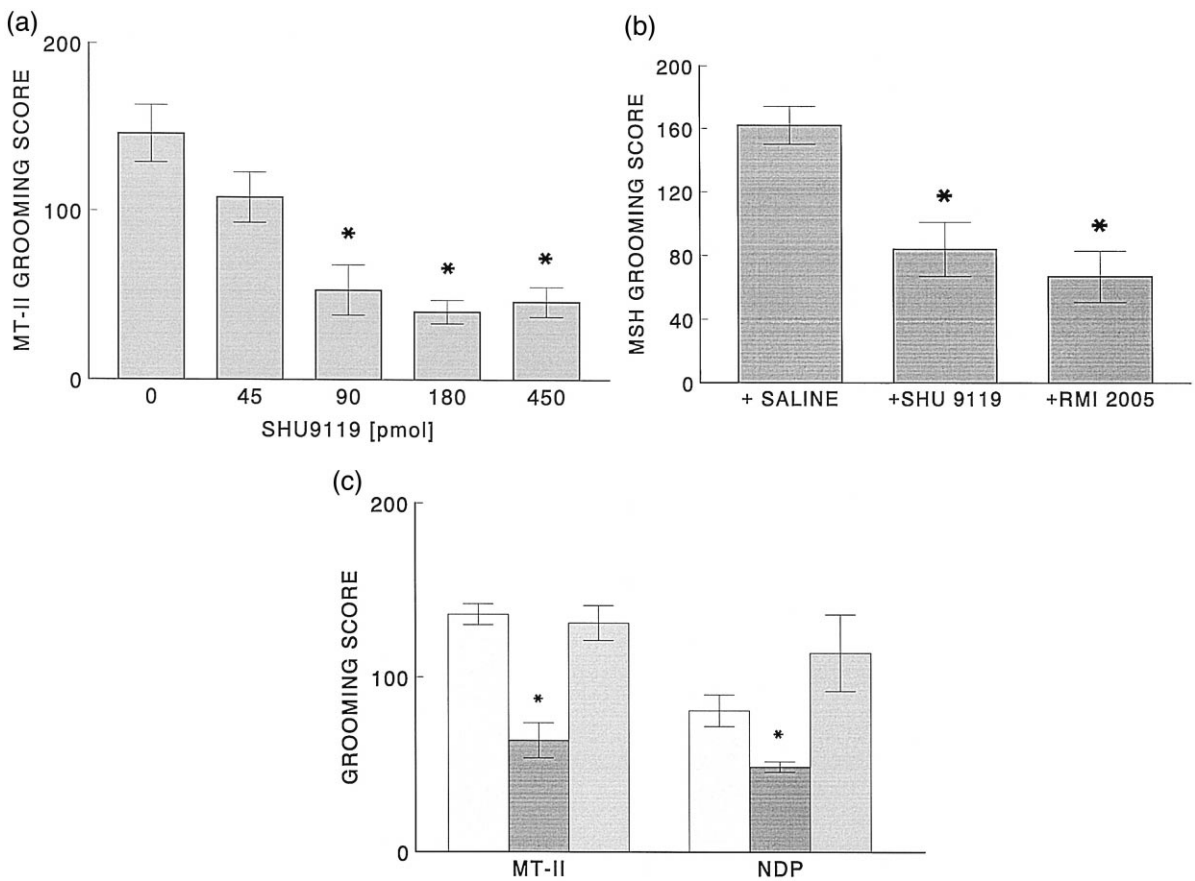


Fig. 5. Inhibition of melanocortin-induced grooming behavior. Efficacy of SHU9119 to block melanotan-II-induced grooming. (A) Rats received i.c.v. injections with a mixture of a submaximal dose of melanotan-II (9 pmol per rat) and different doses of SHU9119. At 15 min after the injection grooming was scored. An asterisk indicates statistical significant difference as compared to injection of 9 pmol melanotan-II alone ($P < 0.05$; $n = 8$). (B) Rats received i.c.v. injections with a mixture of α-MSH (900 pmol per rat) with either saline, 90 pmol SHU9119 (SHU 9119) or 90 pmol RMI-2005 (RMI-2005). At 15 min after the injection grooming was scored. An asterisk indicates statistical significant difference as compared to injection of 900 pmol α-MSH alone ($P < 0.05$; $n = 6$). (C) Competitive inhibition of SHU9119 on melanocortin receptor agonist induced grooming. Rats received i.c.v. injections with melanocortin receptor agonists (9 pmol melanotan-II or 2.5 pmol [Nle$^4$-D-Phe$^7$]α-MSH (NDP) per rat; open bars), mixtures of these doses of melanocortin receptor agonists and 90 pmol SHU9119 (light grey bars), or mixtures of higher doses of melanocortin receptor agonists (45 pmol melanotan-II or 9 pmol [Nle$^4$-D-Phe$^7$]α-MSH with 90 pmol SHU9119 (dark grey bars)). Grooming was scored starting 15 min after the injections. An asterisk indicates statistical significant difference as compared to injection of agonist only ($P < 0.05$; $n = 6$).

the grooming assay, whereas Nle-γ-MSH did not induce grooming (Fig. 3).

### 3.5. Grooming behavior induced by i.v. injections of potent melanocortins

Rats were injected i.v. with 100 μg α-MSH, [Nle$^4$-D-Phe$^7$]α-MSH, melanotan-II, RMI-2001, or RMI-2004 and grooming behavior was recorded (Fig. 4). Melanotan-II potently induced grooming behavior, whereas [Nle$^4$-D-Phe$^7$]α-MSH and RMI-2001 under these experimental conditions were less active but still induced significantly more grooming behavior than i.v. injection of saline. Systemic injection of RMI-2004 or α-MSH did not stimulate grooming behavior.

### 3.6. Interaction of the melanocortin receptor antagonist SHU9119 with the α-MSH derived agonists in the grooming assay

The potency and the receptor selectivity of SHU9119 prompted us to test this peptide in the grooming assay. First, we observed that i.c.v. injection of SHU9119 alone did not induce any signs of toxicity or behavioral abnormalities at doses up to 1 nmol. Secondly, we investigated the effective dose of SHU9119 to block melanocortin-induced grooming. SHU9119 was injected into the brain together with the agonist as a single mixture. Excessive grooming was induced by i.c.v. injection of a submaximal dose (9 pmol) of melanotan-II. Fig. 5a shows the inhibitory dose–response curve for SHU9119 in melanotan-II-induced grooming. 90 pmol of SHU9119 was the lowest effective dose and therefore, this dose was chosen for subsequent experiments. This dose also blocked α-MSH-induced grooming, again, 45 pmol SHU9119 was not effective. Also, RMI-2005 inhibited α-MSH-induced grooming behavior (Fig. 5b).

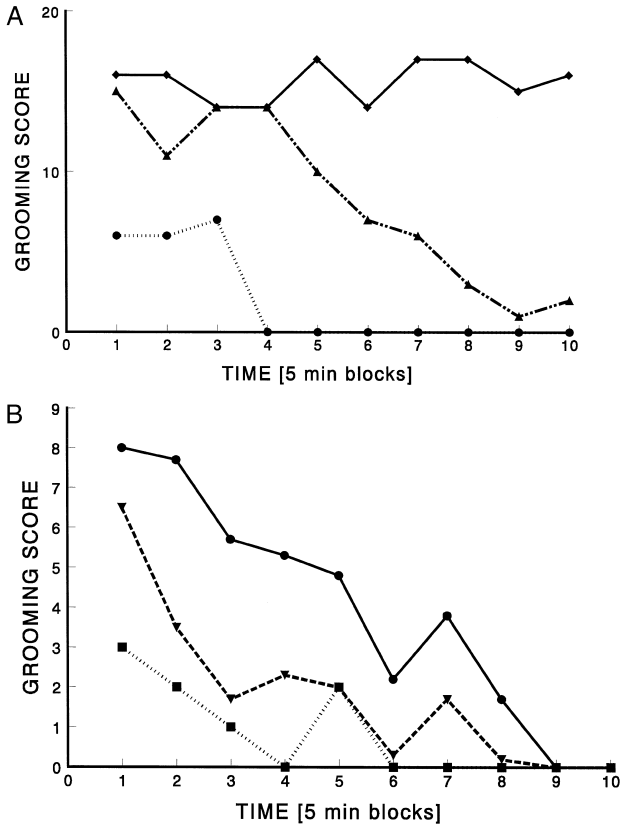


Fig. 7. (A) The time course of SHU9119 antagonism on α-MSH-induced grooming. Rats received an i.c.v. injection per rat with either 900 pmol α-MSH (♦), a mixture of 900 pmol α-MSH and 90 pmol SHU9119 (▲) or were pre-injected with 90 pmol of SHU9119, 15 min before the injection of 900 pmol α-MSH (●). Grooming was scored 15 min following the last i.c.v. injection. (B) The time course of SHU9119 antagonism on novelty-induced grooming. Rats were exposed for the first time to an observation box after receiving an i.c.v. injection with either saline 15 min before the test (♦), 90 pmol SHU9119 per rat 15 min before the test (▲) or 90 pmol SHU9119 per rat 30 min and saline 15 min before the test (●).

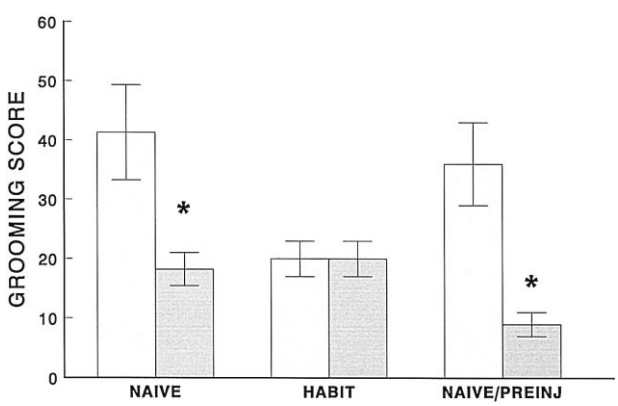


Fig. 6. Melanocortin receptor antagonist SHU9119 inhibits novelty-induced grooming. Rats were exposed either for the first time (NAIVE) or for the fourth time (HABIT) to an observation box after receiving for the first time an i.c.v. injection with either saline (open bars) or 90 pmol SHU9119 per rat (grey bars). Grooming was scored either 15 min (NAIVE and HABIT groups) or 30 min (NAIVE/PREINJ) following i.c.v. injections. An asterisk indicates statistical significant difference as compared to injection of saline only ($P < 0.05$; $n = 6$).

In another set of experiments, we tested whether SHU9119 inhibited the action of melanotan-II in a competitive manner. Therefore, we increased the melanotan-II dose from 9 to 45 pmol, while the dose of SHU9119 remained the same (90 pmol) (Fig. 5c). This reversed the inhibitory effect of SHU9119 (90 pmol). Next, we analysed the antagonistic potency of SHU9119 on grooming induced by another agonist. 90 pmol of SHU9119 inhibited the grooming response when 0.6, 1.5 or 2.5 pmol of [Nle$^4$-D-Phe$^7$]α-MSH were injected. [Nle$^4$-D-Phe$^7$]α-MSH regained activity, when its dose was increased from 2.5 pmol to 4.5 and 9 pmol [Nle$^4$-D-Phe$^7$]α-MSH in the presence of 90 pmol SHU9119. This indicated that SHU9119 acts as a competitive antagonist in the brain. Therefore, we used SHU9119 in further experiments.

### *3.7. The time course of the effect of SHU9119 on novelty and α-MSH-induced grooming*

Next, it was tested whether SHU9119 can block novelty-induced grooming. As shown in Fig. 6, injection of 90 pmol of SHU9119 (injection was at time 0, just prior to placement of a rat into the novel environment, the observation started 15 min later) reduced significantly the grooming response. The grooming score of SHU9119-treated rats ($18 \pm 3$) was similar to that displayed by rats that were habituated to this experimental situation during at least three sessions (score $20 \pm 3$). When we injected SHU9119, 30 min before rats were exposed to novelty, the decrease in the grooming response was even larger ($9 \pm 2$) than grooming displayed by habituated animals.

Fig. 7a shows the time course of SHU9119 inhibition of rat grooming behavior induced by α-MSH. Delivery of α-MSH and SHU9119 as a mixture produced down-regulation of the grooming response which was most pronounced in the second part of the grooming test (25 min after onset of behavioral observation). Similar results were obtained for melanotan-II and [Nle$^4$-D-Phe$^7$]α-MSH induced grooming. When SHU9119 was introduced 30 min before α-MSH (45 min before the start of the behavioral observation) grooming scores were lowered significantly over the whole observation period. However, the grooming response was still present in the first phase (first 20 min of the observation).

Fig. 7b displays the time course of the effect of SHU9119 on novelty-induced grooming. Compared to α-MSH-evoked grooming, novelty-induced grooming had a shorter duration and showed a continuous decline during the observation period. Injection of SHU9119, immediately or 30 min before exposure to novelty, reduced the grooming response.

## 4. Discussion

Here we identify RMI-2001 and [D-Tyr$^4$]melanotan-II as selective agonists which bind with significantly higher affinity to the rat melanocortin $MC_4$ receptor than to the rat melanocortin $MC_3$ receptor. Nle-γ-MSH was identified as a melanocortin receptor agonist with selectivity for the melanocortin $MC_3$ receptor as compared to the melanocortin $MC_4$ receptor. We confirmed that α-MSH, [Nle$^4$-D-Phe$^7$]α-MSH, melanotan-II and SHU9119 do not, or discriminate less between the melanocortin $MC_3$ and the melanocortin $MC_4$ receptor (Hruby et al., 1995). RMI-2004 also did not discriminate between these receptors. RMI-2005 was identified as antagonist for these receptors with slightly higher affinity for melanocortin $MC_4$ receptor than for melanocortin $MC_3$ receptor.

All ligands except α-MSH and Nle-γ-MSH, had a D-enantiomere where L-Phe is present in the natural melanocortins (Fig. 1). It has been demonstrated before that the nature of the residue at this position influenced receptor affinity and activity (Sawyer et al., 1980; De Wied and Jolles, 1982; Hruby et al., 1993, 1995; Hadley et al., 1996). Introduction of D-Phe leads to higher affinity and activity, whereas introduction of D-2-naphthylalanine leads to a higher affinity and antagonism/partial agonism of the melanocortin $MC_3$ receptor and melanocortin $MC_4$ receptor. As shown in Table 1, introduction of D-Phe as in [Nle$^4$-D-Phe$^7$]α-MSH, melanotan-II, RMI-2001 and RMI-2004 leads to a higher increase in activity as would be expected based upon the increase in affinity. This effect was more pronounced for melanocortin $MC_4$ receptor than for melanocortin $MC_3$ receptor. Thus, D-Phe in these ligands appears to stabilize an active receptor conformation. Introduction of a D-Tyr in melanotan-II as in [D-Tyr$^4$]melanotan-II led to a slight decrease in affinity for melanocortin $MC_4$ receptor (being two-fold less), but a large decrease of affinity for melanocortin $MC_3$ receptor (being 30-fold less). Thus, introduction of D-Tyr demonstrates that this position can also contribute to receptor selectivity.

SHU9119 is a derivative of melanotan-II in which D-Phe was replaced with D-2-naphthylalanine at position 7. This substitution changed the melanocortin receptor agonist into a potent competitive antagonist (Hruby et al., 1995). It had been shown that SHU9119 inhibited only the human melanocortin $MC_3$ and $MC_4$ receptors ($pA_2$ value 8.3 and 9.3, respectively) but not human melanocortin $MC_1$ and $MC_5$ receptors (Hruby et al., 1995). We confirmed here that SHU9119 inhibited rat melanocortin $MC_3$ and rat melanocortin $MC_4$ receptor activation (Fig. 2) (Huang et al., 1997). RMI 2005, which has a disulphate bridge between positions 4 and 10 instead of a lactam bridge between positions 5 and 10 (as in SHU9119), also inhibited rat melanocortin $MC_3$ and $MC_4$ receptor activation. Thus, regardless of the type of cyclization, introduction of D-2-naphthylalanine results in antagonism, however, introduction of a disulphate bridge results in (more pronounced) receptor selectivity. This underscores the recent finding by Schioth et al. (1998) who also reported that introduction of D-2-naphthylalanine and cyclization by a disulphate bridge leads in melanocortin analogues leads to antagonism for the melanocortin $MC_3$ and $MC_4$ receptors. Both SHU9119 and RMI-2005 were able to block melanocortin-induced grooming behavior in the rat.

Introduction of a disulphate bridge as in RMI-2001 increased the selectivity for the rat melanocortin $MC_4$ receptor, whereas lactam cyclization as in RMI-2004 did not influence melanocortin $MC_3/MC_4$ receptor selectivity. RMI-2004 had similar affinity as melanotan-II, suggesting that the three N-terminal and the three C-terminal amino acids in RMI-2004 did not influence receptor affinity. However, N- and C-terminal elongation of melanotan-II, as in RMI-2004, decreased the potency on the rat melanocortin $MC_4$ receptor, suggesting that these elonga-

tions influence receptor selectivity for activity more than for affinity.

Although one should keep in mind that in the brain pharmacokinetical properties of peptides may substantially determine their activity, using these ligands in a rat grooming assay suggested that melanocortin-induced grooming behavior is mediated via the melanocortin $MC_4$ receptor. The potency order of ligands able to induce grooming behavior fits very well with that of the potency order of these ligands to bind and to stimulate the rat melanocortin $MC_4$ receptor in vitro. For instance, (1) Nle-γ-MSH did not significantly induce grooming behavior at the highest tested dose, (2) RMI-2001 is more potent than RMI-2004 to stimulate grooming behavior in vivo and the melanocortin $MC_4$ receptor in vitro, whereas these ligands have similar potencies on the melanocortin $MC_3$ receptor, (3) [D-Tyr$^4$]melanotan-II does induce excessive grooming behavior at lower doses than α-MSH, whereas its potency on the rat melanocortin $MC_3$ receptor in vitro is less than α-MSH.

Interestingly, using these potent melanocortin analogs, for the first time also grooming behavior could be elicited following i.v. injection. Melanotan-II was the most potent ligand suggesting that melanotan-II reached more efficiently the sites in the brain mediating melanocortin-induced grooming behavior. The apparent effective passage through the blood brain barrier might be related to the relative low molecular weight and enhanced lipophilic nature of melanotan-II as compared to the other ligands. RMI-2004 and α-MSH, which have a lower potency on the melanocortin $MC_4$ receptor, did not induce grooming behavior when i.v.-injected.

Both the in vitro and the in vivo experiments revealed that SHU9119 inhibited MC receptor activation by agonists in a competitive manner. The lowest effective dose of SHU9119 was able to block melanocortin-induced grooming behavior elicited by submaximal doses of [Nle$^4$-D-Phe$^7$]α-MSH, melanotan-II and α-MSH, suggesting that these ligands act at the same receptor subtype.

Furthermore, we demonstrated here that a potent and selective MC receptor antagonist, SHU9119, inhibited a physiological response of the rat, i.e., grooming evoked by exposure to a novel environment. Since SHU9119 acts as competitive MC receptor antagonist in vivo, this strongly suggests that the exposure to a novel environment activates the melanocortin system. Furthermore, the time course of the effect of SHU9119 to inhibit α-MSH-induced grooming is similar to the time course of SHU9119 inhibiting novelty-induced grooming. In both cases, the effect of SHU9119 is most pronounced in the second phase of the grooming response, which previously was shown to be most susceptible to pharmacological intervention (Gispen and Isaacson, 1986).

Taken together, our present and previous data suggest that the brain melanocortin system has a physiological role in a novelty-induced stress response (grooming). For, (1) SHU9119 blocks novelty-induced grooming (Fig. 6); (2) i.c.v. administration of melanocortin receptor agonists stimulate the activity of the hypothalamus–pituitary–adrenal axis via activation of melanocortin $MC_4$ receptors (Von Frijtag et al., 1998); (3) repeated exposure to a novel environment (habituation) results in less grooming activity (Fig. 6); (4) the grooming activity of a habituated rat is comparable to that when exposure to a novel environment is preceded by an i.c.v. injection with SHU9119 (Fig. 6).

We have pharmacologically characterized melanocortin receptor analogs on the rat melanocortin $MC_3$ receptor and melanocortin $MC_4$ receptor and on melanocortin-induced grooming behavior. In the absence of purely selective ligands, we demonstrate that careful comparison of activity profiles of sets of melanocortin ligands were helpful to delineate which receptor subtype is involved in an effect mediated via melanocortin receptors, namely grooming behavior. These new pharmacological tools may be helpful in delineating how melanocortins regulate physiological processes such as food intake, body temperature and inflammation.

## Acknowledgements

We thank Simone Duis, Linda Nyberg and Mario Lambrechts for their contribution in performing the experiments. This work was supported by David De Wied fellowship to A.W.S. and by NWO 903-49-162 to J.O.

## References

Adan, R.A.H., Cone, R.D., Burbach, J.P.H., Gispen, W.H., 1994a. Differential effects of melanocortin peptides on neural melanocortin receptors. Mol. Pharmacol. 46, 1182–1190.

Adan, R.A.H., Oosterom, J., Ludvigsdottir, G., Brakkee, J.H., Burbach, J.P.H., Gispen, W.H., 1994b. Identification of antagonists for MC3, MC4 and MC5 receptors. Eur. J. Pharmacol. 269, 331–337.

Alvaro, J.S., Tatro, J.B., Quillan, J.M., Fogliano, M., Eisenhard, M., Lerner, M.R., Nestler, E.J., Duman, R.J., 1996. Morphine down-regulates melanocortin-4 receptor expression in brain regions that mediate opiate addiction. Mol. Pharmacol. 50, 583–591.

Brakkee, J.H., Wiegant, V.M., Gispen, W.H., 1979. A simple technique for rapid implantation of a permanent cannula into the rat brain ventricular system. Lab. Anim. Sci. 29, 78–81.

Chen, W., Shields, T.S., Stork, P.J.S., Cone, R.D., 1995. A colorimetric assay for measuring activation of Gs and Gq coupled signaling pathways. Anal. Biochem. 226, 349–354.

Chhajlani, V., Muceniece, R., Wikberg, J.E.S., 1993. Molecular cloning of a novel human melanocortin receptor. Biochem. Biophys. Res. Commun. 195, 866–873.

De Wied, D., Jolles, J., 1982. Neuropeptides derived form pro-opiomelanocortin: behavioral, physiological, and neurochemical effects. Physiol. Rev. 62, 976–1059.

Dunn, A.J., Green, E.J., Isaacson, R.L., 1979. Intracerebral adreno-corticotrope hormone mediates novelty-induced grooming in the rat. Science 203, 281–283.

Fan, W., Boston, B.A., Kesterson, R.A., Hruby, V.J., Cone, R.D., 1997. Role of melanocortinergic neurons in feeding and the agouti obesity syndrome. Nature 385, 165–168.

Ferrari, W., 1958. Behavioural changes in animals after intracisternal injection with adrenocorticotrophic hormone and melanocyte stimulating hormone. Nature (London) 181, 925–926.

Fong, T.M., Mao, C., MacNeil, T., Kalyani, R., Smith, T., Weinberg, D., Tota, M.R., Van der Ploeg, L.H., 1997. ART (protein product of agouti-related transcript) as an antagonist of MC-3 and MC-4 receptors. Biochem. Biophys. Res. Commun. 237, 629–631.

Gispen, W.H., Isaacson, R.L., 1986, Excessive grooming in response to ACTH. In: De Wied, D., Gispen, W.H., Van Wimersma Greidanus, Tj.B. (Eds.), Neuropeptides and Behavior. Vol. 1, Pergamon, Oxford, p. 273.

Gispen, W.H., Wiegant, V.M., Greven, H.M., De Wied, D., 1975. The induction of excessive grooming in the rat by intraventricular application of peptides derived from ACTH: structure–activity studies. Life Sci. 17, 645–652.

Hadley, M.E., Hruby, V.J., Jiang, J., Sharma, S.D., Fink, J.L., Haskell-Luevano, C., Bentley, D.L., Al-Obeidi, F., Sawyer, T.K., 1996. Melanocortin receptors: identification and characterization by melanotropic peptide agonists and antagonists. Pigm. Cell Res. 9, 213–234.

Hruby, V.J., Sharma, S.D., Toth, K., Jaw, J.Y., Al-Obeidi, F., Sawyer, T.K., Hadley, M.E., 1993. Design, synthesis, and conformation of superpotent and prolonged acting melanotropins. Ann. NY Acad. Sci. 680, 51–63.

Hruby, V.J., Lu, D., Sharma, S.D., Castrucci, A.L., Kesterson, R.A., Al-Obeidi, F., Hadley, M.A., Cone, R.D., 1995. Cyclic lactam alfa-melanotropin analogues of Ac-Nle$^4$-cyclo(Asp$^5$,D-Phe$^7$,Lys$^{10}$)alfa-melanocyte stimulating hormone-(4–10)-$NH_2$ with bulkyaromatic amino acids at position 7 show high potency and selectivity at specific melanocortin receptors. J. Med. Chem. 38, 3454–3460.

Huang, Q.H., Entwistle, M.L., Alvaro, J.D., Duman, R.S., Hruby, V.J., Tatro, J.B., 1997. Antipyretic role of endogenous melanocortins mediated by central melanocortin receptors during endotoxin-induced fever. J. Neurosci. 17, 3343–3351.

Huszar, D., Lynch, C.A., Fairchild-Huntress, V., Dunmore, J.H., Fang, Q., Berkemeier, L.R., Gu, W., Kesterson, R.A., Boston, B.A., Cone, R.D., Smith, F.J., Campfield, L.A., Burn, P., Lee, F., 1997. Targeted disruption of the melanocortin-4 receptor results in obesity in mice. Cell 88, 131–141.

Low, M.J., Simerley, R.B., Cone, R.D., 1994. Receptors for the melanocortin peptides in the central nervous system. Curr. Opin. Endocrinol. Diab. 1, 79–88.

Lu, D., Willard, D., Patel, I.R., Kadwell, S., Overton, S., Kost, T., Luther, M., Chen, W., Woychik, R.P., Wilkison, W.O., Cone, R.D., 1994. Agouti protein is an antagonist of the melanocortin-stimulating-hormone receptor. Nature 371, 799–802.

Mountjoy, K.G., Mortrud, M.T., Low, M.J., Simerley, R.B., Cone, R.D., 1994. Cloning and functional characterization of a melanocortin receptor (MC4-R) localized in neuroendocrine and autonomic circuitry in the brain. Mol. Endocrinol. 8, 1298–1308.

Ollmann, M.M., Wilson, B.D., Yang, Y.K., Kerns, J.A., Chen, Y., Gantz, I., Barsh, G.S., 1997. Antagonism of central melanocortin receptors in vitro and in vivo by agouti-related protein. Science 278, 135–138.

Roselli-Rehfuss, L., Mountjoy, K.G., Robbins, L.S., Mortrud, M.T., Low, M.J., Tatro, J.B., Entwistle, M.L., Simerly, R.B., Cone, R.D., 1993. Identification of a receptor for melanotropin and other proopiomelanocortin peptides in the hypothalamus and limbic system. Proc. Natl. Acad. Sci. U.S.A. 90, 8856–8860.

Sawyer, T.K., Sanfilippo, P.J., Hruby, V.J., Engel, M.H., Heward, C.B., Burnett, J.B., Hadley, M.E., 1980. 4-Norleucine,7-D-phenylalanine-α-melanocyte-stimulating hormone: a highly potent α-melanotropin with ultralong biological activity. Proc. Natl. Acad. Sci. U.S.A. 77, 5754–5758.

Schaaper, W.M.M., Adan, R.A.H., Posthuma, T.A., Oosterom, J., Gispen, W.H., Meloen, R.H., 1998. Synthesis of cyclic α-MSH peptides. Letters in Peptide Science 5, 1–4.

Schioth, H.B., Mutulis, F., Muceniece, R., Prusis, P., Wikberg, J.E., 1998. Discovery of novel melanocortin4 receptor selective MSH analogues. Br. J. Pharmacol. 124, 75–82.

Shutter, J.R., Graham, M., Kinsey, A.C., Scully, S., Luthy, R., Stark, K.L., 1997. Hypothalamic expression of ART, a novel gene related to agouti, is up-regulated in obese and diabetic mutant mice. Genes Dev. 11, 593–602.

Spampinato, S., Canossa, M., Carboni, L., Campana, G., Leanza, G., Ferri, S., 1994. Inhibition of proopiomelanocortin expression by an oligonucleotide complementary to beta-endorphin mRNA. Proc. Natl. Acad. Sci. U.S.A. 91, 8072–8076.

Spruijt, B.M., De Graan, P.N.E., Eberle, A.N., Gispen, W.H., 1985. Comparison of structural requirements of αMSH and ACTH for inducing excessive grooming and pigment dispersion. Peptides 6, 1185–1189.

Spruijt, B.M., Van Hooff, J.A.R.A.M., Gispen, W.H., 1992. Ethology and neurobiology of grooming behavior. Physiol. Rev. 72, 825–852.

Tatro, J.B., 1990. Melanotropin receptors in the brain are differentially distributed and recognize both corticotropin and α-melanocyte stimulating hormone. Brain Res. 536, 124–132.

Vergoni, A.V., Bertolini, A., Mutulis, F., Wikberg, J.E., Schioth, H.B., 1998. Differential influence of a selective melanocortin MC4 receptor antagonist (HS014) on melanocortin-induced behavioral effects in rats. Eur. J. Pharmacol. 362, 95–101.

Von Frijtag, J.C., Croiset, G., Gispen, W.H., Adan, R.A.H., Wiegant, V.M., 1998. The role of central melanocortin receptors in the activation of the hypothalamus–pituitary–adrenal-axis and the induction of excessive grooming. Br. J. Pharmacol. 123, 1503–1508.