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Transgenic MSH overexpression attenuates the metabolic effects of a high-fat diet

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Lee M, Kim A, Chua SC Jr, Obici S, Wardlaw SL. Transgenic MSH overexpression attenuates the metabolic effects of a high-fat diet. *Am J Physiol Endocrinol Metab* 293: E121–E131, 2007. First published March 20, 2007; doi:10.1152/ajpendo.00555.2006.—To determine whether long-term melanocortinergic activation can attenuate the metabolic effects of a high fat diet, mice overexpressing an NH₂-terminal POMC transgene that includes α - and γ ₃-MSH were studied on either a 10% low-fat diet (LFD) or 45% high-fat diet (HFD). Weight gain was modestly reduced in transgenic (Tg-MSH) male and female mice vs. wild type (WT) on HFD ($P < 0.05$) but not LFD. Substantial reductions in body fat percentage were found in both male and female Tg-MSH mice on LFD ($P < 0.05$) and were more pronounced on HFD ($P < 0.001$). These changes occurred in the absence of significant feeding differences in most groups, consistent with effects of Tg-MSH on energy expenditure and partitioning. This is supported by indirect calorimetry studies demonstrating higher resting oxygen consumption and lower RQ in Tg-MSH mice on the HFD. Tg-MSH mice had lower fasting insulin levels and improved glucose tolerance on both diets. Histological and biochemical analyses revealed that hepatic fat accumulation was markedly reduced in Tg-MSH mice on the HFD. Tg-MSH also attenuated the increase in corticosterone induced by the HFD. Higher levels of *AgRP* mRNA, which might counteract effects of the transgene, were measured in Tg-MSH mice on LFD ($P = 0.02$) but not HFD. These data show that long-term melanocortin activation reduces body weight, adiposity, and hepatic fat accumulation and improves glucose metabolism, particularly in the setting of diet-induced obesity. Our results suggest that long-term melanocortinergic activation could serve as a potential strategy for the treatment of obesity and its deleterious metabolic consequences.

melanocyte-stimulating hormone; melanocortins; proopiomelanocortin; obesity; hepatic steatosis; diabetes

THE HYPOTHALAMIC MELANOCORTIN SYSTEM is a major regulator of energy homeostasis with effects on feeding behavior as well as energy expenditure (13, 14, 51, 58). This system consists of the proopiomelanocortin (POMC)-derived MSH peptides, including α - and γ ₃-MSH, the MSH antagonist, agouti-related peptide (AGRP), and the melanocortin-3 and -4 receptors (MC3R and MC4R). α -MSH inhibits feeding and stimulates energy expenditure, whereas AGRP is orexigenic and decreases energy expenditure (42, 45, 46). α -MSH and AGRP interact with both the MC3R and MC4R, whereas γ ₃-MSH selectively activates the MC3R, which exerts effects on feed efficiency and adiposity (2, 48). Several studies (6, 13) have demonstrated that inactivation of the melanocortin system in both humans and in rodent models leads to an obese phenotype with

insulin resistance. Inactivating mutations of MC4R are considered, to date, to be the most common cause of human monogenic obesity (19, 57). Similarly, MC4R knockout mice have been shown to be hyperphagic and obese (28). MC3R knockout mice, although not hyperphagic, possess a higher feed efficiency and have increased adiposity as well as reduced lean body mass (7, 11). Mice lacking both MC3- and MC4R are significantly heavier than mice with only the MC4R knockout, suggesting that these two receptors play nonredundant roles in mediating energy balance (11).

Disruption of the melanocortin system via POMC deficiency also results in obesity. In humans, POMC mutations with subsequent deletion of POMC and its MSH cleavage products leads to a syndrome of early-onset obesity, adrenal insufficiency, and red hair pigmentation (32). Mice with targeted deletion of the POMC gene are obese despite profound adrenal insufficiency (10, 60). These mice, when treated with α -MSH, experience substantial levels of weight loss, indicating that restoration of melanocortinergic tone can ameliorate the obesity phenotype (60). Increased susceptibility to obesity has been noted in both POMC^{+/-} mice and in humans heterozygous for POMC null mutations (10, 18). There is ample evidence that short-term use of melanocortin receptor agonists in rodents can reduce food intake and increase energy expenditure (23, 26, 30, 40, 46). Results with longer-term administration of either α -MSH itself or several MSH peptide agonists have been more variable with evidence of tachyphylaxis to some of the effects of α -MSH in some studies (23, 30, 46). In addition, results have been complicated by problems with conditioned taste aversion with some MSH agonists (4). Thus, whether long-term, chronic melanocortinergic activation is effective as a means of promoting weight loss and treating obesity is an area still in need of further investigation.

To study the effects of long-term activation of the melanocortin system on energy homeostasis, we generated mice on a C57BL/6 background that overexpress an NH₂-terminal POMC transgene under the control of the cytomegalovirus (CMV) promoter (Tg-MSH). The transgene contains the sequences for NH₂-terminal POMC, joining peptide and α -MSH. We have chosen to express only the NH₂-terminal portion of POMC, which is processed to α - and γ ₃-MSH in tissues that normally process POMC, to avoid problems with ACTH or β -endorphin (β -EP) overexpression that could affect energy balance. We have previously reported that overexpression of Tg-MSH has a modest effect in reducing body weight and adiposity and improving fasting insulin levels in male mice fed

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a regular chow and in reducing body weight in leptin-receptor deficient db^{3J}/db^{3J} mice (50). It was unknown, however, whether overexpression of MSH would protect against more common forms of diet-induced obesity and ameliorate the associated metabolic derangements. Therefore, we have now studied the effects of MSH overexpression on weight gain, adiposity, hepatic fat deposition, and glucose metabolism in mice fed either a 10% low-fat (LF) diet or a 45% high-fat (HF) diet after being weaned. Oxygen consumption was studied in a subset of mice on the HF diet. We have also examined the effects of diet and MSH overexpression on POMC and AGRP gene expression in the hypothalamus.

MATERIALS AND METHODS

Animals and treatment protocols. Transgenic mice were generated as described previously to overexpress NH₂-terminal POMC under the control of the CMV promoter (50). The transgene contained part of the 5'UTR, the signal sequence, the sorting sequence, γ_3 -MSH, the joining peptide, and α -MSH, including the COOH-terminal glycine necessary for amidation. The transgene was expressed in multiple tissues, including hypothalamus, where levels of α - and γ_3 -MSH were increased twofold. Gel filtration and RIA confirmed that overexpression of α -MSH was limited to tissues that normally process POMC (50). Mice from one line were backcrossed to a coisogenic C57BL/6J line, C57BL/6J- A^{mJ} strain for six generations, and used for the current studies. This is a white-bellied agouti-colored line, which was used instead of black mice to be able to visualize the darkening effect of MSH on coat color. All studies were performed in transgenic homozygous mice with control mice being wild-type (WT) animals generated from the backcross at the N6 generation.

All animals were housed under barrier conditions with a 12:12-h light-dark cycle and were housed together by group with 3–4 animals/cage. Mice were placed on either LF (10% by kcal) or HF (45% by kcal) diet upon being weaned at 4 wk of age and provided ad libitum access to food and water. LF (D12450B) and HF (D12451) diets were purchased from Research Diets. Mice were killed by decapitation after brief exposure to CO₂ between 39 and 60 wk of age. All protocols were approved by the Columbia University Institutional Animal Care and Use Committee and were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Measurement of body weight, body composition, and food intake. Mice were weighed weekly ($n = 15$ –21/group). Body composition was assessed by dual-energy X-ray absorptiometry scan at ages 16–18 wk and again at 37–41 wk with 10–21 animals/group (Lunar PIXIMUS; Lunar, Madison, WI). To assess food intake, mice were housed in cages of 1–4 animals of the same genotype, sex, and diet. Food intake was measured over a 5-day period, and the average daily intake per animal was calculated and expressed as kcal·mouse⁻¹·24 h⁻¹ ($n = 15$ –24 animals/group, 7–10 cages/group).

Indirect calorimetry. Indirect calorimetry was performed on HF-fed male WT and Tg-MSH mice ($n = 4$ /group, age 51–54 wk) as previously described (44). Mice were individually housed in the calorimeter cages and acclimated to the respiratory chambers for 2 days prior to gas exchange measurements. Indirect calorimetry was performed using a computer-controlled Oxymax open-circuit calorimetry system (Columbus Instruments, Columbus, OH). Oxygen consumption and CO₂ production were measured for each mouse at 6-min intervals over a 24-h period. The respiratory quotient (RQ) was calculated as the ratio of CO₂ production over O₂ consumption. Measurements were attained in both fasting and fed states.

Fasting insulin and glucose tolerance tests. At 38–44 wk, tail blood was collected from animals the morning after a 16-h fast ($n = 10$ –12 males/group, 15–20 females/group). The mice were lightly

restrained, and only the first 5 μ l were used for glucose measurements. Glucose was measured using the glucose oxidase method (Glucometer Elite; Bayer, Elkhart, IN). Serum insulin was measured using a commercial RIA kit from Linco Research (St. Louis, MO).

At 20–26 wk, glucose tolerance was assessed following intraperitoneal injection of glucose (2 mg/g body wt) after a 16-h fast ($n = 6$ –12 mice/group). Blood glucose was measured from tail blood collected at baseline and at 20, 40, 60, and 90 min after glucose administration.

Liver weight and hepatic lipid content. Male and female mice were killed between 39 and 48 wk of age. A subset of HF male Tg-MSH and WT mice were killed between 54 and 60 wk of age after a 16-h overnight fast. Immediately upon death, livers were weighed and snap-frozen in liquid nitrogen after a section of liver was taken for histological examination with hemotoxylin and eosin (H&E) stain. Frozen liver samples were stored at -80°C prior to homogenization for lipid assessment. Hepatic lipid was isolated by chloroform-methanol extraction in a modified Folch et al. (21) protocol. Immediately upon isolation, hepatic lipid was assayed for triglycerides (TG) and free fatty acids (FFA). TG levels were quantified using the Infinity TG kit from Sigma-Aldrich (St. Louis, MO). FFA levels were measured using an enzymatic colorimetric assay from Wako Diagnostics (Richmond, VA). Results are expressed per gram of liver.

Plasma FFA, TG, leptin, thyroid hormone, and corticosterone levels. FFA, TG, leptin, and thyroid hormone (T₄) were measured in plasma from trunk blood collected at the time of death. FFA and TG were quantified using commercial kits from Wako Diagnostics and Sigma, respectively. Leptin was assayed with an RIA kit from Linco Research, and T₄ was determined with an RIA kit from Diagnostic Products. Plasma corticosterone was measured by RIA from ICN Biomedicals (Costa Mesa, CA).

Isolation of hypothalamic RNA and quantification of Pomc and Agrp mRNA by solution hybridization assay. The medial basal hypothalamus was dissected as described previously in the rat using a mouse brain matrix (50). A 3-mm coronal section caudal to the optic chiasm was used. Total RNA was extracted with the RNeasy minikit (Qiagen, Valencia, CA) according to the manufacturer's instructions and quantified by spectrophotometry. RNA used to generate standard curves and ³²P-labeled RNA probes were synthesized using commercial transcription kits (Promega). Sense and antisense mRNAs were synthesized from plasmid vectors containing T3 and T7 polymerase promoters and the appropriate mouse cDNA fragment: 923-bp *Pomc*, 254-bp *Agrp*. The *Pomc* plasmid was linearized to yield an antisense probe that targeted the sequence after the sequence included in the transgene so that only changes in endogenous *Pomc* expression were detected. Sense RNAs were quantified spectrophotometrically and used to generate standard curves in the hybridization assays. Hybridizations with *Pomc* and *Agrp* riboprobes were performed together in the same tube followed by the addition of S1 nuclease. Samples were then phenol-chloroform extracted, precipitated, and electrophoresed on a 4% acrylamide gel. The protected *Pomc* and *Agrp* bands were quantified by phosphorimager analysis and compared with the standard curve. Because the protected hybrids were smaller than the cellular transcripts, results were normalized to the full-length RNA species: 0.7 kb for full-length mouse *Agrp* cytoplasmic RNA, 1.0 kb for full-length mouse *Pomc* cytoplasmic RNA. Results are expressed as picograms of cytoplasmic RNA per microgram total RNA.

Statistical analysis. Statistical analysis was performed with Student's *t*-test when only two groups were compared. Two-way ANOVA was used to determine the effects of transgene and diet or sex. Fisher's protected least squares difference test was used to evaluate the significance between groups in such cases. ANOVA for repeated measures was used when values over different times were analyzed. $P < 0.05$ was considered statistically significant. Results are reported as means \pm SE.

RESULTS

Effect of Tg-MSH on body weight, body composition, and food intake. Both WT and Tg-MSH male and female mice ($n = 15\text{--}21/\text{group}$) gained significantly more weight with HF feeding ($P < 0.001$; Fig. 1). However, on the HF diet, the weight gain was significantly less in the Tg-MSH male and female mice compared with WT mice ($P < 0.001$; Fig. 1). On the LF diet there was no significant difference in body weight between WT and Tg-MSH males until 36 wk of age, when Tg-MSH mice weighed slightly less than WT mice, and a significant effect of genotype over time emerged ($P = 0.004$). In contrast, Tg-MSH females on LF diet weighed slightly more than WT females ($P = 0.02$; Fig. 1). At 16–18 wk of age, significant differences in body composition were detected in Tg-MSH mice compared with WT mice on the LF diet despite similar body weights. Body fat percentage was reduced by 14.4% in male and by 10.3% in female Tg-MSH mice on the LF diet ($P < 0.05$; Fig. 2). Lean body mass was also higher in LF Tg-MSH female mice (18.5 ± 0.4 vs. 17.5 ± 0.2 g, $P < 0.05$). At 16–18 wk, the reduction in adiposity was even greater with HF feeding: -30.1% in male and -21.1% in female Tg-MSH mice ($P < 0.001$). Moreover, the adiposity of Tg-MSH male and female mice after 3 mo of HF feeding was no different than when they were fed a LF diet. In contrast, HF WT mice had significant increases in percent body fat: $+35\%$ in males ($P < 0.001$), $+27\%$ in females ($P < 0.01$). By 37–41 wk, the decrease in adiposity in Tg-MSH vs. WT mice was less pronounced: -12% in HF males ($P = 0.003$), -19% in HF females ($P < 0.01$), -16% in LF males ($P < 0.05$; Fig. 2). Although there was no longer a significant decrease in adiposity in older Tg-MSH females on LF diet, they continued to

possess greater lean body mass (18.6 ± 0.4 vs. 17.4 ± 0.2 g, $P < 0.01$). Notably, there was no significant difference in caloric intake between Tg-MSH and WT males on either LF or HF diet, nor was there any difference in caloric intake between Tg-MSH and WT females on the LF diet, although on the HF diet Tg-MSH females consumed significantly less than WT female mice ($P < 0.05$; Table 1).

Effects of Tg-MSH on $\dot{V}O_2$ consumption and RQ. $\dot{V}O_2$ consumption, measured by indirect calorimetry, and RQ were studied in a subset of male mice on the HF diet. Tg-MSH male mice were found to have a significantly higher $\dot{V}O_2$ than WT in both fed ($2,752 \pm 166$ vs. $2,427 \pm 23$ ml \cdot kg $^{-1}\cdot$ h $^{-1}$) and fasted ($2,245 \pm 93$ vs. $1,936 \pm 52$ ml \cdot kg $^{-1}\cdot$ h $^{-1}$) states ($P < 0.01$, 2-way ANOVA; Fig. 3). Resting RQ decreased as expected with fasting. Overall, fed and fasted Tg-MSH mice had a significantly lower resting RQ than WT animals ($P < 0.05$, 2-way ANOVA; Fig. 3). Calculated heat production was, however, not significantly different.

Effect of Tg-MSH on liver weight and hepatic lipid accumulation. On the LF diet, there was no difference in liver weight between WT and Tg-MSH animals (Fig. 4). However, on HF diet, the livers of Tg-MSH mice weighed less than those of WT mice: 1.53 ± 0.17 (Tg-MSH males) vs. 2.11 ± 0.13 g (WT males), $P < 0.05$; 1.13 ± 0.03 (Tg-MSH females) vs. 1.62 ± 0.12 (WT females), $P < 0.001$ (Fig. 4). Liver weight as percentage of body weight was significantly lower in Tg-MSH male ($3.4 \pm 0.27\%$) and female ($3.4 \pm 0.15\%$) vs. WT male ($4.1 \pm 0.15\%$) and female ($4.2 \pm 0.31\%$) mice on the HF diet ($P = 0.002$). A marked increase in hepatic lipid accumulation on the HF diet was noted on histological examination of H&E-stained liver sections from WT mice; this was attenuated

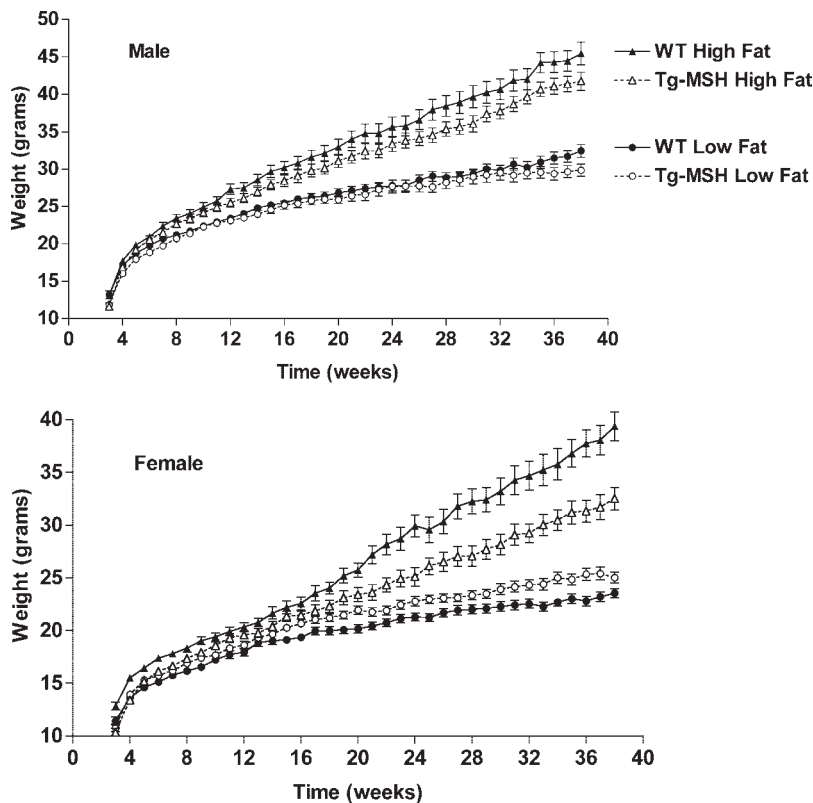


Fig. 1. Effect of diet and transgenic (Tg-MSH) on body weight in male (top) and female (bottom) mice. Both wild-type (WT) and Tg-MSH male and female mice gained more weight on high-fat (HF) diet ($P < 0.001$). On HF diet, Tg-MSH males ($n = 17$) weighed less than WT males ($n = 19$, $P < 0.001$). On low-fat (LF) diet, there was no difference in weight between WT ($n = 23$) and Tg-MSH ($n = 19$) males until 36 wk of age, when Tg-MSH mice weighed slightly less than WT mice, and a significant effect of genotype over time emerged ($P = 0.004$). On HF diet, Tg-MSH females ($n = 19$) weighed less than HF WT females ($n = 15$, $P < 0.001$). On LF diet, Tg-MSH females ($n = 19$) weighed slightly more than LF fed WT females ($n = 20$, $P = 0.02$).

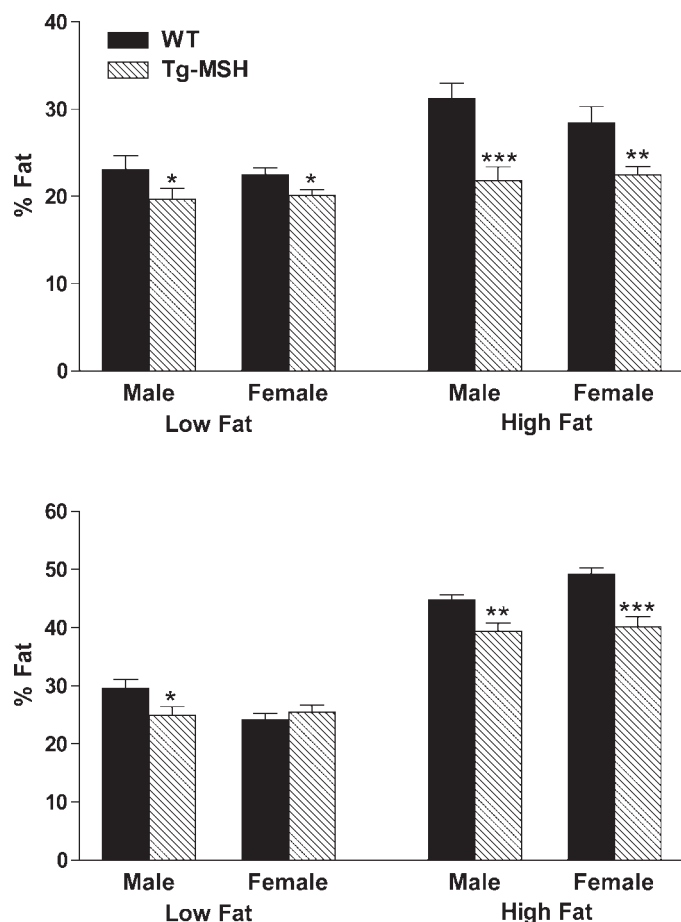


Fig. 2. Effect of diet and Tg-MSH on adiposity in mice at 16–18 wk of age (top) and 37–41 wk of age (bottom). Top: on LF diet, body fat percentage was lower in Tg-MSH male (–14%, $n = 12$) and female (–10%, $n = 14$) mice ($P < 0.05$) compared with WT males ($n = 16$) and females ($n = 15$). These differences in adiposity were more striking on the HF diet –30% in Tg-MSH male ($n = 14$) and –21% in Tg-MSH female mice ($n = 15$, $P < 0.001$) compared with HF WT males ($n = 15$) and females ($n = 10$). Bottom: the protective effect of Tg-MSH on adiposity was less pronounced in older mice: –16% in LF Tg-MSH males ($n = 19$) vs. LF WT ($n = 21$, $P < 0.05$), –12% in HF Tg-MSH males ($n = 17$) vs. HF WT ($n = 17$, $P = 0.003$), and –19% in HF Tg-MSH females ($n = 19$) vs. HF WT ($n = 15$, $P < 0.01$). Although there was no longer any difference in adiposity between LF Tg-MSH ($n = 19$) and WT females ($n = 15$) by 41 wk, LF Tg-MSH female mice continued to demonstrate significantly greater lean body mass than WT females.

in Tg-MSH mice and in some cases, as shown in Fig. 5, was almost completely prevented. Hepatic TG content was significantly lower in male (–32%, $P = 0.009$) and female (–55%, $P = 0.004$) Tg-MSH mice vs. WT mice on the HF diet (Fig. 4).

Table 1. Caloric intake of Tg-MSH and WT male and female mice on HF and LF diets

Genotype/Sex	LF Diet, kcal	HF Diet, kcal
Tg-MSH male	11.8 ± 0.8	13.6 ± 0.5
WT male	11.8 ± 0.8	13.6 ± 0.9
Tg-MSH female	10.6 ± 0.4	8.9 ± 0.5*
WT female	10.6 ± 0.4	13.6 ± 0.5

Values are means ± SE; caloric intake is expressed in kcal/mouse per 24-h period. Tg, transgenic; WT, wild type; HF, high fat; LF, low fat. * $P < 0.05$ compared with WT female mice.

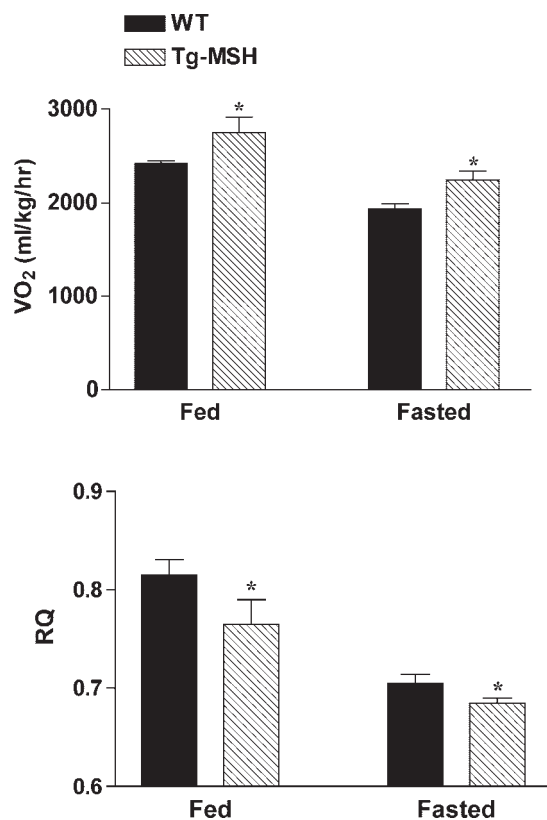


Fig. 3. Effect of Tg-MSH on energy expenditure and respiratory quotient (RQ) in male mice on a HF diet. Top: VO₂ measured by indirect calorimetry was significantly higher in Tg-MSH ($n = 4$) compared with WT ($n = 4$) mice in both the fed and fasted states (* $P < 0.01$, 2-way ANOVA for genotype). Bottom: overall, fed and fasted Tg-MSH mice had a significantly lower resting RQ than WT animals (* $P < 0.05$, 2-way ANOVA for genotype).

There was also a trend toward lower hepatic FFA levels in HF Tg-MSH vs. WT males (7.8 ± 0.4 vs. 9.1 ± 0.5 $\mu\text{M/g}$ liver, $P = 0.08$) and females (8.8 ± 0.5 vs. 10.6 ± 1.5 $\mu\text{M/g}$, $P = 0.18$), but this was not significant. On the LF diet, there was no significant difference in hepatic FFA or TG levels between WT and Tg-MSH females. These measurements were not made in LF male mice. Among females, hepatic TG was compared within each genotype by diet. This analysis revealed that the transgene prevented any significant diet-induced increase in liver TG. In HF female WT mice, hepatic TG increased by 136% vs. LF ($P = 0.0007$), whereas there was no significant diet-induced increase in hepatic TG in Tg-MSH females, 50.2 ± 6.3 vs. 42.1 ± 3.2 mg/g, despite significant increases in adiposity and body weight. Plasma TG levels were not significantly different between WT and Tg-MSH mice, except in the LF female groups where TG levels were lower in Tg-MSH mice (61 ± 2.5 vs. 78 ± 6.1 mg/dl, $P < 0.05$). (Table 2).

Effect of Tg-MSH on glucose homeostasis. Fasting plasma insulin levels were lower in Tg-MSH vs. WT male mice on both LF (0.41 ± 0.03 vs. 0.59 ± 0.04 ng/ml, $P = 0.002$) and HF diets (1.18 ± 0.21 vs. 2.20 ± 0.16 ng/ml, $P = 0.001$) (Fig. 6). In females, there was no significant difference in fasting insulin levels between Tg-MSH and WT mice on the LF diet. However, on the HF diet, fasting insulin levels were lower in female Tg-MSH compared with WT mice (0.42 ± 0.03 vs. 0.78 ± 0.11 ng/ml, $P = 0.001$). Notably, the transgene completely

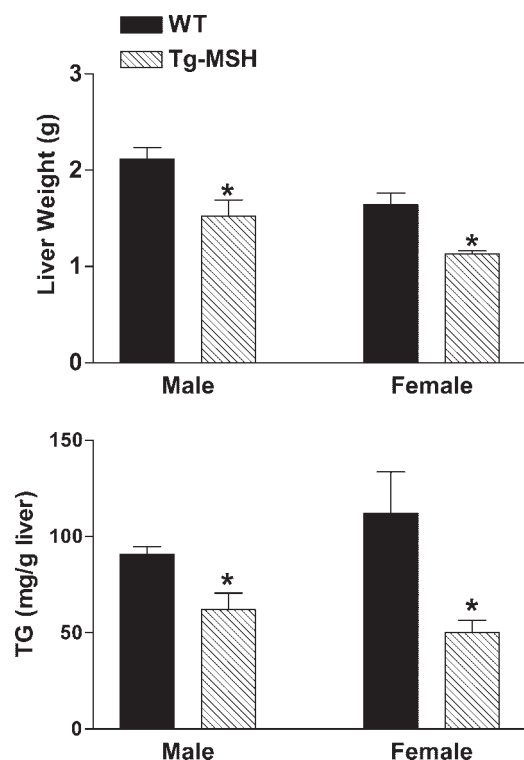


Fig. 4. Effect of Tg-MSH on liver weight and hepatic triglyceride (TG) content on HF diet. *Top*: the mean liver weight was significantly less in both Tg-MSH male ($n = 8$) vs. WT male ($n = 8$) mice ($P < 0.05$) and Tg-MSH female ($n = 9$) vs. WT female ($n = 5$) mice ($P < 0.001$). *Bottom*: hepatic TG content was significantly lower in Tg-MSH male ($P = 0.009$) and female ($P = 0.004$) mice compared with WT animals.

prevented the diet-associated elevation of fasting insulin among females. Glucose tolerance at 18 wk as assessed by intraperitoneal glucose tolerance test was also significantly improved in Tg-MSH mice (Fig. 7): Tg-MSH males ($n = 6$) vs. WT males ($n = 12$), $P = 0.029$ (repeated measures ANOVA) for LF diet; Tg-MSH males ($n = 7$) vs. WT males ($n = 11$), $P = 0.001$ for HF diet; Tg-MSH females ($n = 8$) vs. WT females ($n = 11$), $P = 0.002$ for LF diet; Tg-MSH females ($n = 10$) vs. WT females ($n = 9$), $P = 0.05$ for HF diet. The area under the glucose response curve was reduced in Tg-MSH vs. WT mice by 22 and 27% in LF and HF males, respectively, and by 19 and 25% in LF and HF females, respectively. This was the case for Tg-MSH vs. WT females on the LF diet despite similar body weight and adiposity, indicating that transgenic overexpression of MSH can improve glucose homeostasis via a mechanism that is independent of the degree of adiposity.

Effect of Tg-MSH on plasma leptin, T_4 , and corticosterone levels. Fasting leptin levels were lower in Tg-MSH males compared with WT on HF diet (19.1 ± 2.4 vs. 37.9 ± 5.3 ng/dl, $P = 0.006$). Plasma leptin was also lower in nonfasted Tg-MSH females on HF compared with WT (50.1 ± 5.3 vs. 112.6 ± 11.2 ng/dl, $P < 0.0001$). However, there was no significant difference in leptin between Tg-MSH and WT mice on LF diet in either male or female mice (Table 2).

Compared with WT mice, T_4 values were higher in both Tg-MSH males (4.7 ± 0.5 vs. 3.8 ± 0.2 $\mu\text{g/dl}$) and females (4.4 ± 0.2 vs. 4.1 ± 0.2 $\mu\text{g/dl}$) on the LF diet ($P < 0.05$, 2-way

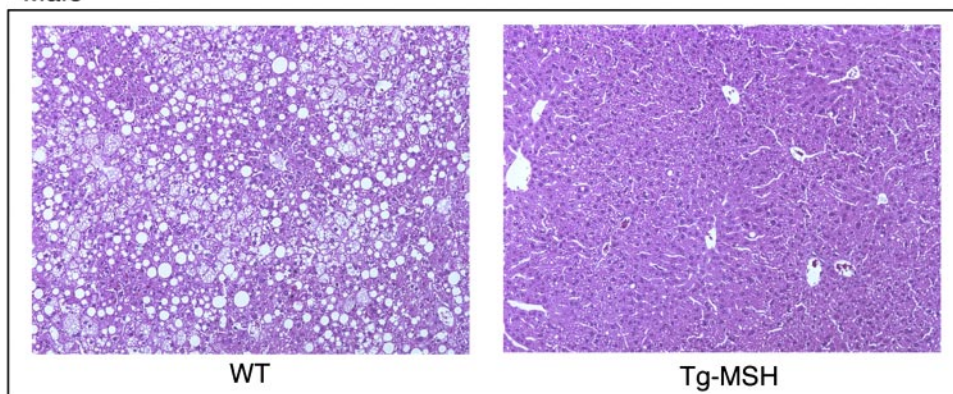
ANOVA). This was not the case among mice on the HF diet, where T_4 levels between Tg-MSH and WT were similar. However, overall there was a significant effect of diet on T_4 levels with HF mice demonstrating significantly lower plasma T_4 than LF mice ($P < 0.0001$, 2-way ANOVA; Fig. 8). Overall, corticosterone levels tended to be lower in Tg-MSH compared with WT mice ($P = 0.068$, 2-way ANOVA). There was also a significant effect of diet on plasma corticosterone, with HF mice demonstrating higher levels than LF mice ($P < 0.005$, 2-way ANOVA; Fig. 8). When analyzed separately on the HF diet, corticosterone levels were significantly lower in Tg-MSH mice ($P = 0.005$).

*Effects of Tg-MSH on hypothalamic *Pomc* and *Agrp* mRNA levels.* There was no significant effect of Tg-MSH or of diet on endogenous *Pomc* gene expression in the hypothalamus between groups. In contrast, both Tg-MSH and diet had significant effects on *Agrp* gene expression. *Agrp* mRNA levels were significantly higher in Tg-MSH male and female mice compared with WT mice on the LF diet ($P < 0.05$; Fig. 9). This effect was not observed on the HF diet, where *Agrp* levels were no longer significantly different between Tg-MSH and WT animals. Overall, *Agrp* levels were significantly lower on the HF diet compared with the LF diet ($P = 0.006$, 2-way ANOVA), and the ratio of *Pomc/Agrp* was also significantly higher on HF vs. LF diets ($P = 0.003$, 2-way ANOVA). A sex difference was also detected, with females demonstrating significantly higher *Agrp* levels than males in all groups ($P = 0.001$).

DISCUSSION

These data show that MSH overexpression reduced weight gain, adiposity, and hepatic steatosis in mice exposed to a HF diet. Even on the LF diet, Tg-MSH mice had less body fat and equivalent or improved lean body mass. These changes occurred in the absence of significant feeding differences in most groups, indicating that effects on energy expenditure and partitioning are the likely mechanisms for the reduced adiposity and body weight. This is consistent with the higher oxygen consumption and lower RQ found in Tg-MSH mice on the HF diet. Tg-MSH also improved glucose metabolism on both the LF and HF diets. Effects of Tg-MSH on body composition and metabolism were found in young animals and persisted in older animals despite changes in hypothalamic *Agrp* gene expression that might tend to counteract the effects of MSH overexpression. Although multiple studies have examined the effects of short-term pharmacological activation of the melanocortin system (23, 26, 30, 40, 46), only a few studies (34, 35, 41, 50) have investigated the effects of long-term melanocortin activation on energy balance. Results of these chronic studies have been more variable and are complicated by problems with long-term peptide delivery and by evidence of conditioned taste aversion with some MSH agonists (4, 23, 30, 46). Tachyphylaxis to effects of prolonged MSH administration has also been reported. Two studies (34, 41) have used either transgenic or viral gene delivery to overexpress *Pomc* in the hypothalamus of leptin receptor-deficient mice and rats and show reduced adiposity and improved glucose metabolism in animals known to have reduced *Pomc* expression in the hypothalamus. The current study shows for the first time that life-long exposure to increased levels of MSH peptides can protect normal

Male



Female

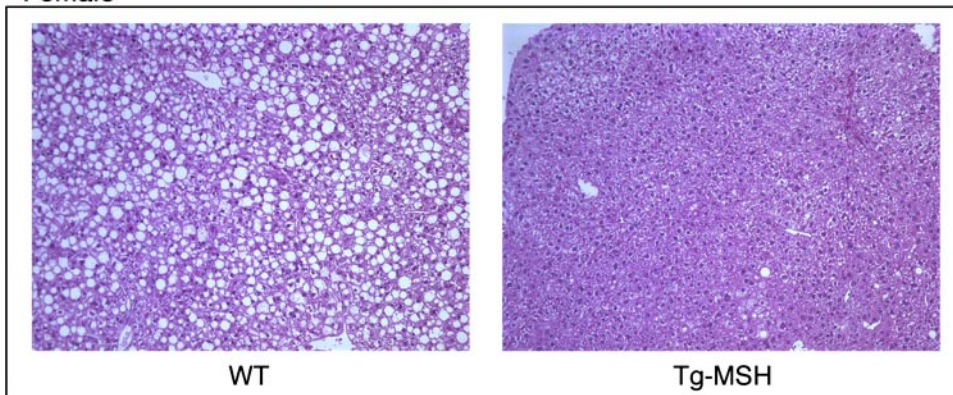


Fig. 5. Liver histology: hematoxylin and eosin stain of liver sections taken from WT and Tg-MSH male (*top*) and female (*bottom*) mice on a HF diet, demonstrating a marked increase in lipid accumulation in the livers of WT compared with Tg-MSH male and female mice.

mice from diet-induced obesity and some of the associated metabolic complications.

The NH₂-terminal POMC transgene expressed in Tg-MSH mice includes the sequences for α - and γ_3 -MSH, both of which may contribute to the changes in body composition and energy balance reported in this study. α -MSH interacts with the MC3R and MC4R and has well-defined effects on food intake, energy expenditure, and body composition. The role of γ_3 -MSH is less clear, but γ_3 -MSH can selectively activate the MC3R, which exerts effects on feed efficiency and adiposity. The transgene contained only NH₂-terminal POMC sequences terminating with α -MSH, rather than the entire molecule, thus avoiding problems with increased ACTH and adrenal stimulation and with increased β -EP that could affect energy balance via interactions with brain opioid receptors. As shown previously (50), although Tg-MSH was expressed in

multiple tissues, it was processed to α - and γ_3 -MSH only in tissues that normally process POMC. Increased levels of α - and γ_3 -MSH were measured in the hypothalamus and brainstem of Tg-MSH mice and specifically in dissections of the arcuate nucleus and paraventricular nucleus, areas known to be important for the central regulation of energy balance. However, increased levels of α - and γ_3 -MSH were also detected in pituitary and blood and could potentially affect energy balance by interactions with peripheral MCRs. MCRs are expressed by adipocytes and macrophages, but little is known about the role of these receptors in mediating effects of MSH on energy balance or fat metabolism. A striking peripheral effect of the transgene, as reported previously, was the uniform, dose-dependent darkening of coat color characteristic of the Tg-MSH mice (50). Thus, although many of the findings reported in this study in Tg-MSH mice are consistent with chronic central activation of the melanocortin pathway, a peripheral site of action cannot be excluded.

Studies were done in mice on the C57BL/6 background that are prone to diet-induced obesity. As expected, both male and female mice demonstrated increased weight gain and adiposity on the HF diet, and MSH overexpression attenuated both the weight gain and increase in body fat. Percent body fat was also less in Tg-MSH mice on the LF diet. Moreover, by 41 wk the transgene continued to attenuate the adiposity in HF male and female mice and in LF male mice. At 41 wk, LF Tg-MSH females no longer had less body fat compared with WT but continued to have greater lean body mass. Notably, the changes in body weight and adiposity in Tg-MSH mice occurred

Table 2. Serum Leptin, TG, and FFA levels

Diet/Genotype/Sex	Leptin, ng/ml	TG, mg/dl	FFA, mM
HF Tg-MSH male	19.1 ± 2.4*	81.96 ± 5.8	0.76 ± 0.03
HF WT male	37.9 ± 5.3	85.68 ± 4.9	0.74 ± 0.04
HF Tg-MSH female	50.1 ± 5.3†	80.44 ± 3.8	0.67 ± 0.03
HF WT female	112.6 ± 11.2	81.40 ± 2.8	0.67 ± 0.03
LF Tg-MSH female	23.3 ± 1.8	61.20 ± 2.5‡	0.67 ± 0.03
LF WT female	26.2 ± 2.9	77.90 ± 6.1	0.60 ± 0.04

Values are means ± SE. TG, triglyceride; FFA, free fatty acid. All levels for male mice are fasting; all levels for female mice are nonfasting. * $P = 0.006$ compared with HF WT males. † $P < 0.0001$ compared with HF WT females. ‡ $P = 0.01$ compared with LF WT females.

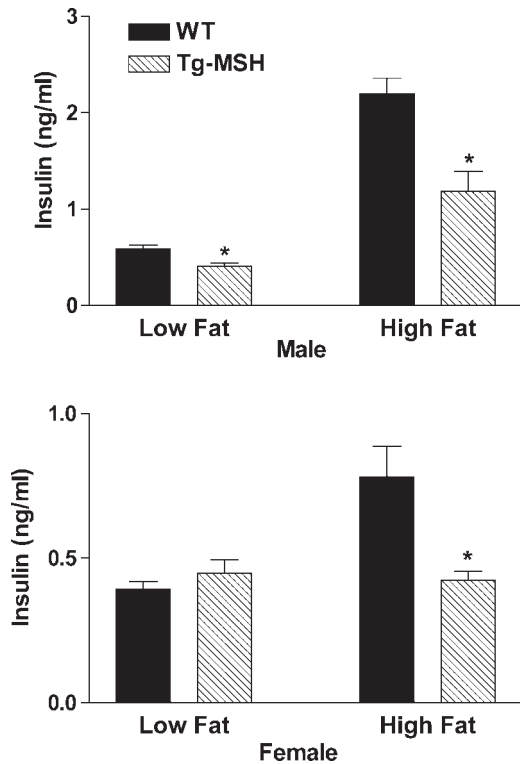


Fig. 6. Effect of Tg-MSH and diet on fasting insulin levels (age 38–44 wk) in male (top) and female (bottom) mice. * $P < 0.002$, Tg-MSH vs. WT. Insulin was lower in Tg-MSH males on LF diet ($n = 11$) vs. WT on LF diet ($n = 12$, $P = 0.002$) and in Tg-MSH males on HF diet ($n = 11$) vs. WT on HF diet ($n = 10$, $P = 0.001$). On LF diet, there was no difference in insulin levels between Tg-MSH ($n = 19$) and WT ($n = 20$) female mice. However, on HF diet, Tg-MSH females ($n = 19$) had significantly lower insulin than WT females ($n = 15$, $P = 0.001$).

without significant changes in food intake in all groups except for the older Tg-MSH female mice on HF diet, who ate less than WT mice but also had the largest decrease in body weight compared with WT mice. It is possible that a decrease in food intake in the other groups of Tg-MSH mice could have occurred earlier, before the feeding studies were performed, or that the methods used to measure food intake were not sensitive enough to detect subtle differences. Tachyphylaxis to the anorectic effect of α -MSH has been shown both in short-term pharmacological experiments as well as in a longer-term study using centrally delivered *Pomc* gene via a recombinant adeno-associated virus (23, 30, 34, 46).

It is likely, given the absence of significant feeding differences in most groups of Tg-MSH mice, that the effects on energy balance are primarily due to changes in energy expenditure and partitioning. Indirect calorimetry studies demonstrated higher oxygen consumption in Tg-MSH compared with WT mice on the HF diet. However, the calculated heat production was not significantly different. Tg-MSH animals also had a lower RQ, indicating that a greater proportion of energy was derived from fat as opposed to carbohydrate. The preferential increase in fat oxidation could explain the reduced adiposity in Tg-MSH animals. A similar increase in resting $\dot{V}O_2$ and decrease in RQ has been reported in mice with genetic *Agrp* deficiency presumably secondary to unopposed melanocortin signaling (59). Effects on energy expenditure and fuel

oxidation have also been demonstrated with short-term administration of α -MSH agonists. MTII increased $\dot{V}O_2$ in lean and obese mice and rats compared with pair-fed controls and caused a reduction in RQ in lean and obese Zucker rats (15, 23, 26, 29, 46). Conversely, both *Pomc*^{-/-} and *Mc4r*^{-/-} mice have reduced resting $\dot{V}O_2$ (3, 10, 54). Furthermore, increased adiposity is seen with pharmacological or genetic reduction of melanocortin signaling even when hyperphagia is prevented (1, 31, 54). *Mc4r*^{-/-} mice also fail to exhibit normal diet-induced thermogenesis in response to increased dietary fat (8). One mechanism by which α -MSH might increase energy expenditure is via modulating sympathetic output to brown adipose tissue, resulting in increased thermogenesis (16, 24, 49). In addition, there is evidence (53) that central MCRs may modulate sympathetic outflow to white adipose tissue, which could affect lipid mobilization. α -MSH could also stimulate lipolysis via direct interaction with MCRs expressed by adipocytes (5). Regardless of the site of action, an increase in serum FFA levels might be expected with enhanced melanocortin induced lipolysis. However, no difference in FFA was detected between WT and Tg-MSH mice. There were also no significant differences in TG levels except for LF Tg-MSH females, who had lower serum TG than WT mice. Other investigators (46) have reported both increased and decreased FFA levels with different MSH injection protocols. Similarly, hypothalamic *Pomc* gene delivery was reported to increase FFA levels in obese Zucker rats but to decrease FFA and TG levels in aged rats

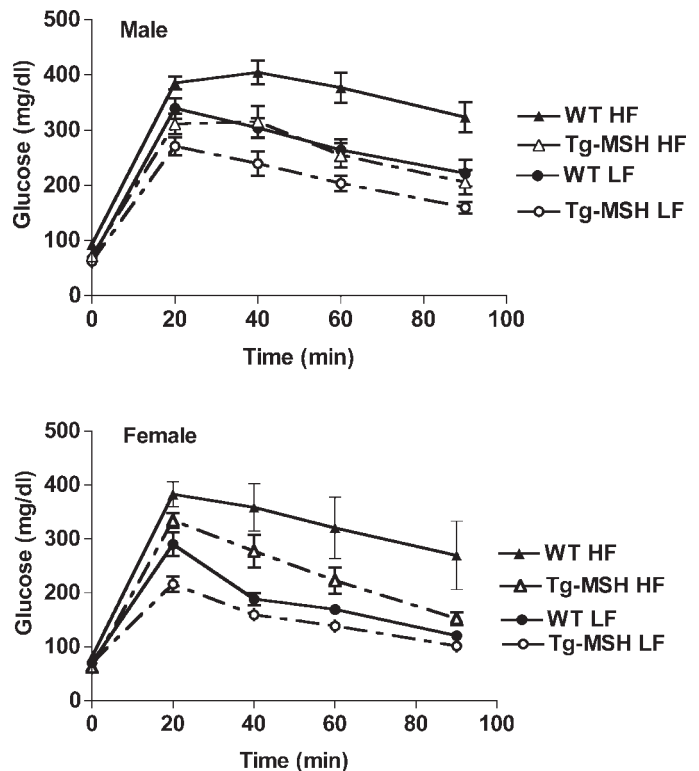


Fig. 7. Effect of Tg-MSH and diet on glucose tolerance test (2 mg/g ip) at 20–26 wk of age. Glucose tolerance was significantly improved in Tg-MSH males (top) and females (bottom) on both LF and HF diets compared with WT animals. Tg-MSH males ($n = 6$) vs. WT males ($n = 12$), $P = 0.029$ for LF diet; Tg-MSH males ($n = 7$) vs. WT males ($n = 11$), $P = 0.001$ for HF diet; Tg-MSH females ($n = 8$) vs. WT females ($n = 11$), $P = 0.002$ for LF diet; Tg-MSH females ($n = 10$) vs. WT females ($n = 9$), $P = 0.05$ for HF diet.

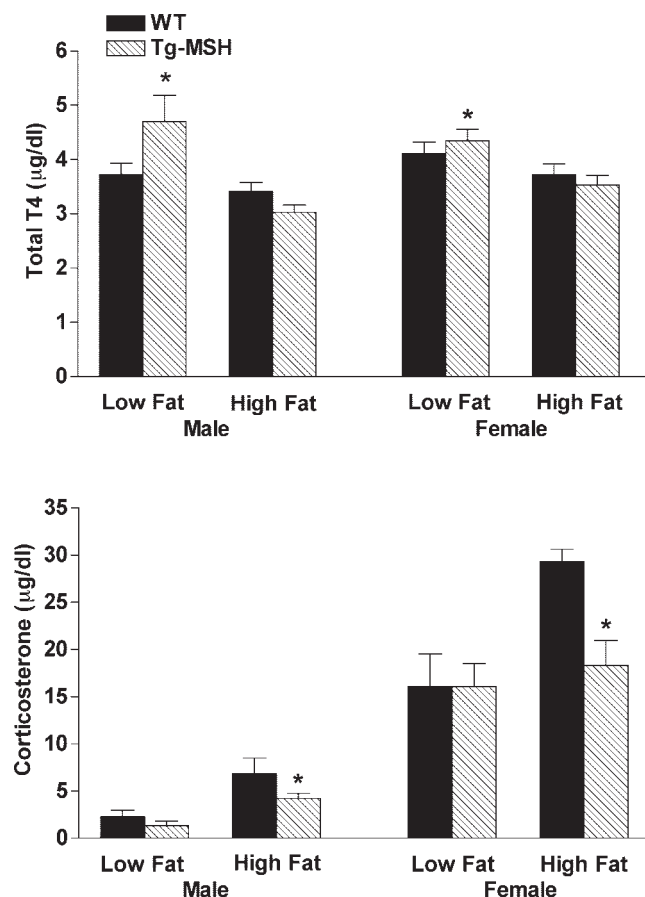


Fig. 8. *Top*: effect of Tg-MSH and diet on plasma thyroid hormone (T₄) levels. On the LF diet, T₄ levels were significantly higher in Tg-MSH male ($n = 5$) and female ($n = 16$) compared with WT male ($n = 7$) and female ($n = 15$) mice ($*P < 0.05$, 2-way ANOVA for genotype). On HF diet, T₄ values were similar between Tg-MSH male ($n = 8$) and female ($n = 19$) and WT male ($n = 8$) and female ($n = 14$) animals. However, there was a significant effect of diet on T₄ levels, with HF-fed mice demonstrating significantly lower plasma T₄ than LF-fed mice ($P < 0.0001$, 2-way ANOVA). *Bottom*: effect of Tg-MSH and diet on plasma corticosterone levels. Overall, corticosterone was lower in Tg-MSH compared with WT mice ($P = 0.068$, 2 way ANOVA). There was a significant effect of diet on plasma corticosterone with HF mice demonstrating higher levels than LF mice ($P < 0.005$, 2 way ANOVA). When analyzed separately on the HF diet, corticosterone levels were significantly lower in Tg-MSH mice ($*P = 0.005$, 2 way ANOVA for genotype).

(34, 35). The lack of change in FFA and TG levels in Tg-MSH mice could be ascribed to the increased utilization of lipids as a fuel source. Indeed, the reduced adiposity, decreased hepatic steatosis, and lowered RQ of Tg-MSH mice suggest that enhanced lipolysis is associated with increased fatty acid utilization.

Another mechanism by which MSH may affect energy balance is via modulation of the hypothalamic-pituitary-thyroid axis. Both α -MSH and AGRP nerve terminals innervate TRH neurons in the paraventricular nucleus (PVN), and these neurons can be stimulated or suppressed by α -MSH and AGRP, respectively (20, 33, 39). Consistent with these findings, Tg-MSH mice on the LF diet had higher T₄ values than WT mice. A similar increase in plasma T₄ was reported in *Agrp* knockout mice (59). In contrast, *Pomc* null mice are reported to have decreased plasma T₄ levels, but results have been conflicting (10, 38). Plasma T₄ levels on the HF diet, in

contrast to the LF diet, were equivalent in Tg-MSH and WT mice and thus could not explain their leaner phenotype. The reason for this difference on the two diets is unclear, but it is noteworthy that animals on the HF diet had significantly lower T₄ levels regardless of genotype. This is consistent with a previous report (56) showing lower plasma T₄ levels in rats on a HF diet. Plasma corticosterone levels also increased on the HF diet, and this was attenuated in Tg-MSH mice. There is some evidence that the hypothalamic melanocortin system may modulate the activity of the hypothalamic-pituitary-adrenal (HPA) axis. POMC neurons innervate corticotropin-releasing hormone (CRH) neurons in the PVN, and α -MSH has been shown to inhibit CRH release (37, 55, 61). Our results are consistent with inhibitory regulation of the HPA axis by α -MSH. In contrast, removal of this inhibition, as is seen in POMC null mice with selective transgenic restoration of pituitary POMC, was shown to increase plasma corticosterone levels (52). The lower corticosterone levels noted in the Tg-MSH mice on the HF diet could contribute to the effects on energy balance. However, it should be noted that these blood levels were obtained at the time of death. A more careful study of the HPA axis in these mice is warranted to determine the role of Tg-MSH in modulating HPA activity.

A striking finding of this study was the ability of Tg-MSH to attenuate the hepatic steatosis induced by the HF diet. Notably, the reduction in hepatic fat was out of proportion to changes in body weight. The mechanism by which Tg-MSH prevents hepatic steatosis is unknown, and it is at present unclear whether this is centrally or peripherally mediated. The melanocortin pathway can modulate the expression of liver enzymes involved with both the synthesis and oxidation of fat (3, 9, 36). Intracerebroventricular injection of MTII decreased hepatic expression of stearoyl-CoA desaturase-1, a lipogenic

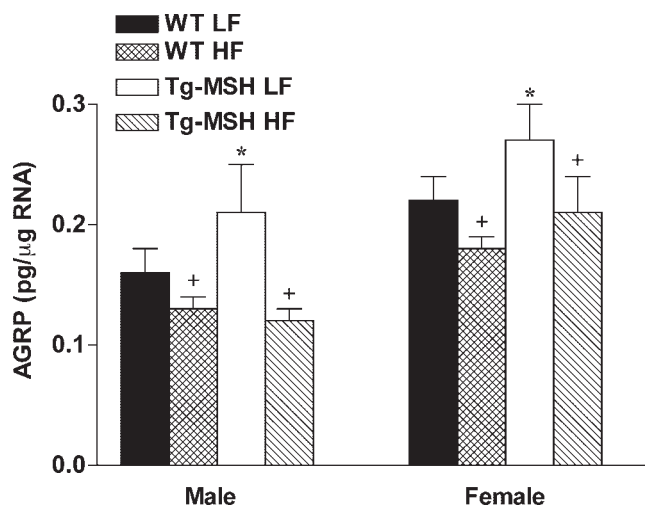


Fig. 9. Effect of Tg-MSH and diet on hypothalamic expression of agouti-related peptide (*Agrp*) mRNA. *Agrp* mRNA levels were significantly higher in Tg-MSH male ($n = 5$) and female ($n = 15$) mice compared with WT male ($n = 7$) and female ($n = 14$) mice on LF diet ($*P < 0.05$). On HF diet, *Agrp* levels did not differ between Tg-MSH male ($n = 8$) and female ($n = 18$) vs. WT male ($n = 9$) and female ($n = 14$) animals. Overall, there was a diet effect with significantly lower *Agrp* levels on HF compared with LF diet ($+P = 0.006$, 2-way ANOVA) and a sex effect with significantly higher AGRP levels in females compared with males in all groups ($P = 0.001$, 2-way ANOVA). There was no significant effect of Tg-MSH or diet on endogenous *Pomc* gene expression.

enzyme (36), and peripheral injection of MTII increased hepatic expression of carnitine palmitoyltransferase I, which is involved in lipid oxidation (9). Hepatic steatosis has been documented in *Pomc* null mice and is worsened by corticosterone replacement (12, 52). There is also evidence (36) that leptin can prevent hepatic steatosis and that this is mediated by central MCRs. There is accumulating evidence (47) for crosstalk between the brain and liver with respect to nutrient sensing and nutrient production. It is possible that modulation of vagus nerve activity by Tg-MSH could play a role in attenuating hepatic steatosis on a HF diet. Tg-MSH could act in the hypothalamus to activate autonomic projections that synapse on the dorsal motor nucleus of the vagus, leading to increased vagal outflow to the liver, or alternatively, Tg-MSH in the brainstem could act directly at MC4Rs on the dorsal vagal complex to increase vagal outflow to the liver. There may also be indirect autonomic effects of Tg-MSH not mediated by the hepatic vagus. In addition, neuroendocrine or peripheral actions of Tg-MSH may alter hepatic fat deposition. Further study is necessary to characterize the mechanisms by which Tg-MSH acts to prevent hepatic lipid deposition.

A key finding of the present study was the effect of Tg-MSH on glucose metabolism and insulin sensitivity. Fasting insulin levels were lower in Tg-MSH male and female animals on the HF diet. On the LF diet, Tg-MSH males had lower fasting insulin than WT males who were of similar body weight; no difference was seen in LF females. Glucose tolerance, however, was improved in all groups of Tg-MSH mice on both diets. This improvement was noted even when Tg-MSH and WT animals on LF diet were matched for adiposity, indicating that overexpression of MSH has beneficial effects on glucose metabolism that are independent of fat mass. These data are consistent with our previous study showing that fed and fasted insulin levels were decreased in Tg-MSH mice on a regular chow diet (50) and with previous pharmacological studies (25, 43). Obici et al. (43) demonstrated that chronic intracerebroventricular infusion of α -MSH enhanced both insulin-stimulated glucose uptake and suppression of hepatic glucose production. In contrast, acute activation of the melanocortin pathway has been reported to cause suppression of plasma insulin, enhanced glucose disposal, and stimulation of gluconeogenesis (17, 22, 25). Transgenic neuronal expression of *Pomc* has been shown to decrease fasting insulin and improve glucose tolerance in *ob/ob* mice. This effect on glucose tolerance was independent of changes in food intake and body weight, but it is unclear whether it was independent of changes in adiposity. No changes in blood glucose or insulin were reported in lean mice with neuronal *Pomc* overexpression. Central viral *Pomc* gene delivery also reduced fasting insulin levels, but this was in the setting of decreased food intake and body weight. The current study shows that long-term melanocortin activation improves glucose tolerance even without significant body weight or adiposity differences. This likely occurs via a central mechanism, as indicated by the study of Obici et al. (43). The downstream mechanisms by which MSH modulates peripheral insulin sensitivity remain to be elucidated but may involve autonomic output to the liver, fat, and skeletal muscle.

A limitation to transgenic studies is the potential for developmental compensation. There was concern that the efficacy of long-term melanocortin activation in promoting weight loss

and body fat reduction could be reduced by a compensatory increase in *Agrp* expression or a decrease in endogenous *Pomc* expression. There were no changes in endogenous hypothalamic POMC mRNA levels in Tg-MSH mice on either the LF or HF diet. *Agrp* mRNA levels, however, were significantly higher in Tg-MSH mice but only on the LF diet. Such an increase could serve to maintain body weight and fat stores in a lean animal. However, despite the increase in *Agrp*, LF Tg-MSH animals still had lower body fat percentage than WT controls. In contrast, on the HF diet, there was no difference in *Agrp* expression in Tg-MSH compared with WT animals. There was, however, a significant dietary effect on *Agrp* expression in all groups, with *Agrp* levels being lower in the HF mice compared with the LF group. Suppression of *Agrp* levels on the HF diet likely reflects a homeostatic response to excessive energy intake and is consistent with the findings of others, although this study extends their findings by demonstrating that such suppression is sustained long term (27, 62). Finally, a sex difference in *Agrp* expression was also noted with females displaying higher *Agrp* levels than males.

In summary, the data presented here show that MSH overexpression reduced weight gain, adiposity, and hepatic steatosis in mice exposed to a HF diet. Additional study is necessary to address the site of action and mechanisms by which Tg-MSH exerts these beneficial weight reduction and metabolic effects. Nonetheless, the results of the current study offer cogent support for long-term melanocortinergic activation as a viable and effective means of treating both obesity and its associated metabolic derangements.

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