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Hypothalamic γ -melanocyte stimulating hormone gene delivery reduces fat mass in male mice

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Abstract

γ -Melanocyte stimulating hormone (γ -MSH) is an endogenous agonist of the melanocortin 3-receptor (MC3R). Genetic disruption of MC3Rs increases adiposity and blunts responses to fasting, suggesting that increased MC3R signaling could be physiologically beneficial in the long term. Interestingly, several studies have concluded that activation of MC3Rs is orexigenic in the short term. Therefore, we aimed to examine the short- and long-term effects of γ -MSH in the hypothalamic arcuate nucleus (ARC) on energy homeostasis and hypothesized that the effect of MC3R agonism is dependent on the state of energy balance and nutrition. Lentiviral gene delivery was used to induce a continuous expression of γ -*Msh* only in the ARC of male C57Bl/6N mice. Parameters of body energy homeostasis were monitored as food was changed from chow (6 weeks) to Western diet (13 weeks) and back to chow (7 weeks). The γ -MSH treatment decreased the fat mass to lean mass ratio on chow, but the effect was attenuated on Western diet. After the switch back to chow, an enhanced loss in weight (–15% vs –6%) and fat mass (–37% vs –12%) and reduced cumulative food intake were observed in γ -MSH-treated animals. Fasting-induced feeding was increased on chow diet only; however, voluntary running wheel activity on Western diet was increased. The γ -MSH treatment also modulated the expression of key neuropeptides in the ARC favoring weight loss. We have shown that a chronic treatment intended to target ARC MC3Rs modulates energy balance in nutritional state-dependent manner. Enhancement of diet-induced weight loss could be beneficial in treatment of obesity.

Key Words

- ▶ neuropeptides
- ▶ POMC
- ▶ γ -MSH
- ▶ obesity
- ▶ mice

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Introduction

The melanocortin neuropeptide system is essential for the regulation of body weight (BW) and energy balance as evidenced by genetic disruptions of the

melanocortin pathways leading to obesity in both humans and rodents (Fan *et al.* 1997, Huszar *et al.* 1997, Butler *et al.* 2000, Chen *et al.* 2000). The system consists

of pro-opiomelanocortin (POMC)-derived melanocyte stimulating hormones α -, β - and γ -MSH and the inverse agonist, agouti-related peptide (AGRP), which mediate their effects through melanocortin receptors 1-5 (MC1-5R) (Lu et al. 1994, Fan et al. 1997, Winsky-Sommerer et al. 2000, Nijenhuis et al. 2001, Nillni 2007, Pritchard & White 2007). MSH peptides are derived from POMC by protein convertases (PC) 1/3 and/or 2 and bind to MCRs within the central nervous system (CNS) with varying affinities; α -MSH is an agonist of both MC4R and MC3R, whereas γ -MSH is considered mainly to be an agonist of MC3R with low affinity to MC4R (Hruby et al. 1995, Oosterom et al. 1999). The anorexigenic POMC neurons found in the arcuate nucleus (ARC) project to second-order neurons located in the hypothalamus, tegmentum and the brain stem to release α -MSH that binds to local MC4R reducing food intake and increasing energy expenditure (Elmqvist et al. 1999, Williams et al. 2000, Berthoud & Morrison 2008). Another neuronal sub-population in the ARC co-expressing AGRP, Neuropeptide Y (NPY) and gamma-aminobutyric acid (GABA neurons) plays an opposing role on feeding and body energy metabolism by regulating second-order neurons and also by inhibiting POMC neuronal activity (Kim et al. 2000).

MC4R is considered to be the main mediator of the anorexigenic and glucoregulatory effects of MSH peptides in the CNS. *Mc4r*-knockout (KO) mice are severely obese, and SNPs in the *MC4R* gene are associated with higher BW in humans and MC4R agonists have potent anti-obesity effects (Huszar et al. 1997, Vaisse et al. 1998, Marsh et al. 1999, Farooqi et al. 2000, Kim et al. 2000, Kumar et al. 2009, Kievit et al. 2013). Although less evident and less explored, MC3R also plays an important role in the regulation of BW by regulating the mass of muscle. In humans, rare MC3R variants have been associated with childhood obesity (Feng et al. 2005). In mice, *Mc3r*-KO leads to moderate increase in adiposity and decrease in lean mass (LM) due to altered nutrient partitioning and insulin resistance, and the phenotype is augmented on high-fat diet (HFD) (Butler et al. 2000, Chen et al. 2000, Butler 2006). Furthermore, the *Mc3r*-KO augments the obesity phenotype of *Mc4r*-KO mice supporting a separate role for the two MCRs (Chen et al. 2000). MC3R is expressed in both NAG and POMC neurons in the ARC and on second-order neurons in the hypothalamus, ventral tegmental area (VTA) and brain stem (Roselli-Rehfuess et al. 1993, Bagnol et al. 1999, Cone 2005, Berthoud & Morrison 2008). Interestingly, specific MC3R agonists induce food intake (FI) in central and peripheral administrations and inhibit POMC neurons in *ex vivo* coronary slice recordings,

which together suggest an auto-inhibitory function for the MC3R on POMC neurons (Cowley et al. 2001, Marks et al. 2006, Lee et al. 2008). On the other hand, *Mc3r*-KO fails to increase the expression of *Npy* and *Agrp* in the ARC during acute fasting conditions and show attenuated refeeding, which indicates that MC3R is important for the function of NAG neurons (Renquist et al. 2012). The behavioral changes in prolonged hypocaloric state including binge-feeding, food anticipatory activity, entrainment to nutrient availability and feeding-related motivational responses are also impaired in *Mc3r*-KO mice (Butler et al. 2017). Studies using neuron-specific rescue of MC3Rs have indicated that the effects on NAG neuron and feeding responses to hypocaloric state and adiposity are mediated by MC3R expressed in the hypothalamus, insulin sensitivity by MC3R in the ventromedial hypothalamus and motivational responses to food by MC3R in the VTA (Begriche et al. 2011, Girardet et al. 2017). These evidences show that the MC3R plays a role in regulating body energy balance in a caloric state-dependent manner, which may be mediated by changes in the balance between the first-order POMC and NAG neurons or via MC3Rs in downstream neurons (Butler et al. 2017).

In this work, we used viral gene delivery to induce a continuous expression of γ -*Msh* in the hypothalamus to study the chronic effects of MC3R activation. Viral gene delivery has previously been used to show that overexpression of the complete POMC gene in the ARC or in the *nucleus tractus solitarius* of the brain stem (NTS) has anti-obesity effects in rats (Li et al. 2003, 2005, Zhang et al. 2010). Our previous work highlighted the importance of α -MSH in the anti-obesity effects as we showed that chronic lentiviral α -*Msh* overexpression in the ARC in diet-induced obese (DIO) mice can reduce weight gain and adiposity without affecting FI (Eerola et al. 2013). Furthermore, by inducing a 1.3-fold increase in α -MSH in the NTS using the same system, we observed a significant FI- and weight-independent improvement in fat mass (FM) (Eerola et al. 2014). The aim of the current work was to assess the long-term metabolic effects of local γ -*Msh* overexpression alone in the hypothalamus and to test whether the composition of diet plays an impact on the physiological effects. Based on the phenotype of *Mc3r*-KO mice, we hypothesized that chronic activation of MC3Rs in the ARC by γ -MSH could have weight reducing effects. Since fasting and the dietary energy content influence the balance of anorexigenic and orexigenic pathways in the ARC (Renquist et al. 2012, Wu et al. 2014), and MC3R is expressed in both the anorexigenic POMC and orexigenic NAG neurons (Bagnol et al. 1999), we hypothesized that

by increasing the levels of γ -MSH in the ARC, it would be possible to modulate the balance between these neurocircuits in different states of energy balance. We were especially interested to simulate a diet-induced weight loss situation, where activation of orexigenic and inhibition of anorexigenic pathways aims to regaining the set weight, thus hampering the efficacy of many anti-obesity therapies.

Materials and methods

Experimental setting and animals

The experiment was divided into three parts (Fig. 1A): an initial 6-week chow diet period (chow), a 13-week Western diet (Western) and a 7-week weight loss chow diet period (weight loss chow). The macronutrient content of the standard rodent chow used was 9kcal% fat, 69kcal% carbohydrates, 22kcal% protein (Special Diet Services, Essex, UK) and the Western diet was 41% kcal fat, 43% kcal carbohydrate, 17% kcal protein (D12079B, Research

Diets, New Brunswick, NJ, USA). Measurements of FI, BW and body composition were used to divide the 2-month-old C57BL/6N male mice (Harlan Laboratories B.V. Venray, The Netherlands) into two single-housed equally matched groups that received either LVi- γ -MSH-EGFP- (γ -MSH $n=18$) or the control LVi-EGFP-virus (EGFP $n=19$) (Supplementary Fig. 1, see section on [supplementary data](#) given at the end of this article). Ten and nine mice were excluded based on lack of EGFP expression in the target area of γ -MSH and EGFP groups, respectively.

The mice were kept in an animal room maintained at $21 \pm 1^\circ\text{C}$ with a fixed 12-h light/12-h darkness cycle. Animal care was conducted in accordance with the guidelines of the International Council of Laboratory Animal Science (ICLAS), and all experimental procedures were authorized by the National Animal Experiment Board.

Lentiviral construct validation

The γ -MSH construct used to create the gene delivery vector LVi- γ -MSH-EGFP was generated using mouse cDNA as a template with cloning primers in

Figure 1

(A) The timetable of the study including all measurements in relation to the three diet periods including a chow diet, Western diet and weight loss chow period. (B) A schematic presentation of the γ -MSH construct and overview of DNA plasmid used for the production of lentiviral vectors. The construct was designed to include the signal and sorting sequences of pro-opiomelanocortin and the endogenous cleavage sites for appropriate intracellular processing. Enzymatic cleavage of the N-terminal POMC by protein convertases (PC) 1/3 and 2 produces γ -MSH. The vector uses the vesicular stomatitis virus (VSV-G) glycoprotein envelope in combination with the ubiquitous human elongation factor alpha (hEF1- α) promoter and induces expression almost exclusively to neurons (Jakobsson et al. 2003). The main components of the lentiviral gene vector are EF1-alpha-promoter, gene construct, enhanced green fluorescent protein (EGFP) followed by the internal ribosome entry site and the Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element (WPRE), which enhances gene expression. (C) Dose-response curve of [D-Trp⁶]- γ -MSH vs the amount of bound cAMP in HEK293 cells expressing human MC3R (hMC3R-cells). (D) Stimulation of cAMP release measured as a decrease in bound cAMP in hMC3R cells by growth media from non-infected HEK293 cells (Control, $n=5$), and cells infected with the EGFP- ($n=5$) and γ -MSH viruses ($n=6$). **** $P < 0.0001$ pairwise t -test adjusted for multiple comparisons with Bonferroni adjustment following one-way ANOVA.

Supplementary Table 1. The construct containing the N'-terminal *Pomc* with signal and sorting sequences and the endogenous cleavage sites for appropriate intracellular processing of γ -MSH was then sub-cloned into the pWPI-EGFP plasmid (Fig. 1B). The second-generation lentiviral vectors were produced according to standard protocols as previously described (Eerola *et al.* 2013). The ability of the virus to produce biologically active peptide was tested in HEK293 cells transfected with human MC3R (hMC3R) (Grieco *et al.* 2002) by treating the hMC3R cells with collected and stored growth media from γ -MSH-infected HEK293T cells or synthetic [D-Trp8]- γ -MSH for positive control (a detailed protocol in Supplementary Appendix 1). Receptor stimulation was measured as a decrease in bound cAMP levels using a commercial kit (PerkinElmer).

Stereotaxis

Buprenorphine (0.1 mg/kg) (Temgesic Schering-Plough, NJ, USA) analgesia was administered intraperitoneally 20 min prior to the injections conducted under isoflurane anesthesia. 6×10^5 TU of EGFP or γ -MSH virus in a similar volume (1 μ L) at 0.1 μ L/min were delivered bilaterally to the ARC by methods described previously (Eerola *et al.* 2013). The stereotaxic coordinates or the point of the injection according to bregma were anterior-posterior (AP) 1.70 mm, dorsal-ventral (DV) -5.90 mm and medial-lateral (ML) ± 0.13 mm in a 10° angle toward the midline in order to avoid secondary target sites of ARC neurons in the hypothalamus (Franklin & Paxinos 1997). The success of the stereotaxic injection was verified postmortem by analyzing EGFP expression and γ -MSH immunoreactivity in the target area (Supplementary Fig. 2). Mice, nine in the EGFP and ten in the γ -MSH group, with misplaced injections according to needle tract and EGFP expression located outside the ARC were excluded from the study.

FI, BW and composition

BW and FI were measured weekly. The FI results are shown as daily mean of total weekly FI. Food spillage was accounted for by carefully checking the bedding (Tapvei aspen) to exclude fallen food crumbs from total FI. FM and LM in grams was assessed utilizing the EchoMRI-700 quantitative nuclear magnetic resonance whole body composition analyzer (Echo Medical Systems, Houston, TX, USA) as described earlier (Eerola *et al.* 2014).

Voluntary running wheel activity

Low-profile wireless running wheels for mice (Med Associates, Inc. Vermont, USA) were used to measure the voluntary running wheel activity on chow and Western periods over 24 h in the home cage.

Baseline and fasting-induced refeeding

Ad libitum feeding during the first 2 h of the darkness period was measured 24 h before the refeeding analysis. Animals were fasted during the light period (12-h) prior to food introduction at the start of dark. Food was weighed manually at 2, 12 and 24 h from food presentation.

Basal glucose and glucose tolerance test (GTT)

Basal glucose and glucose tolerance (glucose 1 mg/kg i.p.) were analyzed after 4-h fast from tail vein blood with a glucose analyzer as previously described (Eerola *et al.* 2013).

Tissue collection and preparation

At the end of the experiment, the mice were killed after a 4-h fast. Terminal blood was collected with intracardiac puncture under ketamine (75 mg/kg i.p. Ketalar, Pfizer Oy, Finland) and medetomidine (1 mg/kg i.p. Domitor, Orion Oy, Finland) anesthesia. A total of 500 μ L blood per mouse was collected in serum separation tubes. White adipose tissue (WAT) pads were separately weighted and the brains, adrenal glands and a single lobe of interscapular brown adipose tissue (BAT) were collected, snap frozen and stored at -80°C until analysis.

Fresh frozen sections and laser capture microdissection (LCM)

Coronal 10 μ M fresh frozen sections of the hypothalamus were collected on Superfrost Plus slides (Menzel, Braunschweig, Germany) using a Leica 3050S cryostat (Leica GmbH) intended for IHC analysis and 50 μ M sections were collected for the LCM procedure and stored at -80°C until processing. All brains collected were first analyzed visually for injection site EGFP expression to decide on inclusion in analysis. Seven samples from each group were used for the LCM procedure, where the ARC was isolated from cryostatic coronal sections (Lee *et al.* 2012) (for more detail see Supplementary Appendix 1).

Gene expression analyses

RNA was isolated from the ARC samples using the PicoPure RNA isolation kit (Catalog # KIT0202) by adding extraction buffer (XB) to a total volume 50 μ L before incubation at 42°C in protocol C and from other tissues using TRIzol Reagent (Cat. 15596026, Invitrogen) or RNeasy Mini Kit (Qiagen GmbH) with DNase treatment (RNase-Free DNase Set, Qiagen GmbH). The isolated RNA was converted to cDNA with a High-Capacity RNA-to-cDNA Kit (Applied Biosystems) on a Applied Biosystems 2720 Thermal Cycler (Applied Biosystems), and qPCR was performed with SYBR Green method using Kapa Sybr Fast qPCR Kit (Kapa Biosystems, Woburn, MA, USA) and 7300 Real Time PCR System (Applied Biosystems). The primers used in the assay are shown in Supplementary Table 1. Beta-actin (*Bact*) was used as an endogenous control. Formula $2^{-\Delta\Delta CT}$ was used for calculating the gene expression, and the expression levels were presented relatively to the expression levels of EGFP-treated mice.

Biochemical analyses

Serum triglycerides were quantified with Triglyceride Reagent (T2449; Sigma Diagnostics) and the non-esterified free fatty acids (NEFA) with NEFA-HR (2) (Wako Chemicals GmbH, Neuss, Germany) according to the manufacturers instructions. Serum insulin levels were measured with an ELISA kit (Mercodia AB, Uppsala, Sweden).

Statistical analysis

Parameters presented over time were analyzed using two-way ANOVA for repeated measures followed by pairwise *t*-tests adjusted for multiple comparisons with Bonferroni adjustment for different time points.

One-way ANOVA followed by pairwise *t*-tests adjusted for multiple comparisons with Bonferroni adjustment was used to compare treatment effect in hMC3R cells. The parametric unpaired *t*-test was used for comparing normally distributed single-parameters and Mann–Whitney test for parameters not passing the normality test. Statistical analyses were conducted with GraphPad Prism 6.0 (GraphPad Software Inc.). Data are presented as means $\pm$ S.E.M. The results were considered statistically significant at $P < 0.05$.

Results

Lentiviral construct validation

Synthetic [D-Trp8]- γ -MSH diluted in cell culture media from non-infected cells decreased bound cAMP indicating increased cAMP production in hMC3R dose dependently (Fig. 1C). Similarly, cell media from γ -MSH-infected HEK293T cells significantly decreased bound cAMP compared to cell media from EGFP-infected or non-infected cells ($F_{(2,13)} = 418.6$, **** $P < 0.0001$, one-way ANOVA) (Fig. 1D). Furthermore, immunohistochemical staining of the hypothalamus of LVi- γ -MSH-EGFP-injected animals showed γ -MSH immunoreactivity in the ARC indicating that the peptide was produced *in vivo* until the end of the 26-week experiment (Supplementary Fig. 2). This was supported by EGFP indicating that the transgene was expressed until termination.

Effects on BW, composition and feeding on the chow and Western diets

The *in vivo* experiment compared the effects of γ -MSH and control EGFP treatments during three different diet

Figure 2

Chow period (A) mean daily food intake (g) of a week, (B) mean daily food intake in kilocalories per gram of weight, (C) body weight, (D) fat mass change, (E) lean mass change and (F) fat mass (FM)/lean mass (LM)-ratio in single-housed EGFP- ($n = 10$) and γ -MSH-treated mice ($n = 8$). * $P < 0.05$ two-way ANOVA followed by pairwise *t*-tests adjusted for multiple comparisons with Bonferroni adjustment.

periods (Fig. 1A). There were no statistically significant differences in absolute FI or in kilocalories consumed per weight during the chow period (Fig. 2A and B) and pre-treatment BW was regained similarly within 2 weeks post surgery and remained similar to the end of the chow period (Fig. 2C). There was no statistically significant difference in FM (γ -MSH: 3.6 ± 0.8 g vs EGFP: 5.4 ± 0.7 g, $t=1.754$, $df=14.67$, $P=0.10$, Student's t -test), FM change (Fig. 2D), LM (γ -MSH: 21.0 ± 0.5 g vs EGFP: 20.5 ± 0.4 g, $t=0.673$, $df=15.8$, $P=0.511$, Student's t -test) or in LM change (Fig. 2E). There was a statistical tendency for attenuated increase in fat mass-to-lean mass (FM/LM) ratio in the γ -MSH-group during the chow period (treatment $F_{(1,16)}=4.475$, $P=0.050$, two-way ANOVA) and *post hoc* analysis showed a significant effect at week 6 (Fig. 2F). The γ -MSH-treated animals also showed a non-statistical tendency toward lower basal glucose levels (γ -MSH: 7.6 ± 0.2 vs EGFP: 8.4 ± 0.4 mmol/L, $t=1.770$, $df=13.75$, $P=0.098$ Student's t -test).

During the Western-period, the FI decreased over the first 5 weeks and then plateaued in both treatment groups (Fig. 3A). The adjustment was slower in γ -MSH-treated animals as the amount of kilocalories eaten per gram of BW was higher during weeks five and six (Fig. 3B). Changes in BW (Fig. 3C) and body composition (Fig. 3D, E and F) did not show statistically significant differences between the groups during the Western period. There were no differences in total FM (γ -MSH: 19.1 ± 1.2 vs EGFP: 19.9 ± 0.7 g, $t=0.610$, $df=16$, $P=0.550$, Student's t -test) or total LM (γ -MSH: 22.6 ± 0.6 vs EGFP: 22.6 ± 0.5 g, $t=1.754$, $df=14.67$, $P=0.974$, Student's t -test) or glucose (γ -MSH: 9.3 ± 0.3 vs EGFP: 9.0 ± 0.3 mmol/L, $t=0.4616$, $df=15.52$, $P=0.650$, Student's t -test) in the end of the Western period.

Voluntary running wheel activity during the chow and Western diets

During the chow period, the peak activity was achieved at 4 h into the dark cycle with no statistically significant differences between the treatments (Fig. 4A). Accordingly, the mean activity during dark hours (Fig. 4B) and mean activity over 24 h (Fig. 4C) were similar between treatments. Western diet reduced activity in both groups and the two-way ANOVA analysis on the hourly data over 24 h did not reveal statistically significant difference between the treatments (Fig. 4D, Treatment $F_{(1,16)}=1.142$, $P=0.30$), but comparing mean activity during the dark hours showed increased activity in γ -MSH-treated animals (Fig. 4E).

Baseline and fasting-induced refeeding during the chow and Western diets

During the chow period, the 12-h food restriction prior to refeeding reduced BW similarly in both treatment groups (Fig. 5A). There was no difference in non-fasted 2-h FI in the beginning of dark (Fig. 5B) or in the 2-h refeeding (Fig. 5C). However, the γ -MSH treatment did increase the 2–12-h FI, which remained higher at 24 h ($F_{(1,16)}=4.902$, $P=0.0417$ Two-way ANOVA) (Fig. 5C). During the Western diet, there were no differences between the treatments in fasting-induced BW change (Fig. 5D), basal 2-h feeding (Fig. 5E) or in refeeding over 24 h (Fig. 5F).

Effects on diet-induced weight loss in obese mice

During the 7-week weight loss chow period, weekly FI increased back to the level of initial chow period in 3 weeks and was not statistically different between the treatment groups during the weight loss period (Fig. 6A)

Figure 3

Western period (A) mean daily food intake (g) of a week, (B) mean daily food intake in kilocalories per gram of weight, (C) body weight, (D) fat mass change, (E) lean mass change and (F) FM/LM ratio in single-housed EGFP- ($n=10$) and γ -MSH-treated mice ($n=8$). * $P<0.05$ two-way ANOVA followed by pairwise t -tests adjusted for multiple comparisons with Bonferroni adjustment.

Figure 4

Running wheel activity during (A) 24 h of the chow period, (B) chow period mean dark time activity, (C) chow period mean 24-h activity, (D) 24 h of the Western period, (E) Western period mean dark time activity, (F) Western period mean 24-h activity in EGFP- ($n=10$) and γ -MSH-treated mice ($n=8$). *** $P<0.001$ Student's t -test.

or over the course of the experiment (Supplementary Fig. 3A). However, cumulative FI was significantly lower in the γ -MSH group at the end of the period (Fig. 6B). Similarly to the Western period, the γ -MSH group seemed to be unable to totally adjust feeding for decreased BW as kilocalories consumed per gram of BW tended to be higher over time ($F_{(1,16)}=2.877$, $P=0.11$ Two-way ANOVA) (Fig. 6C). The hypocaloric state induced weight loss in both groups (Fig. 6D), however, BW ($F_{(1,16)}=9.124$, $P=0.008$ Two-way ANOVA), FM ($F_{(1,16)}=9.266$, $P=0.008$ Two-way ANOVA) and FM/LM ratio ($F_{(1,16)}=8.123$, $P=0.012$ Two-way ANOVA) decreased significantly more in the γ -MSH

group (Fig. 6E, F and H), but LM remained similar between the treatments ($F_{(1,16)}=0.033$, $P=0.857$ Two-way ANOVA, Fig. 6G). Basal glucose levels in the γ -MSH-treated animals decreased significantly over the course of the weight loss period (Treatment $F_{(1,17)}=6.271$, $P=0.023$ Two-way ANOVA, Fig. 6I and Supplementary Fig. 3B), but there was no treatment effect on glucose clearance in GTT ($F_{(1,15)}=1.567$, $P=0.218$ Two-way ANOVA, Supplementary Fig. 3C).

In the end, the γ -MSH-treated mice had decreased BW (Supplementary Fig. 3D) and FM (Fig. 6F), and fitting with this the weights of mesenteric, subcutaneous,

Figure 5

(A) Twelve-hour fasting-induced weight change, (B) 2-h unrestricted food intake after light switch and (C) 24-h refeeding measurements in the chow period and (D) 12-h fasting-induced weight change, (E) 2-h unrestricted food intake after light switch and (F) 24-h refeeding measurements in the Western period in EGFP- ($n=10$) and γ -MSH-treated mice ($n=8$). Two-way ANOVA treatment effect (* $P<0.05$) followed by pairwise t -tests adjusted for multiple comparisons with Bonferroni adjustment (** $P<0.01$).

Figure 6

Weight loss chow period (A) mean daily food intake (g) of a week, (B) cumulative food intake, (C) mean daily food intake in kilocalories per gram of weight, (D) body weight, (E) body weight change, (F) fat mass change, (G) lean mass change, (H) FM/LM ratio and (I) basal glucose change in single-housed EGFP- ($n=10$) and γ -MSH-treated mice ($n=8$). * $P<0.05$, ** $P<0.01$, *** $P<0.01$ two-way ANOVA followed by pairwise t -tests adjusted for multiple comparisons with Bonferroni adjustment.

retroperitoneal and inguinal WAT depots were lower than those in EGFP-treated mice (Table 1). There were no statistically significant differences in the weights of other isolated tissues or serum insulin or serum lipids (Table 1).

BAT and adrenal activity

The mRNA expression of *Ucp1* in BAT (γ -MSH: 0.96 ± 0.12 vs EGFP: 1.00 ± 0.09 , $t=0.2161$, $df=16$, $P=0.832$, Student's t -test) or *Th* in adrenal gland (γ -MSH: 0.94 ± 0.15 vs EGFP: 1.00 ± 0.23 , $t=0.1820$, $df=15$, $P=0.858$, Student's t -test) did not differ.

t -test) or *Th* in adrenal gland (γ -MSH: 0.94 ± 0.15 vs EGFP: 1.00 ± 0.23 , $t=0.1820$, $df=15$, $P=0.858$, Student's t -test) did not differ.

Neuropeptide mRNA levels in ARC

There was a non-statistical tendency for higher expression of endogenous *Pomc* mRNA ($P=0.10$) in γ -MSH-treated animals (Fig. 7A), whereas the expression of the inverse

Table 1 Weights of isolated white adipose tissue depots, interscapular brown adipose tissue (BAT), the liver, the heart and the kidneys as well as serum triglycerides, non-esterified fatty acids (NEFA) and insulin in EGFP- ($n=10$) and γ -MSH-treated mice ($n=8$).

	EGFP ($n=10$)	γ -MSH ($n=8$)	P Value
Fat depot (g)			
Mesenteric	0.95 ± 0.05	0.64 ± 0.12	$<0.05^*$
Retroperitoneal	0.71 ± 0.04	0.42 ± 0.09	$<0.05^*$
Epigonadal	1.34 ± 0.08	0.93 ± 0.17	0.06
Subcutaneous	0.70 ± 0.05	0.46 ± 0.11	$<0.05^*$
Inguinal	0.65 ± 0.04	0.43 ± 0.09	$<0.05^*$
Total fat	4.35 ± 0.21	2.88 ± 0.56	$<0.05^*$
Tissue weight (g)			
BAT	0.31 ± 0.02	0.24 ± 0.04	0.12
Liver	2.02 ± 0.13	1.65 ± 0.14	0.06
Heart	0.15 ± 0.01	0.15 ± 0.01	0.68
Kidney	0.21 ± 0.01	0.21 ± 0.01	0.87
Serum			
Triglycerides (mg/mL)	0.13 ± 0.02	0.12 ± 0.01	0.65
NEFA (mmol/L)	0.36 ± 0.01	0.37 ± 0.02	0.53
Insulin (μ g/L)	1.09 ± 0.28	0.68 ± 0.10	0.24

* $P<0.05$ Student's t -test.

Figure 7

Relative mRNA expression data from (A) *Pomc*, (B) *AgRP*, (C) *AgRP* vs *Pomc* DCt ratio, (D) *Cart*, (E) *Npy*, (F) *Mc3r*, (G) *Mc4r* and (H) *Y2r* in isolated ARC samples of EGFP- ($n=7$) and γ -MSH-treated mice ($n=7$). * $P < 0.05$ Student's *t*-test.

agonist of MCRs, *AgRP*, was not changed (Fig. 7B). However, the DCt ratio between *AgRP* and *Pomc* mRNA expression, which inversely correlates with mRNA expression, was significantly increased in γ -MSH-treated animals (Fig. 7C). The levels of neuropeptides coexpressed in the POMC and AGRP neurons, *Cart* and *Npy*, respectively, were not changed in ARC (Fig. 7D and E). The expressions of *Mc3r* and *Mc4r* mRNA were similar between the treatments (Fig. 7F and G), but the Y receptor expressed in both POMC and NAG neurons, *Y2r*, was elevated in the ARC samples (Fig. 7H).

Discussion

In this study, we utilized a previously successfully used method for the overexpression of single melanocortin peptides in the neurons of the CNS (Eerola et al. 2013, 2014). By using the *in vitro*-tested lentiviral vector carrying and producing only γ -MSH of the POMC sequence, we targeted the ARC in the hypothalamus of mice in order to assess the metabolic impact of the γ -MSH-treatment in response to different dietary situations over 26 weeks. γ -MSH is an agonist of the MC3R, which based on studies in *Mc3r*-KO mice plays a role in the regulation of nutrient partitioning, insulin sensitivity and responses to hypocaloric state. In this study, γ -MSH-treated mice showed quite small changes when feeding *ad libitum* on the chow or the Western diet. However, γ -MSH had significant effects on adjustment to different dietary changes.

Although γ -MSH exerts a weak interaction on the MC4R, the phenotype of the genetic knockout of the *Mc3r*, in many aspects, is opposite to the genetic γ -Msh manipulation shown here suggesting that the effects were mediated by MC3R activation. The *Mc3r*-KO mice present a unique metabolic phenotype showing altered nutrient partitioning without affecting total BW, FI or energy expenditure, but display an increased FM/LM ratio due to impaired fatty acid oxidation and decreased locomotor activity regardless of diet macronutrient composition (Butler et al. 2000, Chen et al. 2000). Fitting with this, the γ -MSH-treated mice had slightly but significantly decreased FM/LM ratio without changes in weight gain or FI during the initial chow period. The modulation of body composition in the *Mc3r*-KO has been reported to be dependent on an impaired WAT lipolysis, which drives amino acid mobilization and influences lean and fat mass development (Butler et al. 2000, Chen et al. 2000, Renquist et al. 2012). In addition, decreased ability to oxidize fat from the diet accompanied by an impaired lipolysis augments BW and FM on HFD in the *Mc3r*-KO and is related to increased nutrient partitioning (Butler et al. 2000). Accordingly, the γ -MSH-treatment could have increased lipolysis and the availability of fatty acids as an energy source during the recovery from surgery and thus decreased the need for amino acid mobilization, which would have led to the attenuated change in FM/LM ratio.

Interestingly, the *Mc3r*-KO mice display a blunted fasting-induced refeeding response (Renquist et al. 2012). In contrast, the γ -MSH-treated mice exhibited an increased refeeding-response on the chow diet.

Food restriction elevates activity in orexigenic over anorexic circuits (Bi *et al.* 2003, Shimokawa *et al.* 2003, Lauzurica *et al.* 2010). Release of NPY and GABA by NAG neurons mediates the acute refeeding effect, whereas AGRP modulates the long-term response (Krashes *et al.* 2013). The impaired refeeding and the decreased NPY and AGRP expressions under fasting conditions in *Mc3r*-KO suggests that MC3R is required to sustain the feeding response mediated by NAG neurons (Renquist *et al.* 2012). Refeeding in γ -MSH group was increased only after the first 2 h, a time period that does not fit the activation pattern of NAG neurons, which returns to the fed levels an hour after reintroduction to food (Krashes *et al.* 2013, Wu *et al.* 2014). The result may suggest that γ -MSH attenuated the rise in *Pomc* activity occurring within 1–2 h after initiation of refeeding (Krashes *et al.* 2013), and thus, favor the auto-inhibition of POMC neurons by MC3Rs in the γ -MSH-treated mice.

The Western diet attenuated the initial changes in body composition in the γ -MSH-treated mice, suggesting that the effect of MC3R stimulation by ARC neurons is not sufficient to affect the regulatory mechanisms governing body energy homeostasis during HFD, which desensitizes the mechanisms of nutrient sensors in the brain (Ryan *et al.* 2012). The introduction of HFD induces caloric overingestion and shifts the neuropeptide balance acutely toward anorexigenic predominance (Torri *et al.* 2002). Surprisingly, there was no difference between groups in FI following diet change to the Western, although some minor differences could potentially have been missed as FI was measured only weekly. However, FI was slowly reduced to match the weight-adjusted caloric intake of the chow period in both groups, but more slowly in the γ -MSH-treated group, which could again reflect a change in the balance between POMC and NAG neurons. Voluntary running wheel activity was increased specifically on the Western period, which is opposite to decreased running wheel activity in *Mc3r*-KO mice (Butler *et al.* 2000). Increased activity could explain why increased FI did not lead to increased weight gain.

When the Western diet was changed again to chow in order to induce weight loss and to perturb energy balance, the weight of γ -MSH-treated mice started to decrease more, and this was dependent on a significant reduction in FM (37% compared to 12%). Decrease in FM was supported by decreased weights of WAT depots and basal glucose levels, but no improvement was detected in serum lipids. The mechanism for decreased FM in γ -MSH-treated animals during weight loss period can only partly result from a small decrease in feeding. In fact, food consumed

per weight tended to be higher in the late stages of the period. Therefore, changes in energy expenditure or nutrient partitioning must be responsible for augmented fat mass loss. SNS-driven BAT thermogenesis analyzed as *Ucp1* mRNA and SNS activity in adrenal gland measured as *Th* mRNA (the rate-limiting enzyme in catecholamine synthesis) were unchanged, which does not support the concept of increased energy expenditure by SNS activity. Based on the phenotype of *Mc3r*-KO mice, increased lipolysis and fat oxidation are plausible mechanisms for the adiposity reducing effect, although in the end of the experiment, the anorexigenic response had plateaued and could explain the lack of difference in lipolytic markers between the treatments.

MC3Rs are expressed on both NAG and POMC neurons that control energy metabolism and FI in the ARC (Bagnol *et al.* 1999, Cone 2005), but also in other regions of the hypothalamus and in the tegmentum (Roselli-Rehfuess *et al.* 1993). The sites mediating the effects of MC3Rs on nutrient partitioning are not established, but hypothalamic expression plays a role (Girardet *et al.* 2017). To test whether the decrease in FM was mediated by the effect of γ -MSH in the ARC, the expression levels of key players were analyzed. There was a decrease in the *Agrp/Pomc* ratio suggesting a shift in the melanocortin system toward anorexigenic predominance favoring augmented fat mass loss. The other neurotransmitters of NAG and POMC neurons, i.e. *Npy* and *Cart*, or melanocortin receptors were unchanged. Interestingly, *Y2r*, another presynaptic receptor controlling these neurons, was upregulated. Activation of Y2R inhibits NAG and stimulates POMC neurons also favoring weight loss (Batterham *et al.* 2002, Ghamari-Langroudi *et al.* 2005). Therefore, the beneficial effect of ARC γ -MSH expression on the diet-induced weight loss in obesity may be mediated by the effects of γ -MSH on the ARC neurons that inhibit the orexigenic predominance aiming to re-gain the set weight. However, it is possible that effects on downstream targets also contribute.

Gene delivery using lentiviral vectors offers a unique opportunity to study the long-term effects of different genes in secluded areas of the CNS as seen in this 26-week study. The approach has clear advantages compared to long-term peptide infusions and the development of transgenic animals. In addition, the construct used in this study requires the enzymatic cleavage and presence of the two forms of protein convertases that cleave the POMC propeptide into active γ -MSH, which limits the expression to cells with native POMC expression. EGFP fluorescence and analysis of target site production of the

gene of interest enable efficient representation of the physiological effects with matched treatments in both experimental groups.

We have shown here that a continuous expression of γ -MSH in the ARC induces either anorexigenic or orexigenic effects depending on the nutritional status, which proposes a dynamic role for γ -MSH by influencing the ARC neuropeptide systems in the regulation of body metabolism. Increased fasting-induced refeeding underlines the importance of MC3R in the fasting response of ARC neurons (Renquist *et al.* 2012). Improved FM/LM ratio on chow fits with the *Mc3r*-KO phenotype, but our results are not in line with increased DIO in *Mc3r*-KO mice (Butler *et al.* 2000, Chen *et al.* 2000), which suggests that the levels of γ -MSH induced by the treatment in the ARC were not sufficient for DIO resistance or the brain region affecting DIO is different. Interestingly, the augmentation of diet-induced weight loss in obese mice points to potential therapeutic applications for γ -MSH.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/JOE-18-0009>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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