Peripherally administered desacetyl α-MSH and α-MSH both influence postnatal rat growth and associated rat hypothalamic protein expression

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Wu, Chia-Shan Jenny, David R. Greenwood, Janine M. Cooney, Dwayne J. Jensen, Michele A. Tatnell, Garth J. S. Cooper, and Kathleen G. Mountjoy. Peripherally administered desacetyl α-MSH and α-MSH both influence postnatal rat growth and associated rat hypothalamic protein expression. Am J Physiol Endocrinol Metab 291: E1372–E1380, 2006. First published July 25, 2006; doi:10.1152/ajpendo.00480.2005.—Desacetyl α-MSH predominates over α-MSH during development, but whether it is biologically active and has a physiological role is unclear. We compared the effects of 0.3 μg·g⁻¹·day⁻¹ desacetyl α-MSH with that of 0.3 μg·g⁻¹·day⁻¹ α-MSH on postnatal body growth by administering the peptides subcutaneously daily for postnatal days 0–14 and also used a two-dimensional gel electrophoresis gel-based proteomic approach to analyze protein changes in hypothalami, the relay center for body weight and growth regulation, after 14 days of treatment. We found that the growth rate between days 1 and 10 was significantly decreased by desacetyl α-MSH but not by α-MSH, but by day 14, a time reported for development of a mature pattern of hypothalamic innervation, both peptides had significantly increased neonatal growth compared with PBS-treated control rats. Desacetyl α-MSH significantly increased spleen weight, but α-MSH had no effect. α-MSH significantly decreased kidney weight, but desacetyl α-MSH had no effect. Both desacetyl α-MSH and α-MSH significantly decreased brain weight. By 14 days, both peptides significantly changed expression of a number of hypothalamic proteins, specifically metabolic enzymes, cytoskeleton, signaling, and stress response proteins. We show that peripherally administered desacetyl α-MSH is biologically active and induces responses that can differ from those for α-MSH. In conclusion, desacetyl α-MSH appears to be an important regulator of neonatal rat growth.

The pivotal role of the melanocortin system in regulation of apetite, metabolism, body size, and body weight is demonstrated clearly by the human melanocortin-4 receptor (MC4R) (24, 65) and proopiomelanocortin (POMC) (34) variants, three knockout mouse models (POMC, MC3R, and MC4R) (10, 14, 29, 70), the spontaneously occurring dominant agouti mouse (68), and a mouse ectopically overexpressing agouti gene-related peptide (26), all of which promote obesity. Still unresolved, though, are the roles of each POMC-derived peptide and the central melanocortin-signaling pathways. The melanocortin peptides α-melanocyte-stimulating hormone (α-MSH) and desacetyl α-MSH are two endogenous peptides derived from a precursor protein, POMC, through posttranslational processing (46). NH₂-terminal acetylation of desacetyl α-MSH to form α-MSH occurs in secretory vesicles just prior to exocytosis (42, 66), but not all desacetyl α-MSH is acetylated, since desacetyl α-MSH is present in the circulation and brain of rodents and humans. The major form of plasma immunoreactive MSH is α-MSH in rodents and desacetyl α-MSH in humans (22, 40), and the relative abundance of these two MSH peptides is developmentally regulated (11, 23). Desacetyl α-MSH predominates in rodent and human brains and fetuses (23, 54).

Desacetyl α-MSH is virtually inactive compared with α-MSH for some biological activities, inducing pigmentation, arousal, memory, attention, excessive grooming, and inhibiting food intake (11, 51, 64). It is generally believed today that desacetyl α-MSH is less efficacious than α-MSH because it is more rapidly degraded compared with α-MSH. This reasoning is based primarily on three studies: one in rabbit plasma (57) and two studies where rat brain homogenates were shown to degrade desacetyl α-MSH much faster than α-MSH (27, 50). However, rapid degradation is difficult to reconcile with the fact that desacetyl α-MSH exists in the brain and circulation and has been reported to have biological activities in vitro and in vivo at a point when α-MSH has little or no response. For example, desacetyl α-MSH is more effective than α-MSH at blocking opiate analgesia and receptor binding (51), stimulating adrenocortical function (28), increasing body weight, muscle, and adipose tissue (39, 61, 62), inducing slow-wave sleep (13), and inducing lipolysis in rainbow trout (69).

In vitro, both α-MSH and desacetyl α-MSH share similar binding affinities for four melanocortin receptor subtypes (MC1R, MC3R, MC4R, and MC5R) (58, 59) and similarly couple these receptors to adenylyl cyclase and the mobilization of intracellular calcium (44, 45). The molecular mechanism(s) for the in vivo differences for these two peptides is therefore not clear. Given that desacetyl α-MSH predominates early in life and activates four melanocortin receptors in vitro similarly to α-MSH, a physiological role for desacetyl α-MSH during development cannot be discounted.

We undertook the present study to test the hypothesis that peripherally administered desacetyl α-MSH is biologically active in vivo. We compared the biological activity of desacetyl α-MSH with α-MSH administered to neonatal rats. Each peptide was subcutaneously injected daily for 14 days and assessed first for chronic effects on body and organ weights and second for chronic effects on hypothalamic protein
expression. The hypothalamus has long been known to be the site for relaying and integrating peripheral and central signals in body weight and growth regulation (9, 41, 67), and therefore, we used a two-dimensional gel electrophoresis (2-DE) gel-based proteomic approach to analyze hypothalamic protein changes at the end of the 2-wk melanocortin peptide treatment period.

MATERIALS AND METHODS

Reagents and Materials

\(\alpha\)-MSH and desacetyl \(\alpha\)-MSH were purchased from Bachem California (Torrance, CA). Prior to use, \(\alpha\)-MSH and desacetyl \(\alpha\)-MSH were converted to their hydrochloride salts (16), lyophilized, and stored as aliquots at \(-80^\circ\)C. The integrity of the peptides after this procedure was confirmed by 1) matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MS) (Voyager DE-Pro; Applied Biosystems, Foster City, CA) and 2) assessment of peptide biological activity following exposure to HEK293 cells stably expressing MC4R and measurement of adenylyl cyclase activity, as previously described (45). All gels and apparatus used in 2-DE were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). All chemicals were purchased from BDH (Biolab, Auckland, New Zealand), BSA fraction V (BSA) was purchased from Sigma (Biolab), and sequencing grade trypsin was purchased from Promega (Madison, WI). All animal procedures undertaken were approved by the Animal Ethics Committee at the University of Auckland.

Subcutaneous Injections into Neonatal Rats

To compare peripheral effects of \(\alpha\)-MSH and desacetyl \(\alpha\)-MSH on neonatal rat growth, nine pups from each litter were subcutaneously injected with equal doses (0.3 \(\mu\)g/g body wt \(1^{-1}\) day \(-1\)) of \(\alpha\)-MSH (3 litters), desacetyl \(\alpha\)-MSH (3 litters), or vehicle control (4 litters) for 14 days. These same doses had previously been used to determine significant effects of these peptides on neonatal rat body growth (39). Adult female Wistar rats were housed in plastic cages and kept on a 12:12-h dark-light cycle. Animals received rat pellets (NRM, Auckland, New Zealand) and tap water ad libitum and were mated with males of the same strain. Each litter of newborn Wistar rats was culled to nine pups per mother by eliminating the smallest and largest pups from the litter, and each litter was assigned to one of three treatment groups: vehicle, \(\alpha\)-MSH, or desacetyl \(\alpha\)-MSH. Rats were weighed at birth and then every 2 days. On each treatment day peptides were dissolved in vehicle consisting of 0.1 M phosphate-buffered saline (PBS; 137 mM NaCl, 27 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.4) containing 1% BSA (PBS) and placed on ice until subcutaneously injected (40 \(\mu\)l) daily for 14 days. PBS was injected as vehicle control. On postnatal day 14, \(1.5\) h after injection, rats were euthanized with an overdose of pentobarbital sodium and stored at \(-80^\circ\)C.

2-DE Gel Electrophoresis and Analysis

Randomly selected hypothalamic tissues from PBS- (\(n = 9\)), \(\alpha\)-MSH- (\(n = 8\)), and desacetyl \(\alpha\)-MSH-treated (\(n = 8\)) rat pups were subjected to 2-DE analysis, resulting in 9, 8, and 8 2-DE-gels, respectively. The methods used for analytic 2-DE gels have previously been described for brain tissue (21, 60). Briefly, in our study, hypothalamic tissue was weighed and homogenized with 3.5 \(\mu\)l of lysis buffer [9 M urea, 2% vol/vol Triton X-100, 2% vol/vol Phosphate, pH 3–10 (Amersham Pharmacia Biotech, Uppsala, Sweden), 0.13 M dithiothreitol, 8 mM phenylmethylsulfonyl fluoride] per mg tissue, using a microprobe in an Eppendorf tube. The homogenates were centrifuged at 7,000 g for 5 min at room temperature and supernatants stored at \(-80^\circ\)C. The constituent proteins were separated on nonlinear pH 3–10 Immobiline DryStrips (Amersham Pharmacia Biotech) in the first dimension and 12–14% gradient SDS-PAGE gels in the second dimension. Silver-stained gels were digitized with consistent image size and settings using a Transparent Media Adapter on a Scanner E6 scanner (Microtek, Hsinchu, Taiwan). The images were imported into Melanie II software (Bio-Rad, Hercules, CA), and the protein spots on each gel were detected and quantitated. All gels were aligned and matched to a reference gel (the gel containing the most protein spots) so that features from each gel that were paired with a spot in the reference gel would form a group. The volume of each protein spot (area \(\times\) optical density) was measured and normalized as percentage of total volume of all spots detected in a given gel (%volume of spots = the sum of intensity values of every pixel in the area outlined for the spot as a percentage of the total staining intensity volume of the gel), which was then exported to Statistica for statistical analysis.

Identification of Proteins of Interest

Sample preparation. For preparative gels, protein samples (100 \(\mu\)l) were loaded via the in-gel rehydration method (Amersham Pharmacia Biotech) and separated as described above for our analytic 2-DE-gels, stained with Colloidal Coomassie Blue stain (1.3 M ammonium sulfate, 34% vol/vol methanol, 3% vol/vol phosphoric acid, 1.2 mM Coomassie Blue R-250) for 24 h at 21°C, and then destained with 5% vol/vol acetic acid until a clear background was achieved. Proteins of interest were excised and then digested in gel with trypsin using a modified method previously reported by Rosenfeld et al. (56). We used a lower salt concentration (25 mM NH4HCO3) and less trypsin (0.2–0.3 \(\mu\)g) than specified in the original method.

Liquid chromatography-MS-MS analysis. Peptide from trypsin-digested proteins was extracted with water and then twice with 50% acetonitrile-5% formic acid in an ultrasonic bath for 15 min and then analyzed using an LCQ Deca ion trap mass spectrometer fitted with a nanospray ESI interface (Thermo Finnigan, San Jose, CA) coupled to a Surveyor HPLC. Peptide samples (20 \(\mu\)l) were injected onto a reversed-phase column (Inertsil ODS-3, C18, 300 \(\mu\)m ID \(\times\) 15 cm, 3 \(\mu\)m; LC Packings, San Francisco, CA). The tryptic peptides were separated at a flow rate of 4 \(\mu\)l/min with a linear gradient of the solvents 0.1% aqueous formic acid (A) and acetonitrile-0.1% formic acid (B) run over 50 min. The linear gradient started at 98% A-2% B and ended at 20% A-80% B by 50 min. The column flow rate was produced by splitting the primary flow rate of 40 \(\mu\)l/min from the Surveyor HPLC system via an Acurate flow splitter (LC Packings). The nanospray interface was used with a 30-\(\mu\)m-ID fused-silica standard coated PicoTip (New Objective, Woburn, MA), and the spray voltage was supplied directly to the coated needle tip at 2.2 kV. The mass spectrometer was operated in the positive-ion mode, and the mass range acquired was between mass-to-charge ratio 300–2,000. The heated capillary temperature was set at 210°C. Data were acquired using a triple-play experiment in data-dependent mode with dynamic exclusion enabled.

Analysis of MS data. The MS-MS data were searched for matches against sequences in a subset of the GenPept nr database (NCBI). MS-MS spectra were interrogated using elements of the BioWorks 3.1 analysis package (Thermo Finnigan). DTA files were created in the Sequest Browser from the raw data files using a threshold value of 1e7 and were searched against indexed rat fasta sequences that had been digested virtually in silico with trypsin using the Sequest search algorithm. Peptide matches were deemed significant when the raw cross-correlation score was above the threshold values of 1.5 (M1+), 2 (M2+), and 2.5 (M3+), the preliminary raw score was above 300, and the delta correlation score was above 0.1 (36). The quality of each match was confirmed by analysis of the MS-MS spectra with the Fuzzy Ion de novo sequencing function of the Sequest Browser software. Selected DTA files were further analyzed by Lutefisk, a
program for the de novo interpretation of MS-MS spectra (63). Proteins with at least two peptide matches and >10% sequence coverage were considered significant.

**Statistics**

The growth curves for the three treatment groups based on body weight data collected over 14 days of treatment were analyzed by a General Linear Model (SAS system) using a nested-within-nested design with repeated measures to separate sources of variation between litters and between individual rats. The independent factors were treatment effects, litter (treatment) effects, and rat (litter × treatment) effects. Nine pups from each litter were assigned to a treatment group, thus forming a double-nested design. The “individual rat” variable was nested with the “litter” variable, which in turn was nested with the “treatment group” variable. The postnatal body weights measured sequentially on different days were treated as

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**Fig. 1.** Effect of melanocortin peptides on newborn rat body weights (9 pups/litter) after subcutaneous injection of PBS (control), α-MSH or desacetyl (des) α-MSH (0.3 μg/g body wt⁻¹·day⁻¹). A: growth rate over 14 days was significantly decreased in des α-MSH-treated pups compared with PBS (P < 0.05), but not in α-MSH-treated pups. General Linear Model (GLM) repeated-measures ANOVA, PBS (n = 36), α-MSH (n = 27), des α-MSH (n = 27). B: body weights (means ± SE) of rat pups on days 0, 2, 4, 6, 8, 10, 12, and 14. Significant differences on day 14 for peptide-treated groups compared with PBS control. *P < 0.05, 1-way ANOVA, PBS (n = 36), α-MSH (n = 27), des α-MSH (n = 27) followed by Bonferroni post hoc test.
repeated measures. Organ weight data were analyzed by first determining the correlation between organ weights and final body weights for each group. Data for organs that significantly correlated with body weight were normalized for body weight (g/100 g body wt). Body weight and normalized organ weight data from day 14 were analyzed by one-way ANOVA with Bonferroni post hoc test (Systat10 package; SPSS, Chicago, IL). Body and organ weights are presented as means ± SE.

The significance of treatment effects on protein spots in 2-DE gels was assessed by analyzing the normalized data (%vol) of individual protein spots of each group using the nonparametric Mann-Whitney U-test (60). A P value < 0.05 was considered statistically significant for all statistical methods used.

RESULTS

Differential Effects of α-MSH and Desacetyl α-MSH on Body Weight Change in Neonatal Rats

Desacetyl α-MSH significantly reduced the days 1–14 postnatal growth curve for newborn rats (P < 0.05) during daily subcutaneous peptide injection compared with a PBS control group (Fig. 1A). This contrasted with no significant effect on postnatal growth curve seen with α-MSH compared with the PBS-treated control group. However, on postnatal day 14, when body weights were measured ~1.5 h after injection, both α-MSH and desacetyl α-MSH-treated pups were significantly heavier (P < 0.05) than the PBS-treated control animals (Fig. 1B).

Differential Effects of α-MSH and Desacetyl α-MSH on Organ Weights

The correlation between organ weight and body weight on day 14 in control (PBS) and treatment (α-MSH and desacetyl α-MSH) groups was significant (P < 0.05) for brain, spleen, heart, kidney, and liver, but not for lung. Comparison of normalized organ weights (g/100 g body wt) between treatment and PBS control groups showed that organ weight was significantly decreased (P < 0.05) for brain by both α-MSH and desacetyl α-MSH and for kidney by α-MSH only and significantly increased for spleen by desacetyl α-MSH only (Table 1).

Peripheral Injection of α-MSH and Desacetyl α-MSH Induced Different Patterns of Protein Changes in the Hypothalamus

α-MSH significantly changed protein expression levels for 32 of 1,209 matched protein groups compared with PBS control rats (Fig. 2A), and desacetyl α-MSH significantly changed 33 of 1,212 matched protein groups compared with PBS control rats (Fig. 2B). Some of these protein spots were increased and others decreased (indicated in Fig. 2, A and B). Only nine proteins (p1079, p1327, p1445, p1458, p1505, p1520, p1610, p1790, and p1854) with significantly altered expression compared with PBS control were common to both α-MSH and desacetyl α-MSH-treated groups (Fig. 2, A and B).

Identification of Proteins of Interest

We identified proteins of interest using liquid chromatography (LC)-MS-MS analysis, employing collision-induced dissociation to yield peptide fragmentation spectra that were used to query the NCBI nr database using the Sequest algorithm. This analysis identified 24 of the 65 protein spots that were significantly changed by peptide treatments (Table S1; supplemental data for this article may be found at http://ajpendo.physiology.org/cgi/content/full/00480.2005/DC1), with more than two peptides matching to each identified protein. Using extent of protein coverage as an indication of confidence of protein identification, 12 of the identified proteins had more than 10% protein coverage, and 4 of 12 of these were increased by both peptide treatments (Table 2 and Table S2, supplemental data). The proteins were grouped according to their main cellular functions, such as cytoskeletal, metabolic, signaling, and stress response proteins, and the significant effect of each peptide treatment indicated (Table 2).

Two proteins, p1610 and p1588, were identified as stathmin. The observation that these two proteins appeared as different spots of similar molecular mass but different pI on the 2-DE gels suggested that p1588 could be a phosphorylated isof orm of stathmin. A phosphate group would add negative charge to the protein, resulting in a shift towards the acidic side of the gel. To test this, the MS-MS data files from both p1610 and p1588 from which tryptic peptide sequences were ascertained were analyzed using a differential modification to serine (Ser), threonine, or tyrosine residues of +79.98 corresponding to the mass of a phosphate group. Indeed, a data file from p1588 matched the sequence ESVPEFPLS*PPK, with a phosphate group added to the second Ser, corresponding to the Ser at position 38 of the amino acid sequence of rat stathmin. Re-analysis clearly showed that the modified peptide can, on its own, explain the gel shift observed. No other phosphorylated peptides were seen for stathmin.

DISCUSSION

We show here that 0.3 μg·g⁻¹·day⁻¹ desacetyl α-MSH, peripherally administered daily for the first 2 wk of life to neonatal rats, is biologically active and induces responses that differ from those for α-MSH similarly administered. When the growth curves for the three treatment groups were compared,

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Brain</th>
<th>Heart</th>
<th>Kidney</th>
<th>Liver</th>
<th>Lung</th>
<th>Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle PBS</td>
<td>3.70±0.04</td>
<td>0.54±0.01</td>
<td>1.11±0.01</td>
<td>3.30±0.04</td>
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<td>0.52±0.01</td>
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<td>α-MSH</td>
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<td>0.53±0.01</td>
<td>1.06±0.01*</td>
<td>3.22±0.06</td>
<td>0.60±0.01</td>
<td>0.53±0.01</td>
</tr>
<tr>
<td>Desacetyl α-MSH</td>
<td>3.49±0.06*</td>
<td>0.54±0.01</td>
<td>1.11±0.01</td>
<td>3.22±0.07</td>
<td>0.58±0.02</td>
<td>0.59±0.01*</td>
</tr>
</tbody>
</table>

Values are means ± SE in g/100 g body wt for PBS (n = 36), α-MSH (n = 27), and desacetyl α-MSH (n = 27) groups. Organs were weighed (mg) on day 14, organ weights were normalized with body weight (g/100 g body wt) for all except lungs (which did not show a significant correlation with body weight), and treatment groups were compared with vehicle PBS group. *P < 0.05, significantly different from PBS group; 1-way ANOVA followed by Bonferroni post hoc test.
desacyl \( \alpha \)-MSH significantly slowed neonatal rat growth, whereas \( \alpha \)-MSH had no effect. Desacyl \( \alpha \)-MSH significantly increased spleen weight, but \( \alpha \)-MSH had no effect. \( \alpha \)-MSH, on the other hand, significantly decreased kidney weight, and desacyl \( \alpha \)-MSH had no effect. Both desacyl \( \alpha \)-MSH and \( \alpha \)-MSH significantly decreased brain weight and increased body weight at postnatal day 14.

Results from this study demonstrate for the first time that desacyl \( \alpha \)-MSH and \( \alpha \)-MSH show different effects on rat neonatal growth. Circulating levels of desacyl \( \alpha \)-MSH have been reported to be more abundant than \( \alpha \)-MSH at birth, but in rodents this is reversed after birth with \( N \)-acetyltransferase activity in rat pituitary peaking at approximately 2 mo of age and then declining (11). It is possible that the relative abundance of \( \alpha \)-MSH and desacyl \( \alpha \)-MSH peptides influences neonatal growth, with increased peripheral desacyl \( \alpha \)-MSH slowing growth in the neonate. In contrast to our findings that desacyl \( \alpha \)-MSH and \( \alpha \)-MSH both increased rat body weight at postnatal day 14 but not prior to this, Mauri et al. (39) reported significant trophic effects from rat postnatal days 2–14.
with peripheral administration of the same dose of desacetyl α-MSH, whereas the same dose of α-MSH had no significant effect. Trophic effects of α-MSH were seen only with a 10-fold higher dose from postnatal days 4 to 14. Some of their observed peptide effects on various organ weights also differed from our observations here. These differences between our results and those of Mauri et al. might be due to different rodent genetic backgrounds (their rat strain was not stated), for results and those of Mauri et al. might be due to different rodent genetic backgrounds (their rat strain was not stated), for example, and in the relative abundance of these two peptides. Another possibility is the statistical model used for data analysis (see Statistics); their experimental design and statistical analysis were not stated.

In adult mice, peripherally administered desacetyl α-MSH has been found to significantly increase body weight, muscle and adipose tissue weight, and food intake (62). It is not surprising that these results differ from our data in the neonatal rat, since the hypothalamus, which is critical for regulation of appetite, body weight, and metabolism in the adult, is not fully developed until after postnatal day 14 in rodents (53). The physiological roles of leptin are also different in neonate compared with adult rats. In adults, leptin is an adipocyte-derived hormone that signals the hypothalamus about energy stores and regulates key metabolic pathways involved in energy homeostasis. However, in newborn rats, leptin is critical for the development of neuron projections in the hypothalamus (7, 8, 52). The leptin signaling network in the hypothalamus is immature in the early postnatal period, and there is a sequential pattern of projections developing from the arcuate nucleus to other parts of the hypothalamus, with the mature pattern of innervation established between postnatal days 8 and 10. Our observation here that desacetyl α-MSH, but not α-MSH-treated pups promoted slower growth compared with control animals between postnatal days 0 and 10 might indicate that desacetyl α-MSH induces catabolic pathways in peripheral tissues that result in mobilization of fat stores. Disparate effects on fat mobilization have been reported in rainbow trout, where desacetyl α-MSH but not α-MSH significantly stimulated fat mobilization (69). It is interesting that, in our study, the catch-up growth for desacetyl α-MSH-treated pups and the trophic effects observed for α-MSH-treated pups compared with control animals between postnatal days 10 and 14 coincided with the reported time for the initial appearance of a mature pattern of hypothalamic innervation.

It is unknown whether the effects of these MSH peptides on body and organ weights are direct and/or indirect, mediated through melanocortin receptors in the periphery and/or in the brain. In adult rats, ~0.001% of an initial dose of α-MSH has been demonstrated to cross the blood-brain barrier (17), and to our knowledge it is unknown how much crosses the blood-brain barrier in neonatal rats. Two melanocortin receptors, MC3R and MC4R, are expressed in the brain and elsewhere (15, 43), and mRNA for these receptors are expressed early in rat development (43) (Mountjoy KG, unpublished data), and therefore, these peptides could be acting through either or both of these receptors peripherally or centrally. Both peptides also similarly couple MC1R and MC5R in vitro (47), and thus the neonatal responses we see here could also involve either one or both of these receptors. Although the mechanism of action of α-MSH and desacetyl α-MSH on organ growth is unclear, their similar (decreased brain weight) and different (decreased kidney and increased spleen weights) effects demonstrate specific and sometimes disparate in vivo biological roles for each peptide.

Our hypothalamic proteomic analysis shows that chronic peripheral injections of α-MSH and desacetyl α-MSH change the expression of a number of proteins, and these effects could be indirect or direct. The hypothalamus is the site for relaying and integrating central and peripheral signals maintaining body weight, and therefore, we would expect changes in body weight, such as those induced by peripheral administration of melanocortin peptides, to be reflected in changes in hypothalamic proteins. The protein changes we observed can be subdivided into four categories: metabolic enzymes, cytoskeletal proteins, signaling proteins, and stress response proteins (Table 2). Both peptides increased levels of ATP synthase, stathmin, coflin, β-tubulin, and a protein similar to protein kinase C inhibitor. Gel spots corresponding to truncated proteins were present at reduced molecular mass and often altered pl values from those expected or observed for the intact protein. These truncations retained some of the peptide complement of the parent protein and may not be artifacts of the sample preparation; they may be natural degraded forms of enzymes reflecting accelerated turnover. α-MSH increased a fragment of ATP synthase (p1351), whereas desacetyl α-MSH increased a fragment of ATP synthase (p1339). ATP synthase couples ATP generation to respiratory chain oxidation, and “uncoupling” of this process leads to proton leakage, resulting in energy dissipated as heat (1, 32, 55). Interestingly, ATP synthase itself has been shown to be a target protein for an anorectic peptide, enterostatin (6). In this study, α-MSH also increased protein expression of adenylate kinase-1 (AK1; p1360), a member of the adenylate kinase family of phosphotransfer enzymes essential in the maintenance of cellular energetics in tissues with highly variable energy demand, such as muscle and brain (30, 31). Genetic ablation of AK1 disturbs

<table>
<thead>
<tr>
<th>Protein</th>
<th>Spot No.</th>
<th>α-MSH</th>
<th>Desacetyl α-MSH</th>
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<tbody>
<tr>
<td>Peroxiredoxin</td>
<td>P1317</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>ATP synthase H+ transporting</td>
<td>P1351</td>
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<td>Adenylate kinase-1</td>
<td>P1360</td>
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<td>β-Tubulin</td>
<td>P1520</td>
<td>2.1</td>
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<tr>
<td>Coflin</td>
<td>P1542</td>
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<td></td>
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<tr>
<td>Stathmin</td>
<td>P1610</td>
<td>1.5</td>
<td>1.8</td>
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<tr>
<td>Similar to PKC inhibitor</td>
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<td>Similar to UMP-CMP kinase</td>
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</table>

Proteins identified in Table S2 that were significantly affected by α-MSH or desacetyl α-MSH treatment compared with control rats are shown grouped by cellular function with direction of peptide effect indicated. Fold change values were calculated on the basis of mean %vol values of protein spots quantitated using Melanie II software. Significant differences for peptide-treated group compared with PBS control were determined using the nonparametric Mann-Whitney U-test, and a P value <0.05 was considered significant.
muscle energetic economy and decreases tolerance to metabolic stress (30). These hypothalamic protein changes suggest that MSH peptides are associated with mitochondrial metabolism and energy utilization.

We show here that peripherally administered α-MSH and desacetyl α-MSH also change hypothalamic expression levels of several cytoskeletal proteins, such as cofilin, tubulin, and stathmin. Both peptides increased levels of a fragment of β-tubulin polypeptide, cofilin, and stathmin. Cofilin, also known as the actin depolymerization factor ADF, has been shown to bind directly to actin filaments and promote their disassembly (38, 49). Stathmin, also known as oncoprotein 18, is an ubiquitous cytoplasmic protein that interacts with tubulin dimers and increases the catastrophe rate (the transition from growing to shrinking) of microtubules (3, 4). Both brain-derived neurotrophic factor and nerve growth factor (NGF) have been reported to phosphorylate stathmin in PC12 cells, mostly on Ser25, which corresponds to the major site phosphorylated by mitogen-activated protein kinase in vitro (12, 20), and blocking the expression of stathmin using antisense oligonucleotides prevented NGF-induced differentiation of PC12 cells into sympathetic-like neurons (19). Stathmin is expressed at higher levels in neonatal rat brain compared with adult brain (25) and is involved in neurogenesis in adult mouse brain (33). In our study, desacetyl α-MSH increased levels of a phosphorylated isoform of stathmin (p1588), and the MS-MS data indicate that this is phosphorylated at Ser38. Interestingly, phosphorylation of stathmin at Ser38 is thought to be via cyclin-dependent kinases during mitosis and results in reduced microtubule-destabilizing activity of stathmin (5, 35, 37). There are at least 14 isoforms of stathmin that migrate on 2-DE gels, two unphosphorylated and 12 increasingly phosphorylated proteins (48, 71), and it has been suggested that regulation of microtubule dynamics by stathmin phosphorylation could be involved in fundamental processes associated with the reorganization of the cytoskeleton, such as neuronal differentiation or synaptic plasticity. The observation that desacetyl α-MSH increased the Ser38-phosphorylated isoform of stathmin suggests that desacetyl α-MSH may reduce the microtubule destabilizing effect and oppose the action of α-MSH, which increases stathmin expression but not phosphorylation.

Reorganization of the cytoskeleton through modification of microtubule dynamics is important in neuronal differentiation and synaptic plasticity, and some of the changes in hypothalamic protein expression we observed might be associated with direct or indirect roles for both MSH peptides in synaptic plasticity. Leptin plays a critical neurotrophic role in the hypothalamus during the neonatal period studied here (8, 52), and leptin also regulates desacetyl α-MSH and α-MSH levels (27). It is possible, therefore, that leptin’s neurotrophic effects are in part mediated though the MSH peptides. α-MSH improves short-term memory in rats (18), and the behavioral effects of α-MSH are permanent in rats treated peripherally postnatally but only transitory in animals treated as adults (2). Hence, the hypothalamic protein changes observed from chronic treatment of α-MSH and desacetyl α-MSH imply a modified neuronal network that might lead to behavioral changes in adult rats.

Our proteomic analysis has produced a snapshot of the peptide changes induced by α-MSH and desacetyl α-MSH peptide treatments. We have identified several hypothalamic proteins, using 2-DE gel-based proteomic techniques, that have not previously been associated with melanocortin peptide signaling. Moreover, we show that peripherally administered desacetyl α-MSH, but not α-MSH, to newborn rats slows their growth over 10 days, and then their body weights catch up and overtake control rats. α-MSH, on the other hand, stimulates body weights between postnatal days 10 and 14. After 14 days of peripheral peptide treatment, both peptides induced changes in hypothalamic protein expression, and although many of the changes were similar for the two peptides, there were also some differences. We have clearly shown that peripherally administered desacetyl α-MSH, like α-MSH, is biologically active in vivo and that it differs from α-MSH in some of its responses. Finally, we demonstrate the application of 2-DE gel-based proteomics as a discovery system to identify hypothalamic proteins that are directly, or indirectly, responsive to either α-MSH or desacetyl α-MSH stimulation in vivo.

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