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Review

Multimeric α-MSH has increased efficacy to activate the melanocortin MC4 receptor ☆

Birgitte Tiesjema, Myrte Merkestein, Keith M. Garner, Mariken de Krom, Roger A.H. Adan *

Rudolf Magnus Institute of Neuroscience, Department of Neuroscience and Pharmacology, University Medical Center Utrecht, The Netherlands

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Abstract

α-Melanocyte stimulating hormone (α-MSH) has a relatively low affinity for the melanocortin MC4 receptor. Constructs of multimeric α-MSH varying from one to eight subunits were synthesized to test whether they displayed an improved ability to bind to and activate the human melanocortin MC4 receptor. α-MSH subunits were coupled by a flexible linker and placed in front of an IRES-eGFP sequence. Efficacy for activation of the melanocortin MC4 receptor increased with every extra subunit, resulting in a 100 fold lower EC50 value of α-MSH8 when compared with α-MSH1. Furthermore, supernatant of cells transfected with α-MSH8 proved to have an increased affinity to the melanocortin MC4 receptor when compared to cells transfected with the other multimers. Together, these data show that multimeric α-MSH has improved ability to activate the human melanocortin MC4 receptor *in vitro*.

Keywords: α-MSH; Melanocortin; Melanocortin MC4 receptor

Contents

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* Corresponding author. Rudolf Magnus Institute of Neuroscience, Department of Neuroscience and Pharmacology, University Medical Center Utrecht, Str. 5.203, Universiteitsweg 100, 3584 CG Utrecht, The Netherlands. Tel.: +31 88 75 68517; fax: +31 88 75 69032.
E-mail address: r.a.h.adan@umcutrecht.nl (R.A.H. Adan).

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1. Introduction

Melanocortins are a family of peptides derived from the precursor protein proopiomelanocortin (POMC). The melanocortin system plays a key role in the central control of energy homeostasis and feeding. Situations where POMC expression is low, as in leptin- or leptin receptor-deficient mice, are associated with obesity. In addition, mutations in the POMC gene itself, in genes necessary for the processing of POMC and in the genes encoding the melanocortin MC3 or melanocortin MC4 receptor result in obese phenotypes (Chen et al., 2000; Huszar et al., 1997; Jackson et al., 1997; Krude et al., 1998; Yaswen et al., 1999). Mice that are POMC deficient are obese, due to hyperphagia, reduced basal oxygen consumption and an altered lipid metabolism (Challis et al., 2004; Smart and Low, 2003; Yaswen et al., 1999). In line with this, transgenic overexpression of POMC under control of the NSE promoter in mice results in a slight decrease in body weight gain and also in reduced fasting-induced hyperphagia (Mizuno et al., 2003). Moreover, transgenic overexpression of (N-terminal) POMC or hypothalamic injection of recombinant adeno-associated virus (rAAV) encoding POMC ameliorates the obese phenotype of aged rats and genetically obese leptin- or leptin receptor-deficient mice and rats (Li et al., 2003, 2005; Mizuno et al., 2003; Savontaus et al., 2004).

It is hypothesized that the ameliorating effects of POMC on obesity and metabolic function are mediated by the melanocortins α-melanocyte stimulating hormone (α-MSH) and β-MSH, products of POMC. Central α-MSH injections for 6 days in wildtype mice transiently reduce food intake and body weight (McMinn et al., 2000). In addition, administration of an α-MSH analog reduces body weight of POMC$^{-/-}$ mice (Yaswen et al., 1999). Also β-MSH inhibits food intake in fasted and non-fasted rats (Abbott et al., 2000; Kask et al., 2000), and γ-MSH is, because it is a strong agonist for the MC3 receptor, also thought to play a role in the regulation of energy balance. Nevertheless, another cleavage product of POMC is β-endorphin, which in contrast to the melanocortins, increases food intake, making the control of energy homeostasis by POMC complicated (McKay et al., 1981). Recently, it was demonstrated that rats with AAV-mediated overexpression of the POMC cDNA in the arcuate nucleus display a slightly reduced amount of perirenal fat on normal chow, but surprisingly increased energy intake and gained more body weight when exposed to a high fat diet (Li et al., 2007). Although compensatory mechanisms may counteract increased melanocortin signaling in these rats, one cannot exclude that the increased body weight gain on a high fat diet is the result of increased signaling by β-endorphin. Opioid signaling increased fat intake on choice diets (Welch et al., 1994). In order to further clarify the specific role of melanocortin receptors in energy balance it would therefore be necessary to chronically increase melanocortins, without altering concentrations of the other POMC products, in particular β-endorphin. Because the affinity of α-MSH for the melanocortin MC4 receptor is rather low and the half-life of α-MSH is relatively short, high concentrations of α-MSH are needed to elicit effects via the melanocortin MC4 receptor. In addition, long-term infusions of a ligand locally in the brain are not feasible, making it difficult to explore the site-specific effects of α-MSH in the regulation of energy balance. To overcome this problem recombinant adeno-associated viral (rAAV) particles encoding α-MSH can be used, which, once injected into the brain, will result in a stable, long-term overexpression of the transgene (Tal, 2000).

Multivalant ligands often have increased binding affinity for their targets (Kiessling et al., 2000). Firstly, multivalent ligands increase the local concentration of binding elements, thereby increasing the chance that the ligand will bind its receptor. Secondly, subsequent binding to a second binding site of an oligomeric receptor can be facilitated when a ligand has more binding elements (avidity). Finally, multivalent ligands can promote receptor clustering, which can be necessary to activate signaling pathways (Kiessling et al., 2000). Melanocortin receptors have been proposed to occur as constitutively preformed dimers (Kopanchuk et al., 2006; Mandrika et al., 2005). In addition, there is evidence that the melanocortin receptors have interacting binding sites and that they display by cooperative binding (Kopanchuk et al., 2005; Kopanchuk et al., 2006). Therefore, they form an excellent receptor type to target with multimeric ligands. Indeed, oligomers of NDP-MSH fragments were found to bind with a higher affinity to both the MC1 and the melanocortin MC4 receptor than monomeric NDP-MSH (Brandenburger et al., 1999; Vagner et al., 2004).

To be able to study the contribution of a chronic α-MSH overexpression in distinct nuclei of the hypothalamus to melanocortinergic regulation of energy balance, we have built rAAV viral vectors encoding multimers of α-MSH cDNA. In this study, the synthesis of these multimers is described. Further, we evaluated the *in vitro* ability of these multimers to bind to and activate the melanocortin MC4 receptor.

2. Materials and methods

2.1. Cloning of α-MSH multimers

A signal sequence (belonging to the Von Willebrand factor), followed by an HA-tag was synthesized using the following (partly overlapping) primers: forward1: 5′GGTGCTGCTTGCTCTGGCCCTCATTTTGCCAGGGACCCTTTGTTACCCCTACGACG 3′, forward2: 5′TGTCCTCGAGGGCCATGATTCCTGCCAGATTTGCCGGGGTGCTGCTTGCTCTGG 3′ and reverse1: 5′CATCTGAGCATGTCAAAATCTGGCCAGGCGTAGTCGGGCACGTCGTAGGGGTAAC 3′. Primers were ligated, filled in using Klenow (USB Corporation, Cleveland, Ohio) and then cloned into pCRscript (Stratagene, La Jolla, California).

α-MSH monomeric cDNA was synthesized with primers (forward: 5′ACGCACCGGTCTCACCCCGCCTGGTTCTTCATCCTATTCCATGGAACACTTCAGGTGGGGA 3′ and reverse: 5′GAATTCACGTCTCCGGGGTGGAGGGTTTAGGCACAGGCTTTCCCCACCTGAAGTGTTCCAT 3′) that are complementary to rat α-MSH cDNA including the hinge region of immunoglobulin heavy G3 chain as linker (Pro-Lys-Pro-Ser-Thr-Pro-Pro-Gly-Ser-Ser). Primers were ligated,

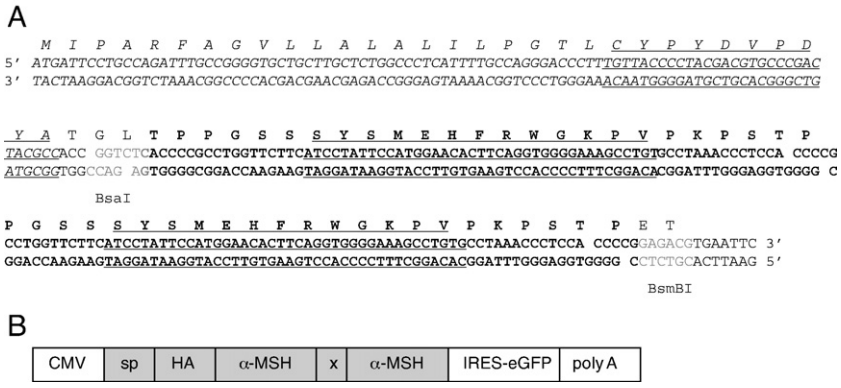

Fig. 1. A. Nucleotide and aminoacid sequence of α-MSH2. Nucleotides and aminoacids in italic encode the signal peptide, nucleotides and aminoacids in italic and underlined encode the HA-tag, nucleotides and aminoacids in bold encode the linker sequence and nucleotides and aminoacids in bold and underlined encode an α-MSH subunit. In grey the recognition sites for BsaI and BsmBI are depicted. Interspaces depict the restriction sites and separation of the two α-MSH monomers. B. Structure of the pIRES2-α-MSH2 multimer construct, containing a CMV promoter, the signal sequence of the Von Willebrand factor, an HA-tag and the α-MSH subunits, coupled by a linker (x), followed by an IRES, eGFP and SV40 late polyadenylation signal (SV40 pA signal).

filled in and then cloned into pCRscript. Monomeric α-MSH was then cloned behind the signal peptide-HA-tag construct using AgeI and EcoRI. α-MSH multimers were synthesized by inserting BsaI/BsmBI digestion products of the monomer in a monomer linearized with BsmBI, resulting in α-MSH1, α-MSH2, α-MSH4 and α-MSH8 (Fig. 1A).

The monomer and multimers were then cloned into pIRES2-eGFP (USB Corporation, Cleveland, Ohio), using XhoI and EcoRI. (Fig. 1B).

2.2. Cell culture and transfection

Human embryonal kidney (HEK)293 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Paisley, Scotland) supplemented with 10% (v/v) fetal calf serum (FCS, Integro, Zaandam, The Netherlands), 2 mM glutamine (Gibco, Paisley, Scotland), 100 units/ml penicillin, 100 units/ml streptomycin and non-essential aminoacids (NEAA, Gibco, Paisley, Scotland). Cells were cultured in a humidified atmosphere and 5% CO_2 at 37 °C.

DNA was transfected into cells with a standard calcium phosphate precipitation protocol. HEK293 cells cultured in 10 cm dishes were transfected with 10 μg of DNA of the α-MSH multimers or pIRES2. One day after transfection, medium was replaced by DMEM supplemented with 2% FCS, 2 mM glutamine, 100 units/ml penicillin, 100 units/ml streptomycin and non-essential aminoacids. Two days later, supernatant of cells transfected with the α-MSH multimers was harvested, concentrated 10 times using YM-3 Centricon filter units (Milipore, Billerica, Massachusetts) aliquotted and stored at −20 °C until further use. For the reporter gene assay HEK293 cells cultured in 10 cm dishes were cotransfected with 50 ng human melanocortin MC4 receptor DNA and 10 μg of cAMP-Responsive Element (CRE)-LacZ construct. For the binding assay, the melanocortin MC4 receptor was expressed in HEK293 cells by transfecting cells cultured in 10 cm dishes with 10 μg of DNA.

2.3. Western blot

For the preparation of cell lysates, cells transfected with the α-MSH multimers or pIRES2 were washed twice with PBS and incubated with M-PER protein extraction reagent (Pierce, Rockford, Illinois) for 5 min at room temperature. Cell debris was removed from the samples by centrifugation at 13,000 g for 5 min and supernatant was collected, aliquotted and stored at −20 °C until further use.

Samples and standards (Kaleidoscope polypeptide standards, Bio-Rad laboratories, Hercules, Canada) were run on a 12% polyacrylamide gels using the tris tricine buffer system (Schagger and von Jagow, 1987). Separated proteins were transferred to nitrocellulose membranes (Hybond C, Amersham Biosciences, Freiburg, Germany). Blots were blocked with 10% (w/v) non-fat milk powder, 0.05% Tween-20 in tris buffered saline (TBS) at room temperature while shaking and incubated overnight at 4 °C with 1:1000 diluted mouse-anti-GFP antibody (Roche Diagnostics, Penzberg, Germany). Immunoreactivity was visualized using a peroxidase-conjugated secondary antibody and SuperSignal West Dura Extended Duration Substrate (Pierce, Rockford, USA) on a BioRad Fluor-S Multi-imager and analyzed with Quantity-One (BioRad, Tokyo, Japan).

2.4. Radioligand binding assay

IC_{50} values were determined by displacement of ^{125}I-[Nle^4, D-Phe^7]-MSH (NDP-MSH, PerkinElmer, Brussels, Belgium). Transfected HEK293 cells growing in 24-well plates were washed with TBS supplemented with 2.5 mM calcium chloride

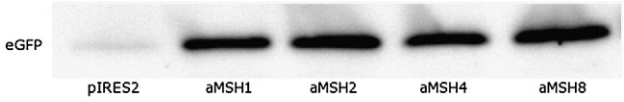

Fig. 2. Western blot of cell lysates of HEK-293 cells transfected with pIRES2 or α-MSH multimers and stained using an eGFP antibody.

Table 1
IC_{50} values and EC_{50} values of the α-MSH multimers

Multimer	Binding	Activation
	IC_{50} (LOG dilution)	EC_{50} (LOG dilution)
α-MSH1	nd	−0.9439
α-MSH2	nd	−2.015
α-MSH4	nd	−2.310
α-MSH8	−1.026	−3.024

IC_{50} values were obtained by displacement of iodinated NDP-MSH. EC50 values were determined in a *LacZ* reporter gene assay. IC_{50} values and EC_{50} values are given as LOG values of the dilution of the supernatant. nd: not possible to determine due to limited displacement.

and incubated for 30 min at RT with ^{125}I-NDP-MSH and various concentrations of the multimers diluted in HAM's F10 medium (Gibco, Paisley, Scotland) supplemented with 2.5 mM calcium chloride, 0.25% BSA (ICN, Aurora, USA) and 200 KIU/ml aprotinin (Sigma, Steinheim, Germany). Cells were rinsed twice with ice-cold TBS supplemented with 2.5 mM calcium chloride to remove non-bound tracer and lysed in 1 M sodium hydroxide. Samples were then counted in a γ-counter.

2.5. Reporter gene assay

Activation of melanocortin MC4 receptors was determined using LacZ as a reporter gene (Chen et al., 1995). One day after transfection, cells were dispensed into 96-well plates. After two days, cells were incubated at 37 °C with α-MSH or with the concentrated supernatant containing the multimers diluted in half-LOG units in serum-free medium (DMEM containing 0.2% BSA (ICN, Aurora, USA). After 5–6 h, the assay medium was aspirated and replaced by 40 µl of lysis buffer (PBS containing 0.1% Triton-X-100 (Boehringer, Mannheim, Germany)). 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 measured in a Victor^2 microplate reader (PerkinElmer, Brussels, Belgium).

2.6. Data analysis

Data of the binding assay and the reporter assay were analyzed using GraphPad Prism (GraphPad Software Inc, San Diego, California). Competition curves were fitted from 6 duplicate data points (12 for α-MSH) using the sigmoidal dose–response curve (variable slope) classical Eq. for non-linear regression analysis and IC_{50} values were calculated. Differences in maximal displacement data were assessed using one-way ANOVA, followed by Tukey's post-hoc tests. In the reporter assay for each curve 12 duplicate data points were collected and EC_{50} values were determined by fitting the data to a sigmoidal dose–response curve with variable slope.

3. Results

3.1. Transfection of HEK293 cells with α-MSH multimers

HEK293 cells were transfected with the different α-MSH multimers or pIRES2-eGFP. Supernatant was collected and used in a binding assay and a reporter gene assay. The cells were lysed and analyzed for eGFP expression. Fig. 2 shows a Western blot of the cell lysates of the cells transfected with the α-MSH multimers. Cell lysates of cells transfected with the multimers contained a single band that ran at the same height as the eGFP band from cell lysate of the cells transfected with pIRES2-eGFP. All the multimers showed a similar expression of eGFP, which was higher than the eGFP expression of pIRES2-eGFP. This indicates that similar amounts of vector were introduced in cells expressing the multimers.

3.2. Binding properties of multimeric α-MSH

Competition binding showed that supernatant from cells transfected with α-MSH1, α-MSH2 and α-MSH4 displayed similar (low) binding properties to the human melanocortin MC4 receptor, whereas supernatant from cells transfected with α-MSH8 had a slightly higher affinity (data not shown). No IC_{50} values for α-MSH1, α-MSH2 and α-MSH4 could be

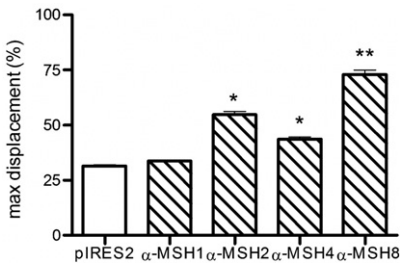

Fig. 3. Displacement of human melanocortin MC4 receptor bound ^{125}I-NDP-MSH by a 2-fold dilution of concentrated supernatant of cells transfected with the multimers. *P<0.05 vs pIRES2 and α-MSH1, **P<0.01 vs all other multimers.

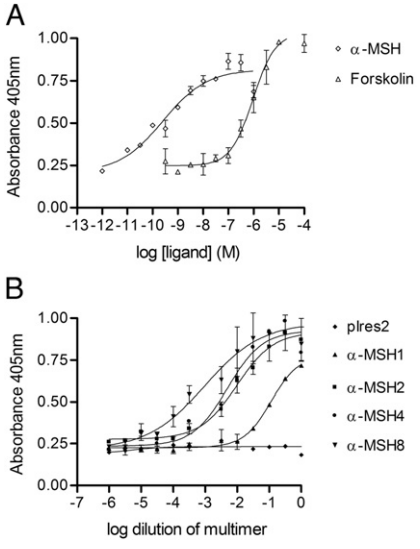

Fig. 4. Dose–response curves of human melanocortin MC4 receptor activation by α-MSH, forskolin (A) or α-MSH multimers (B), measured by CreLacZ reporter gene assay.

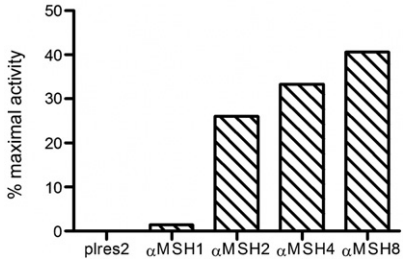

Fig. 5. Activation of human melanocortin MC4 receptors at a 100 fold dilution of the concentration that results in 20% displacement of ^{125}I-NDP-MSH, as percentage of maximal activity.

calculated, since the highest concentration of supernatant containing these multimers was not able to reach maximal displacement. Unfortunately, it was technically not feasible to further increase the concentration of multimers without non-specifically interfering with the binding assay. The IC_{50} values for α-MSH8 are presented in Table 1. The percentage of displacement reached by a two-fold dilution of supernatants is presented in Fig. 3, and was analyzed using one-way ANOVA. This revealed that displacement properties of α-MSH1 were not significantly greater than pIRES2 supernatant. All the other multimers however did show improved displacement when compared to pIRES2 or α-MSH1 supernatant ($P<0.05$). Furthermore, supernatant containing the highest concentration of α-MSH8 was able to displace most ^{125}I-NDP-MSH ($P<0.001$ compared to all other supernatants).

3.3. Melanocortin MC4 receptor activation

Dose–response curves of all α-MSH multimers were obtained and efficacy for activation of the melanocortin MC4 receptor was compared with α-MSH. Whereas supernatant of HEK293 cells transfected with pIRES2-eGFP was not able to activate the human melanocortin MC4 receptor, all α-MSH multimers were capable to activate the human melanocortin MC4 receptor (Fig. 4). Moreover, the highest concentration that was tested of all α-MSH multimers except α-MSH1 was capable to activate the human melanocortin MC4 receptor to the same extent as the highest concentration of α-MSH. However, with the addition of every α-MSH subunit, the EC50 value of the multimer decreased (Table 1), resulting in a 100 fold higher affinity of α-MSH8 when compared to α-MSH1.

Furthermore, activation induced by a 100 fold dilution of the concentration that resulted in 20% displacement in the binding assay gradually increased with each subunit (Fig. 5).

4. Discussion

The present study described the synthesis of multimeric forms of α-MSH, and their ability to bind to and activate the human melanocortin MC4 receptor. We have shown that with the addition of each α-MSH subunit, the affinity of the multimer for the melanocortin MC4 receptor increases. Interestingly, when similar dilutions of concentrations of multimers that gave equal displacement in a binding assay were tested for activity, constructs with more α-MSH subunits were clearly more effective than expected based upon binding. This indicates that, independent from the affinity for the melanocortin MC4 receptor, also the ability to activate signaling pathways downstream the melanocortin MC4 receptor increased with each extra subunit.

Oligomerization of ligands can increase their affinity. For NDP-MSH fragments, affinity for the melanocortin MC4 receptor has been reported to increase stepwise from monomer to trimer (Vagner et al., 2004). The length and structure of a linker can influence the binding affinity of oligomers (Kessler et al., 1988). Dimers separated by an ideal length can display an affinity of 150 times higher than the monomer (Kessler et al., 1988). To prevent the linker to interfere with the binding of a ligand to its receptor, the linker must be hydrophilic and small (Vagner et al., 2004). Indeed, multimers of NDP-MSH fragments with various short linkers have been shown to have an increased affinity for the MC1 and melanocortin MC4 receptor (Brandenburger et al., 1999). With the linker length, also the IC_{50} value of the multimer increased (Vagner et al., 2004). In addition, the linker should not be too flexible, because this could increase the entropic cost to bind to a second binding site, thereby diminishing the avidity of the multimer (Vagner et al., 2004). Vagner et al. showed that linkers of the same length, but with variations in flexibility have different effects on the improved binding capabilities of NDP-MSH fragment oligomers (Vagner et al., 2004).

Our results indicate that also multimers of full length α-MSH, coupled by a relatively long linker can increase the affinity to bind to the melanocortin MC4 receptor, as shown by the competitive binding assay. Cells transfected with α-MSH8 had a lower IC_{50} value compared to the rest of the multimers. In addition, α-MSH8 was able to displace significantly more ^{125}I-NDP-MSH from the human melanocortin MC4 receptor than a similar concentration of α-MSH4 and α-MSH2, which, on their part, were more capable in displacing ^{125}I-NDP-MSH than α-MSH1.

Furthermore, also the capability to activate signaling pathways downstream the melanocortin MC4 receptor was increased, which was at least partly independent from the increased binding properties. Whereas the highest concentration of α-MSH8 that we tested was only able to displace 2 times as much ^{125}I-NDP-MSH from the human melanocortin MC4 receptor than α-MSH1, the EC_{50} value had decreased 100 times. It is known that activation of the melanocortin MC4 receptor by its agonist *in vitro* is associated with time-dependent and concentration-dependent internalization (Gao et al., 2003; Shinyama et al., 2003). Reduced internalization of the melanocortin MC4 receptor due to binding to multiple subunits of an α-MSH multimer could very well explain the increased activation properties of α-MSH8 compared to the binding affinities.

Despite the fact that rigid linkers have been proven to result in the highest increase in affinity, rAAV vector-derived multimers are built up from aminoacids, which are flexible. Even though flexible linkers can decrease avidity, due to loss of entropy, they still increase the local concentration of ligand.

Furthermore, they may still promote clustering of receptors. Based upon rhodopsin, which is the only G-protein-coupled receptor (GPCR) for which the crystal structure is available, the distance between the centers of the binding sites of dimeric GPCRs is about 38 Å (Fotiadis et al., 2003). The distance between two α-MSH subunits in our multimers in the maximal extended form is 70.4 Å, which is therefore enough for a multimer to bind simultaneously to both binding sites of a melanocortin MC4 dimer. Since melanocortin receptors display co-operative binding (Kopanchuk et al., 2005; Kopanchuk et al., 2006), these properties will still favour multimeric α-MSH over the monomer. Although short, rigid linkers may be the best solution for fragmental ligands, for full length α-MSH, which represents the best physiological situation, a flexible linker may be better to provide the folding necessary to bind a receptor dimer simultaneously.

α-MSH has a relatively low affinity for the melanocortin MC4 receptor. Nevertheless, the weight reducing effects of POMC overexpression are ascribed to the actions of α-MSH (McMinn et al, 2000), although recent evidence provides evidence for β-MSH in body weight regulation (Biebermann et al., 2006). However, besides melanocortins, also other cleavage products of POMC, especially β-endorphin, can affect energy balance when injected into the brain (McKay et al., 1981). Combining the increased receptor binding and activation properties of the α-MSH multimers described in this study with the long-term local overexpression of rAAV-delivered transgenes would be an excellent tool to increase the knowledge of site-specific increased melanocortin signaling in energy balance.

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