Hypothalamic neural networks in control of glucose homeostasis
Yi, C.X.

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.

UvA-DARE is a service provided by the library of the University of Amsterdam (http://dare.uva.nl)
Pmch expression during early development is a critical determinant of energy homeostasis

The American Journal of Physiology – Endocrinology and Metabolism (under revision)
Joram D. Mul, Chun-Xia Yi, Pim W. Toonen, Martine C.J. van der Elst, Bart A. Ellenbroek, Andries Kalsbeek, Susanne E. la Fleur, and Edwin Cuppen

Abstract
Postnatal development and puberty are times of strong physical maturation and require large quantities of energy. The hypothalamic neuropeptide Melanin-Concentrating Hormone (MCH) is known to stimulate nutrient intake and regulate energy homeostasis, but the underlying mechanisms are largely not understood. Here we used a novel rat knockout model in which the \textit{Pmch} gene has been inactivated to study the effects of loss of MCH on energy regulation in more detail. \textit{Pmch}^{-/-} rats were found to be lean, confirming earlier findings in MCH knockout mice. While endocrine parameters were changed in \textit{pmch}^{-/-} rats, endocrine dynamics were normal, indicating an adaptation to new homeostatic levels rather than disturbed metabolic mechanisms. Detailed analysis of feeding behavior revealed that \textit{pmch}^{-/-} animals are hypophagic during early development and switch to hyperphagia after entering adulthood. Moreover, our data show that loss of \textit{Pmch} results in a 20% lower set point for body weight that is determined solely during the first 8 postnatal weeks and remains unchanged during adulthood. Although the final body weight depends on the diet, the \textit{Pmch} knockout effect is similar for all diets tested in this study. Current findings show an important role for \textit{Pmch} in energy homeostasis determination during early development, and indicate that the MCH-Melanin-Concentrating Hormone Receptor 1 (MCH1R)-system is a plausible target for childhood obesity treatment, currently a major health issue in first world countries.

Introduction
Childhood obesity is now widely recognized as a severe public health issue. 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 three postnatal weeks. 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 (day 40) and young-adult (day 60) male rats to environmental cues like stress and cold. 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. 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.

MCH has been shown to be a critical mammalian hypothalamic effector of energy homeostasis by various genetic and pharmacological studies. The MCH precursor gene (Pmch) is expressed predominantly in neurons of the lateral hypothalamic area (LHA) and the incerto hypothalamic area (IH), which project throughout the brain. Pmch is also expressed in some peripheral tissues, such as the testis, although at lower levels than in brain. Processing of the preprohormone Pmch results in the production of three neuropeptides: neuropeptide glycine-glutamic acid (N-GE), neuropeptide glutamic acid-isoleucine (N-EI) and MCH. However, most studies have focused on MCH. Pmch mRNA is up regulated after fasting or leptin deficiency, and third ventricle ICV injections of MCH increase food intake and body weight. Pmch knockout mice are lean due to a decreased food intake and an increased metabolic rate, and overexpression of MCH causes obesity. In rodents MCH binds to MCH1R, a G-protein coupled receptor expressed throughout the brain. MCH1R is particularly enriched in the nucleus accumbens shell (AcbSh), thus forming a potential hypothalamic-limbic circuit modulating the hedonic, or rewarding, aspects of feeding. Rodents only express MCH1R, whereas humans also express a second MCH receptor, MCH2R. Recent studies 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 ENU-driven target-selected mutagenesis approach. Preliminary studies in young-adult animals showed that the caloric intake of pmch−/− rats was unchanged compared to control littermates when nutrient intake data was normalized for body weight. Following this initial observation we have analyzed the metabolic characteristics of this knockout (KO) rat model on 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. 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 the diet.

**Methods**

**Animals**
The Animal Care Committee of the Royal Dutch Academy of Science approved all experiments according to the Dutch legal ethical guidelines. The Pmch KO rat (Pmch-1Hubr) was generated by target-selected ENU-driven mutagenesis (see 562). Briefly, high-throughput resequencing of genomic target sequences in progeny from mutagenized rats revealed an ENU-induced premature stop codon in exon 1 (K50X) of 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 approximately 28.6 x10^6 bp 536 and the used ENU treatment resulted in a mutation frequency of 1 per 1.5x10^6 bp 562, approximately 19 mutations can be expected in 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 (NIMs) is much lower than 19, i.e. 3 563. However, as part of the donor chromosome harboring the Pmch mutation is still present after six backcrosses 564, we cannot fully exclude the presence of tightly linked confounding mutations in our model. To further control for possible contributions of confounding mutations, we have repeated several measurements in different outcross generations and could replicate previous findings in each generation. Additionally, we have always generated experimental KO and wild type animals by crossing pmch +/- animals. Experimental animals were obtained at the expected Mendelian frequency. Furthermore, littermates (with similar genetic backgrounds) were used as much as possible for experiments. Pmch +/- rats were viable into adulthood and fertile, and appeared phenotypically normal despite their decreased body weight. Two rats were housed together, unless noted otherwise, under controlled experimental conditions (12 h light/dark cycle, light period 0600-1800, 21±1°C, ~60% relative humidity). Standard fed diet (semi high-protein chow: RM3, 27% CP and 12% F, 3.33 kcal/g AFE, SDS, Witham, United Kingdom) and water was provided ad libitum unless noted otherwise (maintenance chow: RM1, 17% CP and 7% F, 3.29 kcal/g AFE, SDS, Witham, United Kingdom; high-fat chow: 45%-AFE, 20% CP and 45% F, 4.54 kcal/g AFE SDS, Witham, United Kingdom). Only male animals were used in the present study.
Genotyping
Genotyping was done using the KASPar SNP Genotyping System (KBiosciences, Hoddesdon, United Kingdom) using gene-specific primers (forward common, TTAAT ACATT CAGGA TGGGG AAAGC CTTT; reverse wild type, GAAGG TGACC AAGTT CATGCT CGATC TTTCT GCGGT ATCTT CCTT; reverse homozygous, GAAGG TCGGA GTCAA CGGAT TCGAT CTTTC TGCGG TATCT TCCTA). All pups were genotyped during week 3. Genotypes were reconfirmed when experimental procedures were completed.

Northern Blot analysis
Northern Blot analysis 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: ATTCT CCTTC GGCTT TACG; reverse primer: TCCAG AGAAG GAGCA ACAAC.

Immunohistochemistry
A detailed description of the immunohistochemistry procedure is provided in the Supplemental Material and Methods section.

Body weight and nutrient intake monitoring
Pups were housed individually 3 weeks after birth. Until weaning, pups had access to SHP diet in their maternal home cage. Body weight, water intake, and food intake was monitored biweekly for 18 weeks (n = 10-16 per group). Water was freely available, and food (M, SHP, or HF diet) was provided ad libitum. At 8 and 17 weeks of age, nutrient intake was measured for 6 days at 06:00 (dark phase intake) and at 18:00 (light phase intake).

Body composition
A 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, the whole mesenteric WAT pad), liver, adrenals, and the thymus were isolated from 26-week old animals (n = 3-6 per group).

Jugular vein catheter placement
At 22 weeks of age, animals were anaesthetized with isoflurane and equipped with a jugular vein catheter (headpiece: Connector Pedestal 20GA, Plastics One, Roanoke, VA, USA). Before surgery, animals received one dose of Temgesic (0.05 mg/kg, subcutaneous; Schering-Plough, Utrecht, the Netherlands). Animals were allowed to recover for 7 days during which they were handled to minimize stress.
Six time point blood plasma measurements

Blood samples (0.3 ml) were obtained at 24:00, 04:00, 08:00, 12:00, 16:00, and 20:00 (n = 9-13 per group). Blood was collected in EDTA tubes (BD Vacutainer tubes, Plymouth, United Kingdom) containing 20µl aprotinin (Sigma-Aldrich, Zwijndrecht, the Netherlands). Samples were collected on ice and instantly centrifuged at 2150 rcf for 15' at 4°C. Samples were then aliquoted and stored at -80°C until analysis. Plasma leptin levels were determined in duplo using a leptin ELISA (EZRL-83K, Linco Research, St. Charles, Missouri, USA). The assay sensitivity limit was 0.04 ng/ml. The intra- and interassay coefficients of variation were 2.17 and 3.40%, respectively. Plasma insulin levels were determined in duplo using an insulin ELISA (EZRMI-13K, Linco Research, St. Charles, Missouri, USA). The assay sensitivity limit was 0.2 ng/ml. The intra- and interassay coefficients of variation were 1.91 and 7.63%, respectively. Plasma glucose levels were determined in duplo using an OneTouch® Ultrameter® (LifeScan Benelux, Beere, Belgium). Plasma corticosterone levels were determined in duplo using a Corticosterone EIA (DSL Deutschland GMBH – Benelux, Assendelft, NL). The assay sensitivity limit was 1.6 ng/ml. The intra- and interassay coefficients of variation were 3.23 and 4.77%, respectively.

Hyperinsulinemic-euglycemic clamp

A detailed description of the hyperinsulinemic-euglycemic clamp procedure is provided in the Supplemental Material and Methods section.

Statistics

Data are expressed as mean ± S.E.M. Fig. 2B-E, 3A, 3E, 4A-D, S1A-D, S2, S4A, and S6F were analyzed using a two-tailed Students’ t-test. In Fig. 2A, 3B-D, S3A-B, S4B, S5A-C, and S6A overall results were analyzed using a two-way repeated-measure ANOVA with a Tukey-HSD post hoc correction for multiple comparisons, followed by a two-tailed Students’ t-test analysis for each time point if the ANOVA showed mean differences. All data were analyzed using a commercially available statistical program (SPSS for Windows, version 15.0). The null hypothesis was rejected at the 0.05 level.

Results

Generation of the Pmch KO rat

In a large ENU-driven target-selected mutagenesis screen we identified a rat carrying a heterozygous mutation in Pmch562. 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-/- animals, most likely as a result of nonsense-mediated decay (Fig. 1B). Furthermore, there is a gene dose-dependent reduction in Pmch expression in pmch+- rats. The KO 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−/− animals (Fig. 1C).

**Pmch KO rats are lean and hypophagic**
The body weight of pmch−/− animals and wild type siblings was monitored on three different diets (M, SHP, and HF) for 18 weeks starting at postnatal week 3. Pmch−/− animals showed a reduced body weight and a lean phenotype compared to wild type siblings on all three different diets. A difference in body weight between genotypes was not present at birth, or between birth and the 3rd postnatal week (data not shown), and only became visible approximately 3 weeks after birth. When the monitoring was

![Figure 1](image-url)

**Figure 1** Confirmation of the Pmch KO rat. A, Sequencing revealed an induced premature stop codon in the first exon (K50X) in the MCH precursor gene (indicated in schematic overview). The light grey bar indicates the probe used for the Northern Blot analysis. B, Northern Blot analysis of whole brain tissue demonstrated that the premature stop codon results in almost complete loss of Pmch mRNA in animals homozygous for the mutation, and showed a gene dose-dependent reduction in Pmch expression in heterozygous animals (53% expression compared to WT). C, Immunohistochemistry (250x enlargement) revealed that all three neuropeptides derived from the Pmch precursor, N-GE, N-EI, and MCH, are absent in hypothalamic sections derived from pmch−/− animals. Abbreviations: 3V, third ventricle; ic, internal capsule; f, fornix; IHy, incerto hypothalamic area; LHA, lateral hypothalamic area.
stopped after 18 weeks, relative body weight of the pmch\textsuperscript{+/+} animals was 78\% (M), 79\% (SHP), and 82\% (HF) compared to wild type siblings (Fig. 2A). Interestingly, not only wild type animals on HF diet showed a strong increase in body weight compared to wild type animals on M or SHP diet (109\% and 112\% increase, respectively), also pmch\textsuperscript{+/+} animals on HF diet showed a strong increase in body weight compared to pmch\textsuperscript{+/+} animals on M or SHP diet (114\% and 117\%, respectively), thus indicating that pmch\textsuperscript{+/+} animals are capable of increasing their body weight when presented with a HF diet (Fig. 2A). Caloric intake (24-hr) was recorded at 8 and 17 weeks of age showing that pmch\textsuperscript{+/+} animals are significantly hypophagic at both ages on all three diets (Fig. 2B). The reduction in caloric intake occurred both during the light and dark phase (see Supplementary Data, Fig. S1A). Water intake was also monitored showing that pmch\textsuperscript{+/+} animals drink significantly less water at 8 weeks on all diets (Fig. 2D, S1C). However, at 17 weeks of age, water intake was unchanged (M diet), decreased (SHP diet), or increased (HF diet) in pmch\textsuperscript{+/+} animals (Fig. 2D, S1C). Furthermore, pmch\textsuperscript{+/+} animals showed a small but significant decrease in naso-anal body length at 6 and 13 weeks (M, SHP diet) and 12 weeks (HF diet) of age (Fig. S2), suggesting a slightly impaired growth that could be a secondary effect of the decreased caloric intake.

**Body analysis and endocrine profile of Pmch KO rats**

Body composition analysis of 26-week old pmch\textsuperscript{+/+} animals (M, SHP, and HF diet) revealed significantly decreased WAT fat pad weights and liver weights (Fig. 3A). However, no difference was found when liver weights were normalized for body weight, indicating that liver weights are proportional to the body weight (Fig. 3A). Six-time point analysis in 24-week old pmch\textsuperscript{+/+} animals revealed strongly decreased plasma leptin concentrations on all three diets (Fig. 3B). Pmch\textsuperscript{+/+} animals on M diet did not have altered plasma glucose concentrations, whereas pmch\textsuperscript{+/+} animals on SHP diet showed slightly elevated but not significantly changed plasma glucose concentrations, and pmch\textsuperscript{+/+} animals on a HF diet showed significantly elevated plasma glucose concentrations (Fig. 3C). Plasma insulin concentrations were significantly decreased in pmch\textsuperscript{+/+} animals on M or SHP diet, whereas no differences in insulin concentrations were found between pmch\textsuperscript{+/+} and pmch\textsuperscript{+/+} animals on HF diet (Fig. 3D). Interestingly, plasma insulin concentrations in pmch\textsuperscript{+/+} animals were strongly decreased on all three diets during the end of the dark phase (04:00; Fig. 3D). A hyperinsulinemic-euglycemic clamp study revealed no significant difference between body weight-matched pmch\textsuperscript{+/+} and pmch\textsuperscript{+/+} animals in basal plasma glucose levels (pmch\textsuperscript{+/+}: 5.73 ± 0.09 mmol/L; pmch\textsuperscript{+/+}: 5.50 ± 0.12 mmol/L; \(P = 0.14\) by two-tailed Students’ \(t\)-test, \(n = 6\) per group) and basal insulin levels (pmch\textsuperscript{+/+}: 1.23 ± 0.15 ng/ml; pmch\textsuperscript{+/+}: 1.00 ± 0.18 ng/ml; \(P = 0.36\) by two-tailed Students’ \(t\)-test, \(n = 6\) per group) during an equilibrium state in the early afternoon. However, pmch\textsuperscript{+/+} animals did show a significant lower basal endog-
nenous glucose production (EGP) compared to pmch<sup>+/+</sup> animals, reflecting a decreased metabolic clearance rate (MCR) (Fig. 3E). When basal insulin was clamped at approximately two fold of the normal level (pmch<sup>+/+</sup>: 2.16 ± 0.08 ng/ml; pmch<sup>−/−</sup>: 2.24 ± 0.09 ng/ml; P = 0.25 by two-tailed Students’ t-test, n = 6 per group), both pmch<sup>−/−</sup> and pmch<sup>+/+</sup> animals showed a similar EGP (Fig. 3E) and similar rate of glucose disappear-
ance (Rd) \( (pmch^{+/+}: 94.21 \pm 5.43 \mu\text{mol/kg.min}; \ pmch^{+/-}: 99.01 \pm 5.74 \mu\text{mol/kg.min}; \ P = 0.56 \) by two-tailed Students’ \( t \)-test, \( n = 6 \) per group). Additionally, insulin suppression on the EGP also showed no difference \( (pmch^{+/+}: -63.07 \pm 3.67\%; \ pmch^{+/-}: -62.13 \pm 2.86\%; \ P = 0.84 \) by two-tailed Students’ \( t \)-test, \( n = 6 \) per group). This indicates that \( pmch^{+/-} \) animals have a functional and dynamic insulin system for maintaining the basal glucose production and utilization. In line with this, intravenous insulin-tolerance tests (IVITT) revealed no difference between genotypes in whole-body insulin sensitivity (Fig. S3A). Interestingly, intravenous glucose-tolerance tests (IVGTT) showed a trend towards a slightly delayed glucose removal in response to a glucose bolus in \( pmch^{+/-} \) animals on SHP or HF diet (Fig. S3A). The Hypothalamic-Pituitary-Adrenal (HPA) axis activity was also investigated in the 26-week old \( pmch^{-/-} \) animals, and the thymus weight of \( pmch^{-/-} \) animals on SHP diet was found to be decreased \( (pmch^{+/+}: 0.351 \pm 0.018 \text{g}; \ pmch^{+/-}: 0.263 \pm 0.014 \text{g}; \ P < 0.05 \) by two-tailed Student’s \( t \)-test; \( n = 3-4 \) per group) but did not differ 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 08:00 and 20:00 also showed no differences between \( pmch^{+/+} \) and \( pmch^{-/-} \) animals on M diet (data not shown).

**Figure 2** \( Pmch \) KO rats show a decreased body weight and decreased 24-hr caloric intake on three different diets. A, Male \( pmch^{-/-} \) animals showed a decreased body weight over time compared to \( pmch^{+/+} \) control animals on M diet (squares; \( P < 0.001 \)), SHP diet (triangles; \( P < 0.001 \)), and HF diet (circles; \( P < 0.001 \)). HF diet increased body weight over time compared to animals on M or SHP diet, both in \( pmch^{+/+} \) and \( pmch^{-/-} \) animals (WT HF vs. WT M; \( P < 0.001 \); HOM HF vs. HOM M; \( P < 0.001 \); WT SHP vs. WT M; \( P < 0.001 \); HOM SHP vs. HOM M; \( P < 0.001 \)). Animals on M or SHP diet showed no difference over time in body weight within genotype (WT M vs. WT SHP: \( P = 0.23 \); HOM M vs. HOM SHP: \( P = 0.29 \)). All overall longitudinal body weight data were analyzed using a repeated-measure ANOVA. \( Pmch^{+/-} \) animals started showing a reduced body weight per individual measurement after 22 days of age (SHP and HF diet; \( P < 0.05 \) by two-tailed Students’ \( t \)-test) or 26 days of age (M diet; \( P < 0.05 \) by two-tailed Students’ \( t \)-test). B, \( Pmch^{-/-} \) animals ingested fewer calories compared to control animals (M: -26\% and -21\%, 8 and 17 weeks of age, respectively; SHP: -24\% and -17\%, 8 and 17 weeks of age, respectively; HF: -22\% and -15\%, 8 and 17 weeks of age, respectively). C, The hypophagic character of \( pmch^{-/-} \) animals fades at a younger age and completely disappears at an older age when data are normalized for body weight (M: -6\% and +0\%, 8 and 17 weeks of age, respectively; SHP: -3\% and +5\%, 8 and 17 weeks of age, respectively; HF: -2\% and +2\%, 8 and 17 weeks of age, respectively). D, \( Pmch^{-/-} \) animals consumed less water than control animals, except for 17-week old rats on HF diet (M: -24\% and -7\%, 8 and 17 weeks of age, respectively; SHP: -19\% and -6\%, 8 and 17 weeks of age, respectively; HF: -13\% and +10\%, 8 and 17 weeks of age, respectively). E, \( Pmch^{-/-} \) animals drink equal or increased amounts of water compared to control animals when data are normalized for body weight (M: -3\% and +18\%, 8 and 17 weeks of age, respectively; SHP: +2\% and +19\%, 8 and 17 weeks of age, respectively; HF: +8\% and +31\%, 8 and 17 weeks of age, respectively). \( *P < 0.05 \) by two-tailed Student’s \( t \)-test. Data are expressed as mean \pm S.E.M. (\( n = 10-16 \) per group).
Basal locomotor activity and body core temperature

Basal locomotor activity measured during 72 hours in 10-week old (M, SHP, HF) or 19-week old (HF) animals using a home-cage monitoring system did not differ between pmch<sup>+/+</sup> and pmch<sup>−/-</sup> animals (Fig. S4A). Body core temperature measured using telemetry did not reveal a significant difference between adult pmch<sup>+/+</sup> and pmch<sup>−/-</sup> animals on SHP diet (Fig. S4B).

Pmch KO rats show relative hypophagia during early development and relative hyperphagia during adulthood

The hypophagic characteristic of the pmch<sup>−/-</sup> rats is obvious when comparing caloric intake data (Fig. 2B, S1A). However, if caloric intake data are normalized per body weight, this clear pattern disappears and although the differences are very small, pmch<sup>−/-</sup> animals seem slightly hypophagic at 8 weeks of age, and somewhat hyperphagic at 17 weeks of age (Fig. 2C, S1B). As the 24-hr monitoring only showed caloric intake behavior during two short time windows, we decided to longitudinally monitor caloric intake of male pmch<sup>−/-</sup> animals and wild type siblings on M and SHP diet, starting at weaning at 3 weeks of age and ending during adulthood at 18 weeks of age.

In line with our initial data, caloric intake corrected for body weight data of pmch<sup>−/-</sup> animals on M diet showed a decreased caloric intake compared to control animals.

Figure 3 Pmch KO rats show a changed endocrine profile on three different diets although dynamics are intact. A, Pmch<sup>−/-</sup> animals showed decreased body-, total WAT fat pad-, and liver weights compared to pmch<sup>+/+</sup> animals on three different diets (M, SHP, and HF) (n = 3-6 per group). Relative body weight is 78% (M), 80% (SHP), and 77% (HF). Relative total WAT fat pad weight is 48% (M), 51% (SHP), and 54% (HF). Relative liver weight is 71% (M), 75% (SHP), and 74% (HF). However, if liver weights were normalized for body weight, no difference was found between genotypes. B, Pmch<sup>−/-</sup> animals showed decreased plasma leptin levels during 24 hrs on M, SHP, and HF diet (§, P < 0.05 by repeated-measure ANOVA; *P < 0.05 by two-tailed Students’ t-test; n = 8-12 per group). C, Pmch<sup>−/-</sup> animals showed unchanged plasma glucose levels on M diet (P = 0.98 by repeated-measure ANOVA), an elevated trend on SHP diet (P = 0.06 by repeated-measure ANOVA), and elevated glucose levels on HF diet (§, P < 0.05 by repeated-measure ANOVA; *P < 0.05 by two-tailed Students’ t-test) (n = 9-13 per group). D, Pmch<sup>−/-</sup> animals showed decreased plasma insulin levels on M and SHP diet (§, P < 0.05 by repeated-measure ANOVA; *P < 0.05 by two-tailed Students’ t-test), but unchanged insulin levels on HF diet (P = 0.24 by repeated-measure ANOVA) (n = 8-12 per group). E, Pmch<sup>−/-</sup> animals body weight-matched to pmch<sup>+/+</sup> animals showed a decreased basal endogenous glucose production (EGP) and a decreased basal metabolic clearing rate (MCR) compared to pmch<sup>+/+</sup> animals during a hyperinsulinemic-euglycemic clamp analysis (n = 6 per group). EGP levels under hyperinsulinemic conditions did not differ significantly between genotypes (P = 0.33 by two-tailed Students’ t-test; n = 6 per group). *P < 0.05 by two-tailed Students’ t-test. Data are expressed as mean ± S.E.M. The black bars on the x-axes in panels’ B, C, and D indicate the dark phase.
**Figure 4** Pmch KO animals show a switch in caloric intake and a stabilization in relative body weight during development. A, Pmch<sup>−/−</sup> animals showed a switch in relative caloric intake normalized for body weight compared to pmch<sup>+/+</sup> animals on M diet (n = 10-12 per group). Average relative caloric intake from day 40 till day 57 is 94%. However, average relative intake from day 61 till day 145 is 103%. B, Pmch<sup>−/−</sup> animals showed a switch in relative water intake normalized for body weight compared to pmch<sup>+/+</sup> animals on M diet (n = 10-12 per group). Average relative water intake from day 33 till day 57 is 95%. However, average relative intake from day 61 till day 145 is 118%. C, Pmch<sup>−/−</sup> animals showed a switch in relative caloric intake normalized for body weight compared to pmch<sup>+/+</sup> animals on SHP diet (n = 10-11 per group). Average relative caloric intake from day 40 till day 57 was 96%. However, average relative intake from day 61 till day 145 was 104%. D, Pmch<sup>−/−</sup> animals showed a switch in relative water intake normalized for body weight compared to pmch<sup>+/+</sup> animals on SHP diet. Average relative water intake from day 33 till day 57 was 98%. However, average relative intake from day 61 till day 145 was 113%. E, Pmch<sup>−/−</sup> animals showed a stabilization of relative body weight difference during week 8 (day 50-56) on all three diets (M, SHP, and HF) compared to pmch<sup>+/+</sup> animals (n = 10-16 per group). *P < 0.05 by two-tailed Students’ t-test. Data are expressed as mean ± S.E.M.
before week 8, but an increased caloric intake compared to control animals after week 8 (Fig. 4A). A same 'biphasic' pattern was observed for relative water intake (Fig. 4B). These changes result in increased relative nutrient intake behavior in pmch−/− animals compared to wild type siblings during adulthood. The same 'biphasic' patterns were also observed for animals on SHP diet (Fig. 4C, 4D). Remarkably, the observed switch in nutrient intake occurred around the moment the young male rats enter sexual adulthood (approximately between day 55 and day 65). Although caloric intake measurements obtained from animals on HF diet started at day 45 and were not collected on regular intervals as the obtained data from the other two diets, a ‘switching’ pattern resembling the pattern observed with animals on M and SHP diet was also observed with the animals on HF diet (data not shown). However, water intake normalized for body weight at day 45 of male pmch−/− animals on HF diet was already strongly increased compared to wild type siblings, increasing further till week 8, stabilizing during week 8 and remaining strongly elevated after week 8 (data not shown).

Pmch KO rats have an altered energy balance set point

The relative body weight of pmch−/− animals on all diets decreases with approximately 20% during the first 7 weeks compared to wild type siblings, but this difference stabilized quite abruptly during week 8 (Fig. 4C). After this abrupt stabilization the relative body weight difference stayed stable during the remainder of the study and the average remained approximately 79%, 79%, and 83% (M, SHP, and HF respectively; Fig. 4C). Surprisingly, the observed stabilization occurred exactly during the same week of age, postnatal week 8 (days 50-56), with all three diets. These results indicate that the energy balance is set differently in pmch−/− animals when entering adulthood and is maintained at a lower level during adulthood. Interestingly, the observed deviation in body weight between pmch+/+ and pmch−/− animals during the first 8 weeks is mirrored by the weekly body weight growth rate, which is strongly decreased in pmch−/− animals during the first 8 weeks compared to pmch+/+ animals on all three diets, but approaches the level of pmch+/+ animals during adulthood (Fig. S5A, B, C). This indicates that the body weight growth rate is decreased in pmch−/− animals during the first 8 weeks, again suggesting that Pmch has a critical role during this period, and that loss of Pmch during this period lowers body weight growth.

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 were observed around the end of rat puberty, 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−/− animals compared to sham-operated pmch+/+ and pmch−/− animals (Fig. S6A). Both orchiectomized
PMCH+/+ and PMCH−/− animals show a decrease in relative body weight compared to sham-operated animals, although no clear stabilization pattern is observed around week 8 (Fig. S6B, S6C). Sham-operated PMCH−/− animals showed a clear stabilization of relative body weight compared to sham-operated PMCH+/+ animals around week 8, confirming observations from untreated animals (Fig. 4C, S6D). Interestingly, orchiectomized PMCH+/+ animals also showed a stabilization of relative body weight compared to orchiectomized PMCH+/+ animals around week 8 (Fig. S6E). Serum free testosterone levels showed no difference between PMCH+/+ and PMCH−/− animals on SHP diet around day 40, while orchiectomy resulted in almost undetectable levels in both genotypes (Fig. S6F).

**Discussion**

Here, we show that loss of PMCH in the rat causes relative hypophagia during early rat development and puberty, resulting in a 20% decreased energy balance that is maintained during adulthood. While the role of MCH in food intake regulation is well established, it should be noted that the entire PMCH gene is inactivated in our model and that also the less well-characterized neuropeptides N-GE and N-EI are not expressed in these animals. 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, increases luteinizing hormone (LH) release, and stimulates grooming, locomotion, and rearing in male rats. These additional neuropeptides could perform as of yet unknown functions, thereby contributing to phenotypes observed in this study.

In this study, we show that PMCH−/− animals have a changed endocrine profile while its dynamics are intact. As PMCH−/− rats are lean and hypophagic with strongly decreased adipose tissue, the decreased plasma leptin and insulin levels are as expected. Pissios and colleagues recently 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. It is also interesting to note that basal insulin levels seem to be lowered especially at the end of the dark phase. This could be the effect of a combination of decreased caloric intake and a loss of MCH-stimulated insulin release. The hyperinsulinemic-euglycemic clamp showed decreased basal EGP levels in the PMCH−/− animals compared to PMCH+/+ animals (78% in PMCH−/− as compared to PMCH+/+ animals). Interestingly, under hyperinsulinemic conditions EGP levels did not differ significantly anymore between both genotypes, although mean levels were still lower in PMCH−/− animals (84% compared to PMCH+/+ animals under hyperinsulinemic conditions). However, as the inhibition of EGP levels by the hyperinsulinemic conditions was equal between genotypes, these data do not support the idea that PMCH−/− animals are slightly insulin resistant. Moreover, IVITT studies...
also showed no difference between genotypes. Because the observed decreased basal EGP level (22%) is not body weight related, as animals were body weight-matched, it is therefore tempting to speculate that the basal EGP levels in \( pmch^{+/+} \) animals reflect the energy balance level, which is also decreased by approximately 20%. As plasma leptin, insulin, and glucose levels showed normal circadian patterns in general and insulin sensitivity seemed to be unchanged between genotypes, this suggests that the system dynamics in \( pmch^{+/+} \) animals are intact and functional, although adapted to new homeostatic levels. Additionally, thymus weight, adrenal weight, and plasma corticosterone levels showed no clear differences between \( pmch^{+/+} \) and \( pmch^{-/-} \) animals, suggesting that the HPA axis is not severely affected in male \( Pmch \)-deficient rats.

In mice it was observed that the loss of \( Pmch \) (on a mixed genetic background) resulted in hypophagia\(^{548}\); however this was characterized in adult mice only. More detailed analysis of feeding behavior in \( Pmch \)-deficient rats revealed a more complex phenotype. When caloric intake is normalized for body weight, only a slight hypophagia is detectable at a young-adult age (8 weeks), while at an adult age (17 weeks), the hypophagia disappeared completely (M and HF diet) or even inverted to hyperphagia (SHP diet). This would indicate that at these ages, \( pmch^{+/+} \) animals consumed approximately an equal amount of calories per kg body weight as wild type animals. While longitudinal food intake experiments confirmed these findings, they also revealed a clear relative hypophagia in \( Pmch \) KO rats, but only until approximately 60 days of age, a time-period including puberty. After leaving puberty, around day 60, \( pmch^{-/-} \) animals started consuming slightly elevated amounts of chow per body weight, indicating that they are actually slightly hyperphagic. The observed switch occurred in the same week for all three diets, suggesting that the effect is a genetic effect, and is not affected by diet. As the relative body weight of wild type and \( Pmch \) KO rats only diverges during the first 8 postnatal weeks and the difference in relative body weight remains stable during the remainder of the study, the observed slight hyperphagia might be indicative of a possible increase in metabolic rate, similar as has been shown for 20-week old mice with a loss of \( Pmch \) and 17-week old transgenic mice with a severe loss of MCH neurons\(^{548, 571}\). As basal locomotor activity was unchanged in \( pmch^{-/-} \) animals, the relative reduced caloric intake of the \( pmch^{-/-} \) animals during the first 8 weeks and an increased metabolic rate rather than increased basal locomotor activity seem to be the main contributors to the lean phenotype in \( Pmch \) KO rats.

The current findings have an interesting correlate in the effects of lesions of the lateral hypothalamus, which also show a reduced body weight\(^{572}\). However, their phenotype is much more extreme than that of the \( pmch^{-/-} \) rat, probably because it involves the loss of a more extended population of orexigenic neurons (i.e. also those containing orexin). Animals with a dorsomedial hypothalamic nucleus (DMH) lesion also regulate and actively defend their body weight at a reduced level as well. In addition,
they show a reduced linear growth, have a normal body composition, hormone levels, metabolism and respond normally to numerous body weight challenges. Because part of the \textit{Pmch} neuron population is found close to the DMH, loss of these neurons during a DMH lesion is a strong candidate for causing the similarities between both phenotypes.

The switch at the end of puberty is very interesting, as both MCH and N-EI are known to stimulate LH release in rats and a functional interaction between \textit{Pmch} and gonadal steroids, such as testosterone, thus seems plausible. However, free testosterone levels measured in \textit{pmch}\textsuperscript{+/+} and \textit{pmch}\textsuperscript{−/−} animals around day 40 did not differ. Additionally, orchiectomy during postnatal week 5 did lower the body weight of orchiectomized animals compared to sham-operated animals within genotypes, but did not affect the observed stabilization in relative body weight between \textit{pmch}\textsuperscript{+/+} and \textit{pmch}\textsuperscript{−/−} animals around week 8. This suggests that testosterone is not essential to induce the observed stabilization of body weight. Although only males were used in the present study, it is important to note that female \textit{pmch}\textsuperscript{−/−} rats also show a stabilization of relative body weight around week 8 compared to female \textit{pmch}\textsuperscript{+/+} rats (data not shown).

Hypothalamic rat \textit{Pmch} mRNA expression increases slowly during early development, increasing more rapidly after weaning, and stabilizes in 8-week-old young adult rats. \textit{Pmch} is also expressed in Sertoli cells in rat testis, increasing strongly between 15-day old rats and adult animals. Both expression gradients could potentially affect gonadal steroid expression directly or indirectly, although regulation of \textit{Pmch} expression levels by sex steroids is possible as well.

Our observations are in line with a role of \textit{Pmch} in increasing energy reserve levels, a process that is important during times of rapid growth like early development and puberty. Interestingly, relative \textit{Pmch} expression in food-restricted as compared to control rats changes during early development, suggesting the presence of a metabolic feedback mechanism during this time period. Moreover, nest-size induced food restriction of pups until day 25 decreases relative body weight, and this difference in relative body weight diminishes again when animals are given \textit{ad libitum} access to chow after day 25. Interestingly, 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. It is however important to note that the body weight of \textit{pmch}\textsuperscript{−/−} rats starts to differentiate during postnatal week 3.

The body weight of adult humans is normally relatively stable, with only a very small variance over a long period of time. 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. In humans, dieting strategies combining energy restriction and physical activity have shown moderate success for the reduction of body weight. 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, although low-fat intake in combination with high activity can successfully slow the regain of weight. Even though ‘short-term’ (≤ 4 weeks) MCH₁R-antagonism studies are successful in decreasing body weight in adult rats and mice, it would be very interesting to see if ‘long-term’ (> 4 weeks) MCH₁R 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 MCH₁R-antagonism on the determination of the energy balance in young animals.
Supplemental data

Supplemental Figure 1 Pmch KO rats show a decreased nutrient intake during light and dark phase on three different diets. A, Caloric intake is decreased in pmch<sup>−/−</sup> animals on three diets (M, SHP, and HF) during both light (L) and dark phase (D) at age week 8 and age week 17. B, The hypophagic character of pmch<sup>−/−</sup> animals is lost if data is normalized for body weight, except for dark phase caloric intake on M or SHP diet at age week 8. C, Pmch<sup>−/−</sup> animals drink less water on all three diets during both light and dark phases at age week 8. However, this pattern is lost at age week 17. Only the water intake during the dark phase on SHP diet remains decreased, while the other differences disappear. Surprisingly, animals on a HF diet at age week 17 drink more water during the dark phase than pmch<sup>+/+</sup> siblings. D, Pmch<sup>−/−</sup> animals drink equal or increased water quantities of water at age week 8 when water intake is normalized for body weight, with the exception of water intake during the dark phase on M diet. At age week 17, pmch<sup>−/−</sup> animals on all three diets drink more water during light and dark phase. *P < 0.05 by two-tailed Students’ t-test. Data are expressed as mean ± S.E.M. (n = 10–16 per group).
Supplemental Figure 2  *Pmch* KO rats have a decreased body length. *Pmch*−/− animals showed a decreased body length compared to *pmch*+/+ animals on three different diets (M, SHP, and HF) at a different age (M, SHP: 6 and 13 weeks; HF: 12 weeks). Relative body length was 94.2% (M) and 94.9% (SHP) at 6 weeks of age, and 93.8% (M), 94.2% (SHP), and 95.4% (HF) at 12/13 weeks of age. *P < 0.05 by two-tailed Students' t-test. Data are expressed as mean ± S.E.M. (n = 10-16 per group).

Supplemental Figure 3  *Pmch* KO rats show no change in whole-body insulin sensitivity. A, No difference in whole-body insulin sensitivity is observed between adult *pmch*+/+ and *pmch*−/− animals on three different diets (M, SHP, and HF) during an IVITT (n = 5-9 per group). B, *Pmch*−/− animals on SHP or HF diet showed a trend towards slower glucose removal during an IVGTT (n = 3-5 per group). Data are expressed as mean ± S.E.M.
**Supplemental Figure 4** *Pmch* KO rats show no change in locomotor activity and body core temperature.

A, Locomotor activity of *pmch*<sup>−/−</sup> animals on three different diets at a different age (M and SHP: 11 weeks; HF: 10 and 19 weeks) during 72 hours was unchanged compared to *pmch*<sup>+/+</sup> animals (n = 7-8 per group). B, No statistical difference was found in body core temperature measured during 24 hours between adult *pmch*<sup>−/−</sup> animals and *pmch*<sup>+/+</sup> animals on SHP diet (*P* = 0.60 by repeated-measure ANOVA; n = 7-10 per group). Measurement is average of 6 days. Data are expressed as mean ± S.E.M. The black bar in panel B indicates dark phase.

---

**Supplemental Figure 5** *Pmch* KO rats show a decreased growth rate during early development and puberty. A, The weekly body weight growth is strongly decreased in *pmch*<sup>−/−</sup> animals on M diet during the first 8 weeks. After week 8, this difference in weekly body weight growth between genotypes fades. The same effect is observed for *pmch*<sup>−/−</sup> animals on SHP diet (B) or HF diet (C). Data are expressed as mean ± S.E.M. (n = 10-16 per group).
Supplemental Figure 6  Testosterone is not critical to induce relative body weight stabilization around week 8. A: Orchiectomy during postnatal week 5 (indicated by the arrowhead) decreased the body weight of pmch+/+ animals compared to sham-operated pmch+/+ animals (\( P < 0.005 \) by repeated-measure ANOVA; \( n = 8-10 \) per group). Orchiectomy also lowered the body weight of pmch-/- animals (\( P < 0.05 \) by repeated-measure ANOVA; \( n = 9-14 \) per group) compared to sham-operated pmch-/- animals. Orchiectomized animals started...
showing a reduced body weight per individual measurement after 49 days of age (pmch+/-; P < 0.05 by two-tailed Students' t-test) or 60 days of age (pmch-/-; P < 0.05 by two-tailed Students' t-test). At 16 weeks of age, the body weight of orchiectomized pmch+/- animals is 81% compared to the body weight of sham-operated pmch+/- animals. During the same week, the body weight of orchiectomized pmch-/- animals is 87% compared to the body weight of sham-operated pmch-/- animals. All overall longitudinal body weight studies were analyzed by repeated-measure ANOVA. B: The relative body weight of orchiectomized pmch+/- animals showed no clear stabilization around week 8 compared to sham-operated pmch+/- animals (n = 8-10 per group). C: The relative body weight of orchiectomized pmch-/- animals showed no clear stabilization around week 8 compared to sham-operated pmch-/- animals (n = 9-14 per group). D: The relative body weight of sham-operated pmch-/- animals showed a stabilization of relative body weight around week 8 compared to sham-operated pmch+/- animals (n = 8-9 per group). E: The relative body weight of orchiectomized pmch+/- animals showed a stabilization of relative body weight around week 8 compared orchiectomized pmch+/- animals (n = 10-14 per group). F: Serum free testosterone levels did not differ between pmch+/- and pmch-/- animals on day 40 (n = 14 per group) or day 60 (n = 8-10 per group), but were decreased on day 120 (P < 0.05 by two-tailed Students' t-test n = 8 per group). Serum free testosterone levels were undetectable in orchiectomized pmch+/- and pmch-/- animals on day 120 (n = 10-14 per group). Data are expressed as mean ± S.E.M.