Peripheral administration of the N-terminal pro-opiomelanocortin fragment 1–28 to *Pomc*$^{-/-}$ mice reduces food intake and weight but does not affect adrenal growth or corticosterone production

Anthony P Coll, Martin Fassnacht[1], Steffen Klammer[2], Stephanie Hahner[1], Dominik M Schulte[2], Sarah Piper, Y C Loraine Tung, Benjamin G Challis, Yacob Weinstein[3], Bruno Allolio[1], Stephen O'Rahilly and **Felix Beuschlein[2]**

Departments of Clinical Biochemistry and Medicine, Cambridge Institute for Medical Research, Addenbrooke's Hospital, Cambridge CB2 2XY, UK

[1]Division of Endocrinology, Department of Internal Medicine I, University Hospital Würzburg 97080, Germany

[2]Division of Endocrinology and Diabetes, Department of Internal Medicine II, Klinikum der Albert-Ludwigs-Universität Freiburg, Hugstetter Str. 55, D-79106 Freiburg, Germany

[3]Department of Microbiology and Immunology, Faculty of Health Sciences, Ben Gurion University of the Negev, Beer Sheva 84105, Israel

(Requests for offprints should be addressed to F Beuschlein; Email: beuschlein@medizin.ukl.uni-freiburg.de)

Abstract

Pro-opiomelanocortin (POMC) is a polypeptide precursor that undergoes extensive processing to yield a range of peptides with biologically diverse functions. POMC-derived ACTH is vital for normal adrenal function and the melanocortin α-MSH plays a key role in appetite control and energy homeostasis. However, the roles of peptide fragments derived from the highly conserved N-terminal region of POMC are less well characterized. We have used mice with a null mutation in the *Pomc* gene (*Pomc*$^{-/-}$) to determine the *in vivo* effects of synthetic N-terminal 1–28 POMC, which has been shown previously to possess adrenal mitogenic activity. 1–28 POMC (20 μg) given s.c. for 10 days had no effect on the adrenal cortex of *Pomc*$^{-/-}$ mice, with resultant cortical morphology and plasma corticosterone levels being indistinguishable from sham treatment. Concurrent administration of 1–28 POMC and 1–24 ACTH (30 μg/day) resulted in changes identical to 1–24 ACTH treatment alone, which consisted of upregulation of steroidogenic enzymes, elevation of corticosterone levels, hypertrophy of the zona fasciculate, and regression of the X-zone. However, treatment of corticosterone-depleted *Pomc*$^{-/-}$ mice with 1–28 POMC reduced cumulative food intake and total body weight. These anorexigenic effects were ameliorated when the peptide was administered to *Pomc*$^{-/-}$ mice with circulating corticosterone restored either to a low physiological level by corticosterone-supplemented drinking water (CORT) or to a supraphysiological level by concurrent 1–24 ACTH administration. Further, i.c.v. administration of 1–28 POMC to CORT-treated *Pomc*$^{-/-}$ mice had no effect on food intake or body weight. In wild-type mice, the effects of 1–28 POMC upon food intake and body weight were identical to sham treatment, but 1–28 POMC was able to ameliorate the hyperphagia induced by concurrent 1–24 ACTH treatment. In a mouse model which lacks all endogenous POMC peptides, s.c. treatment with synthetic 1–28 POMC alone can reduce food intake and body weight, but has no impact upon adrenal growth or steroidogenesis.

Journal of Endocrinology (2006) **190,** 515–525

Introduction

The pro-opiomelanocortin (POMC) gene is actively transcribed within a range of tissues including anterior pituitary corticotrophs, neurons of the hypothalamic arcuate nucleus, cells in the dermis, and the lymphoid system. POMC itself is a functionally inert polypeptide precursor that undergoes extensive and tissue-specific post-translation modification to yield a number of smaller biologically active peptides (Bertagna 1994, Raffin-Sanson *et al.* 2003).

For example, adrenocorticotrophin (ACTH) has a clearly defined role in controlling steroidogenesis in the adult adrenal cortex; α-melanocyte-stimulating hormone (α-MSH) is a key regulator of skin pigmentation, and a rapidly expanding body of work has established that POMC-derived peptides synthesized in hypothalamic neurons play a central role in the control of energy homeostasis (Butler & Cone 2003, Raffin-Sanson *et al.* 2003, Coll 2004*a*, Cone 2005). In addition, there are data on the potential peripheral action of POMC-derived peptides upon fat mass with ACTH, α-MSH, and β-lipotropin all able to stimulate lipolysis in adipocytes (Ramachandran 1976, Richter & Schwandt 1985, Bradley *et al.* 2005). Accordingly, Forbes *et al.* (2001) demonstrated that peripheral administration of α-MSH

DOI: 10.1677/joe.1.06749
0022–0795/06/0190–515
Online version via http://www.endocrinology-journals.org

analogue to obese *ob/ob* mice stimulated lipolysis and increased circulating free fatty acid levels.

In contrast to these detailed observations, the functions of other regions of the POMC precursor peptide remain contentious. A longstanding body of evidence proposes that the amino terminal region sequence of POMC contains a smaller fragment with adrenal mitogenic activity (Estivariz *et al.* 1980, 1982, 1988*a*, Lowry *et al.* 1983, 1984). Although the major circulating N-terminal POMC peptide, progamma MSH (pro-γ-MSH; human 1–76 POMC), does not have a trophic effect on adrenals (Estivariz *et al.* 1980), shorter peptides derived from this region, in particular the first 28 amino acids, have been demonstrated to possess mitogenic activity. This peptide contains a highly conserved region with a defined secondary structure dependent upon two disulphide bridges between cysteine residues 2–24 and 8–20 (Bennett *et al.* 1986, Seger & Bennett 1986, Denef *et al.* 2001), but has no significant sequence homology with the melanocortins. Peripheral administration of 1–28 POMC to intact rats can increase adrenal weight and mitotic index (Estivariz *et al.* 1982). Further, peripheral delivery of purified 1–28 POMC partially prevents the atrophy of regenerating adrenal glands after hypophysectomy (Estivariz *et al.* 1988*b*). More recently, synthetic 1–28 POMC with correctly aligned disulphide bridges has been shown *in vitro* to stimulate mitogenic activity in adrenal tumor cells whilst also bringing about a reduction in steroidogenesis (Fassnacht *et al.* 2003).

To investigate specifically the effects of 1–28 POMC *in vivo*, we have utilized mice with a null mutation in the *Pomc* gene (*Pomc*$^{-/-}$). *Pomc*$^{-/-}$ mice are hyperphagic and develop severe obesity despite having small dysmorphic adrenal glands with undetectable circulating corticosterone. We have previously demonstrated that peripheral administration of 1–24 ACTH to these mice can cause adrenal hyperplasia and restore circulating corticosterone (Coll *et al.* 2004). Further, we have shown that the hyperphagia and obesity in *Pomc*$^{-/-}$ mice are significantly exacerbated by restoration of low physiological levels of corticosterone (Coll *et al.* 2005). In this study, we wished to determine if the administration of synthetic 1–28 POMC, either alone or in combination with 1–24 ACTH, was able to bring about changes in adrenal morphology and glucocorticoid production in a mouse model lacking all endogenous POMC-derived peptides. In addition, since the magnitude of the hyperphagia and obesity in *Pomc*$^{-/-}$ mice appears to be modulated by circulating levels of glucocorticoids, we also determined the impact of administration of these POMC-derived peptides upon food intake and body weight.

Materials and Methods

Animal studies

Pomc null mice were generated on a 129/SvEv background and the genotypes were determined by PCR of DNA from tail tissue as described previously (Challis *et al.* 2004). All mice were maintained under controlled temperature (22 °C) and light (12 h light from 0700 to 1900 h) and had access to water and standard chow (4·5% fat chow; Special Diet Services, Witham, Essex, UK) *ad libitum*. Eight-week-old female mice were used in all the studies and were individually caged throughout the duration of the experiment.

For each of the four peripheral treatment groups, at least six mice of each genotype (wild type and *Pomc*$^{-/-}$) were used. The groups and dosing schedule were as follows: sham (PBS at 0800 and 2000 h), 1–24 ACTH (PBS at 0800 h), 30 μg Synacthen (Alliance Pharmaceuticals, Wiltshire, UK at 2000 h), 1–28 POMC (10 μg at 0800 and 2000 h), and both 1–24 ACTH and 1–28 POMC (10 μg 1–28 POMC at 0800 h and 10 μg 1–28 POMC + 30 μg Synacthen at 2000 h). The total s.c. injection volume was always 100 μl. Injections were continued over 10 days.

Food intake and body weight were measured daily at the time of the 0800 h injection. All the protocols were in accordance with the United Kingdom Home Office.

Corticosterone replacement

Corticosterone replacement was given as supplemented drinking water at final concentration of 25 μg/ml. CORT was purchased from Sigma.

Adrenal sections

Adrenal tissue from each of the s.c. treatment groups (sham, 1–28 POMC, 1–24 ACTH, and 1–28 POMC and 1–24 ACTH) was analyzed. Adrenal glands were rapidly dissected, cleaned of fat, and weighed. Glands that were to be used for histology were left uncleaned and attached to the kidney and placed in 4% paraformaldehyde overnight at 4 °C. The tissues were dehydrated and embedded in paraffin, and 7 μm sections were cut and stained with hematoxylin and eosin (H&E) using standard protocols. For immunohistochemistry, paraffin-embedded sections were rehydrated, blocked with 0·3% H_2O_2 in methanol for 10 min, and incubated overnight with a rabbit polyclonal antibody (PCNA, 1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA; 20-α-hydroxysteroid dehydrogenase (20αHSD), 1:2500, Y Weinstein (Beuschlein *et al.* 2005); 3ßHSD, 1:2500, A Payne) in blocking buffer containing 3% BSA (Roche), 5% goat serum (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), and 0·5% Tween 20. Bound antibody was detected using the Vectastain ABC Kit Standard (Vector Laboratories, Inc., Burlingame, CA, USA) according to the manufacturer's protocol.

Cell counts

Haematoxylin and eosin (H&E)-stained adrenal sections from each s.c. treatment group were examined with a standard light microscope using 1000× magnification. Cell nuclei within

the zona fasciculata of three independent sections from three different animals per group were counted under standardized conditions. Cell counts were expressed as cell number/ high power field (HPF).

Calculation of adrenal areas

H&E-stained adrenal sections from each s.c. treatment group were examined with a standard light microscope using 50× magnification. Areas were quantified using the SPOT software (Version 3.5.5, Diagnostic Instruments, Inc., Sterling Heights, MI, USA). To ensure reliable comparison between the specimens, two adjacent sections from the middle portion of each individual adrenal ($n=3$) were examined.

Immunoblotting

Individual adrenals from each s.c treatment group were homogenized in lysis buffer (50 mM HEPES pH 7·6, 250 mM NaCl, 0·5 mM EDTA, 0·5% Triton-X and protease inhibitors cocktail; Roche). The homogenate was allowed to rotate at 4 °C for 1 h and the protein contents of the high-speed supernatant samples were measured using the Bio-Rad D_c protein assay kit. Protein samples (6 μg) from organ tissue-solubilized fractions were separated by 10% SDS-PAGE minigel and transferred to immunoblot polyvinylidine difluoride (PVDF) membrane (Millipore, Bedford, MA, USA) for immunoblotting. After blocking of non-specific sites, membranes were incubated overnight at 4 °C in blocking buffer (Tris-buffered saline (TBS) containing 5% (w/v) skim milk powder, and 0·05% Tween 20) with primary antibodies to proliferating cell nuclear antigen (PCNA) (1:750, rabbit polyclonal from Santa Cruz Biotechnology), transcription factor Nurr1 (1:750, Santa Cruz Biotechnology) steroidogenic acute regulatory protein (StAR) (1:1000, D M Stocco), side chain cleavage (SCC) (1:2000, W Miller), 3-β-hydroxy steroid dehydrogenase (3βHSD) (1:20 000, A Payne), 11-β-hydroxylase (11βOH) (1:500, C E Gomez-Sanchez), 20-α-hydroxy steroid dehydrogenase (20αHSD) (1:5000, Y Weinstein) or β-actin (1:5000; Sigma). The washed blots were then incubated with suitable secondary antibodies conjugated to horseradish peroxidase (1:7000; Pierce, Rockford, IL, USA). Antibody binding to the membrane was visualized using the ECL plus (Amersham and Pharmacia) chemiluminescent detection system. All the immunoblots were performed at least three times on adrenal samples from at least two different animals.

Corticosterone assay

Blood was collected from each of the four s.c. treatment groups and from mice receiving i.c.v. injections (see below). All the blood samples were collected in the first two hours of the light cycle, within one minute of initial handling in all experiments. Following cervical dislocation, 200 μl of trunk blood were collected into EDTA tubes (Sarstedt, Numbrecht, Germany) and spun at 10 000 r.p.m. for 10 min. Plasma was removed, frozen on dry ice and stored at −80 °C until assayed. Plasma corticosterone was determined using a commercially available kit (Corticosterone EIA; Immunodiagnostic Systems Limited, Tyne and Wear, UK) according to the manufacturer's protocols. Fifteen microliters of plasma were assayed in duplicate for each sample. The sensitivity of the assay was 0·55 ng/ml with 0·02% cross-reactivity with cortisol.

Intracerebroventricular study

All these studies were carried out on 8-week-old female mice. On day 0, drinking water was replaced by supplemented drinking water (CORT, as described above), which remained in place for the duration of the study. In addition, on day 0, mice underwent stereotaxic surgery to place an indwelling guide cannula into the lateral ventricle. Mice were anaesthetized 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, St Albans, Herts, UK) was implanted into the right lateral ventricle using the following co-ordinates: 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, Swindon, Wilts, UK) and a dummy cannula was inserted. All the animals received analgesia (Rimadyl, 5 mg/kg; Pfizer Animal Health, Sandwich, Kent, UK) and antibiotic (Teramycin LA, 60 mg/kg; Pfizer Animal Health) before being returned to their home cage. On day 7, food intake and body weight were measured. If either parameter did not match pre-surgery values, mice were excluded from the study. On day 8, 1 h before the onset of the dark cycle, mice received either 1–28 POMC, α-MSH (Bachem, St Helens, UK) or PBS (sham) in a total volume of 2 μl. This was administered using a Hamilton syringe for over 2 min. Food intake was measured over the next 24 h. At the end of the experiment, animals were killed to collect blood for corticosterone as described above. In addition, accurate cannula placement was confirmed by infusing 2 μl methylene blue dye into the indwelling cannula. Animals failing to show diffusion of the dye throughout the ventricular system were excluded from subsequent analysis. No adrenal tissue was collected from these animals. All the protocols were performed in accordance with the United Kingdom Home Office legislation.

Statistical analysis

All the results are expressed as means ± S.E.M. Statistical comparisons were analysed by ANOVA and Fisher's protective least significant difference test using Stat View 5

(SAS Institute, Inc., Cary, NC, USA). Statistical significance is defined as $P<0{\cdot}05$ and is indicated as a star (*) or cross (†) in the figures.

Results

Synthetic 1–28 POMC given s.c. has no effect upon adrenal morphology or adrenocortical proliferation in Pomc$^{-/-}$ *mice*

Following s.c. administration of 1–28 POMC for 10 days twice daily, there were no changes in macroscopic adrenal appearance or total weight in WT or *Pomc*$^{-/-}$ mice (sham vs 1–28 POMC, WT: $2{\cdot}63\pm0{\cdot}25$ vs $2{\cdot}87\pm0{\cdot}16$ mg, not - significant (n.s.); *Pomc*$^{-/-}$: $0{\cdot}56\pm0{\cdot}04$ vs $0{\cdot}7\pm0{\cdot}16$ mg, n.s.; Fig. 1). When both 1–28 POMC and 1–24 ACTH were administered s.c. together, the final adrenal weight was indistinguishable from that attained following treatment with 1–24 ACTH alone (1–24 ACTH vs 1–24 ACTH and 1–28 POMC, WT: $6{\cdot}52\pm0{\cdot}3$ vs $6{\cdot}28\pm0{\cdot}21$ mg, n.s.; *Pomc*$^{-/-}$: $2{\cdot}93\pm0{\cdot}12$ vs $2{\cdot}8\pm0{\cdot}08$ mg, n.s.; Fig. 1I). The significant increase in adrenal weight following treatment with 1–24 ACTH alone was in keeping with previously published data (Coll 2004*b*).

Microscopically, sham-treated *Pomc*$^{-/-}$ adrenal glands had a dysmorphic adrenal cortex with small adrenal cells and lack of clear zonal distribution (Fig. 1B). Subcutaneous treatment with 1–28 POMC had no impact upon this dysmorphology (Fig. 1F), whereas 1–24 ACTH markedly increased the cortical area (sham vs 1–24 ACTH, WT: $445\pm15\times10^3$ pixels vs $1143\pm66\times10^3$ pixels, $P<0{\cdot}001$; *Pomc*$^{-/-}$: $182\pm40\times10^3$ pixels vs $561\pm80\times10^3$ pixels, $P<0{\cdot}001$; Fig. 1J). In particular, the size of the zona fasciculata was increased in both genotypes, with a restoration of cortical architecture seen in adrenals of *Pomc*$^{-/-}$ mice (Fig. 1D). In addition, 1–24 ACTH treatment of both wild-type and *Pomc*$^{-/-}$ mice resulted in a significant reduction of cells per HPF indicating adrenal hypertrophy (sham vs 1–24 ACTH, WT: $99{\cdot}4\pm13{\cdot}2$ cells/HPF vs $49{\cdot}4\pm1{\cdot}9$ cells/HPF, $P<0{\cdot}008$; *Pomc*$^{-/-}$: $268{\cdot}7\pm13{\cdot}3$ cells/HPF vs $49{\cdot}6\pm12{\cdot}6$ cells/HPF, $P<0{\cdot}001$; Fig. 1K) with changes following combined treatment with both peptides identical to those seen with 1–24 ACTH alone (1–24 ACTH vs 1–24 ACTH and 1–28 POMC, WT: $49{\cdot}4\pm1{\cdot}9$ cells/HPF vs $69{\cdot}8\pm6{\cdot}9$ cells/HPF, n.s.; *Pomc*$^{-/-}$: $49{\cdot}6\pm12{\cdot}6$ cells/HPF vs $54{\cdot}6\pm1{\cdot}5$ cells/HPF, n.s.; Figure 1K).

Qualitative and quantitative changes in the proliferation marker PCNA using immunohistochemistry and western blotting were assessed in four different groups given s.c. treatment. PCNA expression was not significantly affected by this treatment. Whereas treatment with 1–24 ACTH had marked effects on adrenocortical hypertrophy, PCNA expression was not significantly affected by this treatment. In addition, treatment with s.c. 1–28 POMC, either alone or in combination with 1–24 ACTH, did not significantly alter adrenal proliferation as measured by PCNA protein expression levels (data not shown).

Subcutaneous administration of synthetic 1–28 POMC has no effect on the expression levels of adrenal steroidogenic enzymes or corticosterone production

Western blotting demonstrated lower levels of Nurr1, StAR, SCC, 3βHSD, and 11βOH in *Pomc*$^{-/-}$ mice in comparison with wild-type animals (Fig. 1L). 1–24 ACTH caused a marked induction in the levels of these transcription factors and steroidogenic enzymes (Fig. 1L), whereas s.c. 1–28 POMC treatment had no significant effect on adrenocortical protein expression. Accordingly, twice daily administration of 1–28 POMC for 10 days had no significant impact upon circulating corticosterone levels in either WT (sham vs 1–28 POMC: $118{\cdot}3\pm27$ vs $186{\cdot}9\pm34$ ng/ml, n.s.) or *Pomc*$^{-/-}$ (sham vs 1–28 POMC: not detectable vs $2{\cdot}4\pm1{\cdot}7$ ng/ml, n.s.) whilst 1–24 ACTH significantly increased circulating corticosterone levels in both WT and *Pomc*$^{-/-}$ mice (Fig. 1M). Corticosterone levels achieved after administration of both peptides were identical to levels measured following 1–24 ACTH alone (1–24 ACTH vs 1–24 ACTH and 1–28 POMC, WT: 1307 ± 276 vs 1302 ± 386 ng/ml, n.s.; *Pomc*$^{-/-}$: $274\pm75{\cdot}5$ vs 345 ± 112 ng/ml, n.s.; Fig. 1M).

Taken together, these results demonstrate that the applied treatment protocol using synthetic 1–28 POMC given s.c. was not able to significantly affect adrenal morphology, proliferation or steroidogenesis in either *Pomc*$^{-/-}$ or WT mice.

Absence of POMC-derived peptides results in X-zone disorganization whilst treatment with 1–24 ACTH results in X-zone regression

In addition to the overall adrenal weight, zona fasciculata hypertrophy and adrenocortical proliferation, we assessed potential effects of POMC deficiency and treatment with 1–28 POMC alone or in combination with 1–24 ACTH, on X-zone morphology by utilizing the X-zone-specific marker 20αHSD (Beuschlein *et al.* 2005). Interestingly, western blot experiments revealed lower 20αHSD expression levels in adrenals from *Pomc*$^{-/-}$ animals in comparison with WT mice (Fig. 2I). In addition, X-zone-positive cells showed structural disorganization with 20αHSD expressing cells spread throughout the adrenal medulla (Fig. 2B). Further, s.c. treatment with 1–24 ACTH resulted in a marked decrease (*Pomc*$^{-/-}$) or loss (WT) of 20αHSD expression, whilst 1–28 POMC treatment had no effect on X-zone or 20αHSD expression pattern in either genotype (Fig. 2I).

In combination with the morphological evaluation, these data indicate that POMC-derived peptides are required not only for maintenance of zona fasciculata function, but also for normal development of X-zone cells. In addition, 1–24 ACTH has rapid effects on X-zone morphology and function in wild-type mice through induction of X-zone regression.

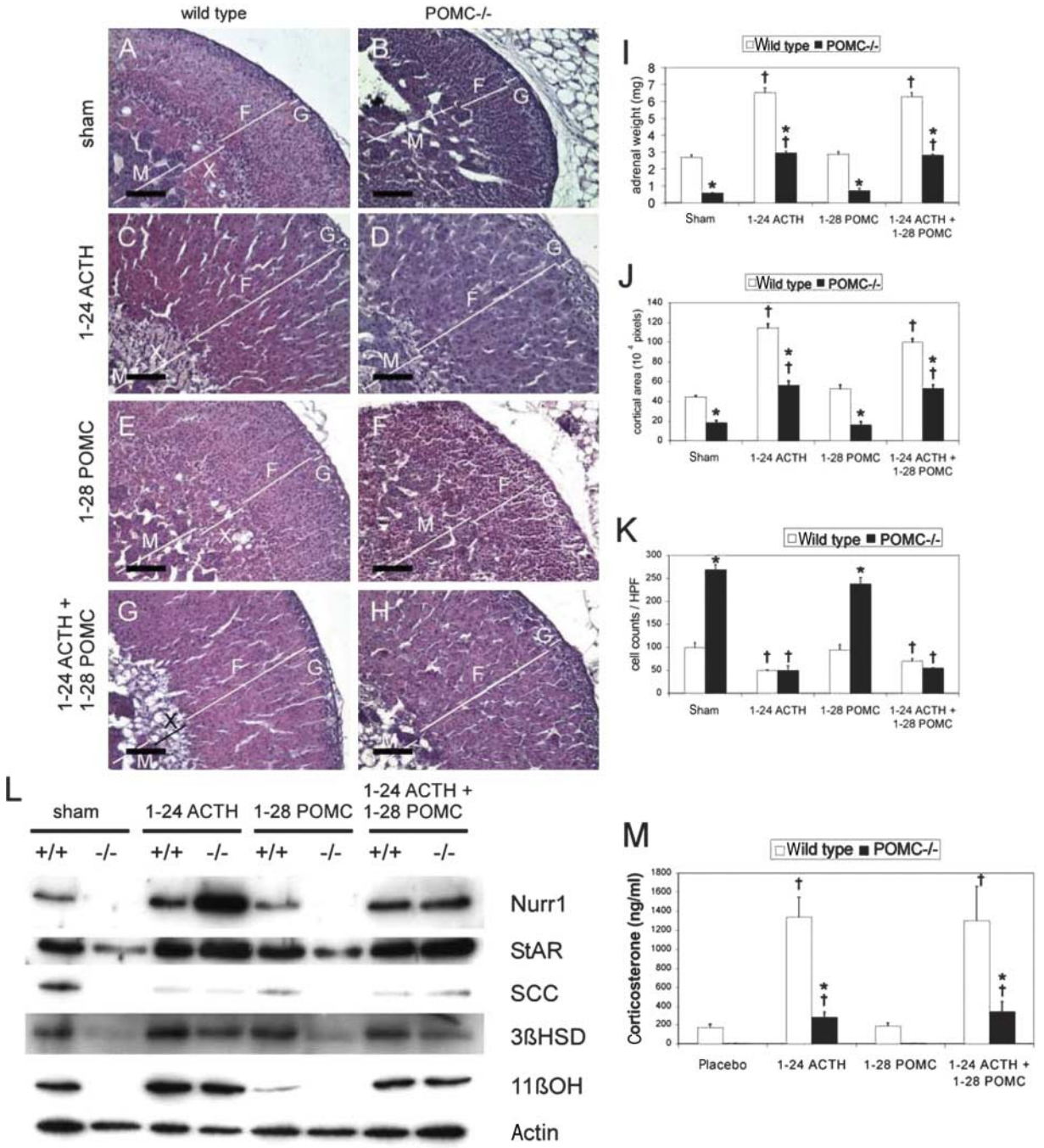


Figure 1 Examples of H&E-stained adrenal sections of wild-type (A, C, E, and G) and *Pomc*$^{-/-}$ mice (B, D, F, and H) after s.c. treatment with sham (A and B), 1–24 ACTH (C and D), 1–28 POMC (E and F) and both peptides (G and H). Adrenal weight (I), cortical areas (J), and cell counts per HPF (K) all indicate induction of adrenocortical hypertrophy by ACTH treatment either alone or in combination with 1–28 POMC, whilst 1–28 POMC itself has no effect on these parameters. Western blot experiments demonstrate lower expression of Nurr1, StAR, SCC, 3βHSD and 11βOH in *Pomc*$^{-/-}$ mice in comparison with wild-type animals and induction of these gene products upon 1–24 ACTH treatment (L). In contrast, 1–28 POMC treatment has no significant effect on adrenocortical transcription profiles (L). Accordingly, 1–24 ACTH induces a significant increase in corticosterone (CORT) secretion in both genotypes, whilst 1–28 POMC treatment does not affect steroidogenesis ($n=6$ per group, M). Bars represent 100 μm. A star (*) refers to significant differences vs other genotype, whilst a cross (†) designates significant differences vs sham-treated controls. $n=6$ per group for adrenal weights and $n=3$ per group for cortical areas and cell counts respectively.

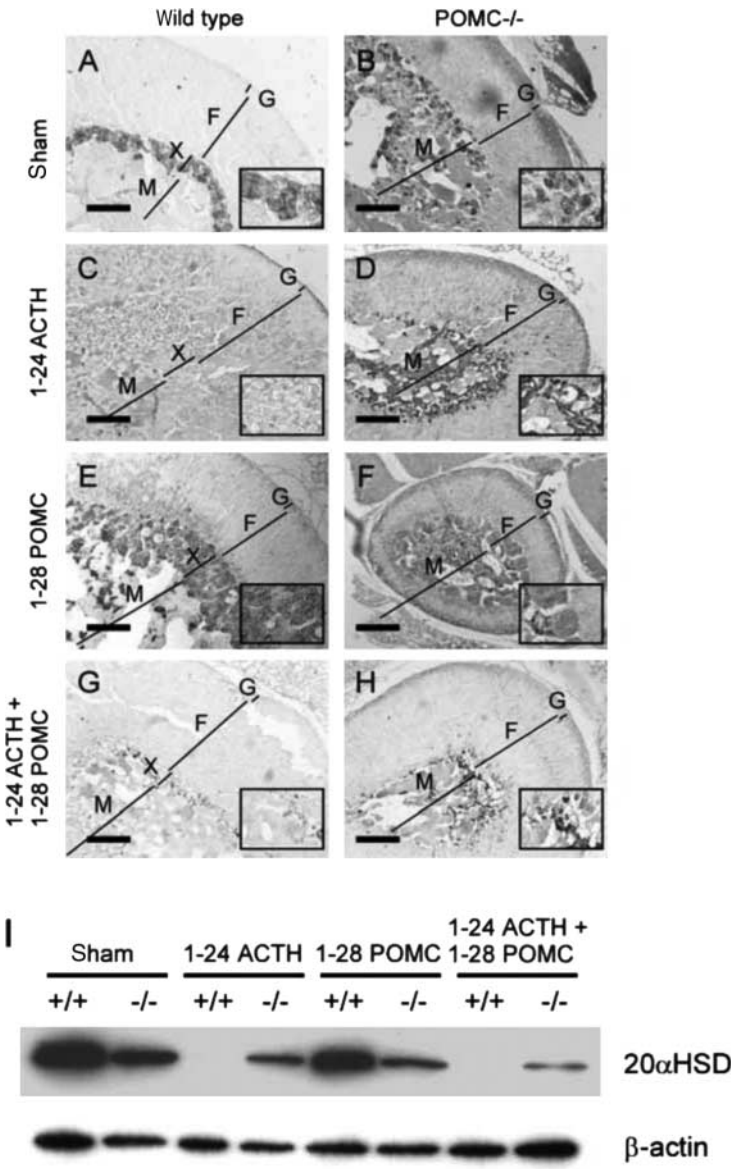
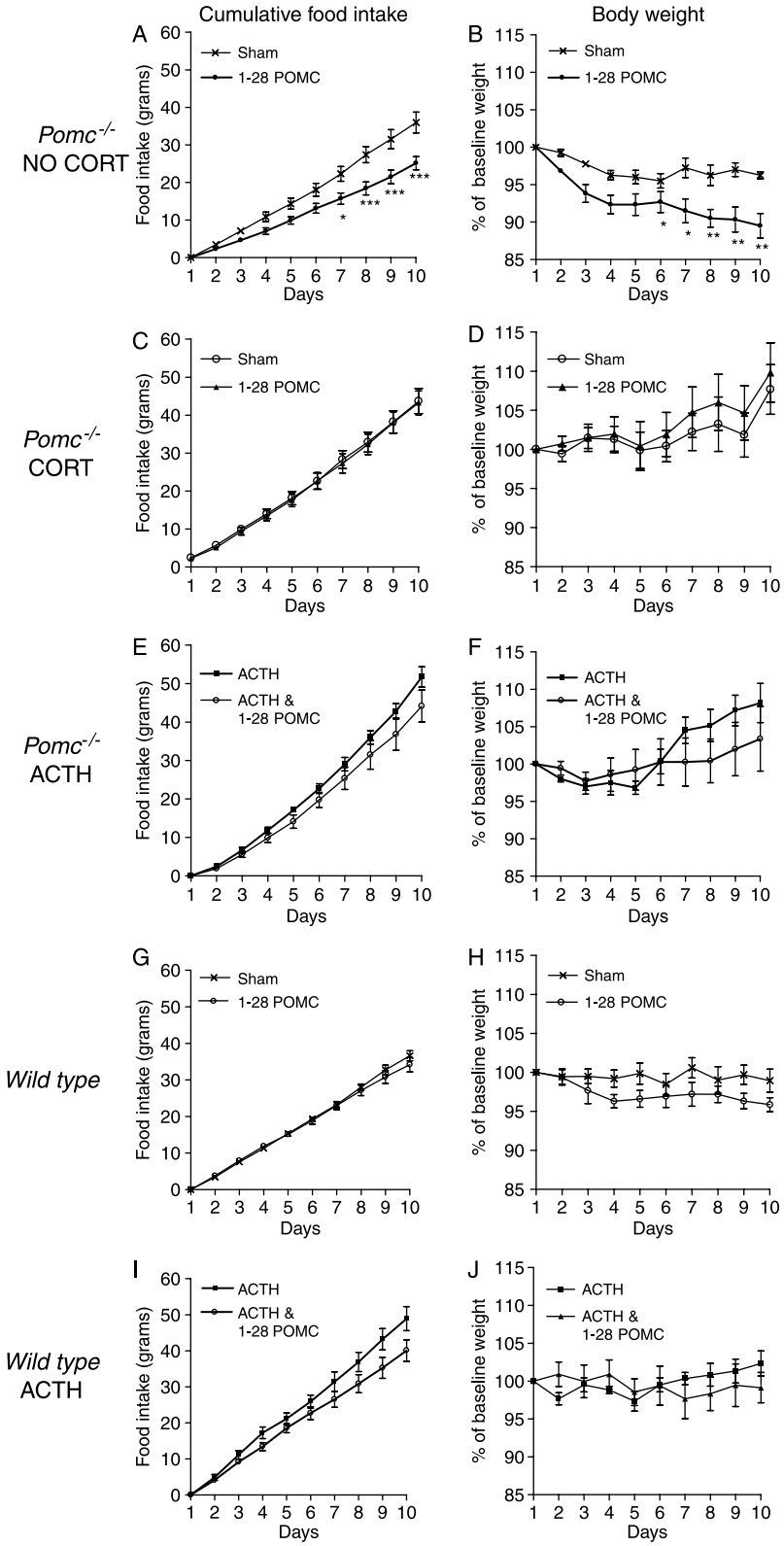


Figure 2 Adrenal sections of wild-type (A, C, E, and G), and *Pomc*$^{-/-}$ mice (B, D, F, and H) after s.c. treatment with placebo (A and B), 1–24 ACTH (C and D), 1–28 POMC (E and F) or both peptides (G and H) stained with the X-zone-specific marker 20αHSD. Whilst untreated wild-type animals display normal X-zone appearance as the innermost adrenocortical zone, *Pomc*$^{-/-}$animals show a disorganization of the X-zone with scattered 20αHSD-positive cells also in the adrenal medulla. In addition, 1–24 ACTH treatment results in a marked reduction in 20αHSD expression and X-zone regression, whilst 1–28 POMC treatment has no additional effect on 20αHSD expression levels or X-zone morphology. Bars represent 100 μm.

Synthetic 1–28 POMC given s.c. can reduce food intake and weight in Pomc$^{-/-}$ *mice, but only in the absence of corticosterone*

Subcutaneous administration of 1–28 POMC to corticosterone-depleted *Pomc*$^{-/-}$ mice resulted in a significant reduction in cumulative food intake and body weight over the 10 days of treatment. By day 7, cumulative food intake was significantly lower in 1–28 POMC-treated *Pomc*$^{-/-}$ mice with a 30% reduction in cumulative food intake on day 10 compared with sham (1–28 POMC vs sham: $25{\cdot}2 \pm 1{\cdot}7$ vs $36{\cdot}0 \pm 2{\cdot}7$ g respectively, $P < 0{\cdot}001$; Fig. 3A). Similarly,



1–28 POMC-treated *Pomc*$^{-/-}$ mice lost significantly more weight than sham (weight change from baseline on day 10, 1–28 POMC vs sham: 89·5±1·6 vs 96·5±1·0% respectively, $P<0·01$; Fig. 3B).

Given that *Pomc*$^{-/-}$ mice have no circulating glucocorticoids, we wished to determine if restoration of corticosterone might influence the anorexigenic effects of 1–28 POMC. In a separate cohort of *Pomc*$^{-/-}$ mice receiving corticosterone-supplemented drinking water (CORT) throughout the 10-day period, we compared the effects of 10 μg of 1–28 POMC given s.c. twice daily with sham treatment. The corticosterone levels achieved in these two treatment groups were in the low physiological range (sham vs 1–28 POMC: 16·6±4·8 vs 16·6±1·0 ng/ml, n.s.). When compared to corticosterone-depleted mice, CORT treatment caused a significant increase in the food intake and body weight of *Pomc*$^{-/-}$ mice. Thus, among the sham-treated *Pomc*$^{-/-}$ mice, those receiving CORT consumed 20% more food and gained significantly more weight than the corticosterone-depleted group (cumulative food intake: 43·7±3·3 vs 36·0±2·7 g respectively, $P<0·05$; weight change from baseline: 107·7±3·2 vs 96·5±1·0% respectively, $P<0·05$).

However, the effects of 1–28 POMC upon food intake and body weight in CORT-treated *Pomc*$^{-/-}$ mice were indistinguishable from sham treatment (1–28 POMC vs sham: food intake, 43·2±3·2 vs 43·7±3·3 respectively, n.s.; body weight as percentage of baseline, 109·8±3·8 vs 107·7±3·2%, n.s.; Fig. 3C and D).

We also examined the effects of 1–28 POMC in *Pomc*$^{-/-}$ mice in which circulating corticosterone had been restored to supraphysiological levels (345 ng/ml) by co-administration of 1–24 ACTH. 1–24 ACTH treatment alone resulted in a significant increase in cumulative food intake and weight change from baseline when compared to sham treatment alone (food intake: 51·7±2·6 vs 36·0±2·7 g respectively, $P<0·01$; percentage of baseline weight: 107·8+2·0 vs 96·5+1·0 respectively, $P<0·05$). When 1–28 POMC was co-administered with 1–24 ACTH, there was close to a 15% reduction in cumulative food intake although this did not reach statistical significance (51·7±2·6 vs 44·2±4·2 g, $P=0·09$). Similarly, weight change following co-administration of both peptides to *Pomc*$^{-/-}$ mice was not significantly different from that seen with 1–24 ACTH administration alone (103·0±3·8 vs 107·8±2·0% respectively, $P=0·3$; Fig. 3E and F).

Trends towards lower food cumulative intake and body weight were seen in wild-type animals receiving s.c. 1–28 POMC, which however failed to reach statistical significance (sham vs 1–28 POMC: food intake, 36·6±1·3 vs 34·2±2·0 g respectively, n.s.; percentage of baseline weight, 99·1±1·7 vs 97·5±1·3% respectively, n.s.; Fig. 3G and H). As was the case in *Pomc*$^{-/-}$ mice, 1–24 ACTH brought about a 25% increase in cumulative food intake in wild-type mice (1–24 ACTH vs sham: 48·9±3·4 vs 36·6±1·3 g respectively, $P<0·01$) although there was no significant change from baseline body weight (1–24 ACTH vs sham: 102·5±1·5 vs 99·1±1·7% respectively, n.s.). Intriguingly, co-administration of 1–28 POMC was able to reduce cumulative food intake in wild-type mice receiving 1–24 ACTH by 18% (total food intake, 1–24 ACTH vs 1–28 POMC and 1–24 ACTH, 48·9±3·3 vs 40·1±3·0 respectively, $P<0·05$) although there was no significant difference in weight change (102·5±1·5 vs 101·7±2·4% respectively, n.s.; Fig. 3I and J).

To further investigate the anorexigenic effect of 1–28 POMC, we attempted to centrally administer this peptide to *Pomc*$^{-/-}$ mice. The POMC peptide, α-MSH, has a more potent anorexigenic action when giving i.c.v. compared with peripheral administration and we wished to determine if this was also the case for 1–28 POMC. However, *Pomc*$^{-/-}$ mice were unable to satisfactorily recover from stereotatic surgery in the absence of corticosterone. If left, corticosterone-depleted *Pomc*$^{-/-}$ mice lost a significant amount of weight post-operatively, reaching less than 80% of their initial weight within 1 week (data not shown). They also became markedly hypophagic, eating less than 50% of their pre-surgery food intake in 1 week (data not shown). We therefore elected not to centrally administer 1–28 POMC to such corticosterone-deficient *Pomc*$^{-/-}$ mice.

However, *Pomc*$^{-/-}$ mice treated with CORT from day 0 were able to readily tolerate stereotaxic surgery and did maintain their weight and food intake post-surgery. In such CORT-treated *Pomc*$^{-/-}$ mice, i.c.v. administration of 1 μg (0·3 nmol) 1–28 POMC 1 h before the onset of the dark cycle had no impact upon cumulative food intake in the following 24 h period (Fig. 4). This was in contrast to the effects of a single equimolar dose of α-MSH, which significantly reduced food intake at all time points (Fig. 4).

Discussion

Subcutaneous administration of synthetic 1–28 POMC to *Pomc*$^{-/-}$ mice had no measurable effects on adrenal morphology, the expression profile of steroidogenic enzymes and corticosterone production. The lack of effects was seen

Figure 3 Effect of s.c. administration of 1–28 POMC on food intake and body weight. The upper three panels show results in *Pomc*$^{-/-}$ mice with no CORT supplementation (NO CORT, A and B), receiving CORT-supplemented drinking water (CORT, C and D) or concurrently receiving 1–24 ACTH (ACTH, E and F) and indicate a more significant reduction in food intake and body weight in CORT-depleted *Pomc*$^{-/-}$ animals. The lower two panels show the results in wild-type mice (G and H), and in wild-type mice concurrently receiving 1–24 ACTH (I and J). Cumulative food intake and change in baseline body weight for over 10 days of treatment indicate that 1–28 POMC has no anorexigenic effect in wild-type animals, but causes a reduction in food intake in mice with ACTH-stimulated CORT excess ($n=6$–7 per group; comparison vs sham: $^{*}P<0·05$, $^{**}P<0·01$, $^{***}P<0·001$).

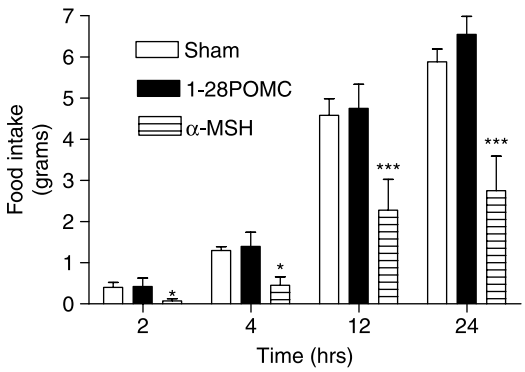


Figure 4 The acute effects of central administration of 1–28 POMC to CORT-treated *Pomc*$^{-/-}$ mice are indistinguishable from sham and in marked contrast to the effects of α-MSH (n=4–5 per group; comparison vs sham; *P<0·05, **P<0·01, ***P<0·001).

when 1–28 POMC was given alone or in combination with 1–24 ACTH. In contrast, s.c. 1–28 POMC significantly reduced food intake and body weight in *Pomc*$^{-/-}$ mice although these effects were ameliorated when circulating corticosterone was restored to a low physiological or supraphysiological level by supplemented drinking water or concurrent 1–24 ACTH respectively.

1–28 POMC was isolated originally from a large-scale purification of human pituitary glands and considered to be an extraction artifact rather than a physiologically formed product (McLean *et al.* 1981). Nevertheless, this fragment has been used previously to investigate the biological role of the highly conserved amino terminal sequence of POMC. Estivariz *et al.* (1982) have demonstrated that purified 1–28 POMC could elicit a dose-dependent increase in the incorporation of thymidine into DNA of dispersed rat adrenal cells. Further, they reported that continuous administration of purified 1–28 POMC to female rats by s.c. pump over 7 days (3 μg/day) resulted in a significant increase in adrenal weight and mitotic index within the adrenal cortex. To determine whether these effects are in part due to untoward contamination with other POMC-derived peptides, the same group examined the adrenal response to synthetic 1–28 POMC by delivering 8 μg of either purified or synthetic 1–28 POMC to intact female rats using osmotic minipumps (Estivariz *et al.* 1988*a*). Both forms of peptide significantly increased mitotic activity in the adrenals of treated animals compared with saline-treated controls, although neither produced a change in plasma corticosterone. Furthermore, both purified and synthetic 1–28 POMC significantly stimulated [^{3}H]thymidine incorporation into DNA of adrenal cells in a dose-dependent manner *in vitro*, although the synthetic peptide was somewhat less potent than the purified peptide in this assay. More recently, Fassnacht *et al.* (2003) demonstrated that synthetic 1–28 POMC is capable of stimulating proliferation of both adrenocortical tumour cells and primary cell cultures of bovine adrenals *in vitro*.

In this study, we were unable to demonstrate any measurable effect upon adrenal function following administration of synthetic 1–28 POMC.

This lack of biological activity of 1–28 POMC in the chosen experimental setting may be interpreted as a result of inadequate amounts of active peptide entering the circulation, although *Pomc*$^{-/-}$ adrenal tissue remains responsive to a similar dosing route and regimen of synthetic 1–24 ACTH (this study and Coll *et al.* 2004*b*). The doses used in the study were chosen to give near equimolar amounts of both peptides (10 nmol 1–24 ACTH vs 6·3 nmol 1–28 POMC) in the pharmacological rather than physiological range exceeding that used in previous published studies using rats (Estivariz 1982, 1988*a*). Thus, this amount of peptides should have been sufficient to elicit a biological response. However, we cannot exclude the possibility that excessive amounts of peptide given chronically for 10 days might have caused a down-regulation of 1–28 POMC-dependent pathways and desensitization within the adrenals. In addition, it is possible that 1–28 POMC might exert a mitogenic activity only in a paracrine fashion and that administered peptides could be degraded in the periphery. We also have no data pertaining to the pharmacokinetics and clearance rates of the administered peptide, which would be valuable in further interpreting the data presented.

A widely accepted hypothesis regarding the post-translational processing of the 16 kDa N-terminal POMC fragment into potential adrenal mitogens involves an adrenal-specific protease (AsP) that cleaves circulating 1–76 POMC to 1–52 POMC which then stimulates adrenal mitogenesis (Bicknell *et al.* 2001) and we acknowledge that 1–28 POMC has not been detected at physiologically relevant levels within the circulation. Although evidence against AsP having either a physiological or pathological role in controlling adrenocortical growth in experimental paradigms other than compensatory adrenal growth has recently emerged (Hansen *et al.* 2004, Hahner *et al.* 2005), this study was not designed to directly address the role of AsP in adrenal biology. Instead, we wished specifically to determine if treatment with the peptide fragment 1–28 POMC might impact upon adrenal function, both in wild-type animals and in those lacking all POMC-derived peptides.

In an attempt to characterize changes in adrenal zonation upon treatment with 1–28 POMC, alone or in combination with 1–24 ACTH, we also assessed the growth kinetics of the murine adrenal X-zone. The physiological role of this particular zone, which becomes evident histologically at 10–14 days of age but subsequently begins to degenerate in males with sexual maturity and in females during the first pregnancy, is uncertain. Although it has been considered as a functional equivalent of the primate zona reticularis, it lacks the ability to produce adrenal androgens. Factors involved in X-zone growth and regression include luteinizing hormone, sex steroids, as well as members of the transforming growth factor-β family of ligands, activin and inhibin (Beuschlein *et al.* 2003). As we show herein, ACTH is to be added to the

factors regulating X-zone growth and regression in two regards. First, lack of POMC-derived peptides results in the failure of proper X-zone development as evident in the altered X-zone morphology and distribution of 20αHSD-expressing cells in virgin female *Pomc*$^{-/-}$ mice. Secondly, treatment with supraphysiological doses of 1–24 ACTH results in the rapid regression of the X-zone and decrease or loss of expression of the marker gene 20αHSD, whilst 1–28 POMC has no such effects in this experimental setting. Although the exact function of the murine X-zone is still to be elucidated these findings add another facet of the clearly zone-specific impact of ACTH on the adrenal cortex.

The significant reduction in food intake and weight loss seen in *Pomc*$^{-/-}$ mice is a novel and unexpected finding. It is worthwhile noting that in the absence of corticosterone, *Pomc*$^{-/-}$ mice treated with sham injection were no longer hyperphagic, having food intake very similar to sham-treated wild-type mice. We have previously observed that cumulative food intake in *Pomc*$^{-/-}$ mice can be affected by even saline injection (Challis *et al.* 2004). This is likely to reflect an increased sensitivity to certain stressors such as handling or s.c. injection, thereby causing stress-induced hypophagia. Similar findings have been reported in A^{y}/a mice, another obese mouse model with disrupted melanocortin signaling (De Souza *et al.* 2000).

Despite this, 1–28 POMC still had a more potent anorexigenic effect than sham injection in *Pomc*$^{-/-}$ animals. One potential mechanism which might explain these findings is the glucocorticoid deficiency seen in *Pomc*$^{-/-}$ mice, as 1–28 POMC had only slight effects upon food intake or body weight in corticosterone-depleted wild-type animals. We were able to restore corticosterone in two ways. Supplemented drinking water and concurrent administration of 1–24 ACTH achieved low physiological and supraphysiological levels of corticosterone respectively. Both strategies resulted in an increase in food intake and body weight in *Pomc*$^{-/-}$ mice compared with corticosterone-depleted animals, with the higher corticosterone levels achieved post-1–24 ACTH resulting in the biggest increase in cumulative food intake and body weight. This is in accordance with our previous published results, which demonstrate that *Pomc*$^{-/-}$ mice are hypersensitive to the adverse metabolic effects of glucocorticoids. More recently, Smart *et al.* (2006) have introduced a POMC transgene that selectively restored peripheral melanocortin and corticosterone secretion in *Pomc* null mice. This also resulted in increased food intake and body fat.

In the present study, when circulating CORT was restored, the effects of peripheral 1–28 POMC on the cumulative food intake and weight change of *Pomc*$^{-/-}$ mice were much reduced. Further, when given as a single dose i.c.v., 1–28 POMC does not have an impact upon food intake. This is in contrast to α-MSH, which is a potent suppressor of appetite when given i.c.v.

Thus, it appears that 1–28 POMC can only bring about significant weight loss in *Pomc*$^{-/-}$ mice, which lack glucocorticoids, although the defined mechanisms remain elusive. Glucocorticoids have multiple sites of action throughout the body, including immunomodulatory effects (Riccardi *et al.* 2002) and it may be that mice lacking corticosterone mount a different stress response to peripherally administered peptide, which leads to anorexia and weight loss. There remains, however, the issue of why 1–28 POMC reduces food intake (albeit only late in the treatment regimen) in wild-type mice also receiving 1–24 ACTH. It may be that the pathological elevation of corticosterone in these wild-type mice (1300 ng/ml) also influences the immune response to repeated peripheral injections.

In summary, in the absence of Corticosterone, synthetic 1–28 POMC has an anorexigenic effect in mice lacking all endogenously derived POMC peptides, but it has no measurable impact upon adrenal growth, steroidogenesis, or proliferation.

Acknowledgements

We are indebted to Dr Douglas M Stocco (Texas Tech University Health Sciences Center, Lubbock, TX, USA), Dr Walter Miller (University of California, San Francisco, CA, USA), Dr Anita Payne (Stanford University School of Medicine, Stanford, CA, USA), and Dr Celso E Gomez-Sanchez (Montgomery Veterans Affairs Medical Center, Jackson, MS, USA) for their generous gift of the anti-StAR antibody, the anti-SCC antibody, the anti-3βHSD antibody and the anti-11βOH antibody respectively. This work was supported by Medical Research Council (UK), the Sackler Foundation (A P C), the Deutsche Forschungsgemeinschaft (Al-201/7-2 to M F and B A), and the Landesstiftung Baden-Würtemberg (P-LS-ASN/5 to F B). The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

References

Bennett HP, Seidah NG, Benjannet S, Solomon S & Chretien M 1986 Reinvestigation of the disulfide bridge arrangement in human pro-opiomelanocortin N-terminal segment (hNT 1-76). *International Journal of Peptide Protein Research* **27** 306–313.

Bertagna X 1994 Proopiomelanocortin-derived peptides. *Endocrinology and Metabolism Clinics of North America* **23** 467–485.

Beuschlein F, Looyenga BD, Bleasdale SE, Mutch C, Bavers DL, Parlow AF, Nilson JH & Hammer GD 2003 Activin induces x-zone apoptosis that inhibits luteinizing hormone-dependent adrenocortical tumor formation in inhibin-deficient mice. *Molecular and Cellular Biology* **23**(11) 3951–3964.

Beuschlein F, Shapiro I & Weinstein Y 2005 The adrenal X zone is involved in progesterone inactivation. *Program of 87th Annual Meeting of the Endocrine Society.* San Diego, CA, USA. Abstract P1–P333.

Bicknell AB, Lomthaisong K, Woods RJ, Hutchinson EG, Bennett HP, Gladwell RT & Lowry PJ 2001 Characterization of a serine protease that cleaves pro-gamma-melanotropin at the adrenal to stimulate growth. *Cell* **105** 903–912.

Bradley RL, Mansfield JP & Maratos-Flier E 2005 Neuropeptides, including neuropeptide Y and melanocortins, mediate lipolysis in murine adipocytes. *Obesity Research* **13** 653–661.

Butler AA & Cone RD 2003 Knockout studies defining different roles for melanocortin receptors in energy homeostasis. *Annals of the New York Academy of Sciences* **994** 240–245.

Challis BG, Coll AP, Yeo GS, Pinnock SB, Dickson SL, Thresher RR, Dixon J, Zahn D, Rochford JJ, White A *et al*. 2004 Mice lacking pro-opiomelanocortin are sensitive to high-fat feeding but respond normally to the acute anorectic effects of peptide-YY(3-36). *PNAS* **101** 4695–4700.

Coll AP, Farooqi IS, Challis BG, Yeo GS & O'Rahilly S 2004*a* Proopiomelanocortin and energy balance: insights from human and murine genetics. *Journal of Clinical Endocrinology and Metabolism* **89** 2557–2562.

Coll AP, Challis BG, Yeo GS, Snell K, Piper SJ, Halsall D, Thresher RR & O'Rahilly S 2004*b* The effects of proopiomelanocortin deficiency on murine adrenal development and responsiveness to adrenocorticotropin. *Endocrinology* **145** 4721–4727.

Coll AP, Challis BG, Lopez M, Piper S, Yeo GS & O'Rahilly S 2005 Proopiomelanocortin-deficient mice are hypersensitive to the adverse metabolic effects of glucocorticoids. *Diabetes* **54** 2269–2276.

Cone RD 2005 Anatomy and regulation of the central melanocortin system. *Nature Neuroscience* **8** 571–578.

De Souza J, Butler AA & Cone RD 2000 Disproportionate inhibition of feeding in A(y) mice by certain stressors: a cautionary note. *Neuroendocrinology* **72** 126–132.

Denef C, Roudbaraki M & Van Bael A 2001 Growth and differentiation factors derived from the N-terminal domain of pro-opiomelanocortin. *Clinical and Experimental Pharmacology and Physiology* **28** 239–243.

Estivariz FE, Hope J, McLean C & Lowry PJ 1980 Purification and characterization of a gamma-melanotropin precursor from frozen human pituitary glands. *Biochemical Journal* **191** 125–132.

Estivariz FE, Iturriza F, McLean C, Hope J & Lowry PJ 1982 Stimulation of adrenal mitogenesis by N-terminal proopiocortin peptides. *Nature* **297** 419–422.

Estivariz FE, Carino M, Lowry PJ & Jackson S 1988*a* Further evidence that N-terminal pro-opiomelanocortin peptides are involved in adrenal mitogenesis. *Journal of Endocrinology* **116** 201–206.

Estivariz FE, Morano MI, Carino M, Jackson S & Lowry PJ 1988*b* Adrenal regeneration in the rat is mediated by mitogenic N-terminal pro-opiomelanocortin peptides generated by changes in precursor processing in the anterior pituitary. *Journal of Endocrinology* **116** 207–216.

Fassnacht M, Hahner S, Hansen IA, Kreutzberger T, Zink M, Aderman K, Jakob F, Troppmair J & Allolio B 2003 N-terminal proopiomelanocortin acts as a mitogen in adrenocortical tumor cells and decreases adrenal steroidogenesis. *Journal of Clinical Endocrinology and Metabolism* **88** 2171–2179.

Forbes S, Bui S, Robinson BR, Hochgeschwender U & Brennan MB 2001 Integrated control of appetite and fat metabolism by the leptin-proopiomelanocortin pathway. *PNAS* **98** 4233–4237.

Hahner S, Fassnacht M, Hammer F, Schammann M, Weismann D, Hansen IA & Allolio B 2005 Evidence against a role of human airway trypsin-like protease—the human analogue of the growth-promoting rat adrenal secretory protease–in adrenal tumourigenesis. *European Journal of Endocrinology* **152** 143–153.

Hansen IA, Fassnacht M, Hahner S, Hammer F, Schammann M, Meyer SR, Bicknell AB & Allolio B 2004 The adrenal secretory serine protease AsP is a short secretory isoform of the transmembrane airway trypsin-like protease. *Endocrinology* **145** 1898–1905.

Lowry PJ, Silas L, McLean C, Linton EA & Estivariz FE 1983 Pro-gamma-melanocyte-stimulating hormone cleavage in adrenal gland undergoing compensatory growth. *Nature* **306** 70–73.

Lowry PJ, Estivariz FE, Silas L, Linton EA, McLean C & Crocombe K 1984 The case for pro-gamma-MSH as the adrenal growth factor. *Endocrine Research* **10** 243–258.

McLean C, Hope J, Salacinski P, Estivariz F & Lowry PJ 1981 Purification and characterization of amino-terminal pro-opiocortin peptides from human pituitary glands. *Bioscience Reports* **1** 843–849.

Raffin-Sanson ML, de Keyzer Y & Bertagna X 2003 Proopiomelanocortin, a polypeptide precursor with multiple functions: from physiology to pathological conditions. *European Journal of Endocrinology* **149** 79–90.

Ramachandran J, Farmer SW, Liles S & Li CH 1976 Comparison of the steroidogenic and melanotropic activities of corticotropin, alpha-melanotropin and analogs with their lipolytic activities in rat and rabbit adipocytes. *Biochimica et Biophysica Acta* **428** 347–354.

Riccardi C, Bruscoli S & Migliorati G 2002 Molecular mechanisms of immunomodulatory activity of glucocorticoids. *Pharmacological Research* **45** 361–368.

Richter WO & Schwandt P 1985 Physiologic concentrations of beta-lipotropin stimulate lipolysis in rabbit adipocytes. *Metabolism* **34** 539–543.

Seger MA & Bennett HP 1986 Structure and bioactivity of the amino-terminal fragment of pro-opiomelanocortin. *Journal of Steroid Biochemistry and Molecular Biology* **25** 703–710.

Smart JL, Tolle V & Low MJ 2006 Glucocorticoids exacerbate obesity and insulin resistance in neuron-specific proopiomelanocortin-deficient mice. *Journal of Clinical Investigation* **116** 495–505.

Received 9 January 2006
Received in final form 28 April 2006
Accepted 28 April 2006
Made available online as an Accepted Preprint
10 May 2006