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Expression of a hypomorphic *Pomc* allele alters leptin dynamics during late pregnancy

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Abstract

Proopiomelanocortin (POMC) neurons in the hypothalamic arcuate nucleus (ARC) are essential for normal energy homeostasis. Maximal ARC *Pomc* transcription is dependent on neuronal *Pomc* enhancer 1 (nPE1), located 12kb upstream from the promoter. Selective deletion of nPE1 in mice decreases ARC *Pomc* expression by 70%, sufficient to induce mild obesity. Because nPE1 is located exclusively in the genomes of placental mammals, we questioned whether its hypomorphic mutation would also alter placental *Pomc* expression and the metabolic adaptations associated with pregnancy and lactation. We assessed placental development, pup growth, circulating leptin and expression of *Pomc, Agrp* and alternatively spliced *Leptin receptor (LepR)* isoforms in the ARC and placenta of $Pomc^{\Delta 1/\Delta 1}$ and $Pomc^{+/+}$ dams. Despite indistinguishable body weights, lean mass, food intake, placental histology and *Pomc* expression and overall pregnancy outcomes between the genotypes, $Pomc^{\Delta 1/\Delta 1}$ females had increased pre-pregnancy fat mass that paradoxically decreased to control levels by parturition. However, $Pomc^{\Delta 1/\Delta 1}$ dams had exaggerated increases in circulating leptin, up to twice that of the typically elevated levels in $Pomc^{+/+}$ mice at the end of pregnancy, despite their equivalent fat mass. $Pomc^{\Delta 1/\Delta 1}$ dams also had increased placental expression of soluble leptin receptor (*LepRe*), although the protein levels of LepRe in circulation were the same as $Pomc^{+/+}$ controls. Together, these data suggest that the hypomorphic $Pomc^{\Delta 1/\Delta 1}$ allele is responsible for the perinatal super hyperleptinemia of $Pomc^{\Delta 1/\Delta 1}$ dams, possibly due to upregulated leptin secretion from individual adipocytes.

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Declaration of Interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Keywords

Pomc; Leptin; Leptin Receptor; Pregnancy; Enhancers

Introduction

Proopiomelanocortin (Pomc) and *Agouti-related protein* (*Agrp*) expressing neurons in the arcuate nucleus (ARC) of the hypothalamus integrate hormonal signals such as leptin (Cowley, et al. 2001), estrogen (Xu, et al. 2011) and insulin to control energy and glucose homeostasis (Varela and Horvath 2012). Leptin targets cells expressing multiple leptin receptor isoforms, including a subpopulation of ARC POMC neurons. The full-length leptin receptor (LepRb) is responsible for intracellular signaling through JAK-STAT signaling pathways (Bates, et al. 2003; Vaisse, et al. 1996) and mediates leptin-induced satiety responses (Bates et al. 2003; Lee, et al. 1996). Although the selective deletion of *LepR* from *Pomc* neurons in adult mice did not alter food intake, it reduced hepatic glucose production during hyperinsulinemic euglycemic clamps and prevented the fasting-induced fall in leptin levels, independent of changes in fat mass (Caron, et al. 2018b).

In addition to LepRb, there are five truncated forms of leptin receptors generated by alternative splicing of *LepR* hnRNA. Of these isoforms, LepRe lacks all intracellular signaling domains (Houseknecht, et al. 1996), is produced from the liver and placenta in rodents and is released into the circulation where it binds with high affinity to leptin (Gavrilova, et al. 1997). The expression of *LepRe* in placenta increases as gestation advances (Challier, et al. 2003; Zhao, et al. 2004). LepRe might buffer leptin signaling by reducing its renal clearance under normal physiological conditions and prevent excessive binding of elevated circulating leptin to LepRb in extreme metabolic conditions, such as late pregnancy (Trujillo, et al. 2011; Tu, et al. 2008). Leptin receptors in the placenta might also influence fetal growth by mediating leptin transport to the fetal circulation or by local leptin bioactivity (Smith and Waddell 2002).

Pregnancy and lactation represent allostatic states in which the neuronal circuits controlling feeding behavior adapt to the high demands for nutrients from the developing fetuses and suckling pups. In particular, lactation is associated with marked hyperphagia, increased expression of orexigenic neuropeptides in the hypothalamus, transient hyperleptinemia and reduced transport of leptin into the brain (Gustafson, et al. 2019). The maternal adaptations to pregnancy are mediated largely by the placenta, which separates the maternal and fetal circulations and secretes hormones including placental lactogens, progesterone and estrogen to maintain pregnancy (Ladyman, et al. 2010; Napso, et al. 2018; Watson and Cross 2005). Similar to *LepR, Pomc* is expressed in both the placenta and hypothalamic neurons (Challier et al. 2003; Chen, et al. 1986). The human *POMC* transcript is localized to trophoblast cells and its expression can be detected from the first to the final trimester with increased levels before parturition (Cooper, et al. 1996). However, the function and regulation of *POMC* expression in the placenta is poorly understood.

Previous work from our lab identified two phylogenetically conserved neuronal *Pomc* enhancers named nPE1 and nPE2, located approximately 12kb and 10kb upstream of the

mouse *Pomc* promoter (de Souza, et al. 2005; Franchini, et al. 2011; Lam, et al. 2015). Both enhancers are required for maximal transcription of *Pomc* in the ARC, but not pituitary corticotrophs or melanotrophs. Individual deletions of nPE1 or nPE2 reduced hypothalamic *Pomc* expression by ~ 70% or ~20%, respectively (Lam et al. 2015). nPE2 is an ancient *Pomc* enhancer that originated more than 166 million years ago in the lineage leading to all extant mammals (Santangelo, et al. 2007), whereas nPE1 is present only in the genomes of placental mammals and originated at least 24 million years later from the exaptation of a distinct retroposon (Franchini et al. 2011). Compared to non-placental animals, e.g., Monotremes, and Marsupials, the existence of a complex placenta allows the nourishment of young during a longer gestational period and thus the birth of more mature and well developed young (Ferner, et al. 2017). Although *Pomc* is highly expressed in the placenta (Zhu and Pintar 1998); it is unknown whether the recent evolutionary association of nPE1 to the *Pomc* gene in placental mammals is coincidental or indicative of a regulatory role for placental *Pomc* transcription.

Because of the importance of leptin and POMC peptides in the central regulation of energy balance in adult animals and the parallels between hypothalamic and placental *LepR* and *Pomc* expression, we hypothesized that deletion of nPE1 from the distal enhancer locus of *Pomc* would alter expression of *Pomc* in the placenta and disrupt the normal metabolic adaptations to pregnancy and lactation. Moreover, previous studies of pregnant rats (Khorram, et al. 1984; Ladyman, et al. 2009; Mann, et al. 1997; Trujillo et al. 2011) showed reduced hypothalamic expression of *Pomc* in late pregnancy and early postnatal days, possibly contributing to hyperphagia by reducing satiety. Therefore, we questioned whether constitutive reductions of *Pomc* expression in the ARC of $Pomc^{\Delta 1/\Delta 1}$ dams would further exacerbate the hyperphagia and weight gain during pregnancy and lactation.

Materials and Methods

Animal Care

All experiments were approved by the University of Michigan's Institutional Animal Care and Use Committee and followed the Public Health Service guidelines for the humane care and use of experimental animals. Mice were housed in ventilated cages under controlled temperature and photoperiod (12-h light/12-h dark cycle), with tap water and laboratory chow (5L0D, LabDiet, St.Louis, U.S.) containing 28.67% protein, 13.38% fat, and 57.94% carbohydrate available ad libitum. Breeding mice were fed with a breeder chow diet (5008, LabDiet, St.Louis, USA) containing 26.53% protein, 16.97% fat, and 56.50% carbohydrates.

Generation and Breeding of Mice

Mice lacking nPE1 ($Pomc^{\Delta 1/\Delta 1}$) were generated by homologous recombination in embryonic stem cells and backcrossed for 12–14 generations onto the C57BL/6J genetic background as described previously (Lam et al. 2015). In the present study, adult (9–20 wk old) virgin female $Pomc^{+/+}$ and $Pomc^{\Delta 1/\Delta 1}$ mice were bred with $Pomc^{+/+}$ male mice and the day of copulation plug detection was counted as day post coitum (dpc) 0.5. The genotypes of offspring from these matings were either $Pomc^{+/+}$ or $Pomc^{\Delta 1/+}$, respectively. A separate cohort of virgin $Pomc^{\Delta 1/\Delta 1}$ females was bred with $Pomc^{\Delta 1/\Delta 1}$ males to generate homozygous

Pomc$^{\Delta 1/\Delta 1}$ offspring and these *Pomc*$^{\Delta 1/\Delta 1}$ dams were denoted as *Pomc*$^{ho\Delta 1/\Delta 1}$ to indicate that all their pups were also homozygous for the hypomorphic allele. Pregnant dams were housed individually and their body weights were recorded on the mornings of dpc3.5, dpc6.5, dpc9.5, dpc12.5 and dpc18.5 with continued measurements at postpartum days (ppd) 0, 3, 6, 9, 12, 15, 18 and 21. Twenty-four-hour food intake was assessed during early pregnancy (dpc3.5-dpc4.5), late pregnancy (dpc17.5-dpc18.5) and mid-lactation (ppd10-ppd11). Body composition of the dams was assessed by nuclear magnetic resonance (NMR; Minispec, Bruker Instruments) before pregnancy, at late pregnancy (dpc17.5 and dpc18.5) and at days ppd0 and ppd21. The number of pups per litter was recorded at birth and the body weight of each pup was measured at ppd0, ppd10 and ppd21.

Plasma Leptin and Soluble Leptin Receptor Measurements

Blood was collected into EDTA tubes from virgin females and dams at dpc17.5 or dpc18.5, ppd0 and ppd21. Plasma leptin levels were determined by ELISA (MOB00, Mouse/Rat Leptin Quantikine ELISA Kit, R&D Systems, Minneapolis, USA) according to the manufacturer's instructions, which included acidification/urea/neutralization steps of the dpc17.5, dpc18.5 and ppd0 samples to fully recover leptin from LepRe interference by denaturation of the binding protein. Soluble leptin receptor (LepRe) was measured by ELISA (DY497, Mouse Leptin R DuoSet ELISA, R&D Systems, Minneapolis, USA) according to the manufacturer's instructions.

Histology

Placentas were dissected at dpc18.5 and post-fixed in 10% neutral buffered formalin overnight at 4 °C. Samples were then washed three times in 1x phosphate buffered saline and transferred to 70% ethanol for short-term storage. Paraffin-embedding, sample sectioning (5 μm) and hematoxylin and eosin (H&E) staining were performed by the University of Michigan Comprehensive Cancer Center Research Histology and Immunohistochemistry Core. Images were acquired using Nikon Eclipse 90i microscope equipped with NIS-Elements AR software.

Quantitative RT-PCR

Total RNA was prepared from arcuate nucleus and placenta with TRIzol (15596026, ThermoFisher Scientific, Grand Island, USA) as described by the manufacturer. cDNA was obtained by reverse transcription of 1 μg RNA with random primers using M-MLV reverse Transcriptase (28025013, ThermoFisher Scientific, Grand Island, USA). qRT-PCR was performed on all samples using a StepOne Real Time PCR System (Applied Biosystems) and SYBR Green Master Mix (4385612, ThermoFisher Scientific, Grand Island, USA). The primers for detection of *Pomc*, *Agrp*, *pan Leptin Receptor* (*LepR*), *LepRa*, *LepRb*, *LepRe*, *Plac1*, *Tpbpa* and reference genes *Hprt* and *Tbp* are in Supplementary Table1. All primers were used at a final concentration of 250 nM. The results were analyzed using the $2^{-\Delta\Delta CT}$ relative quantitation method and graphed as a percentage of the average data from the wildtype control females.

Statistical analysis

Two-tailed Student's t-test or the Mann-Whitney t-test for nonparametric data were used to determine the significant differences between pairs of genotypes for one dependent variable. Two-way ANOVAs followed by Bonferroni's *post-hoc* multiple comparisons tests for pair-wise comparisons were performed to determine the effects of genotype and developmental stages. Linear correlation and regression analyses were performed to determine the relationship between plasma leptin and body fat mass at different physiological stages. Normalized placental weights, placental diameters, fetal weights and crown-rump lengths were calculated by averaging the reading from three measured placentas and fetuses in each litter, divided by the litter size and multiplied by the average litter size for each genotype ($Pomc^{+/+}$: 7.36 and $Pomc^{\Delta 1/\Delta 1}$: 7.27). A detailed description of the statistical method used for each analysis is noted in each figure legend. $P < 0.05$ was considered significant.

Results

Maternal deletion of nPE1 from *Pomc* did not affect the normal temporal patterns or magnitude of changes in body weight or food intake during gestation and lactation.

The weight curves comparing $Pomc^{\Delta 1/\Delta 1}$ to $Pomc^{+/+}$ dams (Figure 1A) were indistinguishable from each other. Pregnancy requires increased food intake to meet the energy requirements for offspring to develop and grow (Makarova, et al. 2010). Compared to dpc3.5, the daily food intake at dpc17.5 increased slightly in both genotypes, but without reaching statistical significance ($Pomc^{+/+}$: 4.1 ± 0.1 to 4.8 ± 0.2 g; and $Pomc^{\Delta 1/\Delta 1}$: 4.5 ± 3.0 to 4.7 ± 0.3 g) (Figure 1B). However, lactation dramatically increased the dams' daily food intake, measured from ppd10 to ppd11 ($F_{2,81} = 92.1$, $P < 0.0001$; two-way ANOVA, main effect of age), but again, there were no significant genotype differences ($Pomc^{+/+}$: 11.1 ± 0.5 g; $Pomc^{\Delta 1/\Delta 1}$: 10.1 ± 0.9 g) or genotype by age interactions (Figure 1B).

Deletion of nPE1 from *Pomc* significantly reduced maternal body fat mass during gestation.

We measured body composition of the females before pregnancy (NP), on the day of parturition (ppd0) and on the day of weaning (ppd21). A two-way ANOVA for lean mass revealed a significant main effect across the three time points ($F_{2, 83} = 80.3$, < 0.0001), but not for genotype or time by genotype interactions (Figure 1C). A significant main effect across time was also detected for fat mass ($Pomc^{+/+}$ vs. $Pomc^{\Delta 1/\Delta 1}$: $F_{2, 81} = 3.5$, $P = 0.03$) (Figure 1D). There was an additional significant main effect of genotype for fat mass ($F_{1, 81} = 8.8$, $P = 0.004$) but no time by genotype interaction. Follow-up *post-hoc* multiple comparisons tests indicated that non-pregnant $Pomc^{\Delta 1/\Delta 1}$ females had 31% greater fat mass ($Pomc^{\Delta 1/\Delta 1}$: 4.15 ± 0.23 g; $Pomc^{+/+}$: 3.17 ± 0.22 g; $P = 0.018$) compared to their sibling $Pomc^{+/+}$ non-pregnant controls as reported previously (Lam et al. 2015) (Figure 1D). Unexpectedly, unlike $Pomc^{+/+}$ dams that had no change in their fat mass during gestation, the $Pomc^{\Delta 1/\Delta 1}$ dams had significantly reduced fat mass by parturition. The preservation of fat mass for $Pomc^{+/+}$ dams during pregnancy, but reduction in fat mass for the $Pomc^{\Delta 1/\Delta 1}$ dams, are shown alternatively with the repeated measures connected by lines for the individual mice in Figure 1E and 1F, respectively. Body composition measurements from an independent cohort of mice at dpc17.5 and dpc18.5 also showed a significant increase of

lean mass (Supplementary Figure 1A) in both genotypes but confirmed a remarkable reduction of fat mass (Supplementary Figure 1B, 1C and 1D) only in *Pomc*$^{\Delta 1/\Delta 1}$ dams during gestation. Dams of both genotypes had similar significant increases in fat mass during lactation (ppd0 to ppd21) (Figure 1D).

Deletion of nPE1 from *Pomc* did not impair placental growth

Because nPE1 is present exclusively in the genome of placental mammals, we tested its physiological significance by evaluating placental weights, diameters, morphology and function at 18.5 days of pregnancy, a time when the placenta is structurally fully developed and at its maximum weight. Deletion of nPE1 from *Pomc* did not change placental weights or diameters (Figure 2A, B). Vertical hemisections of the placenta revealed three layers: the labyrinth zone (Lab), the junctional zone (JunZ) and maternal decidua basalis (Dec). Deletion of nPE1 from *Pomc* did not change the integrity of these three components in the mature placenta (Figure 2C). To further assess the requirements of the nPE1 *Pomc* enhancer on the function of placenta, we measured the expression of *Pomc* and two essential genes for placental growth and development. Relative quantification by RT-PCR indicated that deletion of nPE1 did not change *Pomc* mRNA levels in placenta indicating that nPE1 is not required for placental *Pomc* expression, unlike in the ARC, but similar to pituitary (Figure 2D). *Placenta-specific 1 (Plac1)* expression is almost entirely restricted to trophoblast cells (Cocchia, et al. 2000), and its deficiency leads to placentomegaly and intrauterine growth retardation (Jackman, et al. 2012). Likewise, ablation of *Trophoblast-specific protein alpha (Tpbpa)* positive cells reduced maternal spiral artery sizes and decreased blood flow in the labyrinth and further induced embryonic death after dpc10.5 (Hu and Cross 2011). However, expression levels of *Plac1* and *Tpbpa* in the placenta did not differ by genotype as determined by quantitative RT-PCR (Figure 2E and F).

Constitutive deletion of nPE1 from *Pomc* did not induce any growth alterations in offspring

We next characterized the growth and development of offspring from *Pomc*$^{\Delta 1/\Delta 1}$ and *Pomc*$^{+/+}$ dams. Compared to the *Pomc*$^{+/+}$ pups from *Pomc*$^{+/+}$ dams, the *Pomc*$^{\Delta 1/+}$ pups from *Pomc*$^{\Delta 1/\Delta 1}$ dams had indistinguishable numbers per litter at birth and per sex at weaning (Figure 3A and B), the same body weights from birth (ppd0) until weaning at ppd21 (Figure 3C) and similar crown-rump lengths at birth (*Pomc*$^{+/+}$: 21.4 ± 0.2; *Pomc*$^{\Delta 1/\Delta 1}$: 20.9 ± 0.3 mm) (Figure 3D).

Pomc, Agrp and *LepR* gene expression in the arcuate nucleus

In order to understand possible mechanisms underlying hyperphagia during late pregnancy and lactation, we measured expression of relevant genes in the arcuate nucleus, a crucial site in the hypothalamus for regulating appetite and body weight. A two-way ANOVA indicated a significant main effect of maternal genotype for *Pomc* expression ($F_{1, 73} = 65.57$, $P < 0.0001$) (Figure 4A). Specifically, *Pomc*$^{\Delta 1/\Delta 1}$ dams exhibited an average of 71% reduction in *Pomc* expression, relative to *Pomc*$^{+/+}$ dams collapsed across all of the interrogated developmental stages, which is consistent with our previous report based on virgin mice (Lam et al. 2015). *Agrp* expression was increased ~2.4-fold at dpc18.5 and ~2.9-fold at ppd10 and returned to normal levels at ppd21 in both genotypes compared to their non-pregnant controls (Two-way ANOVA, main effect of physiological stage: $F_{3,73} = 23.28$, P

<0.0001) (Figure 4B). There was a significant increase in *pan-LepR* expression in both genotypes at dpc18.5 compared to both non-pregnant mice and lactating dams at ppd10 (Figure 4C). There were no main effects of genotype, stage or interactions between the two independent variables for either *LepRa* or *LepRb* (Figure 4D and E).

Deletion of nPE1 from *Pomc* significantly increased maternal plasma leptin levels at dpc18.5 independent of fat mass

Pregnancy is associated with increased levels of maternal leptin in women and rats, but the increases are particularly marked in mice (Malik, et al. 2005; Masuzaki, et al. 1997). Moreover, central *Pomc*-expressing neurons directly regulate peripheral leptin release through activation of sympathetic efferents projecting to white adipocytes (Caron et al. 2018b; Rayner and Trayhurn 2001). Therefore, we measured circulating leptin in control $Pomc^{+/+}$ and $Pomc^{\Delta 1/\Delta 1}$ dams and conducted correlation analyses between circulating leptin and fat mass before pregnancy and at dpc18.5 to elucidate possible roles of the central hypomorphic *Pomc* allele on the modulation of circulating leptin levels. Circulating leptin was substantially increased at dpc18.5 in $Pomc^{+/+}$ dams compared to non-pregnant females, remained elevated at ppd0 and dropped to below non-pregnant levels by ppd10 (Figure 5A). $Pomc^{\Delta 1/\Delta 1}$ females exhibited a similar temporal pattern for circulating leptin but the levels were significantly higher in $Pomc^{\Delta 1/\Delta 1}$ mice before pregnancy and at dpc18.5 by ~2-fold compared to the control dams ($Pomc^{+/+}$ vs. $Pomc^{\Delta 1/\Delta 1}$: NP, 4.6 ± 0.4 vs. 10.9 ± 1.5; dpc18.5, 75.8 ± 9.0 vs. 147.8 ± 25.5; ppd0, 72.5 ± 12.2 vs. 97.9 ± 29.5; ppd10, 3.1 ± 0.4 vs. 6.0 ± 1.0 ng/ml). Despite the quantitative differences, linear regression analyses showed that leptin levels were significantly correlated with total fat mass in both $Pomc^{+/+}$ and $Pomc^{\Delta 1/\Delta 1}$ females before pregnancy ($Pomc^{+/+}$: $R^2 = 0.468$, P=0.0291 vs. $Pomc^{\Delta 1/\Delta 1}$: $R^2 = 0.881$, P=0.0017) (Figure 5B). The similar regression line slopes and Y intercepts between the two genotypes before pregnancy indicate a consistent proportionality between total fat mass and circulating leptin levels for $Pomc^{+/+}$ and $Pomc^{\Delta 1/\Delta 1}$ females. However, the slopes of the regression lines were significantly more shallow at dpc18.5 compared to virgin mice for both genotypes ($Pomc^{+/+}$: NP, 0.2221 ± 0.08 vs. dpc18.5, 0.0036 ± 0.005, P =0.0512; $Pomc^{\Delta 1/\Delta 1}$: NP, 0.2419 ± 0.04 vs. dpc18.5, 0.0051 ± 0.004, P = 0.0010) even though total fat mass was not changed between these time points in the wildtype controls (NP, 3.24 ± 0.12 vs. dpc18.5, 3.07 ± 0.13 g) and was actually decreased in the $Pomc^{\Delta 1/\Delta 1}$ dams (NP, 4.83 ± 0.38 vs. dpc18.5, 3.90 ± 0.24 g) (Supplementary Figure 1B).

Increased placental *LepRe* but decreased *pan-LepR* and *LepRb* mRNA in $Pomc^{\Delta 1/\Delta 1}$ dams

In humans and rats, the placenta has been recognized in addition to adipose tissue as one of the sources for circulating leptin (Amico, et al. 1998; Masuzaki et al. 1997). In contrast, mouse placenta does not produce or release leptin as shown by several previous studies (Tomimatsu, et al. 1997; Zhao, et al. 2003). We also failed to detect any leptin transcripts in placenta at dpc18.5 (data not shown) indicating that the placenta is not a direct source of leptin secretion and therefore not responsible for the elevated leptin in pregnant mice. Leptin receptors, on the other hand, are expressed in the placenta across different species (Challier et al. 2003; Smith and Waddell 2002; Zhao et al. 2004). In particular, the soluble form of leptin receptor (LepRe) is produced by the placenta and released into the circulation during pregnancy (Lammert, et al. 2002). $Pomc^{\Delta 1/\Delta 1}$ mice exhibited significantly reduced transcript

levels of placental *pan LepR* and *LepRb* compared to $Pomc^{+/+}$ dams, but *LepRa* mRNA levels were not different between the genotypes (Figure 6). However, the transcript abundance of *LepRe* in placentas from $Pomc^{\Delta 1/\Delta 1}$ dams was ~30% greater than that from $Pomc^{+/+}$ dams and approached statistical significance (P=0.057) (Figure 6).

To investigate whether the elevated transcript levels of *LepRe* led to increased circulating soluble leptin receptor, we measured plasma leptin receptor levels using an ELISA kit which was validated in a previous *in vitro* study involving cell lines transfected with various tagged leptin receptor constructs (Gan, et al. 2012). Compared to non-pregnant mice, dams at dpc18.5 exhibited a highly significant 2.2-fold increase in soluble leptin receptor in both genotypes (ordinary two-way ANOVA: F (1, 30) = 18.03, P = 0.0002, main effect of stage), which is consistent with the previous findings that placenta releases soluble leptin receptor during pregnancy (Yamaguchi, et al. 1998). However, deletion of nPE1 did not induce higher circulating leptin Re compared to wildtype control mice (Supplementary Figure 2). These data revealed the lack of any obvious linear stoichiometry for circulating leptin and LepRe levels between nonpregnant and dpc18.5 dams of either genotype.

Breeding of $Pomc^{\Delta 1/\Delta 1}$ females with homozygous $Pomc^{\Delta 1/\Delta 1}$ males increased circulating leptin and *LepRe* mRNA abundance in placenta at dpc18.5

The placenta is composed of fetal-derived components such as trophoblast cells, and maternal uterine tissue that undergoes a specialized decidualization reaction to support normal placentation (Rossant and Cross 2001; Woods, et al. 2018). Because our breeding strategy (♀ $Pomc^{\Delta 1/\Delta 1}$ x ♂ $Pomc^{+/+}$) yielded all $Pomc^{\Delta 1/+}$ pups, we set up an additional breeding scheme in which female $Pomc^{\Delta 1/\Delta 1}$ were mated with male $Pomc^{\Delta 1/\Delta 1}$ mice to detect any possible effects from the $Pomc^{\Delta 1/\Delta 1}$ fetuses on placental maturation and the maternal circulation. Compared to the previously described $Pomc^{+/+}$ and $Pomc^{\Delta 1/\Delta 1}$ dams (Figure 2A and C), $Pomc^{ho\Delta 1/\Delta 1}$ dams had similar placental size and placental weights (Figure 7A). The combination of $Pomc^{\Delta 1/\Delta 1}$ dams and fetuses did not change placental integrity as indicated by H&E staining (Figure 7B). After normalization to litter size, the fetal lengths and fetal weights were similar between the two genotypes (Figure 7C). The two breeding schemes generated similar numbers of pups per litter (Figure 7D). qRT-PCR analysis of gene expression from the $Pomc^{ho\Delta 1/\Delta 1}$ placentas showed significant reductions in mRNA levels for both *pan LepR* and *LepRa* mRNA compared to $Pomc^{+/+}$ dams. *LepRb* mRNA levels were not changed, but *LepRe* levels were increased by 30% in the placentas of $Pomc^{ho\Delta 1/\Delta 1}$ compared to $Pomc^{+/+}$ dams (Figure 7E), similar to the results shown in Figure 6 for $Pomc^{\Delta 1/\Delta 1}$ dams. There were no genotype differences in placental expression of *Pomc*, *Tpbpa* or *Plac1*. The $Pomc^{ho\Delta 1/\Delta 1}$ dams exhibited a significant increase in circulating leptin at dpc18.5 relative to $Pomc^{+/+}$ dams ($Pomc^{ho\Delta 1/\Delta 1}$: 103.0 ± 12.5 ng/ml; $Pomc^{+/+}$: 44.2 ± 4.1; Mann Whitney two-tailed test; U = 9, P = 0.0003) (Figure 7F), similar to the $Pomc^{\Delta 1/\Delta 1}$ dams described previously in this report (Figure 5A). Blood samples from an independent cohort of $Pomc^{ho\Delta 1/\Delta 1}$ dams between the broader stage range of dpc16.5 to ppd0 confirmed that leptin levels at late pregnancy to parturition were significantly higher in the $Pomc^{ho\Delta 1/\Delta 1}$ compared to $Pomc^{+/+}$ dams ($Pomc^{+/+}$: 28.3 ± 3.5; $Pomc^{ho\Delta 1/\Delta 1}$: 72.0 ± 14.8 ng/ml; Mann-Whitney two-tailed test; U = 16, P = 0.0032).

Discussion

In this study, we investigated the effects of a hypomorphic *Pomc* allele created by a germline deletion of the neuronal-specific *Pomc* enhancer nPE1 on the regulation of maternal metabolism, expression of *Pomc* in the placenta, circulating leptin in the dams, placental growth during pregnancy, litter size and pup growth during lactation. nPE1 originated from an exaptation event of a mammalian-apparent LTR retrotransposon sometime between the metatherian/eutherian split (147 Mya) and the placental mammalian radiation (~90 Mya). Therefore, nPE1 is a placental apomorphy that is located exclusively in the genomes of all placental mammals, but not other vertebrates, including the non-placental mammalian orders Monotremata and Marsupialia (Franchini et al. 2011). The other known *Pomc* enhancer nPE2 appeared earlier in evolution from an independent exaptation event from a different retrotransposon and is found in all mammals (Santangelo et al. 2007). nPE1 has one *bona fide* tandem binding motif for the transcription factors *Islet1* and *Nkx2.1* and both factors are essential for hypothalamic *Pomc* expression during embryogenesis (Nasif, et al. 2015; Orquera, et al. 2019). In addition, nPE1 contains a Stat3 consensus binding site (de Souza et al. 2005) suggesting a possible role for modulation of *Pomc* transcription by leptin-mediated Jak2/Stat3 signaling.

Leptin is a potent stimulator of *Pomc* neuron activity, but also plays vital roles during pregnancy. The *ob/ob* mouse is infertile and administration of leptin restores fertility in both sexes (Chehab, et al. 1996; Mounzih, et al. 1997). Substantial increases of circulating leptin in the last trimester of pregnancy have been reported in animal models and humans (Anim-Nyame, et al. 2000; Malik et al. 2005; Tomimatsu et al. 1997), which result from combinations of species' specific differences in placenta-derived leptin (Masuzaki et al. 1997), increased fat mass, enhanced hormonal stimulation of leptin secretion from fat (such as insulin, estrogen and hCG), increased soluble leptin binding protein (Gavrilova, et al. 1997) or other undetermined factors (Sivan, et al. 1998; Zhao et al. 2003).

The current study showed that at late pregnancy (dpc18.5), the $Pomc^{\Delta 1/\Delta 1}$ and $Pomc^{ho\Delta 1/\Delta 1}$ dams exhibited a 2-fold and 2.3-fold higher level of circulating leptin, respectively, compared to the already elevated leptin levels of $Pomc^{+/+}$ dams (Figures 5A and 7F). Although $Pomc^{\Delta 1/\Delta 1}$ dams had slightly, but not statistically higher fat mass compared to $Pomc^{+/+}$ dams at dpc18.5 ($Pomc^{+/+}$: 3.07 ± 0.13 vs. $Pomc^{\Delta 1/\Delta 1}$: 3.90 ± 0.24 g), the 2-fold increase in circulating leptin cannot be simply attributed to ~0.9 g more fat mass in $Pomc^{\Delta 1/\Delta 1}$ dams, indicating that the quantity of fat is not the major contributor for the exaggerated increase of circulating leptin in $Pomc^{\Delta 1/\Delta 1}$ dams at the end of gestation. Concomitantly, we detected a 1.25-fold and 1.3-fold increase in *LepRe* expression in $Pomc^{\Delta 1/\Delta 1}$ and $Pomc^{ho\Delta 1/\Delta 1}$ placentas, respectively (Figures 6 and 7E). Our lab has shown previously that deletion of both nPE1 and nPE2 from the *Pomc* gene results in obese, hyperleptinemic and infertile mice that exhibit low sympathetic tone in general and reduced renal sympathetic nerve activity specifically (Chhabra, et al. 2017; Chhabra, et al. 2016). It has been demonstrated previously that inhibition of sympathetic activity on white adipocytes increases their leptin production (Caron, et al. 2018a; Rayner and Trayhurn 2001). Since $Pomc^{\Delta 1/\Delta 1}$ mice only maintain ~30 % *Pomc* mRNA expression, it is likely that these mice

also have decreased sympathetic output to adipose tissue, which leads to increased leptin release into the circulation.

Although some previous studies have reported reductions in ARC *Pomc* mRNA levels in pregnant or lactating rats, we did not observe any reductions from nonpregnant levels in our $Pomc^{+/+}$ control dams at dpc18.5 and ppd10. Therefore, the increased expression of ARC *Agrp* without suppression of *Pomc* is apparently sufficient for mediating the hyperphagia that occurs during lactation. Future studies are necessary to confirm this hypothesis and rule out additional neurochemical mechanisms described previously for hyperphagic pregnant rats (Ladyman et al. 2010). On the other hand, constitutive reduction of ARC *Pomc* expression by 70% in $Pomc^{\Delta 1/\Delta 1}$ dams led to a paradoxical decrease of fat mass during pregnancy with no effect on postnatal body weight, body composition or hyperphagia during lactation.

Intriguingly, we also detected a moderate increase of *LepRe* transcript abundance in the placentas of both $Pomc^{ho\Delta 1/\Delta 1}$ and $Pomc^{\Delta 1/\Delta 1}$ dams compared to $Pomc^{+/+}$ controls, although we failed to detect any further increase in circulating soluble leptin receptor protein in $Pomc^{\Delta 1/\Delta 1}$ dams compared to $Pomc^{+/+}$ controls. Because leptin levels were increased by roughly 20-fold from nonpregnant measurements to those at dpc18.5 in both genotypes of mice, while LepRe levels only doubled, there apparently was not a linear stoichiometry between the two molecules. Such nonlinearity has been reported previously, but not nearly to the extent of our data (Murakami, et al. 2001).

One possible explanation for this discrepancy lies in fundamental characteristics of the ELISA itself that we used to measure soluble leptin receptor in plasma. Almost all of the earlier literature studying the complicated relationship between circulating leptin and soluble leptin receptor in pregnant mice was based on leptin radioimmunoassays and/or Western blots of leptin-like immunoreactivity after its affinity purification with Sepharose beads bound to recombinant leptin receptor (Gavrilova et al. 1997; Huang, et al. 2001; Lammert et al. 2002; Yang, et al. 2004). The soluble leptin receptor was quantified by Western blots after its affinity purification with Sepharose beads bound to recombinant leptin or was alternatively estimated from the binding capacity of serum to [^{125}I] recombinant leptin (Gavrilova et al. 1997; Huang et al. 2001; Murakami et al. 2001). Collectively, these foundational studies concluded that 95% of circulating leptin in pregnant mice is bound to the soluble leptin receptor with high affinity; there is a 40-fold increase in the binding capacity of late-pregnancy maternal serum to leptin compared to serum from nonpregnant female mice; and the source of soluble leptin receptor in pregnant mice is mostly from translation of placental *LepRe* (Gavrilova et al. 1997). Moreover, it was shown that elevated soluble leptin receptor interfered with the accurate RIA measurement of leptin because of binding competition between the soluble leptin receptor and the leptin antisera during the immunoprecipitation to separate free from bound [^{125}I] leptin tracer (Gavrilova et al. 1997).

To the best of our knowledge, the soluble leptin receptor ELISA we used is the only current commercially available assay that has been published by literature (Gan et al. 2012; Lee, et al. 2018; Pereira, et al. 2019; Yamada, et al. 2019), but has not been verified previously in studies using pregnant mice. Qualitatively, the assay was sufficient to confirm a significant

increase in plasma soluble leptin receptor from dpc18.5 dams relative to nonpregnant females. However, the increase was only two-fold compared to typical 30 to 40-fold increases in other reports (Gavrilova et al. 1997; Lammert et al. 2002). Therefore, the ELISA may not have been sensitive enough to detect changes associated with the elevated levels of leptin in either genotype of mice at late pregnancy. Another possibility is that the LepRe ELISA kit we used registers lower than expected values for soluble leptin receptor in plasma with greatly elevated leptin due to some unknown interference. However, data provided with the ELISA states that the addition of mouse leptin up to 50 ng/ml does not interfere with the assay. This does not exclude the possibility of assay interference from some other component of the plasma differentially produced in $Pomc^{\Delta 1/\Delta 1}$ vs. $Pomc^{+/+}$ dams. Regardless of these considerations, further attempts to modify performance of the ELISA or to adopt alternative assays for quantifying either leptin or its soluble receptor are beyond the scope of this paper.

We evaluated the physiological importance of *Pomc* nPE1 on the placenta and our results showed that lack of nPE1 did not change the expression of two critical placental genes (*Plac1* and *Tpbpa*) or alter placental morphology based on intact layers of the labyrinth zone, junctional zone and maternal decidua basalis. Compared to *Pomc* expression in ARC, *Pomc* was expressed at a low but detectable level in placenta (Supplementary Figure 3). Although nPE1 is a placental mammalian novelty, placental *Pomc* expression was unchanged in either $Pomc^{\Delta 1/\Delta 1}$ or $Pomc^{ho\Delta 1/\Delta 1}$ mutant mice compared to wildtype mice. Therefore, similar to pituitary corticotrophs and melanotrophs, nPE1 apparently does not play a role in the regulation of *Pomc* expression in placenta. The lack of a prominent altered phenotype in placentas or offspring of nPE1 deletion animals indicates that nPE1 and decreased ARC *Pomc* expression are not essential for pregnancy under *ad libitum* feeding conditions. Our previous work identified a rodent-specific deletion in the nPE1 *core* sequence, suggesting that nPE1 in mice may have different functional activities than in other mammals (Franchini et al. 2011). Moreover, laboratory mice were housed in ventilated cages without any external challenges such as food deprivation or extreme environmental conditions. The existence of nPE1 may facilitate the ability of placental animals to adapt to their environment for improved survival and reproduction, which happens during natural selection and is difficult to replicate using lab mice.

In conclusion, we have demonstrated that the neuronal *Pomc* enhancer nPE1, despite being a placental novelty of mammalian evolution, is dispensable for placental expression of *Pomc* during mouse pregnancy and for the marked metabolic adaptations of the dam during pregnancy and lactation under *ad libitum* access to food. Regardless of the unknown function of *Pomc* expression in placenta, we have revealed that a 70% reduction of *Pomc* expression in the ARC leads to substantial increases of plasma leptin and moderately high placental soluble leptin receptor transcript levels at late pregnancy, independent of total body fat mass, compared to control mice.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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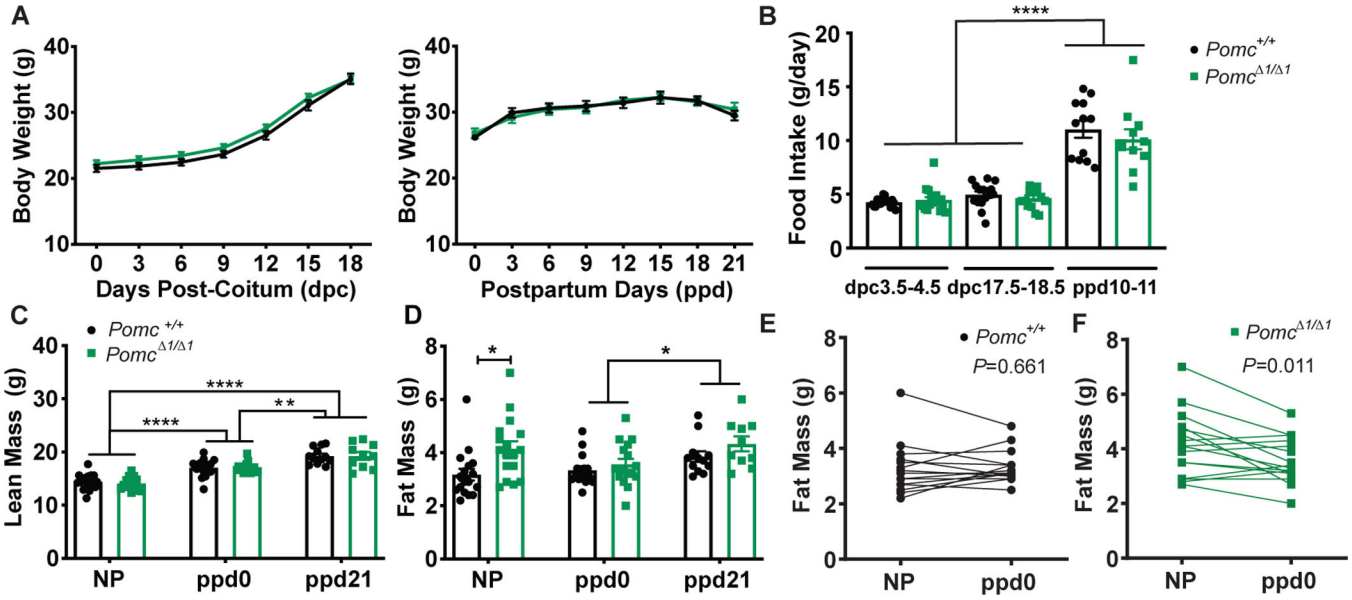

Figure 1: Targeted deletion of neuronal *Pomc* enhancer nPE1 reduced total body fat mass during pregnancy but not body weight during pregnancy or lactation.

A). Comparison of body weight changes across gestation (n=16–17) and lactation (n=6–8) between $Pomc^{+/+}$ and $Pomc^{\Delta 1/\Delta 1}$ dams. B). Comparisons of 24 hr food intake at early pregnancy (dpc3.5-dpc4.5, n=17–18), late pregnancy (dpc17.5-dpc18.5, n=14–15) and mid lactation (ppd10-ppd11, n=11–12). Body composition comparisons between $Pomc^{+/+}$ and $Pomc^{\Delta 1/\Delta 1}$ dams (C and D) at the indicated stages (n=10–17). Two-way ANOVAs followed by Bonferroni's *post-hoc* multiple comparison tests for pair-wise comparisons of food intake, fat mass and lean mass (A, B, C and D). $Pomc^{\Delta 1/\Delta 1}$ dams exhibited a significant reduction in fat mass during gestation from NP to ppd0 (E) while control $Pomc^{+/+}$ dams had no change in fat mass during gestation (F). Paired Student's two-tailed *t*-tests were used to determine the significant genotype differences in fat mass from NP to ppd0 (E and F). NP, non-pregnant; dpc, days post coitum; ppd, postpartum day. Data shown are the mean ± SEM. $*P < 0.05$, $**P < 0.01$ and $****P < 0.0001$.

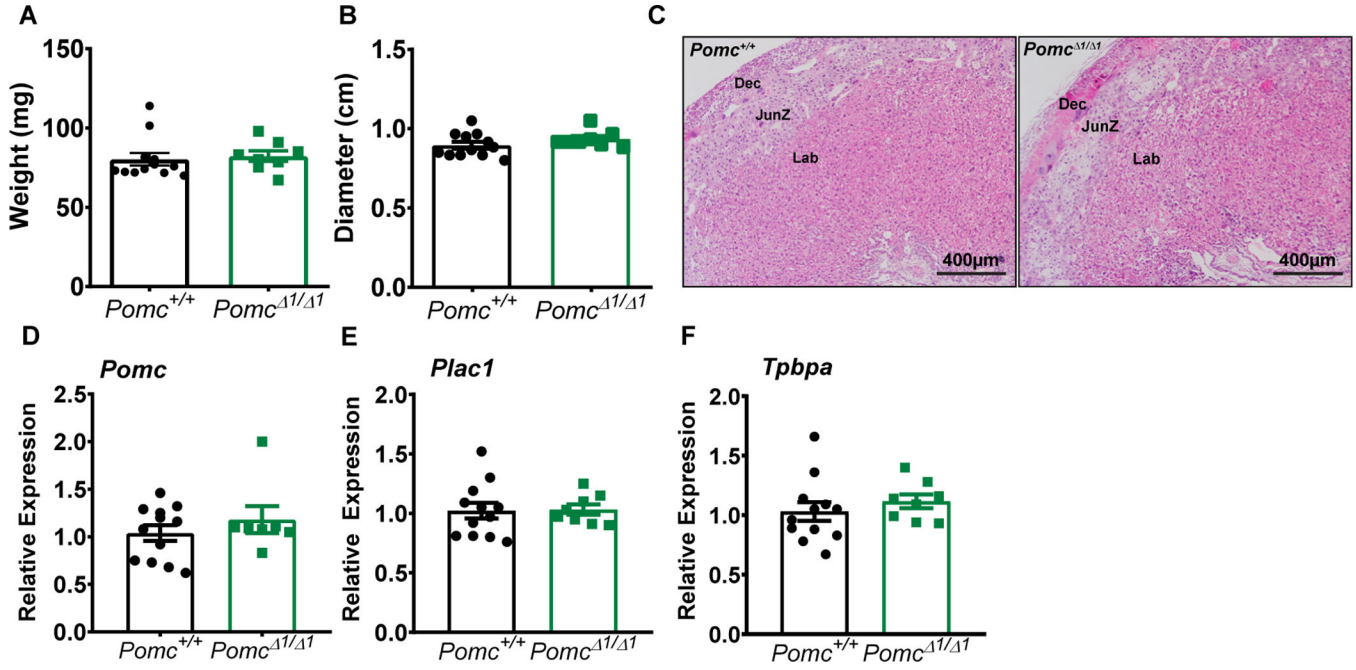

Figure 2. Pregnant mice with neuronal *Pomc* enhancer nPE1 deletion had normal placental morphology and function.

Three placentas from each dam were measured and the average weight (A) and diameter (B) were compared between *Pomc*$^{+/+}$ and *Pomc*$^{\Delta 1/\Delta 1}$ (n=8–12 dams). Placental morphology was characterized by Hematoxylin and Eosin (H&E) staining (C). Dec, Decidua; JunZ, Junctional Zone; Lab, Labyrinth. Quantitative RT-PCR was performed to compare *Pomc* (D), *Plac1 (Placenta specific 1)* (E) and *Tpbpa (Trophoblast specific protein alpha)* (F) gene expression in placenta (n=8–12 dams). Unpaired Student's two-tailed t-tests revealed no significant genotype differences for any of the variables. Data shown are the mean ± SEM.

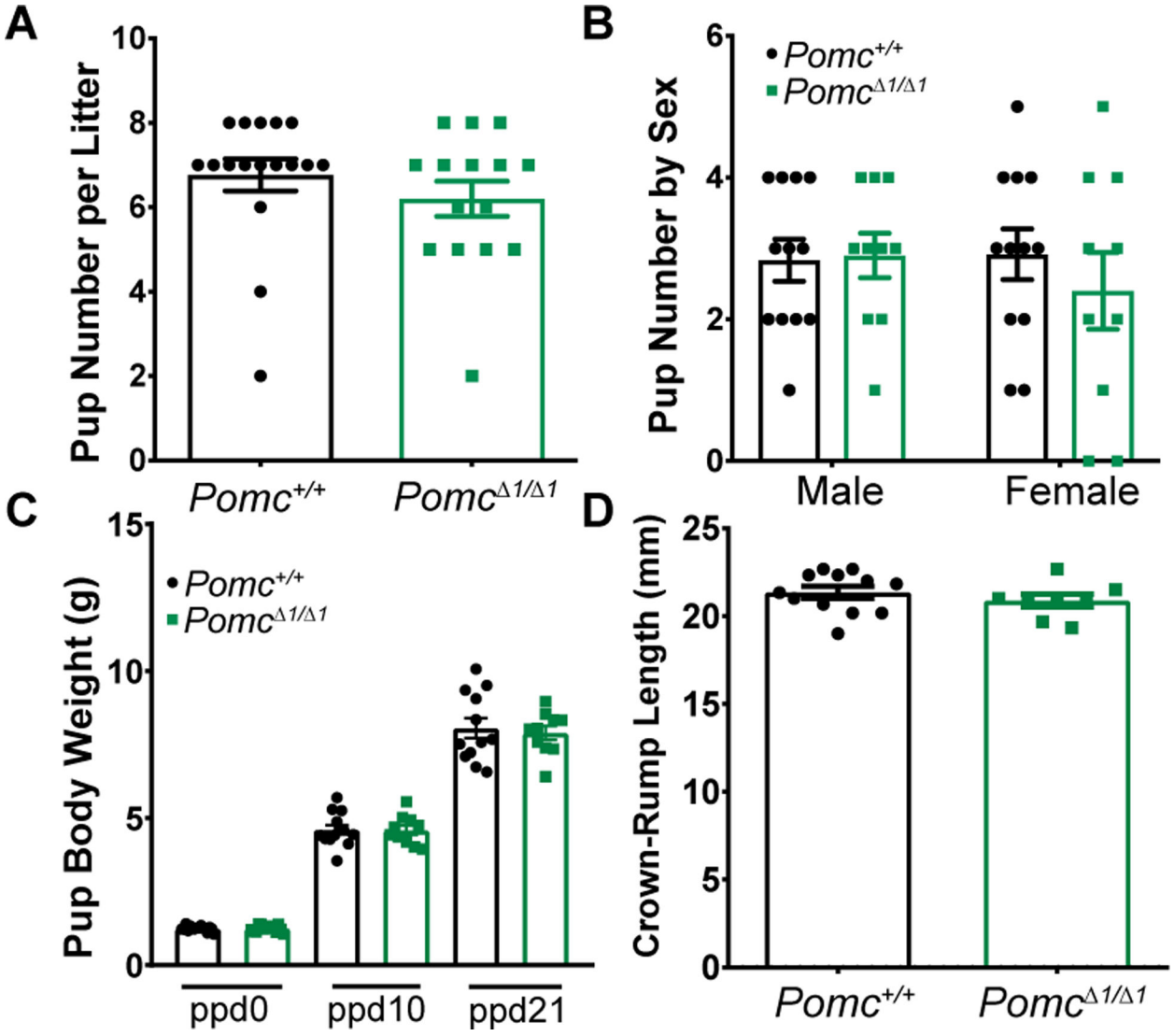

Figure 3. Maternal deletion of neuronal *Pom*c enhancer nPE1 did not cause any growth defects in the offspring.

A) Average number of pups per litter (n=17–19 litters), B) average number of male/female pups per litter (n=10–16 litters), C) body weight of pups (n=10–17 litters) at the indicated postnatal days (ppd) and D) Crown-Rump lengths from dpc18.5 fetuses (n=11–12 litters) were compared between genotypes. Unpaired two-tailed Student's *t*-tests revealed no significant genotype differences for any of the variables. Data shown are the mean ± SEM.

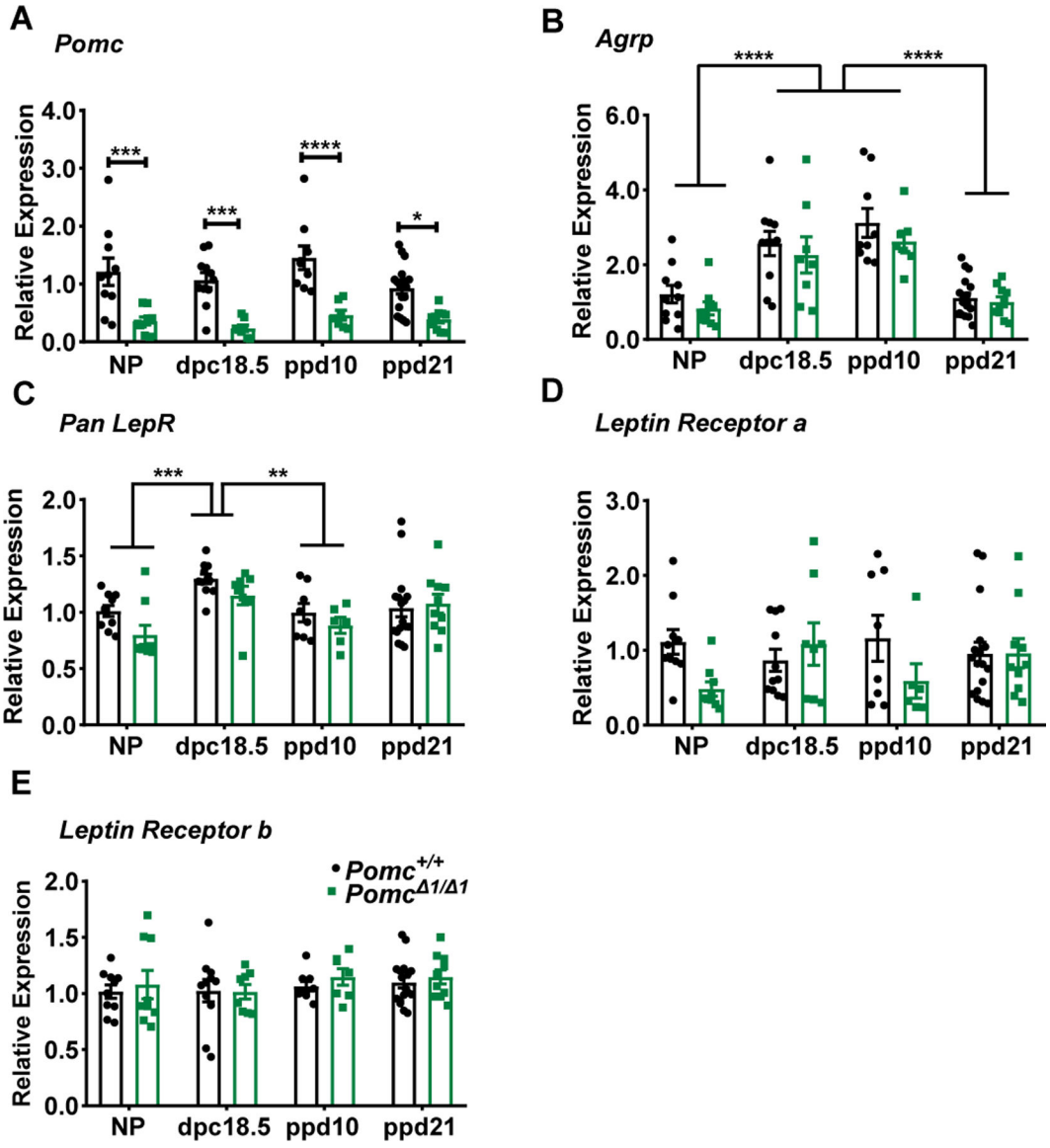

Figure 4. Gene expression in the hypothalamic arcuate nucleus measured by qRT-PCR from *Pomc*$^{\Delta 1/\Delta 1}$ mice relative to *Pomc*$^{+/+}$ controls before pregnancy, at late gestation, at lactation and at weaning.

Comparison of relative gene expression for *Pomc* (A), *Agrp* (B) and *Leptin Receptor isoforms* (C-E) from the two genotypes at indicated stages (n=8–17 dams). NP, not pregnant; dpc, day post coitum; ppd, postpartum day. Two-way ANOVAs followed by Bonferroni's *post-hoc* multiple pair-wise comparison tests were performed. Data shown are the mean ± SEM. *$P < 0.05$, **$P < 0.01$ and ****$P < 0.0001$.

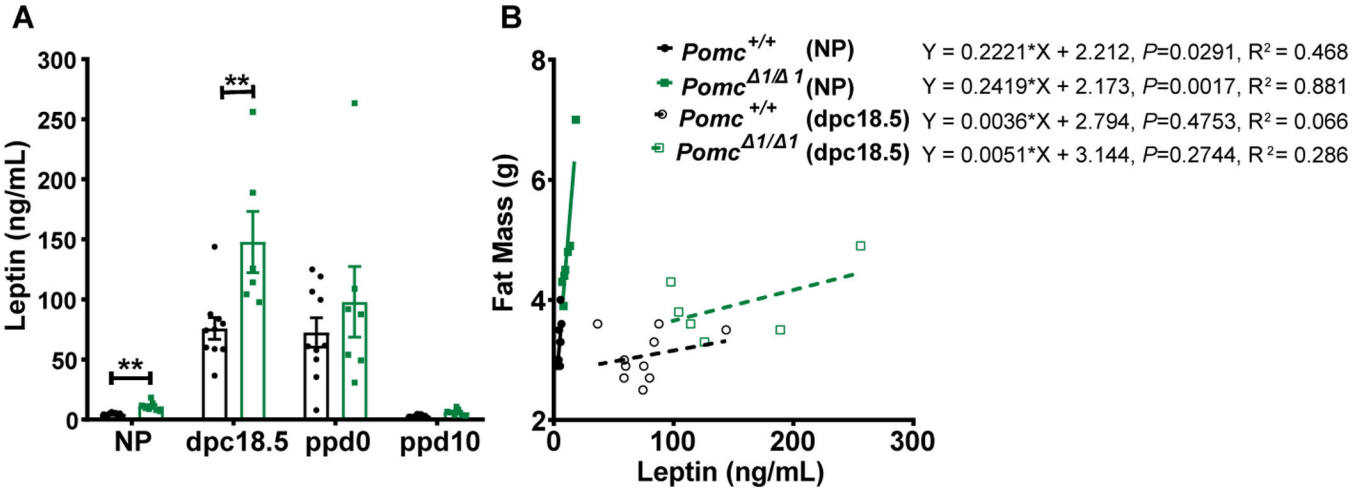

Figure 5. Deletion of neuronal *Pomc* enhancer nPE1 significantly increased plasma leptin levels at dpc18.5 independent of total body fat mass.

(A) Serial plasma leptin levels at indicated experimental stages (n=7–10 dams). Two-way ANOVAs followed by Bonferroni's *post-hoc* multiple pair-wise comparison tests and unpaired two-tailed Student's *t*-tests were performed to evaluate the genotype effect. Data shown are the mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.005$. (B) Correlation and linear regression analyses of plasma leptin with respect to body fat mass in non-pregnant (NP) and dpc18.5 mice (n= 6–10 dams). The *P*-values indicate whether regression line slopes are significantly different from 0.

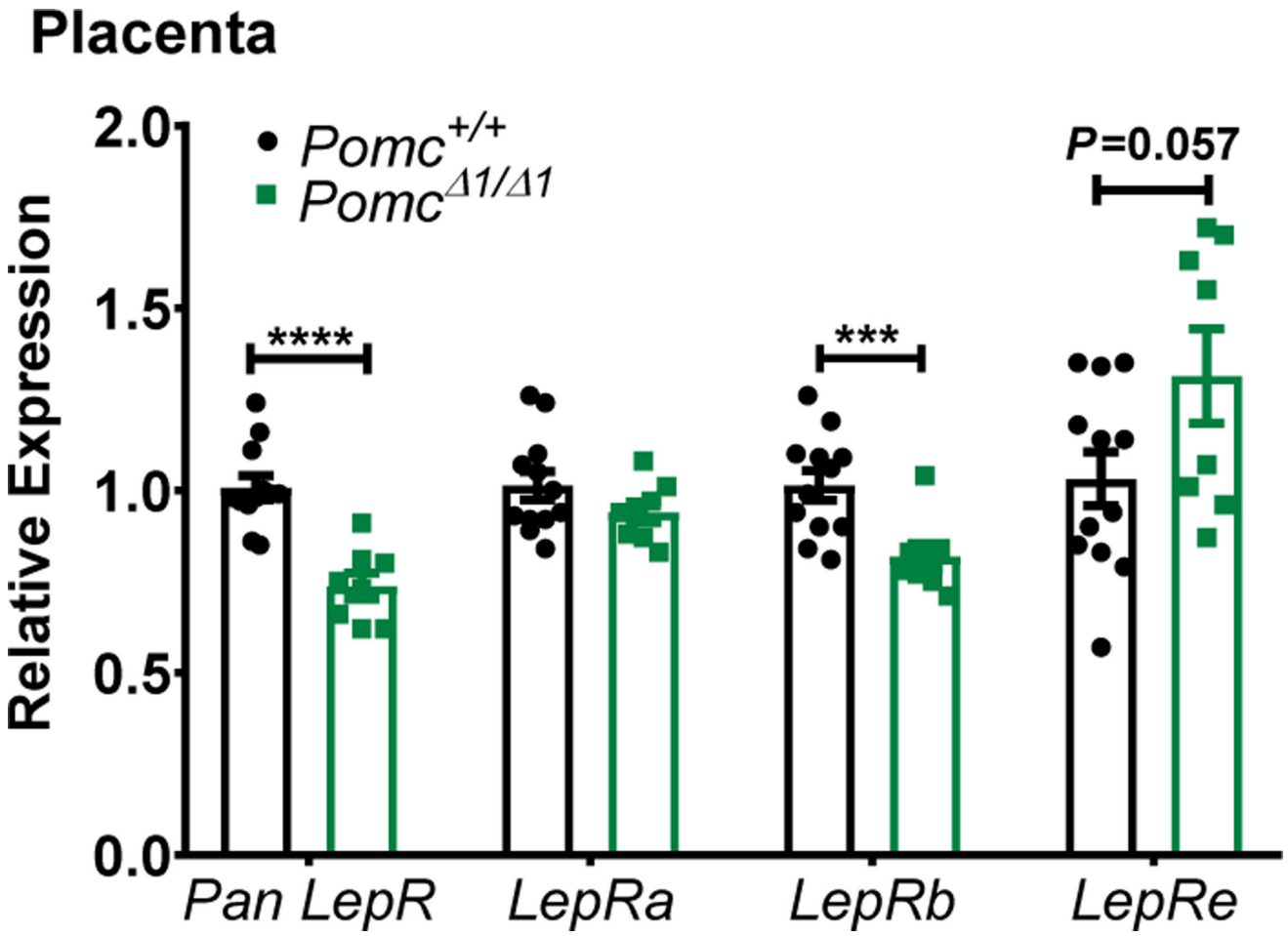

Figure 6. Inactivation of *Pomc* enhancer nPE1 increased *soluble leptin receptor* (*LepRe*) expression in the placenta at late pregnancy stage (dpc18.5).

Quantitative RT-PCR was performed to detect expression of *Leptin Receptor* isoforms in placenta at dpc18.5 (n=8–12 dams). Two-tailed Student's t-tests were performed to determine any significant differences between genotypes. Data shown are the mean ± SEM. *** $P < 0.005$, **** $P < 0.0001$.

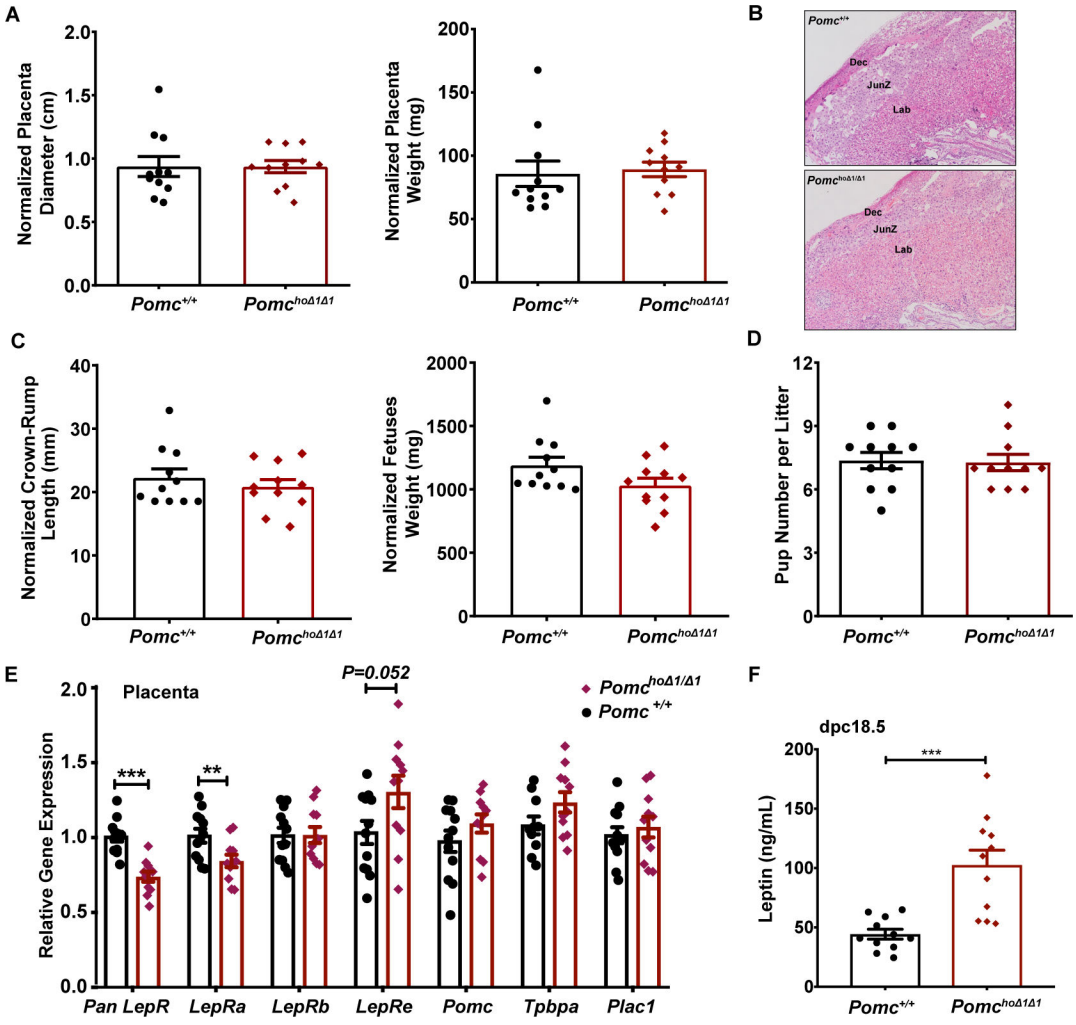

Figure 7. *Pomc*$^{hoΔ1/Δ1}$ dams had increased plasma leptin levels and *LepRe* expression in placenta.

Comparison of normalized placental diameters, normalized placental weights (A) and placental morphology (B) (n=11 dams) between *Pomc*$^{+/+}$ and *Pomc*$^{hoΔ1/Δ1}$ dams. Three placentas from each dam were measured. The normalized value was calculated by averaging three measurements, dividing by the number of pups per litter and multiplying by the average number of pups per litter from each genotype. (C) Normalized Crown-Rump lengths, normalized fetal weights and (D) number of pups per litter (n=11 litter) at dpc18.5 from *Pomc*$^{+/+}$ and *Pomc*$^{hoΔ1/Δ1}$ dams. (E) Gene expression profiles in *Pomc*$^{hoΔ1/Δ1}$ placentas (n=10–11 dams). (F) Comparison of plasma leptin levels between *Pomc*$^{+/+}$ and *Pomc*$^{hoΔ1/Δ1}$ (n=10–11 dams) at dpc18.5. Data outliers were identified and removed using the ROUT method in Prism8. Each data set was analyzed by unpaired two-tail t-tests to identify genotype differences. Data shown are the mean ± SEM. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.005$.