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ORIGINAL ARTICLE

Maternal influence of prolyl endopeptidase on fat mass of adult progeny

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Background: Maternal genotype has lifetime effects on progeny, but few specific genes, and no proteases, are known to underlie maternal effects. Prolyl endopeptidase (PREP) is a serine protease with putative substrates that regulate appetite or milk production.
Objective: To test effects of PREP on obesity phenotypes in mice.
Design: Mice with a gene trap (GT) of PREP ($PREP^{gt/gt}$) on the C57BL/6J (B6) background were generated. Minimal PREP protein was detected by western blot. In Experiment 1, direct effects of PREP were measured in littermate mice derived from intercrosses of heterozygotes ($PREP^{WT/gt}$). In Experiment 2, maternal effects of PREP were measured in reciprocal crosses of heterozygous ($PREP^{WT/gt}$) and wild-type (WT) ($PREP^{WT/WT}$) males and females.
Diets: Mice were fed either low-fat (LF, Experiments 1 and 2) or high-fat (HF, Experiment 1) defined diets.
Measurements: Adiposity index (AI) was calculated from body weight (BW) and weights of four fat depots measured in 120-day-old mice. Fasting plasma glucose, insulin and leptin were measured. *In vivo* plasma α-MSH levels were measured by targeted quantitative peptidomics.
Results: *Experiment 1*—In intercross mice, there were significant diet effects, but few genotype effects. There were no genotype effects on BW or AI in males or females on either diet. *Experiment 2*—In contrast, reciprocal crosses of heterozygous males or females with WT B6 revealed highly significant parent of origin effects on all traits except body length. Progeny (WT and heterozygous genotypes and both sexes) born to female $PREP^{WT/gt}$ heterozygotes had fat pads that weighed as much as ~twofold more at 120 days old than progeny born to male heterozygotes.
Conclusion: Heterozygosity for PREP GT results in highly significant maternal effects, whereas homozygosity for the $PREP^{gt/gt}$ mutation has a much more limited direct effect.
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Keywords: PREP; maternal genetic effects; endopeptidase; mouse

Introduction

Many studies show that maternal diet influences obesity of progeny in mouse models[1,2] and human beings.[3] However, maternal influences on obesity are not restricted to diet: they also include maternal genetic effects. Maternal genetic effects occur when maternal genotype influences phenotypes in progeny independent of progeny genotype. Recently, several quantitative trait loci for maternal genetic effects on the obesity of pups were identified.[4] These studies utilized F2 and F3 populations of Large × Small (LG × SM) mice in which all mice were genotyped, haplotyped, phenotyped and the F3 progeny were cross fostered to other F2 nurse mothers. The results showed that maternal effects are located on several chromosomes. Maternal genetic effects were also identified in studies of congenic strains of mice.[5]

Prolyl endopeptidase (PREP, post-proline cleaving enzyme, also prolyl oligopeptidase) was first isolated from human uterus as an oxytocin-degrading enzyme.[6] Putative substrates for PREP include substance P, TRH, GnRH, arginine-vasopressin, angiotensins I–IV, bradykinin, oxytocin, ß-endorphin, neurotensin, α-MSH, ß-casomorphin,

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LVV-hemorphin, urotensin and octadecaneuropeptide.[7] Quantitative PCR and *in situ* hybridization reveal high levels of PREP mRNA in cerebellum, hypothalamus, pituitary and several other brain nuclei.[8] Enzymatic studies identified PREP activity in plasma. PREP mRNA and protein are also located in testis, thymus and lung. PREP activity in uterus and ovary varies strongly with estrous cycle.[9]

Serum PREP levels are correlated with depression, mania and anorexia.[10,11] PREP expression is also correlated to Alzheimer's disease and neurodegeneration, implicating PREP activity in memory.[12] No earlier studies suggest that PREP influences obesity or report phenotypes of PREP-deficient mice.

We examined the effects of minimal expression of PREP on obesity phenotypes in mice. Analysis of intercross mice derived from heterozygous parents reveals that PREP has few direct genotype effects, primarily body weight (BW) and adiposity index (AI) in females fed low-fat (LF) diet. In contrast, heterozygosity for PREP gene trap (GT) ($PREP^{WT/gt}$) in female mice bred to wild-type (WT) ($PREP^{WT/WT}$) male mice leads to increased BW and fat mass in all progeny, regardless of progeny genotype, when compared with progeny produced from female $PREP^{WT/WT}$ bred to male $PREP^{WT/gt}$ heterozygotes.

Materials and methods

Generation of PREP GT ($PREP^{gt/gt}$) mice

PREP is located on mouse chromosome 10 at 44 MB on the Ensembl version 49 mouse assembly. It has 15 exons, all of which are predicted to include protein-coding sequence. BayGenomics clone RRM213 (NHLBI—Bay Area Functional Genomics Consortium, http://baygenomics.ucsf.edu) was identified as having an insertion in the second intron of the PREP gene in a 129-strain ES cell. As protein coding begins in the first exon, the resulting protein in the GT mice would include the first 40 amino acids of this 710 residue protein before the inserted β-galactosidase. Founder chimeric mice were bred to B6. Progeny were genotyped for β-galactosidase. Mice positive for β-galactosidase were maintained by backcrossing to B6. Presence of the 129-strain congenic DNA surrounding the PREP gene was confirmed at every generation by genotyping three microsatellite markers polymorphic between 129 and B6. The markers bracket the PREP gene: two proximal (D10Mit148 and D10Mit55) and one distal (D10Mit36).

$PREP^{gt/gt}$ mice are born in expected Mendelian numbers in litters of average size for the B6 background. The homozygotes do not exhibit increased mortality or disease up to 120 days of age. We have not retained older mice.

Animal maintenance and diet

B6 mice were initially purchased from The Jackson Laboratory (The Jackson Laboratory, Bar Harbor, ME, USA) and maintained for over 10 years in a breeding colony at UC Davis.

All animals were kept in the same room with a 14-h light/10 h dark cycle, 21 ± 2 (s.d.) °C temperature and >25% humidity. Breeding and nursing mice were in shoe-box cages with deionized water and breeding chow available *ad libitum*. Pups were weaned at 3 weeks of age, separated by gender and housed 3–5 mice in suspended wire cages with either a LF diet (AIN-76A) or a high-fat (HF) diet and deionized water available *ad libitum*. AIN-76A (Research Diets, Inc., New Brunswick, NJ, USA) is a widely used purified control diet that contains 20.8 kcal% protein (20.5 kcal% casein), 67.7 kcal% carbohydrate (51.3 kcal% sucrose) and 11.5 kcal% fat as corn oil. The HF diet (D12492, Research Diets) is a purified diet used in the study of obesity and diabetes. This diet contains 20% kcal% protein (19.7 kcal% casein), 20 kcal % carbohydrate (6.8 kcal% sucrose) and 60% of calories from fat, predominantly lard with some soy oil.

Genetic crosses

In Experiment 1, heterozygous breeders from the cross of $PREP^{gt/gt}$ and $PREP^{WT/WT}$ mice were inter-bred to produce pups that were heterozygous $PREP^{WT/gt}$ (H), GT $PREP^{gt/gt}$ or WT $PREP^{WT/WT}$ for PREP for testing of direct effects of PREP on obesity phenotypes. In Experiment 2, a reciprocal cross of H (m, f) × WT (m, f) was used to test maternal effects of $PREP^{WT/gt}$ on obesity phenotypes.

Food intake

For food intake measurements, a minimum of five mice per genotype were singly housed in wire-bottom cages with either LF or HF diet at 10 weeks of age and acclimated for 4 days before the start of the measurement period. Food intake was measured at the end of 4 days and averaged to determine daily intake.

Body composition and plasma phenotypes

At 120 ± 3 (s.d.) days of age, mice were fasted for ~15 h before collection of blood through the retroorbital sinus within 90 s of initiating ether anesthesia at ~3 h into the light phase of the diurnal cycle (1000 h). Blood samples were collected in iced ethylenediaminetetraacetic acid and plasma kept at −70 °C until analyzed. Anal to nasal length was measured, and mice were killed by cervical dislocation. Four white adipose depots, femoral, gonadal, retroperitoneal and mesenteric, were dissected by the same individual to minimize variation in technique and weighed as described earlier.[13] The weights of the four adipose depots were used to calculate AI (total adipose depot weight/live BW × 100). AI is strongly correlated with percent body fat[14] and is used as a surrogate for body fat percentage.

Plasma samples were analyzed in duplicate for glucose by the glucose oxidase method (Thermo Electron, VIC,

Australia, Cat. #TR15104), insulin by mouse ultrasensitive EIA (ALPCO Diagnostics, Salem, NH, USA, Cat. # 80-INSMSU-E01) and leptin by a mouse leptin ELISA kit (Millipore, St Charles, MO, USA, Cat. # EZML-82 K). An index of insulin resistance was calculated from fasting plasma glucose and insulin using the homeostatic model assessment (HOMA) of insulin resistance (HOMA-IR) that was developed for human beings,[15] but is now used extensively in rodent models.[16–18] The HOMA-IR was calculated using the formula: fasting plasma insulin ($ng\,ml^{-1}$) × fasting plasma glucose ($mmol\,l^{-1}$)/ (22.5×0.0417).[18]

α-MSH plasma level

Plasma samples were suspended in an equal of volume of 0.1% TFA in water. Samples were then centrifuged at $17\,000 \times g$ for 15 min at 4 °C. The supernatants were run through Strata C18-E, 55 μM, 70A (Cat# 8B-S001-FBJ, Phenomenex, Torrance, CA, USA) Sep-Paks according to the manufacturers instructions. Sample were then frozen and lyophilized. Sample extracts were solubilized with 50 μl 2% acetonitrile, 0.1% formic acid and sonicated in an ultra sonic bath for 10 min. 10 μl of samples were analyzed by mass spectrometry using a Thermo TSQ Vantage with a Michrom multidimensional liquid chromatography system and a CTC Pal Autosampler. The column used is a Magic C18 (200 μm × 150 mm) set at a flow rate of $2\,\mu l\,min^{-1}$. Separation of peptides was achieved by using a 90-min step gradient with a mobile phase of varying concentration of acetonitrile (5 to 90%). Scan width was set at 0.002 and Q1 and Q3 peak widths at 0.7. Peak areas for, respectively, 5 and 8 multiple reaction monitoring (MRM) transitions of the prevalent +3 charge state, for both target peptides, α-MSH and γ-MSH, were then calculated using the software Xcalibur version 2.0.7.

Effect of dam BW on progeny

Dam BWs, litter sizes and average pup weights were compared by parity and genotype in dams fed breeder chow.

Western blots

Protein was extracted from a variety of tissue samples. Approximately 50 mg tissue was homogenized in 1 ml Rigor buffer (50 mM KCl, 50 mM Tris, 5 mM EGTA, 2 mM NaN_3, pH 7.5) supplemented with protease inhibitor cocktail (Roche Cat# 1 697 498 001) on ice. The samples were centrifuged at 4 °C for 10 min at $14\,000\,g$. The supernatant was retained and used for western blot analysis. 10 μg of protein was run on NuPAGE 4–12% Bis–Tris Gel and transferred to GE nitrocellulose membrane. We performed Ponceau S staining on unblocked membrane and blocked with 5% milk overnight in a cold room. The membrane was blocked with primary antibody (Abcam ab58988) for 30 min, washed three times with TBST for 10 min, then blocked with secondary antibody (Abcam ab6721) for 30 min and finally washed another four times for 10 min with TBST.

Statistical analyses

Data are presented as means ± s.e. A general linear model was used to assess effects of pup genotype and diet or maternal genotype and pup gender on body composition using JMP (SAS Institute, Cary, NC, USA). *Post hoc* comparisons were by Tukey's HSD. A Bonferroni correction for multiple tests in this study indicates that an ANOVA *P*-value of 0.001 is statistically significant.

Statement of ethics

We certify that all applicable institutional and governmental regulations concerning the ethical use of animals were followed during this research. All animals were housed and cared under conditions meeting National Institutes of Health standards as stated in the Guide for Care and Use of Laboratory Animals and American Association for Accreditation of Laboratory Animal Care accreditation standards. All animal use was conducted according to Institutional Animal Care and Use Committee-approved protocols.

Results

Confirmation of GT

Surveys of mRNA abundance, both BioGPS (https://biogps.gnf.org/) and MPSS GEO (http://www.ncbi.nlm.nih.gov/geo/info/mouse-trans.html)[19] reveal that PREP is expressed in several tissues. Deficiency of PREP protein in GT mice was confirmed by performing a western blot of brain and kidney proteins from $PREP^{WT/WT}$, $PREP^{WT/gt}$ and $PREP^{gt/gt}$ mice fed either the LF or HF diet as shown in Figure 1a and lung from mice fed only the LF diet in Figure 1b. Additional western blots comparing lung, brain, muscle, ovary, uterus and heart in $PREP^{WT/WT}$ and $PREP^{gt/gt}$ mice fed only the LF diet are in Figure 1c. PREP was identified by including recombinant PREP on the western blot. The sample genotypes were verified by PCR. The antibody to PREP was made to a synthetic peptide based on the amino-terminal end of human PREP. PREP protein was present in $PREP^{WT/WT}$ mice, reduced in $PREP^{WT/gt}$ and almost absent in $PREP^{gt/gt}$ mice (Figure 1). There was no detectable effect of diet on the amount of PREP protein in kidney and brain. The amount of PREP in $PREP^{gt/gt}$ homozygotes varied from undetectable in gastrocnemius muscle to easily visible in brain. Detectable PREP protein in $PREP^{gt/gt}$ mice results from alternative splicing to remove the ß-gal containing 'GT' exon. Our data show that efficiency of alternative splicing of PREP varies between tissues, but in all tissues $PREP^{gt/gt}$ exhibit reduced protein.

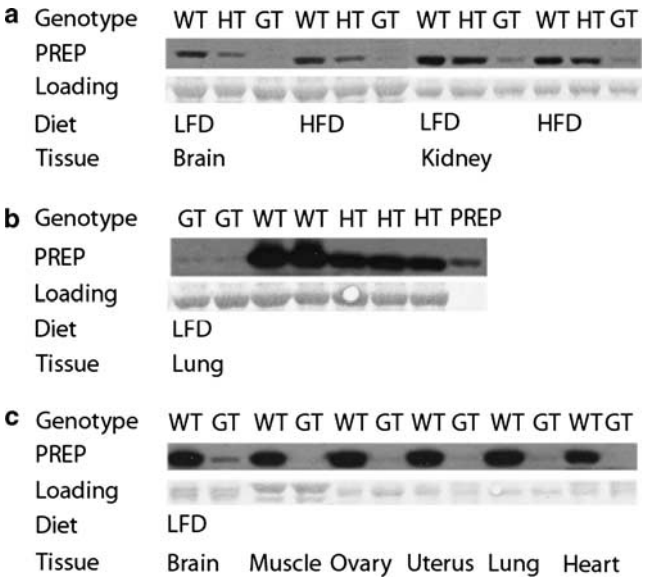

Figure 1 Western blot of PREP protein in tissues of $PREP^{WT/WT}$, $PREP^{WT/gt}$ and $PREP^{gt/gt}$ mice fed either LF or HF diets as well as recombinant PREP protein: (**a**) brain and kidney; (**b**) lung; (**c**) brain, muscle, ovary, uterus, lung and heart. See text for methods. The minimal PREP protein in $PREP^{gt/gt}$ mice indicates that this mutation is a PREP hypomorph rather than a complete deletion.

Direct effects of $PREP^{gt/gt}$ on obesity phenotypes (Experiment 1)
Intercrosses of heterozygous ($PREP^{gt/gt} \times PREP^{WT/WT}$) parents were used to determine direct genotype effects. An intercross colony was maintained by breeding of heterozygous parents. Obesity phenotypes of $PREP^{WT/WT}$, $PREP^{WT/gt}$ and $PREP^{gt/gt}$ littermates maintained on either AIN or HF diet were compared. Few genotype effects of PREP GT were observed in F2 animals. Figure 2 presents BW and AI of female and male littermates of different genotypes and diets from the intercross. Genders were analyzed separately. Both genders show a strong diet effect ($P<0.0001$) for both BW and AI (Figure 2). In female mice, genotype effects for both BW and AI were not statistically significant, although when analyzed individually, BW was lower in $PREP^{gt/gt}$ than in $PREP^{WT/WT}$ mice, whereas AI was lower in $PREP^{WT/gt}$ than in $PREP^{WT/WT}$ mice. There was a significant genotype effect on BW for male mice ($P=0.0003$) with $PREP^{WT/gt}$ mice on the HF diet being heavier than $PREP^{gt/gt}$ However, there were no genotype effects on BW or AI in males fed the LF diet (Figure 2). We also observed recessive genotype effects on kidney weight in females fed the LF diet ($P=0.002$) and BMI in males fed the HF diet ($P<0.0001$), as $PREP^{WT/WT}$ and $PREP^{WT/gt}$ are statistically indistinguishable, whereas $PREP^{gt/gt}$ are significantly smaller (data not shown).

Data on fasting plasma insulin, leptin, glucose and calculated HOMA-IR are presented in Table 1. In females, leptin was significantly higher in mice fed the HF diet: no other diet or genotype effects were observed. In males, insulin, leptin, glucose and HOMA-IR are all higher in mice HF diet fed groups: no genotype effects were observed. In females ($N=30$), leptin was strongly correlated with AI (0.84) and to a lesser extent with insulin (0.58) and HOMA-IR (0.57), and insulin was strongly correlated with glucose (0.76) and HOMA-IR (0.95). In males ($N=36$), leptin was strongly correlated with AI (0.75) and to a lesser extent with insulin (0.68) and HOMA-IR (0.69), and insulin was strongly correlated with glucose (0.69) and HOMA-IR (0.96). There were no significant effects on total cholesterol (data not shown).

Using targeted quantitative proteomics, we measured the active, *N*-acetylated and *C*-amidated α-MSH from the plasma of $PREP^{gt/gt}$ mice and WT littermates. First, using MSMS on α-MSH standard, we identified five MRM transitions that we could then monitor in plasma samples. On account of the variability encountered across the biological samples, we also monitored γ-MSH, another proopiomelanocortin-derived peptide. γ-MSH also results from the digestion of a proopiomelanocortin-derived peptide by proprotein convertase subtilisin/kexin type 2, but it lacks a proline and thus is not a putative PREP substrate. We identified eight MRM transitions using γ-MSH standard and then quantitated γ-MSH in multiplex, that is in the same MS run as α-MSH. We observed a close correlation between the two hormones for each genotype: $R^2=0.93$ and $R^2=0.90$, respectively, for the $PREP^{gt/gt}$ and WT genotypes (Figure 3). When normalizing α-MSH with γ-MSH levels, we detected a statistically significant decrease (1.5-fold) of α-MSH in the plasma of $PREP^{gt/gt}$ mice with a one-tailed Student *t*-test *P*-value = 0.0016 (Figure 4).

Daily food intake was measured in male and female mice of all three genotypes on both diets. There were no significant genotype effects (data not shown).

Reciprocal cross reveals maternal effect on obesity (Experiment 2)
We examined the hypothesis that heterozygotes for the PREP GT mutation will have a parent of origin effect on obesity. We performed reciprocal crosses of $PREP^{WT/gt} \times PREP^{WT/WT}$ using mice that were at generation N8 of backcross. All animals were phenotyped by the same protocol as for the intercross, but using only the LF diet (Figure 5; Table 2). Pup genotype had no effect on phenotypes in either gender (Figure 5) and the pups of both genotypes were combined for analysis of maternal effects. Progeny of heterozygous $PREP^{WT/gt}$ females weighed significantly more and had significant greater fat mass (AI) than progeny of WT $PREP^{WT/WT}$ mothers. Pups born to heterozygous $PREP^{WT/gt}$ mothers also had higher body mass index and organ weights, but not anal nasal length than pups born to WT $PREP^{WT/WT}$ mothers (Table 2).

Comparison of intercross and reciprocal cross data reveals maternal effects
Progeny from the intercross (Experiment 1) were compared with progeny from the reciprocal cross (Experiment 2)

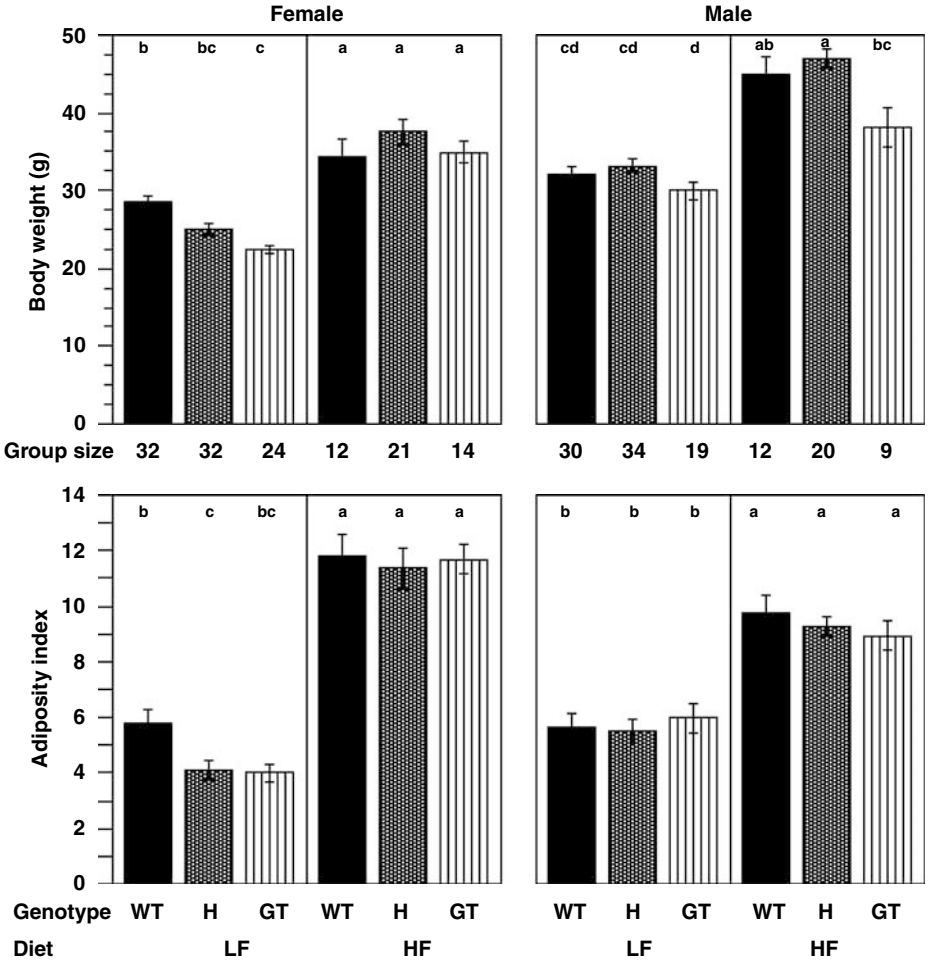

Figure 2 Intercross mice (Experiment 1). BWs and AI of female and male PREP WT ($PREP^{WT/WT}$, WT), heterozygote ($PREP^{WT/gt}$, H) and GT ($PREP^{gt/gt}$) fed LF AIN-76A or HF diets and killed at 120 days of age. AI was calculated as (total adipose depot weight/live BW × 100). Data are presented as means ± s.e. *P*-values were calculated by ANOVA: Bonferroni correction for multiple tests sets significant *P*-value at ⩽0.001. *Post hoc* comparisons were by Tukey's HSD: means not sharing a letter differ significantly ($P \leqslant 0.05$). Genders were analyzed separately by two-way ANOVA. Females: BW effect of diet, $P<0.0001$, effect of genotype, NS; AI effect of diet $P<0.0001$, effect of genotype, NS. Males: BW effect of diet, $P<0.0001$, effect of genotype, $P=0.0003$; AI effect of diet, $P<0.0001$, effect of genotype, NS.

Table 1 Fasting plasma insulin, leptin, glucose and calculated HOMA-IR of mice from the intercross (Experiment 1)

Diet	*Low –fat*			*High fat*			*2-Way ANOVA*[a]		
Genotype	$PREP^{WT/WT}$ [b]	$PREP^{WT/gt}$	$PREP^{gt/gt}$	$PREP^{WT/WT}$	$PREP^{WT/gt}$	$PREP^{gt/gt}$	*Diet*	*Genotype*	*Interaction*
Females									
Insulin ng ml^{-1}	0.14 ± 0.03	0.21 ± 0.03	0.15 ± 0.03	0.22 ± 0.04	0.46 ± 0.11	0.19 ± 0.02	NS	NS	NS
Leptin ng ml^{-1}	10.2 ± 2.4c	8.4 ± 3.2c	3.7 ± 1.0c	22.9 ± 14.1b,c	54.3 ± 8.7a	40.4 ± 9.6a,b	<0.0001	NS	NS
Glucose mmol l^{-1}	5.5 ± 0.8	5.2 ± 0.3	5.7 ± 0.9	6.0 ± 0.8	9.5 ± 1.1	6.6 ± 0.3	NS	NS	NS
HOMA-IR	9.5 ± 2.9	10.9 ± 1.1	10.3 ± 3.8	14.7 ± 3.5	50.6 ± 18.1	13.2 ± 1.4	NS	NS	NS
Males									
Insulin ng ml^{-1}	0.13 ± 0.04b	0.19 ± 0.05b	0.18 ± 0.03b	0.78 ± 0.29a	0.31 ± 0.07b	0.30 ± 0.06 b	<0.0001	NS	NS
Leptin ng ml^{-1}	3.9 ± 1.1c	6.7 ± 3.0c	7.9 ± 3.6c	34.2 ± 4.1a	25.3 ± 5.4a,b	13.0 ± 4.8 bc	<0.0001	NS	NS
Glucose mmol l^{-1}	4.8 ± 0.4c	5.3 ± 0.4b,c	5.6 ± 0.7b,c	10.9 ± 1.7a	8.6 ± 0.5a,b	7.0 ± 2.0 abc	<0.0001	NS	NS
HOMA-IR	6.4 ± 1.9b	10.9 ± 3.3b	11.0 ± 2.4b	101 ± 44a	29.0 ± 6.5b	24.7 ± 9.5 b	<0.0001	NS	0.0004

Data are means ± s.e. [a]*P*-values were calculated by ANOVA: Bonferroni correction for multiple tests sets significant *P*-value at ⩽0.001. *Post hoc* comparisons were by Tukey's HSD: means not sharing a letter differ significantly $P \leqslant 0.05$. [b]Group sizes: females eating low-fat diet $N=6$ each group; eating high-fat diet $N=4$ each group; males eating low-fat diet $N=8$ each group; males eating high-fat diet $PREP^{WT/WT}$, $N=3$; $PREP^{WT/gt}$, $N=5$; $PREP^{gt/gt}$, $N=4$.

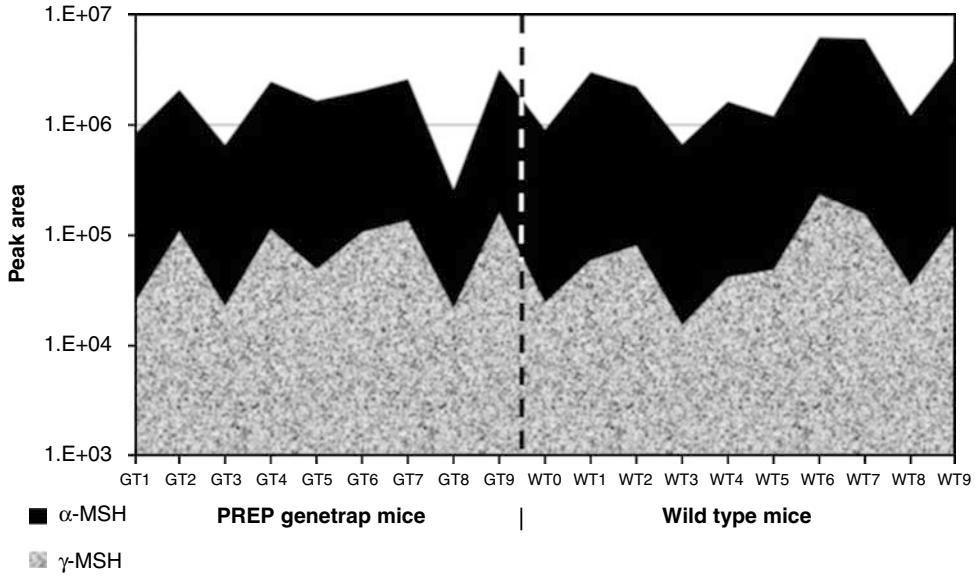

Figure 3 Quantitative-targeted peptidomics of α-MSH and γ-MSH. Peak areas of the MRM transitions are combined and plotted on a log scale for each mouse. Mice are sorted by genotype with the $PREP^{gt/gt}$ mice on the left side (id starting with GT) and the WT mice ($PREP^{WT/WT}$ id starting with WT) on the right. Linear correlation between the two endogenous peptides underlines that γ-MSH, which is not a putative PREP substrate, can be used as an endogenous control to normalize α-MSH quantities across biological replicates.

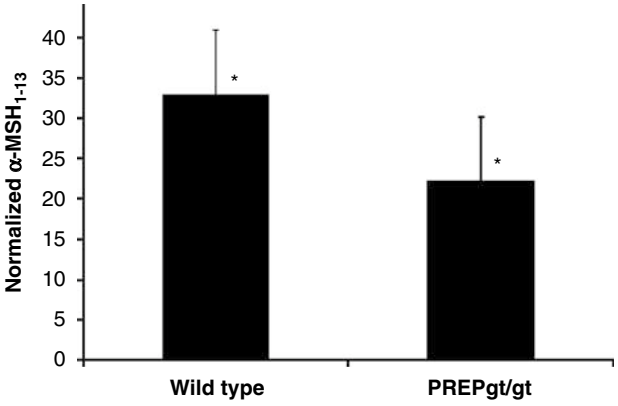

Figure 4 α-MSH plasma level determined by quantitative-targeted proteomics. Average of MRM transitions combined peak areas shows a genotype effect. There is 1.5-fold more α-MSH in the plasma of WT mice ($N = 10$) than in homozygous GT mice ($N = 9$) (Student *t*-test *P*-value = 0.0016).

(Figure 6). Pups of both $PREP^{WT/WT}$ and $PREP^{WT/gt}$ genotypes are combined in this analysis, as there was no significant effect of pup genotype on adiposity. The results support two conclusions: first, obesity because of the PREP GT mutation is either additive or dominant, as effects are observed when breeding heterozygotes and second, data are consistent with maternal effects on obesity. Males cannot be the cause of the obesity phenotype, as they cannot both cause leanness in the reciprocal $PREP^{WT/gt}$ male × $PREP^{WT/WT}$ female cross and obesity in the $PREP^{WT/gt}$ male × $PREP^{WT/gt}$ female intercross.

Dam genotype and BW do not affect litter size or pup birth weight (Table 3). Preliminary data from crosses of $PREP^{WT/WT}$, $PREP^{WT/gt}$ and $PREP^{gt/gt}$ females indicate that dams become heavier with parity, but litter sizes and average pup weights are unaffected.

Discussion

We produced GT mice that express minimal levels of PREP. PREP GT has a strong maternal effect on BW and fat mass: all progeny, regardless of PREP genotype, born to females heterozygous for the PREP mutation ($PREP^{WT/gt}$) are significantly more obese than progeny born to WT ($PREP^{WT/WT}$) mothers mated to $PREP^{WT/gt}$ heterozygous fathers.

Our data do not distinguish whether the maternal effects of PREP GT mutation are pre- or post-natal. The obesity observed in progeny of PREP heterozygous females is not secondary to maternal obesity, as PREP heterozygous females are, if anything, leaner than WT females on the LF diet (see Figure 2). In addition, increased BW in dams fed higher-fat breeder chow did not result in pups of greater average BW at birth (Table 3).

There is no evidence of altered glucose metabolism in the PREP-deficient mice fed the LF diet (Table 1). And in female mice, unlike males, even the HF diet did not result in elevated glucose and insulin levels. This differential regulation of insulin resistance by gender in B6 mice is likely because of gonadal steroid production in females.[20,21] Therefore, it is unlikely that gestational diabetes in the dams could explain the increased obesity of pups born to PREP heterozygous mothers.

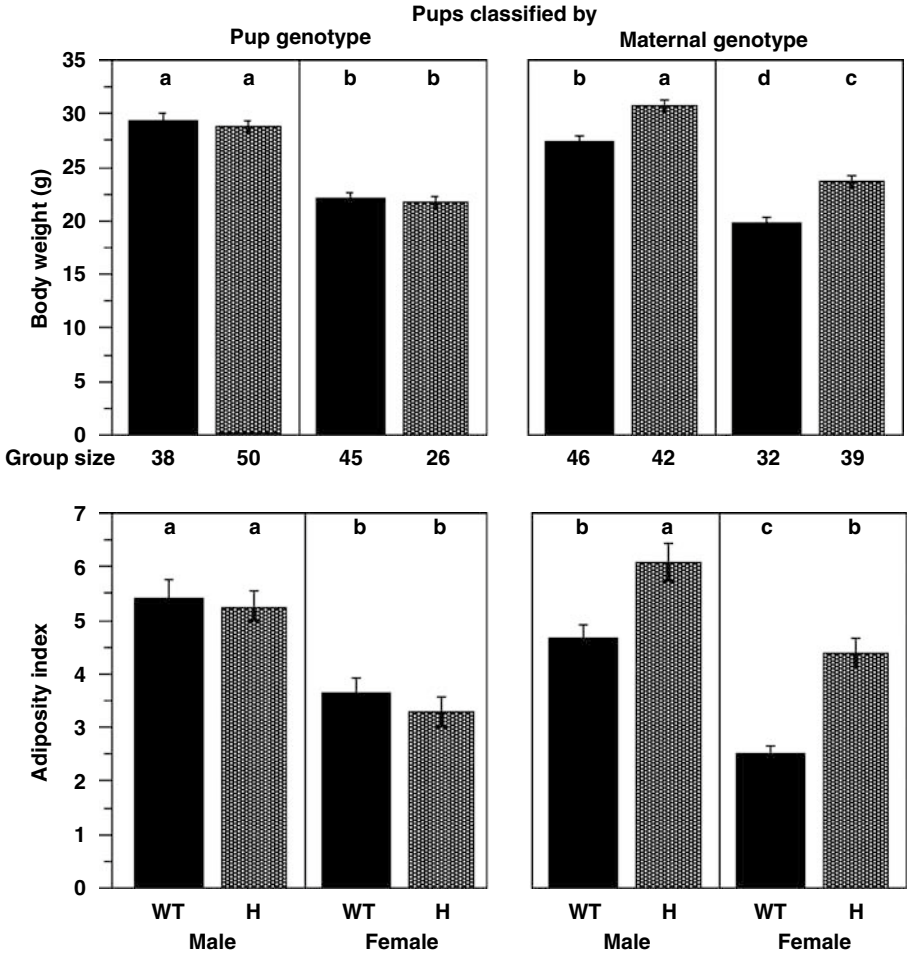

Figure 5 Reciprocal cross (Experiment 2). BW and AI of male and female pups with either WT ($PREP^{WT/WT}$, WT) or heterozygous ($PREP^{WT/gt}$, H) mothers in the reciprocal cross and categorized by either the pup genotype or the maternal genotype. Pups were fed LF diet only. Data are means ± s.e. See Figure 2 legend for methods. Three-way ANOVA for both BW and AI: effect of gender, $P<0.0001$; effect of pup genotype, NS; effect of maternal genotype, $P<0.0001$; all interactions, NS.

Table 2 Body composition phenotypes of male and female pups of either wild-type ($PREP^{WT/WT}$) or heterozygous ($PREP^{WT/gt}$) mothers. Pups were fed LF diet only. Data are means ± s.e.

	Maternal genotype				*Two-way ANOVA*[a]		
	$PREP^{WT/WT}$[b]	$PREP^{WT/gt}$	$PREP^{WT/WT}$	$PREP^{WT/gt}$	*Maternal genotype*	*Gender of pups*	*Interaction*
	Male pups		*Female pups*				
AN length (cm)	9.9 ± 0.0a	10.0 ± 0.0a	9.6 ± 0.1b	9.6 ± 0.0b	NS	<0.0001	NS
Body mass index	0.28 ± 0.00b	0.31 ± 0.00a	0.22 ± 0.00d	0.25 ± 0.00c	<0.0001	<0.0001	NS
Liver weight (g)	0.97 ± 0.02b	1.11 ± 0.03a	0.88 ± 0.02c	1.03 ± 0.03a,b	<0.0001	0.0006	NS
Kidney weight (g)	0.30 ± 0.00b	0.32 ± 0.01a	0.22 ± 0.00d	0.24 ± 0.00c	<0.0001	<0.0001	NS
Spleen weight (g)	0.07 ± 0.00b	0.09 ± 0.00a	0.07 ± 0.00b	0.08 ± 0.00a	<0.0001	NS	NS

[a]*P*-values were calculated by ANOVA: Bonferroni correction for multiple tests sets significant *P*-value at ⩽0.001. *Post hoc* comparisons were by Tukey's HSD: means not sharing a letter differ significantly $P\leqslant0.05$. [b]Group sizes: males, $PREP^{WT/WT}$, $N=46$; $PREP^{WT/gt}$, $N=42$; females, $PREP^{WT/WT}$, $N=32$; $PREP^{WT/gt}$, $N=39$.

When normalizing α-MSH with γ-MSH levels, we detected a genotype effect that was not observable when directly comparing α-MSH between the two genotypes. The decrease of α-MSH in plasma from $PREP^{gt/gt}$ is counter-intuitive, as one could expect accumulation of α-MSH with reduced PREP enzyme, as it digests α-MSH *in vitro*.[22] Our hypothesis is that

feedback inhibition of α-MSH production from the deacetylated form of α-MSH may result from decreased turnover of α-MSH in a PREP-deficient mouse strain. Such a mechanism would involve homeostatic sensing of α-MSH concentrations as suggested in the *pars intermedia* of the marsh frog.[23]

We have also used targeted peptidomics to show that PREP removes the penultimate valine from full-length acetylated α-MSH with 13 amino acids (α-MSH_{1-13}) to produce a 12-amino-acid form of α-MSH_{1-12}.[24] We then show that the substrate/product ratio of α-MSH_{1-13} to α-MSH_{1-12} is increased in pituitaries of $PREP^{gt/gt}$ mice, proving that α-MSH_{1-13} is an *in vivo* substrate for PREP. We have furthermore shown that α-MSH_{1-12} does not stimulate action potentials in melanocortin receptor-4 containing neurons.[25] In addition, the last three amino acids of α-MSH_{1-13}, KPV, have antiinflammatory effects.[26] These results mean that the effect of PREP on α-MSH_{1-13} may influence *in vivo* melanocortin signaling and inflammation.

The putative effects of PREP on vasopressin and oxytocin,[6] which influence milk production, and the earlier presentation that inhibitors of PREP increase vasopressin levels in some brain regions,[8] support the hypothesis that alterations of vasopressin and/or oxytocin by the PREPKO may lead to the maternal obesity effects observed here.

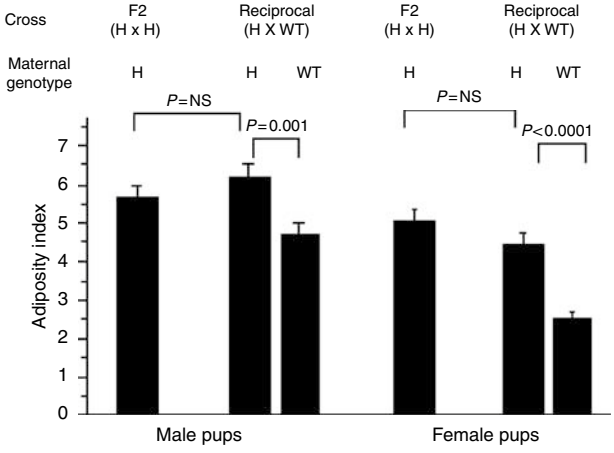

Figure 6 Maternal obesity effects of the PREP GT mutation on AI in male and female pups. Values are mean ± s.e. See Figure 2 legend for methods. There are no significant differences between mice born to $PREP^{WT/gt}$ mothers. The F2 intercross has only $PREP^{WT/gt}$ heterozygous mothers. Pup genotypes were $PREP^{WT/gt}$ or $PREP^{WT/WT}$ and are combined in this analysis, as there was no genotype difference. Male and female heterozygous breeders in the reciprocal cross were littermates of $PREP^{WT/WT} \times PREP^{WT/gt}$ crosses.

Several specific genes exhibit parent of origin effects on obesity. Gs-α influences obesity through an imprinting mechanism in which deletion of the maternal allele causes obesity.[27] Yellow coat color and obesity in the agouti viable yellow mouse (A^{vy}) is due to an intracisternal A particle inserted in the agouti gene cause ectopic expression of agouti.[28] Intracisternal A particle methylation can also be influenced by maternal diet.[29] There are no other known examples of maternal effects genes. Thus, maternal effects because of a PREP GT mutation represent a novel mechanism for maternal effects.

The present data not only illustrate a novel mechanism for maternal genetic effects, but also suggest the possibility of unintentional obesity from the inhibition of PREP in human beings. PREP is a member of a serine protease family that can be inhibited by natural and synthetic compounds. Natural inhibitors were identified in hydrolysates of cheese, fish,[30] wine,[31] casein,[32] unsaturated fatty acids[33] and plant phenolics.[34] A survey of traditional Chinese medicinal plants identified PREP inhibitors. Berberine, an extract from *Rhizoma coptidis*, was identified as a PREP inhibitory molecule.[35] Thus, human beings could already self-administer PREP inhibitors using traditional medicinal extracts.

Several PREP inhibitors have been synthesized and tested in animal models and human beings. Compound S 17092 improves memory in rodents and human beings.[36] Inhibition of PREP by a single dose of compound S 17092 increased hypothalamic α-MSH, although chronic treatment had no effect.[22] Inhibition of PREP by S 17092 also raised levels of arg-vasopressin and TRH in some brain regions.[37] Clinical trials of S 17092 in human beings to test roles in cognitive enhancement and mood stabilization have begun.[38]

Our results show a novel mechanism for maternal genetic effects on obesity of adult progeny through a serine protease. It is unlikely that PREP is the only protease that influences maternal effects. In principle, any gene that influences in *utero* or post-natal nutrition or care could cause maternal effects. As there are many similar serine proteases and as PREP is influenced by environmental factors such as food and traditional and pharmaceutical compounds, these results may

Table 3 Dam body weight, litter size and average pup weight by parity and genotype

	Parity 1 *Dam genotype*			*Parity 2* *Dam genotype*		
	$PREP^{WT/WT}$	$PREP^{WT/gt}$	$PREP^{gt/gt}$	$PREP^{WT/WT}$	$PREP^{WT/gt}$	$PREP^{gt/gt}$
Number of dams	1	4	6	3	4	10
Dam body wt (g)[a]	21.8	18.1 ± 0.7	19.3 ± 0.3	24.3 ± 0.5	23.9 ± 0.7	24.1 ± 0.7
Litter size	8	8.3 ± 0.5	6.7 ± 0.9	8.7 ± 0.7	6.0 ± 0.7	8.6 ± 0.4
Total number of pups	8	33	47	26	24	86
Average pup weight (g per litter)	1.28	1.28 ± 0.04	1.22 ± 0.02	1.29 ± 0.05	1.34 ± 0.03	1.28 ± 0.01

Dams fed breeder chow. [a]Two-way ANOVA: effect of parity, $P<0.0001$. All other *P*-values for table, NS.

illustrate a prevalent protease action mode. This could have broad significance for discovery of additional maternal genetic effects and for the relevance of PREP to obesity.

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References

1 Samuelsson AM, Matthews PA, Argenton M, Christie MR, McConnell JM, Jansen EH *et al.* Diet-induced obesity in female mice leads to offspring hyperphagia, adiposity, hypertension, and insulin resistance: a novel murine model of developmental programming. *Hypertension* 2008; **51**: 383–392.
2 Lambin S, van Bree R, Caluwaerts S, Vercruysse L, Vergote I, Verhaeghe J. Adipose tissue in offspring of Lepr(db/+) mice: early-life environment vs. genotype. *Am J Physiol Endocrinol Metab* 2007; **292**: E262–E271.
3 Davison KK, Birch LL. Obesigenic families: parents' physical activity and dietary intake patterns predict girls' risk of overweight. *Int J Obes Relat Metab Disord* 2002; **26**: 1186–1193.
4 Hager R, Cheverud JM, Wolf JB. Maternal effects as the cause of parent-of-origin effects that mimic genomic imprinting. *Genetics* 2008; **178**: 1755–1762.
5 Diament AL, Warden CH. Multiple linked mouse chromosome 7 loci influence body fat mass. *Int J Obes Relat Metab Disord* 2004; **28**: 199–210.
6 Walter R, Shlank H, Glass JD, Schwartz IL, Kerenyi TD. Leucylglycinamide released from oxytocin by human uterine enzyme. *Science* 1971; **173**: 827–829.
7 Garcia-Horsman JA, Mannisto PT, Venalainen JI. On the role of prolyl oligopeptidase in health and disease. *Neuropeptides* 2007; **41**: 1–24.
8 Bellemere G, Vaudry H, Mounien L, Boutelet I, Jegou S. Localization of the mRNA encoding prolyl endopeptidase in the rat brain and pituitary. *J Comp Neurol* 2004; **471**: 128–143.
9 Ohta N, Takahashi T, Mori T, Park MK, Kawashima S, Takahashi K *et al*. Hormonal modulation of prolyl endopeptidase and dipeptidyl peptidase IV activities in the mouse uterus and ovary. *Acta Endocrinol (Copenh)* 1992; **127**: 262–266.
10 Maes M, Monteleone P, Bencivenga R, Goossens F, Maj M, van West D *et al*. Lower serum activity of prolyl endopeptidase in anorexia and bulimia nervosa. *Psychoneuroendocrinology* 2001; **26**: 17–26.
11 Maes M, Goossens F, Scharpe S, Calabrese J, Desnyder R, Meltzer HY. Alterations in plasma prolyl endopeptidase activity in depression, mania, and schizophrenia: effects of antidepressants, mood stabilizers, and antipsychotic drugs. *Psychiatry Res* 1995; **58**: 217–225.
12 Mantle D, Falkous G, Ishiura S, Blanchard PJ, Perry EK. Comparison of proline endopeptidase activity in brain tissue from normal cases and cases with Alzheimer's disease, Lewy body dementia, Parkinson's disease and Huntington's disease. *Clin Chim Acta* 1996; **249**: 129–139.
13 Warden CH, Fisler JS, Shoemaker SM, Wen PZ, Svenson KL, Pace MJ *et al*. Identification of four chromosomal loci determining obesity in a multifactorial mouse model. *J Clin Invest* 1995; **95**: 1545–1552.
14 Chiu S, Kim K, Haus KA, Espinal GM, Millon LV, Warden CH. Identification of positional candidate genes for body weight and adiposity in subcongenic mice. *Physiol Genomics* 2007; **31**: 75–85.
15 Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* 1985; **28**: 412–419.
16 Schaalan M, El-Abhar HS, Barakat M, El-Denshary ES. Westernized-like-diet-fed rats: effect on glucose homeostasis, lipid profile, and adipocyte hormones and their modulation by rosiglitazone and glimepiride. *J Diabetes Complications* 2009; **23**: 199–208.
17 Herbach N, Rathkolb B, Kemter E, Pichl L, Klaften M, de Angelis MH *et al*. Dominant-negative effects of a novel mutated Ins2 allele causes early-onset diabetes and severe beta-cell loss in Munich Ins2C95S mutant mice. *Diabetes* 2007; **56**: 1268–1276.
18 Chen H, Simar D, Lambert K, Mercier J, Morris MJ. Maternal and postnatal overnutrition differentially impact appetite regulators and fuel metabolism. *Endocrinology* 2008; **149**: 5348–5356.
19 Barrett T, Troup DB, Wilhite SE, Ledoux P, Rudnev D, Evangelista C *et al*. NCBI GEO: archive for high-throughput functional genomic data. *Nucleic Acids Res* 2009; **37** (Database issue): D885–D890.
20 Vasudevan H, Xiang H, McNeill JH. Differential regulation of insulin resistance and hypertension by sex hormones in fructose-fed male rats. *Am J Physiol Heart Circ Physiol* 2005; **289**: H1335–H1342.
21 Yakar S, Nunez NP, Pennisi P, Brodt P, Sun H, Fallavollita L *et al*. Increased tumor growth in mice with diet-induced obesity: impact of ovarian hormones. *Endocrinology* 2006; **147**: 5826–5834.
22 Bellemere G, Morain P, Vaudry H, Jegou S. Effect of S 17092, a novel prolyl endopeptidase inhibitor, on substance P and alpha-melanocyte-stimulating hormone breakdown in the rat brain. *J Neurochem* 2003; **84**: 919–929.
23 Jenks BG, Verburg van Kemenade BM, Tonon MC, Vaudry H. Regulation of biosynthesis and release of pars intermedia peptides in Rana ridibunda: dopamine affects both acetylation and release of alpha-MSH. *Peptides* 1985; **6**: 913–921.
24 Perroud B, Alvarado RJ, Espinal GM, Morado AR, Phinney BS, Warden CH. *In vivo* multiplex quantitative analysis of 3 forms of alpha melanocyte stimulating hormone in pituitary of prolyl endopeptidase deficient mice. *Mol Brain* 2009; **2**: 14.
25 Wallingford N, Perroud B, Gao Q, Coppola A, Gao X-B, Diament A *et al*. Prolylcarboxypeptidase regulates food intake by promoting breakdown of α-MSH. *J Clin Invest* 2009. In press.
26 Brzoska T, Luger TA, Maaser C, Abels C, Böhm M. Alpha-melanocyte-stimulating hormone and related tripeptides: biochemistry, antiinflammatory and protective effects *in vitro* and *in vivo*, and future perspectives for the treatment of immune-mediated inflammatory diseases. *Endocr Rev* 2008; **29**: 581–602.
27 Xie T, Chen M, Gavrilova O, Lai EW, Liu J, Weinstein LS. Severe obesity and insulin resistance due to deletion of the maternal Gsalpha allele is reversed by paternal deletion of the Gsalpha imprint control region. *Endocrinology* 2008; **149**: 2443–2450.
28 Dolinoy DC, Weidman JR, Waterland RA, Jirtle RL. Maternal genistein alters coat color and protects Avy mouse offspring from obesity by modifying the fetal epigenome. *Environ Health Perspect* 2006; **114**: 567–572.
29 Waterland RA, Travisano M, Tahiliani KG, Rached MT, Mirza S. Methyl donor supplementation prevents transgenerational amplification of obesity. *Int J Obes (Lond)* 2008; **32**: 1373–1379.
30 Sorensen R, Kildal E, Stepaniak L, Pripp AH, Sorhaug T. Screening for peptides from fish and cheese inhibitory to prolyl endopeptidase. *Nahrung* 2004; **48**: 53–56.
31 Yanai T, Suzuki Y, Sato M. Prolyl endopeptidase inhibitory peptides in wine. *Biosci Biotechnol Biochem* 2003; **67**: 380–382.

32 Asano M, Nio N, Ariyoshi Y. Inhibition of prolyl endopeptidase by synthetic peptide fragments of human beta-casein. *Agric Biol Chem* 1991; **55**: 825–828.
33 Park YS, Jang HJ, Lee KH, Hahn TR, Paik YS. Prolyl endopeptidase inhibitory activity of unsaturated fatty acids. *J Agric Food Chem* 2006; **54**: 1238–1242.
34 Lee SH, Jun M, Choi JY, Yang EJ, Hur JM, Bae K *et al*. Plant phenolics as prolyl endopeptidase inhibitors. *Arch Pharm Res* 2007; **30**: 827–833.
35 Tarrago T, Kichik N, Segui J, Giralt E. The natural product berberine is a human prolyl oligopeptidase inhibitor. *ChemMedChem* 2007; **2**: 354–359.
36 Morain P, Lestage P, De Nanteuil G, Jochemsen R, Robin JL, Guez D *et al*. S 17092: a prolyl endopeptidase inhibitor as a potential therapeutic drug for memory impairment. Preclinical and clinical studies. *CNS Drug Rev* 2002; **8**: 31–52.
37 Bellemere G, Vaudry H, Morain P, Jegou S. Effect of prolyl endopeptidase inhibition on arginine-vasopressin and thyrotrophin-releasing hormone catabolism in the rat brain. *J Neuroendocrinol* 2005; **17**: 306–313.
38 Morain P, Boeijinga PH, Demazieres A, De Nanteuil G, Luthringer R. Psychotropic profile of S 17092, a prolyl endopeptidase inhibitor, using quantitative EEG in young healthy volunteers. *Neuropsychobiology* 2007; **55**: 176–183.