# *Chapter 3*

# Multimeric $\alpha$-MSH has increased efficacy to activate the MC4 receptor

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# MULTIMERIC α-MSH HAS INCREASED EFFICACY TO ACTIVATE THE MC4 RECEPTOR

**ABSTRACT**

α-MSH has a relatively low affinity for the 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 hMC4 receptor. α-MSH subunits were coupled by a flexible linker and placed in front of an IRES-EGFP sequence. Efficacy for activation of the MC4 receptor increased with every extra subunit, resulting in a 100 fold lower $EC_{50}$ 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 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 hMC4 receptor *in vitro*.

**INTRODUCTION**

Melanocortins (MCs) are a family of peptides derived from the precursor protein proopiomelanocortin (POMC). The MC 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 MC 3 or 4 receptor result in obese phenotypes (97;100;151;322;323). Mice that are POMC deficient are obese, due to hyperphagia, reduced basal oxygen consumption and an altered lipid metabolism (149-151). 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 (131). 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 (131-133;324).

It is hypothesized that the ameliorating effects of POMC on obesity and metabolic function are mediated by the melanocortins 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 (117). In addition, administration of an α-MSH analog reduces body weight of POMC$^{-/-}$ mice (151). Also β-MSH inhibits food intake in fasted and non-fasted rats (86;87), 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 (88).

In order to further clarify the specific role of MC 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 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 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 (257).

Multivalant ligands often have increased binding affinity for their targets (303). Firstly, multivalent ligands increase the local concentration of binding elements, thereby increasing the chance the ligand will bind to 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 (303). Melanocortin receptors have been proposed to occur as constitutively pre-formed dimers (325;326). In addition, there is evidence that the MC receptors have interacting binding sites and that they display by co-operative binding (325;327). 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 MC4 receptor than monomeric NDP-MSH (328;329).

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 MC4R.

MATERIAL AND METHODS

**Cloning of α-MSH multimers**

A signal sequence (belonging to the Von Willebrand factor), followed by an HA tag were synthesized using the following (partly overlapping) primers: forward1: 5'GGTGCTGCTTGCTCTGGCCCTCATTTTGCCAGGGACCCTTTGTTACCCCTACGACG3' , forward2: 5' TGTCCTCGAGGGCCATGATTCCTGCCAGATTTGCCGGGGTGCTGCTTGC TCTGG 3' and reverse1: 5' CATCTGAGCATGTCAAAATCTGGCCAGGCGTAGTCGGGCA CGTCGTAGGGGTAAC 3'. Primers were ligated, filled in using Klenow (USB Corporation, Cleveland, Ohio) and than cloned into pCRscript (Stratagene, La Jolla, California).

α-MSH monomeric cDNA was synthesized with primers ( forward: 5'ACGCACCGG TCTCACCCCGCCTGGTTCTTCATCCTATTCCATGGAACACTTCAGGGGGGA 3' and reverse: 5'GAATTCACGTCTCCGGGGTGGAGGGTTTAGGCACAGGCTTTCCCCACCTG AAGTGTTCCAT 3') that are complementary to rat α-MSH cDNA including a flexible linker (Pro-Lys-Pro-Ser-Thr-Pro-Pro-Gly-Ser-Ser). Primers were ligated, filled in and than cloned

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

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

A

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     M   I   P   A   R   F   A   G   V   L   L   A   L   A   L   I   L   P   G   T   L   C   Y   P   Y   D   V   P   D
5'  ATGATTCCTGCCAGATTTGCCGGGGTGCTGCTTGCTCTGGCCCTCATTTTGCCAGGGACCCTTTGTTACCCCTACGACGTGCCCGAC
3'  TACTAAGGACGGTCTAAACGGCCCCACGACGAACGAGACCGGGAGTAAAACGGTCCCTGGGAAACAATGGGGATGCTGCACGGGCTG


    Y   A   T   G   L   T   P   P   G   S   S   S   Y   S   M   E   H   F   R   W   G   K   P   V   P   K   P   S   T   P
  TACGCCACC GGTCTCACCCCGCCTGGTTCTTCATCCTATTCCATGGAACACTTCAGGTGGGGAAAGCCTGTGCCTAAACCCTCCA CCCCG
  ATGCGGTGGCCAG AGTGGGGCGGACCAAGAAGTAGGATAAGGTACCTTGTGAAGTCCACCCCTTTCGGACACGGATTTGGGAGGTGGGG C
         BsaI

  P   G   S   S   S   Y   S   M   E   H   F   R   W   G   K   P   V   P   K   P   S   T   P   E   T
  CCTGGTTCTTCATCCTATTCCATGGAACACTTCAGGTGGGGAAAGCCTGTGCCTAAACCCTCCA CCCCGGAGACGTGAATTC 3'
  GGACCAAGAAGTAGGATAAGGTACCTTGTGAAGTCCACCCCTTTCGGACACGGATTTGGGAGGTGGGG CCTCTGCACTTAAG 5'
                                                                          BsmBI
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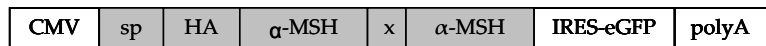
B

| CMV | sp | HA | α-MSH | x | α-MSH | IRES-eGFP | polyA |

**Figure 1:** A. Nucleotide and aminoacid sequence of $\alpha$-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 $\alpha$-MSH subunit. In grey the recognition sites for BsaI and BsmBI are depicted. Interspaces depict the restriction sites and separation of the two $\alpha$-MSH monomers.
B. Structure of the pIRES2-$\alpha$-MSH2 multimer construct, containing a CMV promoter, the signal sequence of the Von Willebrand factor, an HA-tag and the $\alpha$-MSH subunits, coupled by a flexible linker (x), followed by an IRES, EGFP and SV40 late polyadenylation signal (SV40 pA signal).

**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% CO2 at 37 Celsius.

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 $\alpha$-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 MC4R DNA and 10 μg of cAMP-Responsive Element (CRE)-LacZ construct. For the binding assay, the MC4 receptor was expressed in HEK293 cells by transfecting cells cultured in 10 cm dishes with 10 μg of DNA.

**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 minutes at RT. Cell debris was removed from the samples by centrifugation at 13,000 g for 5 minutes 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 (330). Seperated 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 RT 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).

**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-wells plates were washed with TBS supplemented with 2.5 mM calcium chloride and incubated for 30 minutes 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 1M sodium hydroxide. Samples were than counted in a γ-counter.

**Reporter gene assay**

Activation of MC4 receptors was determined using LacZ as a reportergene (316). One day after transfection, cells were dispensed into 96-wells plates. After two days, cells were

incubated at 37 °C with $\alpha$-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 hours, 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, pH7.4 containing 1.6 g/l o-Nitrophenyl β-Dgalactopyranoside (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 Victor² microplate reader (PerkinElmer, Brussels, Belgium).

**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 $\alpha$-MSH) using the sigmoidal dose-response curve (variable slope) classical equation 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.

RESULTS

**Transfection of HEK293 cells with $\alpha$-MSH-multimers**

HEK293 cells were transfected with the different $\alpha$-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. Figure 2 shows a western blot of the cell lysates of the cells transfected with the $\alpha$-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.
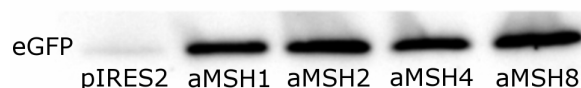


**Figure 2:** Western blot of cell lysates of HEK-293 cells transfected with pIRES2 or $\alpha$-MSH multimers and stained using an EGFP antibody.

**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 hMC4 receptor, whereas supernatant from cells transfected with α-MSH8 had a slightly higher affinity (data not shown). No IC50 values for α-MSH1, α-MSH2 and α-MSH4 could be 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 IC50 value for α-MSH8 is presented in table 1. The percentage of displacement reached by a two-fold dilution of supernatants is presented in figure 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).

**Table 1:** $IC_{50}$ values and $EC_{50}$ values of the α-MSH-multimers. $IC_{50}$ values were obtained by displacement of iodinated NDP-MSH. $EC_{50}$ 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 determined.

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

**MC4 receptor activation**

Dose response curves of all α-MSH multimers were obtained and efficacy for activation of the hMC4 receptor was compared with α-MSH. Whereas supernatant of HEK293 cells transfected with pIRES2-EGFP was not able to activate the hMC4 receptor, all α-MSH multimers were capable to activate the hMC4 receptor (Fig. 4). Moreover, the highest concentration that we were able to test of all α-MSH multimers except α-MSH1 was capable to activate the hMC4 receptor to the same extent as the highest concentration of α-MSH. However, with the addition of every α-MSH subunit, the $EC_{50}$ 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).
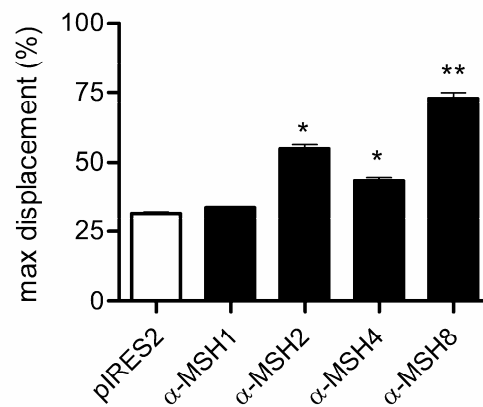
**Figure 3:** Displacement of hMC4 receptor bound ^{125}I-NDP-MSH by a 2-fold dilution of concentrated supernatant of cells transfected with the multimers. $*p<0.05$ vs pIRES2 and $\alpha$-MSH1, $**p<0.01$ vs all other multimers.
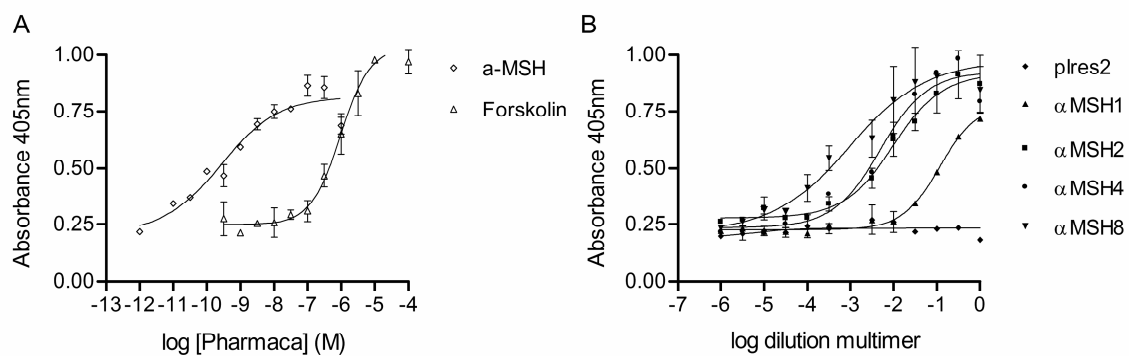


**Figure 4:** Dose-response curves of hMC4 receptor activation by $\alpha$-MSH, forskolin (A) or $\alpha$-MSH multimers (B), measured by CreLacZ reporter gene assay.

## DISCUSSION

The present study described the synthesis of multimeric forms of $\alpha$-MSH, and their ability to bind to and activate the hMC4 receptor. We have shown that with the addition of each $\alpha$-MSH subunit, the affinity of the multimer for the 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 $\alpha$-MSH subunits were clearly more effective than expected based upon binding. This indicates that, independent from the affinity for the MC4 receptor, also the ability to activate signaling pathways downstream the MC4 receptor increased with each extra subunit.

Oligomerization of ligands can increase their affinity. For NDP-MSH fragments, affinity for the MC4 receptor has been reported to increase stepwise from monomer to trimer (329). The length and structure of a linker can influence the binding affinity of

oligomers (331). Dimers separated by an ideal length can display an affinity of 150 times higher than the monomer (331). To prevent the linker to interfere with the binding of a ligand to its receptor, the linker must be hydrophilic and small (329). Indeed, multimers of NDP-MSH fragments with various short linkers have been shown to have an increased affinity for the MC1 and MC4 receptor (328;329). With the linker length, also the IC50 value of the multimer increased (329). 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 (329). 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 (329).
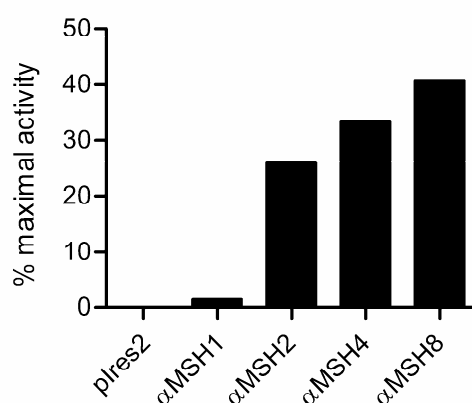


**Figure 5:** Activation of hMC4 receptors at a 100 fold dilution of the concentration that results in 20% displacement of ^{125}I-NDP-MSH, as percentage of maximal activity.

Our results indicate that also multimers of full length α-MSH, coupled by a relatively long, flexible linker can increase the affinity to bind to the 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 hMC4 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 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 hMC4 receptor than α-MSH1, the $EC_{50}$ value had decreased 100 times. It is known that activation of the MC4 receptor by its agonist *in vitro* is associated with time-dependent and concentration-dependent internalization (332;333). Reduced internalization of the 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 (334). The distance between two $\alpha$-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 MC4 dimer. Since MC receptors display by co-operative binding (325;327), these properties will still favor multimeric $\alpha$MSH over the monomer. Although short, rigid linkers may be the best solution for fragmental ligands, for full length $\alpha$-MSH, which represents the best physiological situation, a flexible linker may be better to provide the folding necessary to bind a receptor dimer simultaneously.

$\alpha$-MSH has a relatively low affinity for the MC4 receptor. Nevertheless, the weight reducing effects of POMC overexpression are ascribed to the actions of $\alpha$-MSH (117;151). However, besides melanocortins, also other cleavage products of POMC, especially β-endorphin, can affect energy balance when injected into the brain (88). Combining the increased receptor binding and activation properties of the $\alpha$-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.