A Comparative Study of the Central Effects of Specific Proopiomelancortin (POMC)-Derived Melanocortin Peptides on Food Intake and Body Weight in Pomc Null Mice

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Functional disruption of either MC3R or MC4R results in obesity, implicating both in the control of energy homeostasis. The ligands for these receptors are derived from the prohormone proopiomelanocortin (POMC), which is posttranslationally processed to produce a set of melanocortin peptides with a range of activities at the MC3R and MC4R. The relative importance of each of these peptides α-MSH, γ3-MSH, γ2-MSH, γ1-LPH, lipotropin (γ-LPH) and, in man but not in rodents, β-MSH in the maintenance of energy homeostasis is, as yet, unclear. To investigate this further, equimolar amounts (2 nmol) of each peptide were centrally administered to freely feeding, corticosterone-supplemented, Pomc null (Pomc−/−) mice. After a single dose at the onset of the dark cycle, α-MSH had the most potent anorexigenic effect, reducing food intake to 35% of sham-treated animals. β-MSH, γ-LPH, and γ2- and γ2-MSH all reduced food intake but to a lesser degree. The effects of peptide administration over 3 d were also assessed. Only α-MSH significantly reduced body weight, affecting both fat and lean mass. Other peptides had no significant effect on body weight. Pair-feeding of sham-treated mice to those treated with α-MSH resulted in identical changes in total weight, fat and lean mass indicating that the effects of α-MSH were primarily due to reduced food intake rather than increased energy expenditure. Although other melanocortins can reduce food intake in the short-term, only α-MSH can reduce the excess fat and lean mass found in Pomc−/− mice, mediated largely through an effect on food intake. (Endocrinology 147: 5940–5947, 2006)
affinities to melanocortin receptors (15). Although α-MSH has equal affinity for MC3R and MC4R, β-MSH has the highest affinity for MC4R (16, 17), whereas γ3-MSH preferentially binds to MC3R (18). These differences between the in *vitro* characteristics of the melanocortins are illustrated by a recent study characterizing human MC4R polymorphisms that demonstrated β-MSH remained active at a number of receptors that were not stimulated by other melanocortins (19).

Several *in vivo* studies have compared the response of centrally administered melanocortins on food intake (20–22). α-, β-, and γ-MSH appear to have differential effects upon short-term feeding behavior, both in terms of magnitude and time of onset of effect. However, none of these studies have been carried out in the total absence of endogenous melanocortins, and none have directly compared the ability of the melanocortins to bring about changes in weight.

In this study, we have used a mouse model lacking all endogenous POMC peptides (Pomc−/−) to further investigate the effects of the centrally derived melanocortin peptides upon food intake and body weight. By giving equimolar amounts of peptide intracerebroventricularly (icv) to corticosterone-supplemented Pomc−/− mice, we wished to determine whether α-, β-, and γ-MSH might have differential effects on the hyperphagia and excess fat and lean mass found in this mouse model. In addition, we have also taken into account the species difference in POMC processing and investigated the effects of γ-LPH, the direct precursor of β-MSH, as well as using both γ3-MSH and γ2-MSH.

**Materials and Methods**

**Pomc**−/−** mice**

Pomc null mice were generated on a 129/SvEv background, and genotypes were determined by PCR of DNA from ear tissue using a method previously described (11). All mice were maintained under controlled temperature (22°C) and light (12 h light from 0700–1900 h) and had *ad libitum* access to water and standard chow (4.5% fat chow; Special Diet Services, Witham, UK). Twelve-week-old male mice were used in all the studies and were individually caged throughout the duration of the experiment. All protocols were in accordance with the United Kingdom Home Office.

**icv studies**

On d 0, mice underwent stereotaxic surgery to place an indwelling guide cannula into the lateral ventricle. Mice were anesthetized with a mix of inhaled isoflurane and oxygen and a 26-gauge steel guide cannula (internal diameter 0.24 mm, outer diameter 0.46 mm, length 2 mm; Semat International, Herts, UK) was implanted into the right lateral ventricle using the following coordinates: 1.0 mm lateral from bregma, 0.5 mm posterior to bregma. The guide cannula was secured to the skull using quick-drying cyanoacrylate glue and a dental cement (Associated Dental Products, Wiltshire, UK) and a dummy cannula was inserted. All animals received analgesia (Rimadyl, 5 mg/kg; Pfizer Animal Health, Kent, UK) and antibiotic (Terramycin LA, 60 mg/kg; Pfizer Animal Health) before being returned to their home cage.

In addition, on d 0, drinking water was replaced by corticosterone-supplemented drinking water (as described in Corticosterone replacement), which remained in place for the duration of the study. Our previous studies (23) have shown that, without corticosterone supplementation, Pomc−/− mice are affected by a rapid decline in body weight and food intake in the postoperative period. As the aim of the study was to directly compare the anorexigenic potencies of melanocortin peptides, we elected for continuous corticosterone supplementation.

On d 7, food intake and body weight were measured. If either parameter did not match presurgery values, mice were excluded from the study. On d 8, 1 h before the onset of the dark cycle, mice received either 2 nmol peptide (α-MSH, γ-LPH, β-MSH, γ3-MSH, or γ2-MSH) or PBS.
(sham) in a total volume of 2 μl. This was administered using a Hamilton syringe over 2 min. Food intake was measured over at 2, 4, 12, and 24 h.

On d 9 and 10, peptide was administered in an identical way to d 8, with food intake and body weight measured at 0800 h each d. On the morning of d 11, mice underwent a dual-energy x-ray absorptiometry (DEXA) scan to assess body morphology, and were killed to collect blood and tissue. In addition, accurate cannula placement was confirmed by injecting 2 μl of methylene blue dye into the indwelling cannula. Animals failing to show diffusion of the dye throughout the ventricular system were excluded from subsequent analysis.

Corticosterone replacement

Corticosterone replacement was given as supplemented drinking water at a final concentration of 25 μg/ml. Corticosterone was purchased from Sigma-Aldrich (Poole, UK). Corticosterone concentrations achieved in all six treatment groups were identical (sham vs. α-MSH vs. γ-LPH vs. β-MSH vs. γ3-MSH vs. γ2-MSH: 50.9 ± 23.0 vs. 43.3 ± 16.5 vs. 45.4 ± 18.0 vs. 33.4 ± 4.2 vs. 42.4 ± 23.6 vs. 42.4 ± 23.6, all P = n.s. vs. sham).

Peptides

α-MSH (Ac-YSYMEHRWGTK TVN-H2), β-MSH (H-DEGPYRMMEHRWGPSPK-D-2-MSH (H-YVMGHFW DRF-G-OH) and γ2-MSH (H-YVMGHFW DRF-G-OH) were all purchased from Bachem (St Helens, UK).

Statistical significance was determined using Student’s t test, except for the data presented in Fig. 3, where significance was sought using two-way ANOVA. The PRISM software package (GraphPad, San Diego, CA) was used for all analyses. Results were considered statistically significant at P < 0.05.

Results

All centrally derived melanocortin peptides can reduce food intake

To determine the relative anorexigenic potency of each of the centrally derived melanocortins, we administered 2 nmol of each peptide into the lateral ventricle of Pommec–/– mice who were also receiving corticosterone-supplemented drinking water. All peptides were given 1 h before the onset of the dark cycle with food intake over the subsequent 24-h period recorded.

At all time points, α-MSH caused the biggest reduction in food intake, with the amount consumed at 24 h only a third of the amount eaten by sham-treated animals (sham vs. α-MSH, 8.22 ± 0.73 vs. 2.85 ± 0.74 g, P < 0.001) (Fig. 2). γ3-MSH caused the second largest reduction in food intake, able to reduce food intake at all time points measured across the 24-h period. Interestingly, γ-LPH and β-MSH had similar anorexigenic actions, although both were less effective than α- or γ3-MSH in reducing food intake. Finally, γ2-MSH was the least effective, only bringing about a significant reduction in food intake at 4 h (sham vs. γ2-MSH, 2.35 ± 0.10 vs. 1.63 ± 0.24 g, P < 0.05) (Fig. 1).

Only α-MSH can reduce body weight in Pommec–/– mice

Although all the peptides given as a single dose were able to reduce food intake, none caused a significant reduction in body weight. Therefore, we wished to determine if repeated central administration of melanocortin peptides might be able to impact upon the excess fat and lean tissue mass we have previously reported in corticosterone-treated Pommec–/– mice (24), and in particular, if different peptides might have differential effects upon this phenotype. Therefore, we extended the study by administering equimolar amounts of each peptide for another 2 d, again with each dose given just before the onset of the dark cycle.

Once again, α-MSH had the most potent effect on food intake, with cumulative food intake reduced to less than half that seen in sham animals (sham vs. α-MSH, 31.50 ± 2.38 vs. 13.99 ± 2.20 g, P < 0.001) (Fig. 3A). The other administered peptides also reduced cumulative food intake, but their effects were all of a similar magnitude (γ-LPH vs. β-MSH vs. γ3-MSH vs. γ2-MSH, 23.63 ± 1.72 vs. 24.72 ± 3.00 vs. 24.72 ± 3.00 vs. 24.92 ± 2.00 g, respectively, all P < 0.05 vs. sham) (Fig. 3, B–E).

α-MSH was also the only administered peptide that brought about a significant reduction in weight. Thus, three doses of α-MSH were enough to cause a 6-g weight loss in total body weight (Fig. 4A), representing a reduction of 13% from baseline weight (Fig. 4B). Analysis of body composition...
by DEXA on the day after the third injection demonstrated this loss to be a combination of reduced fat and lean mass (sham vs. α-MSH; fat mass, 15.26 ± 1.15 vs. 12.82 ± 0.63 g, P < 0.05; lean mass, 26.08 ± 0.86 vs. 22.83 ± 0.43 g, P < 0.05) (Fig. 5A). Analysis of anatomical depots showed the fat loss to be from mesenteric (sham vs. α-MSH, 745 ± 46 vs. 582 ± 31 mg, P < 0.01), retroperitoneal (534 ± 90 vs. 344 ± 27 mg, P < 0.05), and sc depots (809 ± 78 vs. 603 ± 34, P < 0.05) without any effect upon the gonadal fat pad (Fig. 5B). Whole liver mass was also significantly reduced after α-MSH treatment (2115 ± 68 vs. 1285 ± 150, P < 0.01) (Fig. 5B).

Fat mass in γ3-MSH-treated mice was reduced to 86% of that seen in sham-treated animals, but in absolute terms, this failed to reach statistical significance (sham vs. γ3-MSH, P < 0.05).
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There are far fewer studies that have directly compared the
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Abbott et al. (21) centrally administered equimolar amounts of
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Intriguingly, this study also reported adverse locomotor ac-
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these particular peptides being studied further. Finally, Millington et al. (22) compared the effects of centrally administered α-MSH, β-MSH, and γ2-MSH on hypothalamic neuronal activation and on food intake in rats fasted for 48 h. In contrast to the study by Abbott et al., this study demonstrated that α- and γ2-MSH, but not β-MSH, suppressed food intake. Furthermore, the two peptides shown to be biologically active brought about their effects over different time courses with α-MSH acting more rapidly than γ2-MSH. Each of the melanocortins used in this study also caused distinct anatomical patterns of activation within the hypothalamus, suggesting the individual peptides may have distinct biological roles (22).

Despite these studies, there remains contention about the physiological hierarchy of the melanocortin peptides. The results of the current study show α-MSH to be the most potent anorexigen, significantly reducing food intake over 24 h after a single dose. Repeated administration of α-reduced both fat and lean mass, but to an identical degree to that seen in pair-fed mice. We have previously reported that the phenotype of Pomc<sup>−/−</sup> mice results from increased food intake and a reduction in resting basal metabolic rate (11). The finding that α-MSH-treated mice did not lose more weight than pair-fed mice indicates that, at least in the paradigm used in this study, administration of α-MSH did not significantly increase energy expenditure but simply ameliorated the hyperphagia.

These results are intriguing in the light of recent data clearly demonstrating functional divergence of the melanocortin pathway. Balthasar et al. (31) engineered a lox P-modified, null Mc4r allele, which can be reactivated by Cre recombinase. Using Sim1 cre transgenic mice, Mc4r expression was restored in the paraventricular hypothalamus and a subpopulation of the amygdala neurons. Sixty percent of the obesity seen in total Mc4r null mice was prevented. This was entirely due to a normalization in food intake with the reduced energy intake typical of Mc4r<sup>−/−</sup> mice unaffected by this targeted reexpression. Thus MC4R in the paraventricular hypothalamic nucleus and/or amygdala control food intake but MC4R elsewhere control energy expenditure. Given that we administered peptide into the lateral ventricle, it is likely that a higher concentration of peptide would have reached the MC4R in the PVN compared with more neuroanatomically distinct sites like the brainstem. This may, in part, explain why the predominant effect of α-MSH was on food intake.

Compared with α-MSH, the physiological roles of β-MSH and its direct precursor, γ-LPH, remain less well characterized. This may be understandable as rodents lack the N-terminal cleavage site necessary for the generation of β-MSH. Therefore, conclusions drawn from rodent data may have resulted in an important role for β-MSH being overlooked. In fact, it has required human genetic studies to bring novel insights into the function of this peptide. Two recent reports have demonstrated that β-MSH is likely to be a physiologically relevant endogenous ligand at the MC4R (32, 33), thereby challenging the canonical view that α-MSH is the primary melanocortin ligand controlling energy homeostasis in humans. Like the previous rodent studies reviewed above, our data also suggest that β-MSH can reduce food intake but
also show that POMC-derived peptide γ-LPH, which is more physiologically relevant to mice, can also significantly reduce food intake to a similar degree. Thus γ-LPH may have a function in modulating appetite and food intake in rodents.

Much of the data derived from rodent studies suggest that the primary role of the γ-MSH species may be in the regulation of the cardiovascular system and in particular integrating the response to dietary sodium excess (34). A high-sodium diet increases the pituitary content of γ-MSH and results in a doubling of plasma γ-MSH concentration. Renal MC3Rs are also up-regulated by a high-salt diet and acting via these receptors, γ-MSH is thought to be able to bring about a marked natriuresis. However, there is as yet no mouse model that solely lacks γ-MSH, with some of the key conclusions regarding the role of this melanocortin drawn from a mouse model functionally lacking γ-MSH because of a lack of the prohormone converting enzyme, PC2 (35). This enzyme is involved in processing a range of other peptides, with the knock-on effect of these unprocessed hormones and the lack of smaller downstream peptides likely to impact upon the key physiological functions within the mouse. The data in the current study show both γ3-MSH and γ2-MSH can both reduce food intake. Although this is in accordance with many of the previous published studies (20–22), a recent report by Marks et al. (36) demonstrated that an analog of γ-MSH (d-Trp⁶-γ-MSH) with high affinity for MC3R can increase food intake. The explanation of this orexigenic action invoked a central mechanism in which the ligand bound to MC3R on arcuate POMC neurons. Acting as an inhibitory autoreceptor, increased activity at MC3R, therefore, could bring about a reduction in melanocortinergic tone and decrease food intake. The data presented in the current study are not able to directly address this putative mechanism of action of γ-MSH, as even if centrally administered peptide were acting upon MC3R on POMC neurons, the very nature of the Pomp⁻/⁻ mouse means melanocortinergic tone is non-existent before any peptide is given. However, it is noteworthy that the peptide analog studied by Marks et al. (36) was given peripherally, not centrally, and as MC3R are found in many other tissues, including adipose tissue and the stomach, it is possible that the effects of d-Trp⁶-γ-MSH seen were from a site of action outside of the hypothalamus.

The current study also demonstrated that γ3-MSH significantly reduced liver mass and showed a trend to reducing total fat mass, indicating that this ligand may have a potential role in controlling fat deposition. This may be via stimulation of the sympathetic system (37, 38) although the nature of the receptor involved cannot be determined by the present study.

One limitation of a pharmacological study is that it can never wholly replicate a complex physiological scenario, with any results undoubtedly an underestimate of the true elegance and subtlety at work within the system. POMC is highly posttranslationally modified and there is still much to understand with respect to the regulation of these processes. Indeed, some intriguing recent data have suggested that leptin can critically alter an important step in melanocortin processing (39). By analysis of the protein content and enzymatic activity within the hypothalamus of wild-type and leptin-deficient ob/ob mice, Guo et al. (39) demonstrated that leptin specifically and rapidly stimulated the generation of acetylated α-MSH. Their data suggested that this is the result of a leptin-dependent increase in the activity of an N-acetyltransferase in hypothalamic POMC neurons. The effects of leptin on energy balance via the central melanocortin pathway now appear to have an additional level of complexity by regulating acetylation of α-MSH. In addition, the fact that there are multiple POMC-derived peptides, but only two centrally expressed melanocortin receptor subtypes through which they can act, brings a further layer of complexity to the system. It will take carefully targeted genetic models to tease apart the system further and ascribe true physiological function to each peptide.

Although all the peptides were administered in equimolar amounts, in keeping with what is known about POMC processing in vivo (14), we have no data pertaining to the pharmacokinetics of peptides after administration. Such data would be valuable in further interpreting the results presented, as it is conceivable that the ability of α-MSH to bring about the biggest change in food intake and body weight result, in part, from an increased resistance to degradation and clearance.

Finally, there are compelling data demonstrating that an intact central melanocortin system is required for normal glucose homeostasis, independent of its role in controlling food intake and body weight (40) and we have previously shown that 10 d of glucocorticoid treatment rendered Pomp⁻/⁻ mice markedly insulin resistant (24). The current study was not designed to compare the ability of centrally administered melanocortin peptides to potentially ameliorate this insulin-resistant state. Such future studies may be informative in resolving unanswered issues surrounding the true physiological roles of the melanocortin peptides.

In summary, although other centrally administered melanocortins can reduce food intake in the short-term, only α-MSH is also able to reduce the excess fat and lean mass found in Pomp⁻/⁻ mice, mediated largely mediated through its effects on food intake.

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