Chloride is now widely recognized as a severe public health issue (43). Treatment with drugs aimed at neural systems involved in the determination of the energy balance could potentially result in a lower energy balance during puberty as well as later in adulthood. Therefore, neuropeptides involved in body weight regulation during early development and puberty are attractive targets for anti-childhood obesity drugs.

The neuronal metabolic systems in humans and primates develop prenatally, while in rodents these systems develop during the first 3 postnatal weeks (5, 21). This results in the activation and optimization of neuronal systems during early rodent development. A second important metabolic period is puberty, a period of major growth, hormonal changes, and sexual maturation. In the rat, puberty is characterized by different responses of young-adolescent [postnatal day (PND) 40] and young-adult (PND 60) male rats to environmental cues like stress and cold (17, 18). In addition to these age-dependent behavioral differences, the amount of food consumed during early rodent life plays an important role in determining subsequent food intake in later life (44). Following this initial observation, many studies have shown that postnatal nutrition is important for the regulation of appetite in adult rodents, suggesting that the energy balance is predominantly determined during early development (40).

Melanin-concentrating hormone (MCH) has been shown to be a critical mammalian hypothalamic effector of energy homeostasis by various genetic and pharmacological studies (46). The MCH-precursor gene (Pmch) is expressed predominantly in neurons of the lateral hypothalamic area and the incerto hypothalamic area, which project throughout the brain (3, 60). Pmch is also expressed in some peripheral tissues, such as the testes, although at lower levels than in the brain (25). Processing of Pmch results in the production of three neuropeptides: neuropeptide glycine-glutamic acid (N-GE), neuropeptide glutamic acid-isoleucine (N-EI), and MCH (41). Pmch mRNA is upregulated after fasting or leptin deficiency (32, 50); third ventricle intracerebroventricular injections of MCH increase food intake and body weight (11, 19, 22, 28, 53); Pmch knockout mice are lean due to a decreased food intake and an increased metabolic rate (31, 59); and overexpression of MCH causes obesity (35). In rodents MCH binds to melanin-concentrating hormone receptor 1 (MCHR1), a G protein-coupled receptor expressed throughout the brain (7, 33, 55, 56). MCHR1 is particularly enriched in the nucleus accumbens shell (33, 47, 56), thus forming a potential hypothalamic-limbic circuit modulating the hedonic, or rewarding, aspects of feeding (16, 47). Recently, it was indeed shown that the MCH

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**Pmch expression during early development is critical for normal energy homeostasis**

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Mul JD, Yi CX, van den Berg SAA, Ruiter M, Toonen PW, van der Elst MCJ, Voshol PJ, Ellenbroek BA, Kalsbeek A, la Fleur SE, Cuppen E. Pmch expression during early development is critical for normal energy homeostasis. Am J Physiol Endocrinol Metab 298: E477–E488, 2010. First published November 24, 2009; doi:10.1152/ajpendo.00154.2009.—Postnatal development and puberty are times of strong physical maturation and require large quantities of energy. The hypothalamic neuropeptide melanin-concentrating hormone (MCH) regulates nutrient intake and energy homeostasis, but the underlying mechanisms are not completely understood. Here we use a novel rat knockout model in which the MCH precursor Pmch has been inactivated to study the effects of loss of MCH on energy regulation in more detail. Pmch−/− rats are lean, hypophagic, osteoporotic, and although endocrine parameters were changed in pmch−/− rats, endocrine dynamics were normal, indicating an adaptation to new homeostatic levels rather than disturbed metabolic mechanisms. Detailed body weight growth and feeding behavior analysis revealed that Pmch expression is particularly important during early rat development and puberty, i.e., the first 8 postnatal weeks. Loss of Pmch resulted in a 20% lower set point for body weight that was determined solely during this period and remained unchanged during adulthood. Although the final body weight is diet dependent, the Pmch-deficiency effect was similar for all diets tested in this study. Loss of Pmch affected energy expenditure in both young and adult rats, although these effects seem secondary to the observed hypophagia. Our findings show an important role for Pmch in energy homeostasis determination during early development and indicate that the MCH receptor 1 system is a plausible target for childhood obesity treatment, currently a major health issue in first world countries.

melanin-concentrating hormone; hypothalamus; childhood obesity; rat knockout model

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MCH regulates energy homeostasis during early development

System affects motivation for feeding or drugs of abuse (9, 42). Rodents only express MCH1R, whereas humans also express a second MCH receptor, MCH2R (54). Recent studies (24, 36, 39, 45, 58) have focused on MCH1R, demonstrating that MCH1R antagonism decreases food intake and weight gain in adult rodents.

Most MCH-related studies using genetic models or MCH antagonists have primarily focused on the function of MCH during adulthood. Therefore, the effect of loss of Pmch expression on energy regulation during early development is largely unexplored. To study nutrient intake during this period, we utilized a novel rat knockout model that was generated recently using an N-ethyl-N-nitrosourea (ENU)-driven target-selected mutagenesis approach (61). Preliminary studies in young-adult animals showed that the caloric intake of pmmch<sup>−/−</sup> rats was unchanged compared with control littersmates when nutrient intake data were normalized for body weight. Following this initial observation, we have analyzed the metabolic characteristics of the Pmch knockout rat model in three different diets [maintenance (M), semi-high-protein (SHP), and high-fat (HF)] by following body weight and food intake during development and adulthood, and by measuring endocrine values. Furthermore, the metabolic profile of pmmch<sup>−/−</sup> rats was analyzed using indirect calorimetry. Our results show that Pmch plays an important role in the energy balance determination during the first 8 postnatal weeks and that loss of Pmch results in a 20% decreased body weight during adulthood regardless of diet.

**MATERIALS AND METHODS**

**Animals.** The Animal Care Committee of the Royal Dutch Academy of Science and the Leiden University Medical Center approved all experiments according to the Dutch legal ethical guidelines. The Pmch knockout rat (Pmch<sup>−/−</sup>) was generated by target-selected ENU-driven mutagenesis (see Ref. 61). Briefly, high-throughput resequencing of genomic target sequences in progeny from mutated rats revealed an ENU-induced premature stop codon in exon 1 (K50X) in Pmch in a rat (Wistar/Crl background). The heterozygous mutant animal was backcrossed to wild-type Wistar background for six generations to eliminate confounding effects from background mutations induced by ENU. Assuming that the total amount of coding DNA in a male rat is ~28.6 × 10<sup>9</sup> bp (10) and the used ENU treatment resulted in a mutation frequency of 1 per 1.5 × 10<sup>6</sup> bp (61), ~19 mutations can be expected in the protein-coding sequences of the founder animal. Backcrossing six times would therefore decrease the total number of random background mutations to 1. Furthermore, the maximal number of nonsense inducing mutations is much lower than 19, i.e., 3 (10). However, as part of the donor chromosome harboring the Pmch mutation is still present after six backcrosses (29), we cannot fully exclude the presence of tightly linked confounding mutations in our rat model. To further control for possible contributions of confounding mutations, we repeated several measurements in different outcross generations and could replicate previous findings in each generation. Additionally, we always generated experimental pmmch<sup>−/−</sup> and pmmch<sup>+/−</sup> rats by crossing pmmch<sup>+/−</sup> rats. Experimental rats were obtained at the expected Mendelian frequency. Furthermore, littersmates (with similar genetic backgrounds) were used as much as possible for experiments. Pmch<sup>+/−</sup> rats were viable into adulthood and fertile and appeared phenotypically normal despite their lower body weight. Two rats were housed together, unless noted otherwise, under controlled experimental conditions (12:12-h light-dark cycle, light period 0600–1800, 21 ± 1°C, ~60% relative humidity). The standard fed diet in our animal facility (semi-high-protein chow: RM3, 26.9% crude protein, 11.5% fat, and 61.6% carbohydrates; 3.33 kcal/g AFE; SDS, Witham, UK) was provided ad libitum together with water, unless noted otherwise (maintenance chow: RM1, 17.5% crude protein, 7.4% fat, and 75.1% carbohydrates; 3.29 kcal/g AFE; SDS; high-fat chow: 45%–AFE, 20% crude protein, 45% fat, and 35% carbohydrates; 4.54 kcal/g AFE SDS). Only male rats were used in the present study.

**Genotyping.** Genotyping was done using the KASPar SNP Genotyping System (KBiosciences, Hoddesdon, UK; as described in Ref. 66) using gene-specific primers (forward common: TTAAT ACATT CAGGA TTGGGG AAAGC CTTT; reverse wild type: GAAGTG AACGTT CATGCT CGAICT TCTT) and reverse homozygous: GAAGAG TCGGA GTCAAT GCATTC CTCTTG CTGGG TATCT TCCTA). All pups were genotyped at 3 wk of age. Genotypes were confirmed when experimental procedures were completed.

**Northern blot analysis.** Northern blot analysis (as described in Ref. 27) was done using a Pmch-specific radiolabeled PCR-derived probe covering the first exon of the gene. The following primers were used for probe generation: forward primer: ATCTCT CCTTCC GTGCT TC; and reverse primer: TCCAG AGAAG GAGGCA AACA.

**Body weight and nutrient intake.** Animals were housed individually at PND 21. Until weaning, animals had access to SHP diet in their maternal home cage. Body weight, water intake, and food intake was monitored biweekly for 18 wk. Food (M, SHP, or HF diet) and water were provided ad libitum. At 8 and 17 wk of age, nutrient intake was measured for 6 consecutive days at 0600 (dark phase intake) and at 1800 (light phase intake).

**White adipose tissue and organ weight.** A white adipose tissue (WAT) fat pad sample (containing the right side of the subcutaneous WAT pad, the whole epididymal WAT pad, the right side of the perirenal WAT pad, and the whole mesenteric WAT pad), liver, adrenals, and the thymus were isolated from 26-wk-old rats.

**Jugular vein catheter.** The 22-wk-old rats were anesthetized with isoflurane and equipped with a jugular vein catheter (headpiece: Connector Pedestal 20GA; Plastics One, Roanoke, VA). Before surgery, rats received one dose of Temgesic (0.05 mg/kg sc; Schering-Plough, Utrecht, the Netherlands). Rats were allowed to recover for 7 days during which they were handled to minimize stress (e.g., GAT CTTTC TGCGG TATCT TCCTA). All pups were genotyped at PND 21. Until weaning, animals had access to SHP diet in their maternal home cage. Body weight, water intake, and food intake was monitored biweekly for 18 wk. Food (M, SHP, or HF diet) and water were provided ad libitum. At 8 and 17 wk of age, nutrient intake was measured for 6 consecutive days at 0600 (dark phase intake) and at 1800 (light phase intake).

**Indirect calorimetry.** Indirect calorimetry was measured in an eight-cage combined, open circuit indirect calorimetry system (LabMaster System, TSE Systems, Bad Homburg, Germany). After a 20-h acclimatization period, parameters of indirect calorimetry [O<sub>2</sub> uptake (V<sub>O2</sub>) and CO<sub>2</sub> production (V<sub>CO2</sub>)] and caloric intake (SHP diet) were measured for 3 consecutive days. Carbohydrate and fat oxidation rates were calculated from V<sub>O2</sub> and V<sub>CO2</sub> using the following formulas: carbohydrate oxidation (cal/h) = (4.585 × V<sub>CO2</sub> - 3.226 × V<sub>O2</sub>) and fat oxidation (cal/h) = (1.695 × V<sub>O2</sub> - 1.701 × V<sub>CO2</sub>) × 9. Total energy expenditure (EE) was calculated from the sum of carbohydrate and fat oxidation. Physical activity was measured using infrared sensor frames. Interruptions of infrared sensor pairs were detected by a control unit and registered by a computer with the relevant software (ActiMo2; TSE Systems). Body composition and bone mass density were measured by dual-energy X-ray absorptiometry (DEXA) using a Norland pDEXA Sabre scanner (Norland Stratec, Fort Atkinson, WI). Fecal samples were collected, freeze-dried, and analyzed for gross energy content using adiabatic bomb calorimetry (IKA Calorimeter System C4000; Heitersheim, Germany). The energetic ratio was calculated as the EE (kcal/day) divided by the metabolizable energy (kcal/day; kcal ingested minus kcal lost in feces). All measurements were done at average PND 40 and 120. At PND 130, an indirect calorimetric analysis was performed during 48-h caloric starvation (no SHP diet; water freely available), followed by 72 h of refeeding.

**Statistical analysis.** Data are expressed as means ± SE. Longitudinal body weight, longitudinal endocrine (leptin, insulin, and glucose), intravenous insulin–tolerance tests (IVITT), intravenous...
glucose-tolerance tests (IVGTT), and longitudinal body core temperature data were analyzed using a repeated-measures ANOVA followed by a Tukey’s honestly significant difference post hoc analysis. The statistical analysis included the within-subjects factors of time (days or hours) and genotype (pmch<sup>+/+</sup>, pmch<sup>−/−</sup>). All other data were analyzed using Student’s t-test. All data were analyzed using a commercially available statistical program (SPSS for Macintosh, version 16.0). The null hypothesis was rejected at the 0.05 level.

**Supplemental materials and methods.** Additional materials and methods for this article can be found in the Supplemental Materials and Methods available online at the Am J Physiol Endocrinol Metab website.

**RESULTS**

**Generation of the Pmch knockout rat.** In a large ENU-driven target-selected mutagenesis screen, we identified a rat carrying a heterozygous mutation in Pmch (61). The mutation (K50X) resulted in a premature stop codon in exon 1 (Fig. 1A). Northern blot analysis showed that Pmch mRNA is almost completely absent in pmch<sup>−/−</sup> animals, most likely as a result of nonsense-mediated decay (Fig. 1B). Furthermore, Pmch expression is gene dose-dependent reduced in pmch<sup>+/−</sup> rats.

The knockout phenotype was confirmed by immunohistochemistry, which showed that all three neuropeptides derived from Pmch, N-GE, N-EI, and MCH, are absent in sections of the lateral hypothalamus of pmch<sup>−/−</sup> animals (Fig. 1C).

**Pmch knockout rats are lean and hypophagic.** The body weight of pmch<sup>+/+</sup> and pmch<sup>−/−</sup> rats was monitored on three different diets (M, SHP, and HF) for 18 wk starting at PND 21. At the end of the study, pmch<sup>−/−</sup> animals showed a lower body weight compared with pmch<sup>+/+</sup> rats on all three diets (Fig. 2, A–C). Body weight did not differ between genotypes at birth, or between birth and the third postnatal week (data not shown), but started to diverge ~3 wk after birth. Furthermore, pmch<sup>−/−</sup> rats showed a reduction in naso-anal body length at 6 and 13 wk (M and SHP diet) and 12 wk (HF diet) of age (Supplemental Fig. S1), suggesting an impaired growth that could be a secondary effect of the decreased caloric intake. When the study was completed, relative body weight of pmch<sup>−/−</sup> rats was 78% (M), 79% (SHP), and 82% (HF) compared with pmch<sup>+/+</sup> rats (Fig. 2, A–C). Pmch<sup>+/+</sup> as well as pmch<sup>−/−</sup> rats on the HF diet showed a higher body weight at the end of the

![Fig. 1. Confirmation of the Pmch knockout rat.](image-url)

**Fig. 1.** Confirmation of the Pmch knockout rat. **A:** sequencing revealed an induced premature stop codon in the first exon (K50X) in the melanin-concentrating hormone (MCH) precursor gene (indicated in schematic overview). Light grey bar indicates the probe used for Northern blot analysis. Light grey bar indicates the probe used for Northern blot analysis. **B:** Northern blot analysis of whole brain tissue demonstrated that the premature stopcodon results in almost complete loss of Pmch mRNA in animals homozygous (HOM) for the mutation and showed a gene dose-dependent reduction in Pmch expression in heterozygous (HET) rats [53% expression compared with wild type (WT)]. **C:** immunohistochemistry (×250 enlargement) revealed that all 3 neuropeptides derived from the Pmch precursor, neuropeptide glycine-glutamic acid (N-GE), neuropeptide glutamic acid-isoleucine (N-EI), and MCH are absent in hypothalamic sections derived from pmch<sup>−/−</sup> animals. 3V, third ventricle; ic, internal capsule; f, fornix; IHy, incerto hypothalamic area; LHA, lateral hypothalamic area.
Data are means on semi-high-protein (SHP) diet (Fig. 2, study compared with the M or SHP diet (109 and 112% respectively; Fig. 2, A–C). This indicates that pmch−/− animals are capable of increasing their body weight when presented with a HF diet. Longitudinal analysis of caloric intake showed that pmch−/− animals were hypophagic on the M diet (Fig. 2D), SHP diet (Fig. 2E), and HF diet (Fig. 2F). Caloric intake measured during 6 consecutive days in 8- and 17-wk-old rats confirmed these observations (Supplemental Fig. S2A) and showed the hypophagia occurred both during the light and dark phase (Supplemental Fig. S2C). Water intake was decreased in 8-wk-old pmch−/− rats on all diets and unchanged (M diet), decreased (SHP diet), or increased (HF diet) in 17-wk-old pmch−/− rats compared with pmch+/+ rats (data not shown).

Body analysis and endocrine profile of Pmch knockout rats. Body analysis of 26-wk-old pmch−/− rats (M, SHP, and HF diet) revealed a decrease in adipose tissue, even if adipose tissue was normalized for total body weight (Fig. 3A). Liver weights were lower but showed no difference when normalized for total body weight, indicating that liver weights were proportional to the body weights (Fig. 3A). Blood analysis in 24-wk-old pmch−/− rats revealed lower plasma leptin concentrations on all three diets compared with the pmch+/+ rats (Fig. 3B). Plasma glucose concentrations did not differ in rats on the M diet and tended to be higher in pmch−/− rats on the SHP diet, while on the HF diet pmch−/− rats showed higher plasma glucose concentrations compared with pmch+/+ rats (Fig. 3C). Plasma insulin concentrations were lower in pmch−/− rats on the M or SHP diet but did not differ between genotypes on HF diet (Fig. 3D). Plasma insulin concentrations in pmch−/− rats seemed lower during the end of the dark phase (0400) on all three diets (Fig. 3D). A hyperinsulinemic euglycemic clamp study in body weight-matched pmch−/− and pmch+/+ rats revealed no difference in basal glucose levels (pmch+/+: 5.73 ± 0.09 mmol/l; pmch−/−: 5.50 ± 0.12 mmol/l; P = 0.14 by Student’s t-test) or insulin levels (pmch+/+: 1.23 ± 0.15 ng/ml; pmch−/−: 1.00 ± 0.18 ng/ml; P = 0.36 by Student’s t-test) during an equilibrium state in the early afternoon. However, pmch−/− rats showed a lower basal endogenous glucose production (EGP) compared with pmch+/+ rats, reflecting a decreased metabolic clearance rate (Fig. 3E). Under hyperinsulinemia (pmch+/+: 2.16 ± 0.08 ng/ml; pmch−/−: 2.24 ± 0.09 ng/ml; P = 0.25 by Student’s t-test), both pmch+/+ and pmch−/− rats showed a reduction in EGP, but no differences were found between groups (Fig. 3E). When comparing hyperinsulinemic to basal values, insulin-mediated suppression on the EGP did not differ between genotypes (pmch+/+: −63.07 ± 3.67%; pmch−/−: −62.13 ± 2.86%; P = 0.84 by Student’s t-test). In addition, glucose disappearance rate (Ra) did also not differ between genotypes (pmch+/+: 94.21 ± 5.43 μmol · kg·1 · min−1; pmch−/−: 99.01 ± 5.74 μmol · kg·1 · min−1; P = 0.56 by Student’s t-test). These data indicate that
Pmch knockout rats have a functional and dynamic insulin system for maintaining the basal glucose production and utilization. In line with this, IVITT revealed no difference between genotypes in whole body insulin sensitivity (Supplemental Fig. S3A). Interestingly, IVGTT showed a trend toward a slightly delayed glucose removal in response to a glucose bolus in Pmch−/− rats on the SHP or HF diet (Supplemental Fig. S3A). The hypothalamic-pituitary-adrenal axis activity was also investigated in the 26-wk-old Pmch−/− rats, finding a decreased thymus weight in Pmch−/− rats on SHP diet (Pmch−/−: 0.351 ± 0.018 g, Pmch+/+: 0.263 ± 0.014 g; P < 0.05 by Student’s t-test; n = 3–4 per group) but no difference on the other two diets (data not shown). Weight of the adrenals did not differ on any diet (data not shown). Plasma corticosterone levels at 0800 and
2000 also showed no differences between pmch-/- and pmch+/- rats on the M diet (data not shown).

Basal physical activity and body core temperature. Basal physical activity measured in 10-wk-old (M, SHP, and HF) or 19-wk-old (HF) rats using a home-cage monitoring system did not differ between pmch+/- and pmch-/- rats (Supplemental Fig. S4A). Body core temperature measured using telemetry revealed no significant difference between 25-wk-old pmch+/- and pmch-/- rats on the SHP diet (Supplemental Fig. S4B). However, pmch-/- rats showed two small peaks in body core temperature during the night phase compared with pmch+/- rats (Supplemental Fig. S4B).

Pmch knockout rats have an altered energy balance set point. The relative body weight of pmch-/- rats on all diets decreased ~20% during the first 7 wk compared with pmch+/- rats, but this difference stabilized quite abruptly during wk 8 (Fig. 4A). After this stabilization, the relative body weight difference stayed stable during the remainder of the study and the average remained ~79, 79, and 83% (M, SHP, and HF, respectively) compared with pmch+/- rats (Fig. 4A). The observed stabilization occurred exactly during the same week of age, postnatal week 8 (PND 50–56), with all three diets. These results indicate that the energy balance is set differently in pmch-/- rats when entering adulthood and is maintained at a lower level during adulthood. The observed deviation in body weight between pmch+/- and pmch-/- rats during the first 8 wk is mirrored by the weekly body weight growth rate, which is decreased in pmch-/- rats during the first 8 wk compared with pmch+/- rats on all three diets, but approaches the level of pmch+/- rats during adulthood (Supplemental Fig. S5, A–C). This indicates that the body weight growth rate is only decreased in pmch-/- rats during the first 8 wk. Body weight gain per calories was increased in 7-wk-old pmch-/- rats compared with pmch+/- rats, although not significantly on the M diet (Supplemental Fig. S5D). The same pattern was observed in 14-wk-old rats (Supplemental Fig. S5E), indicating that although lean, the growth efficiency of pmch-/- rats is improved compared with pmch+/- rats after ingesting the same amount of calories.

Pmch knockout rats show relative hypophagia during early development and relative hyperphagia during adulthood. To investigate the sudden stabilization of relative body weight, we normalized caloric intake for body weight. After normalization for body weight and shown as relative caloric intake, young pmch-/- rats (<PND 55) showed slight hypophagia, while adult pmch-/- rats (>PND 60) showed slight hyperphagia on M diet (Fig. 4B), SHP diet (Fig. 4C), and HF diet (Fig. 4D) compared with pmch+/- rats. Caloric intake measured during 6 consecutive days in 8- and 17-wk-old rats confirmed these observations (Supplemental Fig. S2, B and D). A same “biphasic” pattern was observed for relative water intake on all diets (data not shown).

Hypothalamic gene expression. Because the hypothalamus is an important brain region regulating energy balance, the relative expression of a subset of hypothalamic genes in young (PND 40) and adult (PND 100) rats was investigated. At both PND 40 and 100, expression of Pmch was almost undetectable in pmch-/- rats compared with pmch+/- rats (Fig. 5, A and B). At PND 40, expression of Fatso (Fto) was decreased, while expression of Mchr1, pro-opiomelanocortin (Pomc), cocaine- and amphetamine-regulated transcript (Cartpt), neuropeptide-Y (Npy), agouti-related peptide (Agrp), and hypocretin (Hcrt; also known as orexin) was unchanged in pmch-/- rats compared with pmch+/- rats (Fig. 5A). At PND 100, expression of Hcrt and Fto was increased, expression of Mchr1 and Pomc was decreased, and expression of Cartpt, Npy, and Agrp was unchanged in pmch-/- animals compared with pmch+/- rats (Fig. 5B).

EE in Pmch knockout rats. To investigate how loss of Pmch affects EE, indirect calorimetric analysis was performed using metabolic cages at PND 40 and 120. Pmch-/- rats showed a lower body weight gain over time, again characterized by a
Fig. 5. Hypothalamic gene expression in *Pmch* knockout rats. A: gene expression of a selection of hypothalamic neuropeptides at PND 40. Relative expression of *Pmch* and *Fto* is decreased in *pmch*+/− rats compared with *pmch*+/+ rats (*P < 0.05 by Student's t-test). B: gene expression of a selection of hypothalamic neuropeptides at PND 100. Relative expression of *Pmch*, *Mchrl*, and *Pomc* is decreased, while expression of *Hcrt* and *Fto* is increased in *pmch*+/− rats compared with *pmch*+/+ rats (*P < 0.05 by Student’s t-test). Data are means ± SE (n = 9 per group). *Mchrl*, melanin-concentrating hormone receptor 1; *Pomc*, pro-opiomelanocortin; *Carptt*, cocaine- and amphetamine-regulated transcript; *Npy*, neuropeptide-Y; *Agrp*, agouti-related peptide; *Hcrt*, hypocretin; *Fto*, Fatso.

sudden stabilization of relative body weight difference (Fig. 6A). Lean mass did not differ between genotypes at PND 40 but was decreased at PND 120 (Fig. 6B). Fat mass was decreased in *pmch*−/− rats at both PNDs compared with *pmch*+/+ rats (Fig. 6C). Food intake was lower in *pmch*−/− rats at both PNDs compared with *pmch*+/+ rats (Fig. 6D). After normalization for lean mass, food intake remained lower compared with *pmch*+/+ rats (Fig. 6E). In contrast, if data were normalized for total body mass food intake did not differ between genotypes (Fig. 6F). Absolute EE in *pmch*−/− rats was decreased at PND 40 and 120 compared with *pmch*+/+ rats (Fig. 6G). The decreased EE in *pmch*−/− rats was characterized by a decreased carbohydrate oxidation, while fat oxidation was equal between genotypes (data not shown). EE normalized for lean mass in *pmch*−/− rats was decreased at PND 40 during the light and dark phase and both during the light and dark phase (Fig. 6G). The decreased EE in *pmch*−/− rats was approaching the end of the starvation, while fat oxidation was equal between genotypes (data not shown). The decreased body weight regain trend in *pmch*−/− rats was reflected by an impaired refeeding response compared with *pmch*+/+ rats (Supplemental Fig. S6F). Additionally, both *pmch*+/+ and *pmch*−/− rats showed hyperphagia compared with basal caloric intake levels at PND 120 (87.5 vs. 70.2 and 73.2 vs. 60.9 kcal/day, respectively; Fig. 6D; Supplemental Fig. S6F).

**Testosterone does not induce the observed stabilization of relative body weight.** As the observed stabilization of relative body weight and “switch” in nutrient intake behavior appeared around the end of rat puberty (approximately between PND 55 and 65), we tested the hypothesis that changes in blood testosterone levels induced our observed phenotype. Orchiectomy during postnatal week 5 reduced the body weight of *pmch*+/+ and *pmch*−/− rats compared with sham-operated *pmch*+/+ and *pmch*−/− rats (Supplemental Fig. S7A). Both orchietomized *pmch*+/+ and *pmch*−/− rats showed a decrease in relative body weight compared with sham-operated rats, although no clear stabilization pattern was observed around week 8 (Supplemental Fig. S7, B and C). Sham-operated *pmch*−/− rats showed a clear stabilization of relative body weight compared with sham-operated *pmch*+/+ rats around week 8, confirming observations from untreated animals (Fig. 4C; Supplemental Fig. S7D). However, orchietomized *pmch*−/− rats also showed a stabilization of relative body weight compared with orchietomized *pmch*+/+ rats around week 8 (Supplemental Fig. S7E). Serum free testosterone levels in *pmch*−/− rats showed no difference on SHP diet around PND 40 and a decreased trend on PND 60, and levels were decreased on PND 120 compared with *pmch*+/+ rats (Supplemental Fig. S7F). Orchietomy resulted in almost undetectable levels in both genotypes (Supplemental Fig. S7F).

**Pmch knockout rats develop osteoporosis.** Bone mass density was reduced in *pmch*−/− rats at both PND 40 and 120 compared with *pmch*+/+ rats (Supplemental Fig. S8).

**DISCUSSION**

The key findings of this work are the demonstration that *Pmch* expression during early development and puberty is of critical importance for a normal energy balance and that loss of *Pmch* results in a 20% decreased energy balance that is maintained during adulthood.
While the role of MCH in energy regulation is well established, it should be noted that the entire \textit{Pmch} gene is inactivated in our rat model and that the less well-characterized neuropeptides N-GE and N-EI are not expressed. Although N-GE so far does not seem to have a biological function, N-EI is implicated in modulatory action on anxiety- and sexual-related behavior in female rats (20), increases luteinizing hormone release (2), and stimulates grooming, locomotion, and

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**Fig. 6. Energy expenditure in \textit{Pmch} knockout rats.**

A: body weight is decreased in \textit{pmch}^{-/-} rats compared with \textit{pmch}^{+/+} rats [$F(1,18) = 36.3, P < 0.001$]. Relative body weight stabilizes during week 7 (inset). Metabolic measurements are indicated by a triangle. B: lean mass was decreased in \textit{pmch}^{-/-} rats at PND 120 but not at PND 40 compared with \textit{pmch}^{+/+} rats ($P < 0.05$ by Student’s $t$-test). C: fat mass was decreased in \textit{pmch}^{-/-} rats at PND 40 and 120 compared with \textit{pmch}^{+/+} rats ($P < 0.05$ by Student’s $t$-test). D: food intake was decreased in \textit{pmch}^{-/-} rats at PND 40 and 120 compared with \textit{pmch}^{+/+} rats ($P < 0.05$ by Student’s $t$-test). E: food intake normalized for lean mass (LM) was decreased in \textit{pmch}^{-/-} rats at PND 40 and 120 compared with \textit{pmch}^{+/+} rats ($P < 0.05$ by Student’s $t$-test). F: energy expenditure was decreased in \textit{pmch}^{-/-} rats at PND 40 and 120, both during the light (L) and dark (D) phase compared with \textit{pmch}^{+/+} rats ($P < 0.05$ by Student’s $t$-test). G: energy expenditure normalized for lean mass was decreased in \textit{pmch}^{-/-} rats during the PND 40 light and dark phase ($P < 0.05$ by Student’s $t$-test) but showed no difference during the PND 120 dark phase compared with \textit{pmch}^{+/+} rats. H: energy expenditure normalized for body weight showed increased trends in \textit{pmch}^{-/-} rats during the PND 40 light and dark phase ($P < 0.05$ by Student’s $t$-test) and was equal during the PND 120 dark phase compared with \textit{pmch}^{+/+} rats. I: fecal output (dry wt per day) was decreased in \textit{pmch}^{-/-} rats at PND 40 and 120 compared with \textit{pmch}^{+/+} rats ($P < 0.05$ by Student’s $t$-test). J: fecal energy loss (kcal per day) was decreased in \textit{pmch}^{-/-} rats at PND 40 and 120 compared with \textit{pmch}^{+/+} rats ($P < 0.05$ by Student’s $t$-test). K: energetic ratio (EE per day divided by metabolizable energy per day) showed an increased trend in \textit{pmch}^{-/-} rats at PND 40 ($116\%: P = 0.058$ by Student’s $t$-test) but was equal between genotypes at PND 120 compared with \textit{pmch}^{+/+} rats ($105\%: P = 0.32$ by Student’s $t$-test). Data are means $\pm$ SE ($n = 8$ per group).

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AJP-Endocrinol Metab • VOL 298 • MARCH 2010 • www.ajpendo.org
rearing in male rats (57). These additional neuropeptides could perform as of yet unknown functions, thereby contributing to phenotypes observed in this study.

Pmch−/− rats have a lower body weight and are hypophagic. Plasma leptin levels were lower in pmch−/− rats, correlating with the lower adipose amounts. Two independent studies (48, 65) showed that MCH is a positive regulator of insulin release; thus loss of Pmch expression could explain the decreased basal plasma insulin levels and the delayed glucose clearance in the IVGTT studies. Basal insulin levels were lower especially at plasma insulin levels and the delayed glucose clearance in the slight hyperphagia compared with hypophagia, while adult young phagia, while adult young phenotypes observed in this study.

It is however important to note that loss of Pmch does not mimic temporary caloric restriction with accompanying body weight regain after refeeding, but results in a model of chronic voluntary caloric restriction as rats were allowed to feed ad libitum throughout their life. Our findings suggest that Pmch expression drives energy intake and storage to levels exceeding the minimal need to grow during early development and puberty but that this “overstimulation” disappears around post-natal week 8. During adulthood, Pmch expression remains functional during times of starvation (50). However, our findings indicate that Pmch expression is especially relevant during early development and puberty. This is supported by the observation that the energy balance in pmch−/− rats remained −80% compared with pmch+/+ rats during the remainder of our study. Moreover, nest-size induced food restriction of pups until PND 25 decreases relative body weight, and this difference in relative body weight diminishes again when animals are given ad libitum access to chow after PND 25 (51). However, the catch-up growth is incomplete as relative body weight stabilizes around week 9, reflected by an increased growth velocity in restricted rats until week 8 and no difference in growth velocity after week 8 (51).

The relative body weight of pmch+/+ and pmch−/− rats started to diverge during postnatal week 3, when the pups start to feed at their own, and stabilized around postnatal week 8 for the remainder of the study. Increased oxygen consumption has been shown for 20-wk-old mice with a loss of Pmch and 17-wk-old transgenic mice with a severe loss of MCH neurons (1, 59). It is however important to note that in both situations data were normalized for body weight. As it is unknown if the metabolic activity of WAT is altered in pmch−/− rats and as it has been demonstrated that WAT has a minimal contribution to EE (13), we chose to show EE not normalized, normalized for lean mass, and normalized for body weight. EE data normalized for body weight showed increased or a trend toward increased EE levels in pmch−/− rats compared with pmch+/+ rats, which is in line with findings that oxygen consumption levels are increased in MCH−/− mice if normalized for body weight (1, 59). However, EE not normalized or EE normalized for lean mass showed decreased or a trend toward decreased levels in pmch−/− rats compared with pmch+/+ rats. Therefore, the higher amount of nonmetabolically active WAT in pmch+/+ rats might be a confounding factor underlying the calculation of EE, and normalization for body weight can result in incorrect conclusions.

The energetic ratio, calculated by correcting EE values for the amount of metabolizable energy, i.e., the energy absorbed by the rat, showed an increased trend at PND 40 but did not differ at PND 120. These data explain why young pmch−/− rats (<PND 55) increase their body weight at a slower pace compared with pmch+/+ rats, whereas older pmch−/− rats increase their body weight at a similar pace compared with pmch+/+ rats. We therefore conclude that loss of Pmch decreases rather than increases EE in the rat and that the change in EE is secondary to the change in caloric intake. This hypothesis is strengthened by our observations that body weight loss is equal between genotypes during 48-h starvation and that pmch−/− rats show a refeeding deficit compared with pmch+/+ rats during 72-h refeeding. The lower absolute EE values might be related to the lower caloric intake due to a lower thermic effect of food (12).

Basal locomotor activity did not differ between genotypes at various ages (PND 40 or 120, week 10/11 or 19), with various diets (M, SHP, or HP), and with different techniques (metabolic cage or phenotyper). This indicates that physical activity does not contribute to the lean phenotype of Pmch-deficient rats and supports the idea that loss of Pmch primarily results in a decrease of caloric intake. Interestingly, the Mchr1 knockout

AJP-Endocrinol Metab • VOL 298 • MARCH 2010 • www.ajpendo.org
mice show hyperphagia and an increased physical activity (8, 38). Although loss of Pmch or Mchr1 both produce a lean phenotype, the aberrant behavior resulting in leanness is different (i.e., normal activity vs. hyperactivity, and hypophagia vs. hyperphagia), and no explaining mechanisms have been proposed to date.

Hypothalamic Pmch mRNA expression increases slowly during early development, increasing more rapidly after weaning, and stabilizes in 8-wk-old rats (49). Pmch is also expressed in Sertoli cells in rat testis where expression increased strongly between PND 15 and adulthood (25). Relative hypothalamic Pmch expression was almost undetectable in pmch /−/ rats at PND 40 and PND 100 compared with pmch +/+ rats, confirming our Northern blot analysis. Relative Mchr1 expression did not differ between genotypes at PND 40, while being decreased at PND 100 compared with pmch +/+ rats, suggesting a feedback system affecting Mchr1 expression during adulthood. Relative Fto expression in pmch /−/ rats was decreased at PND 40 and increased at PND 100 compared with pmch +/+ rats, while relative expression of Pomc and Hcrt was normal at PND 40 but was decreased and increased, respectively, in pmch /−/ rats compared with pmch +/+ rats at PND 100. The expression profiles of Npy and Agrp in adult rats confirm findings in adult mice; however, the expression profiles of Pomc and Hcrt either partially agree or disagree with findings in adult mice (1, 59). In summary, the time-related differences in expression profiles and a likely interaction between Pmch and the orexigenic and anorectic systems studied here could offer an explanation to the sudden stabilization of relative body weight but remain to be studied in more detail.

The stabilization of relative body weight during the end of puberty suggested a functional interaction between Pmch and gonadal steroids, such as testosterone. However, free testosterone levels on PND 40 did not differ between genotypes and orchietomy during postnatal week 5 lowered the body weight of orchietomized rats compared with sham-operated rats within genotypes but did not affect the observed stabilization in relative body weight between pmch +/+ and pmch /−/ rats around week 8. This indicates that testosterone affects the energy balance but is not essential to induce the observed stabilization of relative body weight. Moreover, free-testosterone levels were decreased in pmch /−/ rats on PND 120, suggesting that the decreased energy balance level influenced free testosterone levels.

The osteoporotic phenotype in pmch /−/ rats was already observed at PND 40 and confirms the finding that MCHR1 knockout mice develop high bone turnover osteoporosis (4). Energy restriction is known to decrease bone mass density in adult rats (37), indicating that the decreased energy balance in pmch /−/ rats could result in osteoporosis. Hypogonadism is another known induer of osteoporosis (15, 63). However, pmch /−/ rats were already osteoporotic, while serum-free testosterone levels were indifferent compared with pmch +/+ rats at PND 40, suggesting that loss of MCH signaling leads to osteoporosis independently of androgen deficiency.

The body weight of adult humans is normally relatively stable, with only a very small variance over a long period of time (30, 52). Classic studies in rodents have shown that stable body weights are actively maintained when animals receive caloric restriction or when the rat’s body weight is experimentally elevated; animals quickly restored their body weight to the level appropriate for their age and gender when returned to standard conditions (62). In humans, dieting strategies combining energy restriction and physical activity have shown moderate success for the reduction of body weight (23, 26). However, many individuals who have lost weight using a dieting strategy will regain a large proportion or all of the weight lost within 5 years from the end of the treatment (6, 14, 67), although low-fat intake in combination with high activity can successfully slow the regain of weight (34, 68). Even though “short-term” (>4 wk) MCHR1-antagonist studies are successful in decreasing body weight in adult rats and mice (24, 36, 45), it would be very interesting to see if “long-term” (>4 wk) MCHR1 antagonism can chronically alter the energy balance of adult animals successfully. Because our data indicate that loss of Pmch can lower the energy balance and that Pmch expression is important during early development and puberty, it would be even more interesting to study the effect of MCHR1 antagonism on the determination of the energy balance in young animals.

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DISCLOSURES

No conflicts of interest are declared by the author(s).

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AJP-Endocrinol Metab • VOL 298 • MARCH 2010 • www.ajpendo.org
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