

A role for β -melanocyte-stimulating hormone in human body-weight regulation

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Summary

Pro-opiomelanocortin (POMC) expressing neurons mediate the regulation of orexigenic drive by peripheral hormones such as leptin, cholecystikinin, ghrelin, and insulin. Most research effort has focused on α -melanocyte-stimulating hormone (α -MSH) as the predominant POMC-derived neuropeptide in the central regulation of human energy balance and body weight. Here we report a missense mutation within the coding region of the POMC-derived peptide β -MSH (Y5C- β -MSH) and its association with early-onset human obesity. In vitro and in vivo data as well as postmortem human brain studies indicate that the POMC-derived neuropeptide β -MSH plays a critical role in the hypothalamic control of body weight in humans.

Introduction

Increased *POMC* gene expression in the hypothalamic arcuate nucleus represents a key step in the anorexigenic signaling cascade of leptin (Seeley and Woods, 2003). POMC-expressing neurons of the arcuate nucleus project to other hypothalamic nuclei, including the paraventricular nucleus (PVN), where stimulation of the postsynaptic melanocortin-4 receptor (MC4R) promotes negative energy balance, reducing body weight (Cone 2005). The important and nonredundant role of POMC signaling in body-weight homeostasis is demonstrated by the extreme and early-onset obese phenotype in humans and mice with loss-of-function mutations in the *POMC* gene (Krude et al., 1998, Yaswen et al., 1999). Humans heterozygous for mutated *POMC* are overweight or modestly obese (Krude et al., 2003), and heterozygous *Pomc*^{-/+} mice only develop obesity on a high-fat diet (Low 2004, Challis et al., 2004). Individuals heterozygous for mutations of the *MC4R* gene also have intermediate obese phenotypes and represent the most prevalent monogenetic cause of human obesity (Farooqi et al., 2003). POMC-deficient humans and mice are also characterized by secondary reduction of glucocorticoids and red-hair pigmentation since pituitary POMC is the precursor of ACTH, which stimulates glucocorticoid secretion via the MC2R in the adrenal cortex, as well as MSH, which effects hair pigmentation via the MC1R.

Sequential cleavage of human pre-pro-POMC in the hypothalamus results in the intermediate product ACTH via prohormone convertase 1 (PC1), which in turn is cleaved to α -MSH, β -MSH, and γ -MSH by PC2 (Figure 1A) (Pritchard et al., 2002). While α -MSH and β -MSH bind to the MC4R with a comparably high affinity (Schioth et al., 2002), ACTH binds to MC4R with lower affinity (Kask et al., 2000), and γ -MSH binds exclusively to the MC3R. MC3R is believed to be less important for food in-

take control (Chen et al., 2000). Therefore, the anorectic effect of POMC neurons in the hypothalamus is thought to be mediated via α -MSH or β -MSH.

Most research on body-weight regulation has focused on the role of α -MSH, in part because the commonest animal models, rodents, lack the N-terminal cleavage site for β -MSH and are therefore β -MSH deficient (Bennett, 1986). Because the amino acid sequence of β -MSH is highly conserved across species (Takeuchi et al., 1999) and most species contain both cleavage sites for β -MSH, β -MSH may have a key role in the control of energy homeostasis in humans and other species. Although some pharmacological studies have suggested an anorexigenic action of β -MSH, results are conflicting (Abbott et al., 2000; Kask et al., 2000; Millington et al., 2001; Harrold et al., 2003), and relevant genetic data are sparse.

Large mutation-screening studies have failed to identify a human missense mutation in α -MSH or β -MSH (Hinney et al., 1998, Echwald et al., 1999, Miraglia del Giudice et al., 2001, Challis et al., 2002). However, heterozygous mutations have been identified in the *POMC* gene in obese individuals. The mutations are located either in the N-terminal pre-pro-signal region of POMC (Miraglia del Giudice et al., 2001) or are close to the β -MSH peptide sequence itself (Hinney et al., 1998; Challis et al., 2002). One mutation in particular that was identified in different cohorts changes the C-terminal cleavage site of β -MSH, resulting in formation of a fusion peptide containing β -MSH as well as β -endorphin (Challis et al., 2002). This fusion MSH-like peptide was postulated to have a dominant-negative rather than a loss-of-function effect (Challis et al., 2002).

In the present report, we describe a loss-of-function missense mutation within the coding region for β -MSH (Y5C- β -MSH). The association of this mutation with human childhood obesity suggests a role of β -MSH in human body-weight regulation.

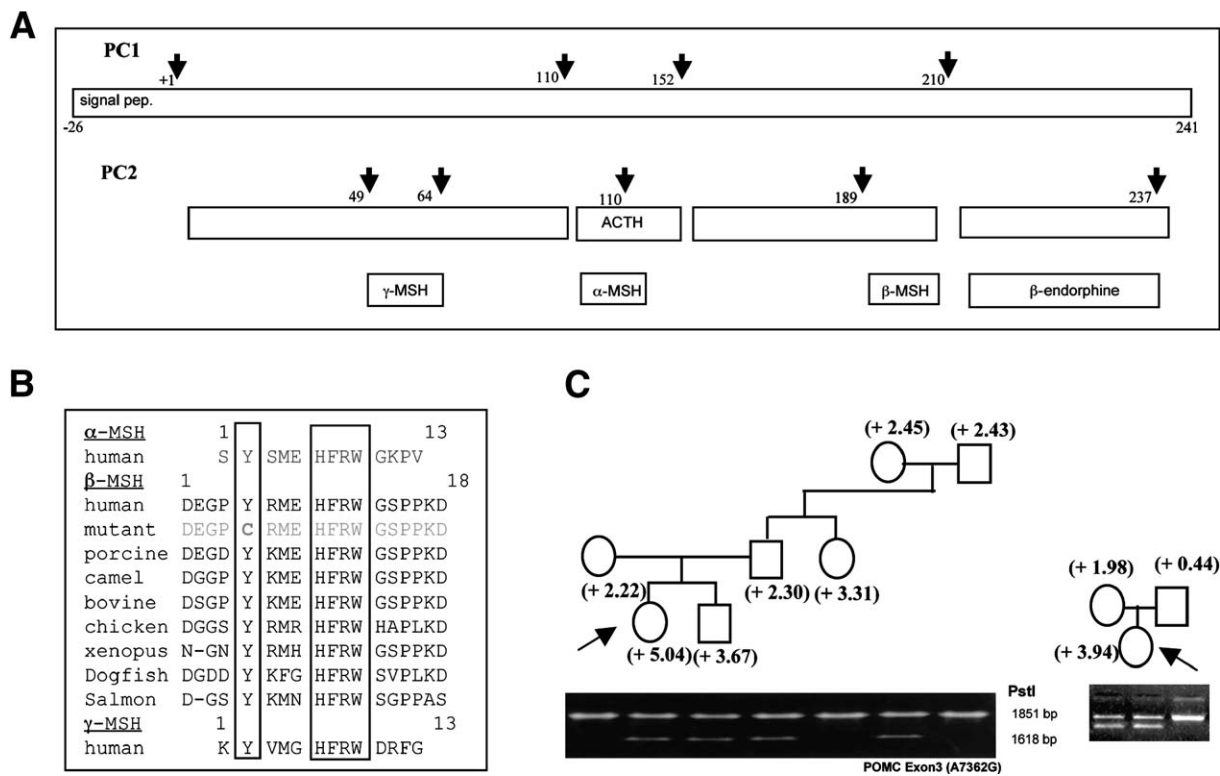


Figure 1. POMC processing and identification of a variation in the region coding for β -MSH
A) Structure of the POMC precursor protein and the respective position of the resulting peptides. Numbers indicate the cleavage sites of PC1 and PC2.
B) Alignment of melanocortin peptides from different species showing the conservation of the mutant amino acid position within all melanocortin peptides.
C) Pedigrees of families with the A7362G mutation (Y5C) in exon 3 as indicated by restriction with PstI. The BMI standard deviation scores (SDS) according to Rolland-Cachera et al. (1991) are given in parenthesis for each family member. The index cases identified in the mutation screening study are marked with an arrow.

Results and discussion

Identification of a β -MSH mutation in obese children

By direct sequencing of the complete coding region of the *POMC* gene in a small cohort of 15 severely obese children, we identified a novel heterozygous nonsynonymous coding mutation within the sequence coding for Tyr in position 5 of β -MSH resulting in a Cys residue substitution (Y5C- β -MSH) in one child. Further investigation revealed the same mutation in family members of the index child, all of whom are obese (Figure 1C). Because other family members who do not carry the Y5C- β -MSH mutation are overweight, the mutation is likely not the only obesity factor in this family. Because several previous studies failed to identify *POMC* gene mutations, we hypothesized that either the indirect methods used in those forgoing studies were insensitive or else the mutation is rare. To evaluate this, we compared our method to indirect mutation-screening methods including SSCP and WAVE and concluded that the Y5C- β -MSH mutation cannot be identified by those techniques. The mutation can be detected, however, by mutation-specific restriction digestion.

To determine the prevalence of the Y5C- β -MSH mutation in obese versus normal-weight children, we performed a study using restriction-enzyme analysis with PstI in 722 obese children and adolescents (BMI \geq 90th BMI percentile) and a control group of 1270 normal-weight children and adolescents. We identified two mutation carriers in the obese cohort and none in the control group. The available parental DNA samples re-

vealed the Y5C- β -MSH mutation in the obese mother (BMI 35) of one patient. Upon fixation of the ratio of obese to normal-weight volunteers (1:1), more than 2200 obese and 2200 controls would be necessary to confirm a difference of the presently estimated allele frequencies (0.00 versus 0.028) at the 5% test level with a power of 80%. Pediatric cohorts of obese children are difficult to generate in this magnitude, and existing cohorts of adult obese patients of this sample size are not informative since it has been shown that the genetic influence of single-gene defects becomes less penetrating in adult life compared to childhood (Hebebrand et al., 2003). Nevertheless, our data suggest, that although the Y5C- β -MSH is rare, the mutation manifests with a relevant penetrance since all mutation carriers identified so far are obese.

Loss of function of the mutant Y5C- β -MSH peptide

Comparing MSH peptides of different species (Figure 1B) reveals five amino acids which are identical in all available species, including the core sequence of His-Phe-Arg-Trp, which represents the receptor binding motif, as well as the Tyr residue in position 5 that is replaced by the identified Y5C mutation. Therefore, we hypothesized that a mutation that changes residue 5 of β -MSH similarly to the Y5C mutation might result in a profound loss of function. We therefore studied the in vitro function of the Y5C- β -MSH mutation using CHO-K1 cells stably expressing the human MC4R. The mutant Y5C- β -MSH peptide revealed a 100-fold reduced binding affinity (Figure 2A) and a 50-fold reduced biological activity—as shown by intracellular cAMP

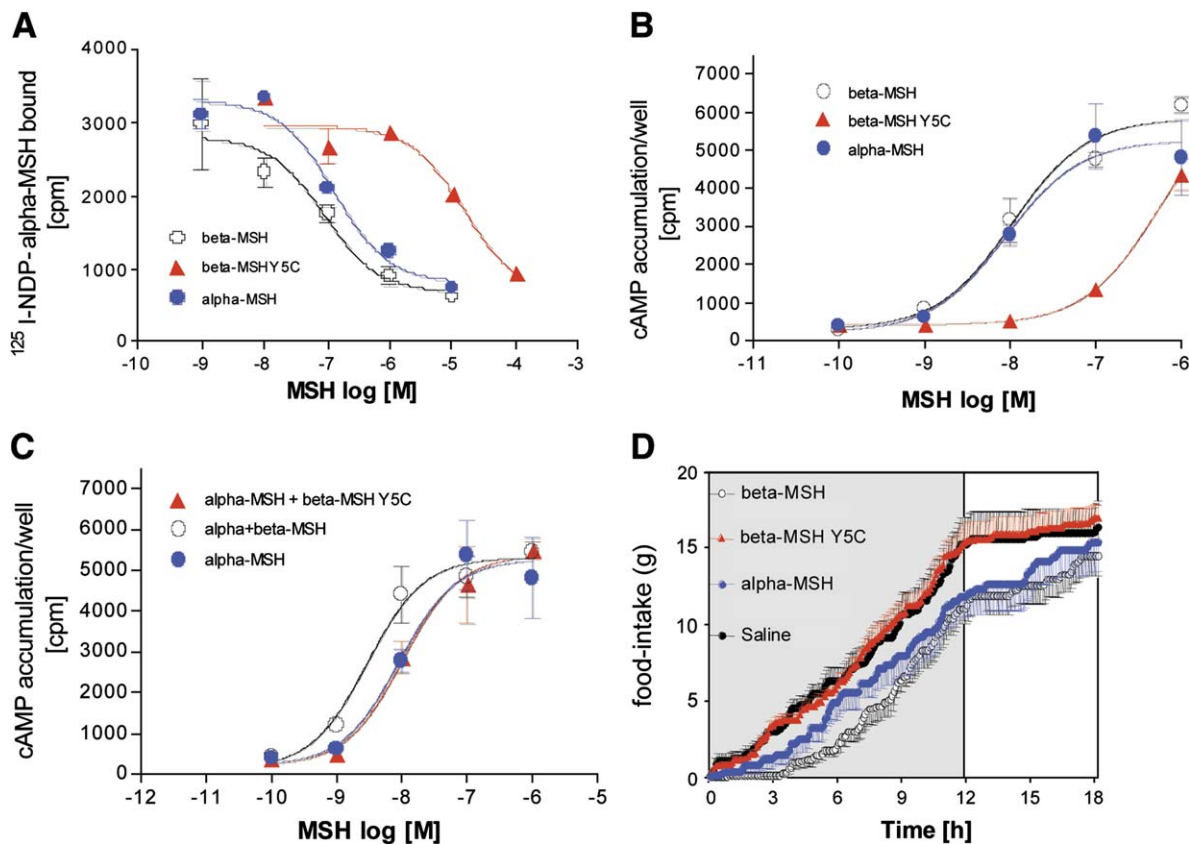


Figure 2. Functional characterization of the A7362G missense mutation (Y5C) in vitro and in vivo. Error bars in (A)–(C) represent means \pm SEM of one out of four independent experiments, each carried out in duplicate. In (D), error bars represent means \pm SEM of food intake in groups of rats tested; the numbers of rats in each group are given in the [Experimental Procedures](#). **A)** Competition binding experiment using CHO-MC4R cells, radiolabeled [^{125}I]NDP- α -MSH, and an increasing amount of unlabeled MSH peptides. The calculated K_i values are: α -MSH: 224 ± 76 nM, β -MSH: 197 ± 75 nM, Y5C- β -MSH: 1375 ± 188 nM. **B and C)** Accumulation of cAMP in CHO-MC4R cells labeled with [^3H]adenine and incubated with increasing amounts of MSH (B) or costimulated with equimolar MSH combinations (C). **D)** Effect of intracerebroventricular administration of MSH peptides on food intake of male rats. The gray zone represents the active dark phase.

generation—compared to the wild-type β -MSH and α -MSH peptides (Figure 2B). These results suggested a loss-of-function effect of the heterozygous Y5C- β -MSH mutation. However, a dominant-negative effect of a mutant peptide was proposed for another *POMC* gene mutation resulting in a fusion peptide of β -MSH and β -endorphin (Challis et al., 2002), thus representing a possible explanation for our findings. We therefore conducted costimulation studies with equimolar concentrations of the mutant and the intact β -MSH peptide. These studies did not reveal a dominant-negative effect (Figure 2C).

Although rodents do not process β -MSH, human β -MSH has been reported to reduce food intake in rats after intracerebroventricular (i.c.v.) administration (Abbott et al., 2000; Kask et al., 2000), presumably due to the homology of the human and rat MC4R. We took advantage of this cross-species effect and compared the in vivo function of the different human MSH peptides in rats. i.c.v. administration of wild-type α - and β -MSH in ad libitum-fed rats resulted in a comparable, significant, and dose-dependent decrease of food intake, whereas i.c.v. Y5C- β -MSH had no effect on food intake (Figure 2D). The lack of an increase of food intake in the Y5C- β -MSH-treated animals shows that the mutant peptide does not interfere with endogenous MC4R agonists, again arguing against a dominant-negative effect of the mutant peptide. Therefore, both

the in vitro and in vivo functional data demonstrate a loss-of-function effect and exclude a dominant-negative effect of the mutant β -MSH peptide.

Expression of POMC-derived peptides in human arcuate nucleus

The observation that a heterozygous loss-of-function mutation in β -MSH can be identified in obese patients implies that β -MSH might play a relevant role in human hypothalamic body-weight regulation.

Postmortem examination of human hypothalamus by HPLC revealed that POMC is processed into β -MSH (Bertagna et al., 1986), but a detailed distribution and expression pattern of β -MSH in human hypothalamus has not been reported. We therefore performed immunohistochemistry in postmortem sections of the human arcuate nucleus (for section selection, see Figure S1 and the corresponding description within the [Supplemental Experimental Procedures](#) in the [Supplemental Data](#) available with this article online). Specificity of the antibodies used against α -MSH, β -MSH, and ACTH was demonstrated in crosscompetition experiments based on histological sections of the arcuate nucleus and hair follicles, which are known to highly express MSH peptides (Figure S2). These crosscompetition experiments revealed a high specificity for each antibody.

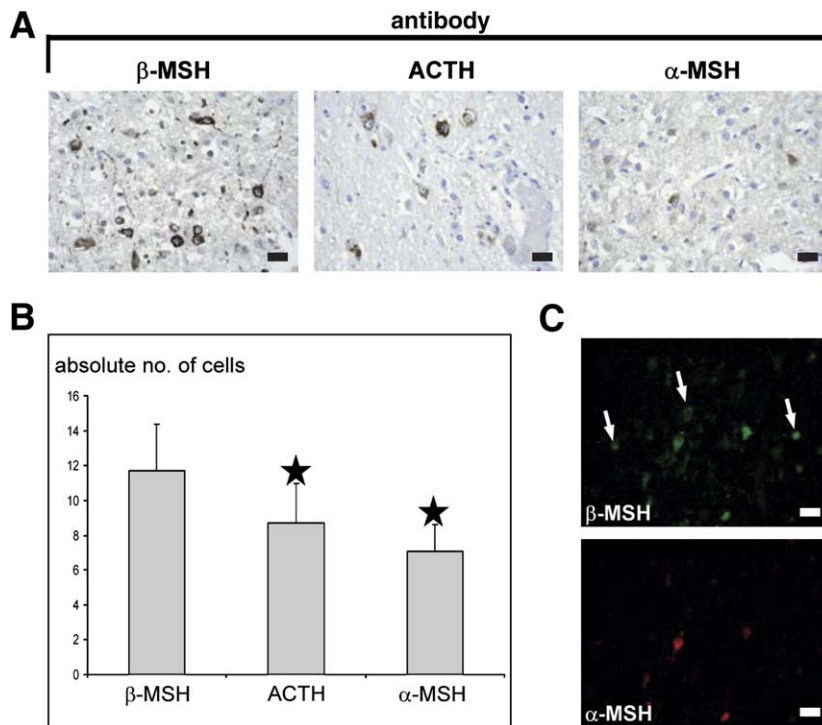


Figure 3. Immunohistochemical determination of melanocortin peptide expression in the human arcuate nucleus

A) Determination of expression of α - or β -MSH and ACTH peptide in human arcuate nucleus sections. Immunopositive neurons and axons are intermingled with immunonegative neurons and glial cells. Original magnification (om) \times 400.

B) Number of α -MSH-, β -MSH-, and ACTH-positive neurons in serial sections of the arcuate nucleus (mean and standard deviation of numbers counted in 300 microscopic fields with $62.5 \mu\text{m}^2$ each).

C) Immunofluorescence double staining of α -MSH (red) and β -MSH (green) on sections of the arcuate nucleus. Most neurons and axons express β -MSH, whereas only a subset of them express α -MSH. Arrows indicate β -MSH-positive and α -MSH-negative neurons (om \times 400). In all figures, the scale bar represents $15 \mu\text{m}$.

We then determined the distribution of α -MSH, β -MSH, and ACTH in arcuate nucleus of five normal-weight individuals (BMI range 24–29) in serial sections. We found a reproducible and consistent pattern of POMC-derived peptide expression in all five specimens (Figure 3A). The mean number of positive neurons taken together from all sections (300 microscopic fields of $62.5 \mu\text{m}^2$ each) significantly differed among the three peptides, with β -MSH having the highest density (11.7 ± 2.7 per field) relative to ACTH-positive (8.7 ± 2.5 per field) and α -MSH-positive (7.1 ± 1.6 per field) neurons (Figure 3B). Immunofluorescence double-labeling experiments revealed that all α -MSH-positive neurons (red) were also positive for β -MSH (green), while several neurons were only positive for β -MSH (Figure 3C). Taken together, these data demonstrate that β -MSH is expressed in POMC neurons in the human arcuate nucleus. Because complete cleavage of POMC via PC1 and PC2 should result in equimolar concentrations of α -MSH and β -MSH within a single neuron, the detection of a higher number of β -MSH-positive neurons compared to α -MSH neurons and the observation of β - but not α -MSH-positive neurons seem to be counterintuitive. It has been reported that α -MSH, in its non-N-acetylated state, is not biologically active as determined with i.c.v. injections in rodents. Since nonacetylated α -MSH is active in *in vitro* receptor assays, this observation can be explained by a reduced biological half-life of nonacetylated α -MSH. Recently, this proposed reduced half-life of nonacetylated α -MSH compared to acetylated α -MSH was proven in a coinubation experiment with brain extracts (Guo et al., 2004). Therefore, the observation of a higher number of β -MSH-positive neurons compared to α -MSH neurons might point to a state of incomplete posttranslational acetylation of α -MSH and subsequent degradation of nonacetylated α -MSH in human arcuate neurons with the consequence of a predominant expression of β -MSH as the functional anorexigenic POMC-derived peptide. However, further possible influences on the im-

munohistological results, like different postmortem stability of the particular peptides, cannot be excluded. HPLC data from microdissected arcuate neurons in nonrodent species may be one way to resolve these open questions. Nevertheless, our data indicate that β -MSH is neither less present nor less functionally relevant for hypothalamic energy-balance regulation in humans than α -MSH.

A role of β -MSH in human body-weight regulation

The POMC-MC4R pathway plays an important, dosage-sensitive role in body-weight regulation in different species as shown by the obese phenotype of several MC4R heterozygous animal models (Huszar et al., 1997). In addition, heterozygous loss of MC4R function represents the most frequent genetic alteration identified in severely obese human individuals (Farooqi et al., 2003). More than 70 different mutations have been located throughout the whole coding region of the MC4R gene. In contrast, missense mutations in the POMC gene are rarely found within obese individuals and are restricted to the β -MSH peptide, as shown in this report as well as in the accompanying paper by Lee et al. (2006) (this issue of *Cell Metabolism*). These findings of β -MSH mutations rather than α -MSH mutations in obese individuals can be explained by the structure of the POMC precursor protein: α -MSH represents the N-terminal receptor binding domain of ACTH (Figure 1A). Inactivating mutations of α -MSH could therefore lead to ACTH deficiency and would most likely lead to secondary hypocortisolism with a dramatic negative effect on survival with a consequent negative selection of those α -MSH mutant alleles. Mutations in the β -MSH peptide, as described in our report as well as in the accompanying publication by Lee et al., could result in an obese phenotype with normal cortisol secretion. This mutation might therefore have the same restricted effect on body weight as reported earlier for MC4R mutations. However, due to the limited number of functionally relevant residues within β -MSH, the likelihood of an

inactivating mutation is very low. This might explain the low frequency compared to MC4R gene mutations.

In summary, immunohistochemistry and functional data from the few currently known heterozygous Y5C- β -MSH mutation carriers presented here suggest that the lack of functional β -MSH seems to reduce critically the amount of MSH peptide in the POMC/MC4R signaling pathway, leading to overweight and obesity. Therefore, although rare, the obese phenotype in Y5C- β -MSH mutation carriers implies a significant effect of endogenous human β -MSH on body weight. Such a previously underestimated role of β -MSH within one of the most essential pathways of body-weight regulation may offer an intriguing new perspective for the treatment of obesity. Drug discovery strategies based on hypothalamic peptides specifically targeting the central melanocortin system may benefit from considering β -MSH as a potential compound. Analogs derived from α -MSH, besides having a strong anorexigenic effect, also exhibit a strong stimulatory effect on sexual behavior (Martin and MacIntyre, 2004). While this might represent another indication for those compounds, it probably excludes them as an option for the treatment of severe obesity. Since β -MSH analogs might have different pharmacological properties compared to α -MSH analogs, they represent a new strategy to develop an anorexigenic compound without the side effects observed with α -MSH (Mayer et al., 2005; Hsiung et al., 2005).

Experimental procedures

Mutational screening

Genomic DNA was isolated from peripheral blood leukocytes from all family members by use of a DNA extraction kit (QIAamp Blood Kit, QIAGEN, Hilden, Germany). DNA samples of obese children were collected either at the Charité outpatient clinic in Berlin or at two obesity clinics in the south of Germany where extremely obese children are treated from different regions of Germany. Exons 2 and 3 of the *POMC* gene were amplified by PCR techniques. Sequencing was performed with BigDye Terminator Cycle Sequencing Ready Reaction Kit (PerkinElmer, Weiterstadt, Germany). The sequencing reactions were analyzed with an automatic sequencer (ABI 3100, Applied Biosystems, Foster City, CA). PCR products of *POMC* exon 3 were further analyzed by restriction-enzyme analysis with PstI followed by separation on a 1.5% agarose gel. All clinical investigation and genetic analyses were conducted in accordance with the guidelines proposed in the Declaration of Helsinki, and informed consent was obtained from all family members.

Functional characterization of the mutant β -MSH in vitro

For investigation of agonist-stimulated cAMP accumulation, CHO-hMC4R cells were seeded in 12-well plates (2×10^5 cells/well) (for details, see Supplemental Data). Labeling with 2 μ Ci/ml [3 H]adenine (31.7 Ci/mmol, Pharmacia, Freiburg, Germany) was performed after 48 hr. One day later, cAMP accumulation assays were performed. Cells were washed once and incubated in serum-free Ham's F12 medium containing 1 mM 3-isobutyl-1-methylxanthine (Sigma Chemical Co., Steinheim, Germany) in the absence or in increasing amount of agonist α -MSH and β -MSH and mutant β -MSH for 1 hr at 37°C. The reactions were stopped by aspiration of medium, and intracellular cAMP was released by incubation of 1 ml 5% trichloric acid, which was measured by anion exchange chromatography.

For cell-surface binding studies, cells were seeded in 48-well plates (1×10^4 cells/well). Twenty-four hours later, cells were incubated overnight in the presence of [125 I]NDP- α -MSH (Amersham Pharmacia, UK; specific activity 2,000 Ci/mmol; 120,000 cpm/well) with increasing amounts of agonist α -MSH, β -MSH, and mutant β -MSH. After washing, specifically bound [125 I]NDP- α -MSH was measured.

MSH peptides were synthesized by Dr. P. Henklein, Institute of Biochemistry, Charité, Humboldt University Berlin; in all experiments, α -MSH was N-acetylated; the integrity of the peptides was controlled by HPLC.

Functional characterization of in vivo i.c.v. effects in rats

The animals were considered fully adapted to cannula implantation into the 3rd cerebral ventricle (i3v) when food intake reached the physiological average of 25 g/24 hr and signs of stress or visceral illness were absent. Rats were placed in a customized indirect calorimetry system combining continuous, automated measurement of feeding, drinking, energy expenditure, respiratory quotient, and locomotor activity (TSE Systems GmbH, Bad Homburg, Germany). Rats were adapted to the calorimetry system and i3v saline injections for at least 48 hr. For the experiment, rats were randomized into four groups and injected i3v with 5 μ l of 0.9% sodium chloride ($n = 7$), α -MSH ($n = 7$, 10 μ M), β -MSH ($n = 6$, 10 μ M), or mutant β -MSH ($n = 7$, 10 μ M) shortly before the onset of dark. Food intake was measured continuously for the following 24 hr (for details, see Supplemental Data).

Postmortem human brain tissue

Autopsies were performed with a postmortem delay of 22.5 ± 1.2 hr following written consent for pathological examination according to the law of Berlin (Sektionsgesetz, Gesetz- und Verordnungsblatt für Berlin, 1996; 52 (32), 237–239). Only subjects with no previous history or evidence of neurological disease were included in the study (3 males, 2 females; mean age 61.8 years; range of age 47–72 years; mean BMI 26.4; range of BMI 24–29). Brains and small slices of the scalp were removed and fixed in buffered 4% formaldehyde. Tissue samples of hypothalamic arcuate nucleus and scalp were routinely paraffin embedded. Subsequently, the samples of the arcuate nucleus were cut in six 4 μ m serial sections every 100 μ m step ($n = 6$) (see Figure S1). All sections were stained with hematoxylin and eosin or were further subjected to immunohistochemistry and immunofluorescence double labeling.

Light microscopy and fluorescence double-labeling sections were observed using a Leica DMRB microscope (Leica, Bad Bensheim, Germany). Immunopositive neurons were counted in ten standardized microscopic fields (62.5 μ m²) of each step, as defined by an ocular grid (Leica). Values in Figure 3B represent the number of cells per microscopic field and are given as means \pm standard deviation out of 300 fields counted. For statistical evaluation, the Kruskal-Wallis analysis of variance with Dunn's test for multiple comparisons was applied. P values less than 0.01 were considered significant.

Immunohistochemistry

Sections were mounted on slides and fixed overnight at 37°C. On the next day, they were dewaxed, rehydrated, and microwaved for 3×4 min in 10 mM citrate buffer (pH 6.5) at 600 W (Bosch, Berlin, Germany). Endogenous peroxidase activity was blocked by incubating sections with 3% hydrogen peroxide in methanol for 15 min at room temperature. Following washing, sections were incubated with a 1:20 dilution of normal goat serum in PBS for 20 min and then incubated with either monoclonal mouse anti-human α -MSH antibody (Bachem, Weil am Rhein, Germany; dilution 1:100), polyclonal mouse anti-human β -MSH antibody (G. Brabant, Hannover, Germany; 1:100 [for crossreactivity, see Supplemental Experimental Procedures]), or monoclonal ACTH antibody (DAKO, Hamburg, Germany; 1:200) in 10% fetal bovine serum in PBS. Sections were washed and treated with a goat anti-mouse IgG (DAKO; 1:100). Immunopositive cells were detected and visualized with the Vectastain ABC Elite kit (Vector Laboratories, Wertheim, Germany) and diaminobenzidine (Sigma Chemical Co., Deisenhofen, Germany). Nuclei were counterstained with hematoxylin. For negative controls, the primary antibody was replaced by including the specific ligand peptide immunogen as a competitor of antibody binding. After omitting primary antibodies, no immunostaining was observed.

For immunofluorescence studies, sections were subjected to microwave treatment and incubated with the mouse anti-human α -MSH antibody (1:20) as described above. Immunofluorescence was performed using goat anti-mouse Cy3 (Dianova, Hamburg, Germany; 1:50). For double labeling, mouse anti-human β -MSH (1:20) was used and visualized by goat anti-mouse Oregon green (MoBiTec, Göttingen, Germany; 1:100) binding.

Supplemental data

Supplemental Data include Supplemental Experimental Procedures and two figures and can be found with this article online at <http://www.cellmetabolism.org/cgi/content/full/3/2/141/DC1/>.

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