OBESITY

Physiological consequences of transient hyperleptinemia during discrete developmental periods on body weight in mice

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Leptin plays a role in central nervous system developmental programs and intercurrent physiological processes related to body fat regulation. The timing and neuromolecular mechanisms for these effects are relevant to the prevention and treatment of obesity. Factors implicated in a body weight "set point" including dietary fat, circulating leptin, and other adipokines tend to covary with adiposity and are difficult to disarticulate experimentally. To dissociate leptin effects from adiposity and diet, we created a transgenic mouse in which leptin expression is regulated by doxycycline exposure. Using this system, we investigated the physiological consequences of developmentally-timed transient hyperleptinemia on subsequent adiposity. We evaluated physiological effects of leptin elevation during adulthood (9 to 29 weeks old), "adolescence" (3 to 8 weeks old), and the immediate postnatal period [postnatal days 0 to 22 (P0 to P22)] on long-term adiposity and susceptibility to gain weight on high-fat diet (HFD) fed ad libitum. We found that inducing chronic hyperleptinemia in adult or "adolescent" mice did not alter body weight when excess leptin was discontinued, and upon later exposure to HFD, weight gain did not differ from controls. However, transient elevation of circulating leptin from P0 to P22 increased weight and fat gain in response to HFD, indicating greater susceptibility to obesity as adults. Thus, transient plasma leptin elevations—mimicking one aspect of transient adiposity—increased later susceptibility to diet-induced obesity, although these effects were restricted to a critical developmental (P0 to P22) time window. These findings may have clinical implications for weight management in infancy.

INTRODUCTION

Childhood obesity is highly correlated with subsequent adult obesity (*1*). Ninety percent of children who are obese at 3 years of age are overweight or obese as adolescents, and infants born large for their gestational age have a 1.5-fold higher risk of being obese as adolescents compared to babies born normal or small for their gestational age (*2*). These correlations of childhood obesity with obesity later in life likely reflect interactions of genetic predisposition (*3*, *4*) with developmental (*5*) and environmental (*3*, *6*) factors. The increasing prevalence of childhood and adult obesity during the past 30 years (*7*, *8*) suggests a substantial impact of nongenetic factors. Accordingly, close attention is being paid to the gestational and early childhood influences mediated by maternal adiposity (*9*) and early feeding practices associated with adiposity (*10*–*12*). The mechanistic bases for such effects (including genetic susceptibility) are difficult to disarticulate experimentally, but these distinctions are critical to developing preventive measures for obesity (*13*, *14*). The studies reported here are based on the premise that the hormone leptin, by virtue of its responsiveness to systemic nutritional status and adiposity (*15*, *16*) and importance in the development of central nervous system (CNS) circuits that subserve energy homeostasis (*17*, *18*), could mediate some of the long-term effects of transient perturbations of adiposity.

Dietary fat and adiposity alter leptin signaling in the CNS (*19*). The extent to which molecular (leptin homeostasis) and neuroanatomic (neuronal circuit alteration) processes involved in the establishment of the neuroregulatory mechanisms of adiposity can be influenced by environmental factors such as adequacy and quality of intrauterine metabolic fuels and endocrine processes, postnatal diet, and intercurrent obesity is unclear. Specifically, can the long-term regulation of adiposity be altered by perinatal factors, and what are the mechanisms underlying such alterations? Dietary fat content (*19*), adiposity (*19*), overfeeding (*20*), and leptin (*21*) have been implicated in such effects, but because these factors are experimentally correlated, isolation of individual factors during development has not been possible. The underlying cellular and molecular mechanisms for maternal programming of adult obesity remain unclear (*22*–*28*).

Leptin is a neurotrophic factor that plays a critical role in the development of hypothalamic feeding circuits (*17*). Leptin deficiency in mice during the first 12 days of life impairs the formation of projections from the arcuate nucleus of the hypothalamus (ARH) to other brain regions involved in energy homeostasis [the paraventricular nucleus (PVH), the dorsomedial hypothalamic nucleus (DMH), and the lateral hypothalamic area (LHA)] (*17*). Other experiments have shown that maternal high-fat feeding or hyperinsulinemia can impair the formation of these circuits (*20*, *29*). We hypothesized that excess leptin during this time period could also influence circuit formation.

Here, we investigated the physiological consequences of developmentally timed transient hyperleptinemia on subsequent adiposity. Our hypothesis was that periods of transiently increased leptin lead to higher subsequent body weight by virtue of effects on neural structures that regulate adiposity and that the timing of this exposure would be an important factor in such responses. We generated a

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doxycycline (dox)–responsive leptin transgenic mouse in which noninvasive induction of hyperleptinemia can be isolated from diet composition, obesity, and its resulting metabolic consequences such as elevated free fatty acids and glucose, insulin insensitivity, and fatty liver. A tetracycline-controlled Tet-On gene expression system enabled transgenic leptin expression regulated by exposure to dox in a dose-responsive manner that can be rapidly turned on and off. We evaluated the physiological effects of transient elevation of leptin in ad libitum chow-fed mice during "adulthood" [9 to 29 weeks old, postnatal days 63 to 203 (P63 to P203)], "adolescence" (3 to 8 weeks old, P22 to P56), and the "postnatal period" (P0 to P22) on subsequent adiposity and susceptibility to gain weight when offered a highly palatable diet ad libitum.

RESULTS

Generation and validation of leptin-overexpressing mice

We generated leptin-overexpressing mouse embryonic stem (ES) cells (KH2-Lep) (fig. S1, A to D) and subsequently used them to create transgenic mice via tetraploid blastocyst injection (*30*). The transgenic mouse generated for the experiments reported in this manuscript required two transgenes for leptin expression in response to dox: the reverse tet-transactivator (*M2rtTA*) at the *Rosa26* locus (R26-*rtTA*) and the leptin transgene in *Col1A1* locus controlled by tetracycline-responsive elements (TREs) and the cytomegalovirus promoter (TRE-*Lep*). Dox-induced leptin-overexpressing mice ("2TG") responded to dox exposure by producing leptin, whereas control mice ("1TG") contained only the TRE-*Lep* transgene and did not respond to dox (fig. S1B).

Induction of leptin in 2TG mice is proportional to dox concentration (Fig. 1A). Circulating leptin in 2TG mice increased at the lowest dox dose of 10 μg/ml (1.9 ± 0.4 ng/ml to 30.6 ± 5.4 ng/ml), whereas 1TG controls maintained proportionality of leptin to fat mass (Fig. 1A). The induction of leptin in 2TG mice ceased rapidly upon withdrawal of dox (Fig. 1B). 2TG mice were exposed to successive intervals of 24 hours of ad libitum access to dox (200 μg/ml) immediately, followed by 24 hours of dox withdrawal. Within 24 hours of dox exposure, circulating leptin in 2TG mice increased from 18.1 ± 5.8 ng/ml to 98.6 ± 11.5 ng/ml and returned to baseline within 24 hours of dox removal (16.2 ± 6.1 ng/ml; Fig. 1B). After two cycles of 24 hours of dox exposure, circulating leptin in 2TG mice returned to concentrations seen in animals provided with dox-free water, but these concentrations were significantly below the baseline values (13.1 ± 5.5 ng/ml versus 18.1 ± 5.8 ng/ml; $P < 0.01$; Fig. 1B), suggesting that overexpression of leptin for 48 hours reduced endogenous leptin production, possibly due to fat loss. To assess the kinetics of leptin overexpression, we gavaged 2TG mice with 400 μg of dox. Plasma leptin concentrations were induced within 4 hours and peaked after 6 to 8 hours (83.62 ± 18.68 ng/ml; Fig. 1C). Within 24 hours after the single dox gavage, plasma leptin concentrations returned to baseline with no residual effects on plasma leptin (Fig. 1C).

Because *rtTA* was inserted in the *Rosa26* locus, its expression was ubiquitous. The primary source of the increased plasma leptin in the dox-induced 2TG mice was the gastrointestinal tract (stomach, duodenum, jejunum, and ileum), which was likely due to both the oral route of dox administration and the capacity of the gut to secrete peptides (fig. S1, E to I). Other tissues expressing transgenic leptin included the adipose tissue (perigonadal, subcutaneous, and brown), liver, kidney, lung, spleen, and the hypothalamus (fig. S1, E to I). Exposing adult 2TG mice to a dox concentration of 200 μg/ml in drinking water for 2 weeks induced circulating leptin concentrations that were at the high end of the physiological range in obese animals (70 to 227 ng/ml) and resulted in reduced body fat (fig. S2), indicating that dox-induced leptin was bioactive and capable of causing physiological responses. When 1TG nursing dams were exposed to dox at parturition, the dams remained euleptinemic, but the 2TG pups had significantly higher circulating leptin concentrations than the 1TG littermates ($P < 0.001$; fig. S3). Thus, bioactive dox is transferred in breast milk.

Hyperleptinemia in adult male mice (P63 to P203)

To investigate whether transient hyperleptinemia affects long-term body weight in adult animals, we exposed 9-week-old 1TG (controls) and 2TG (leptin-overexpressing) mice fed ad libitum chow to increasing concentrations of dox in drinking water containing 5% sucrose (2TG + dox group; Fig. 2A). Dox exposure was continued for 20 weeks. The dox dose was escalated on a biweekly basis to mimic in the 2TG mice the leptin concentration profile seen with high-fat diet (HFD)–induced weight gain (*31*). Circulating leptin concentrations in the 2TG + dox group were significantly higher than the control group starting at the dox dose (12.5 μg/ml; $P < 0.05$) and remained significantly elevated for the remaining 16 weeks until the cessation of dox ($P < 0.01$; Fig. 2B). Body weight, fat mass, lean mass, food intake, water intake, and plasma glucose were not different between the groups during the 20 weeks of hyperleptinemia (Fig. 2, C to H).

After a 20-week period of gradual elevation of circulating leptin concentrations, dox was eliminated from the drinking water, and leptin in 2TG mice declined to concentrations appropriately proportional

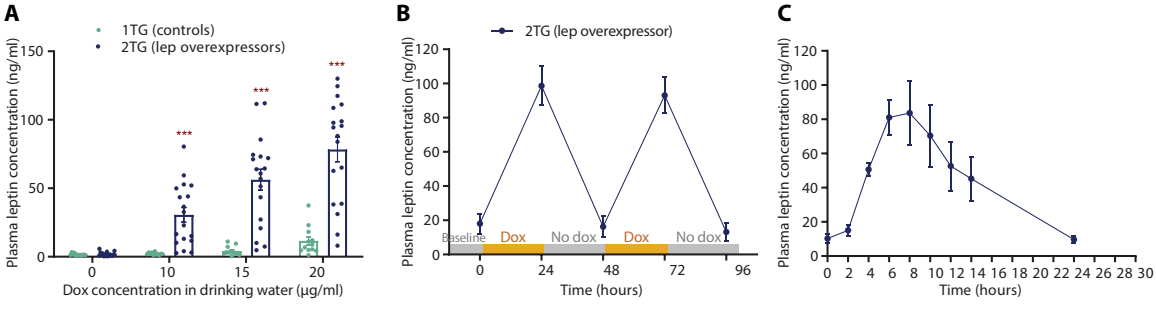

Fig. 1. Validation of leptin-overexpressing mice. (**A**) Dose response of 1TG control and 2TG dox-induced leptin (lep)–overexpressing mice to increasing amounts of dox in drinking water. (**B**) Rapid control of leptin expression with two 24-hour dox exposures in 2TG leptin-overexpressing mice. (**C**) Dynamic induction of circulating leptin after dox gavage 2TG leptin-overexpressing mice. All values are means ± SEM. Student's *t* test, ***$P < 0.001$.

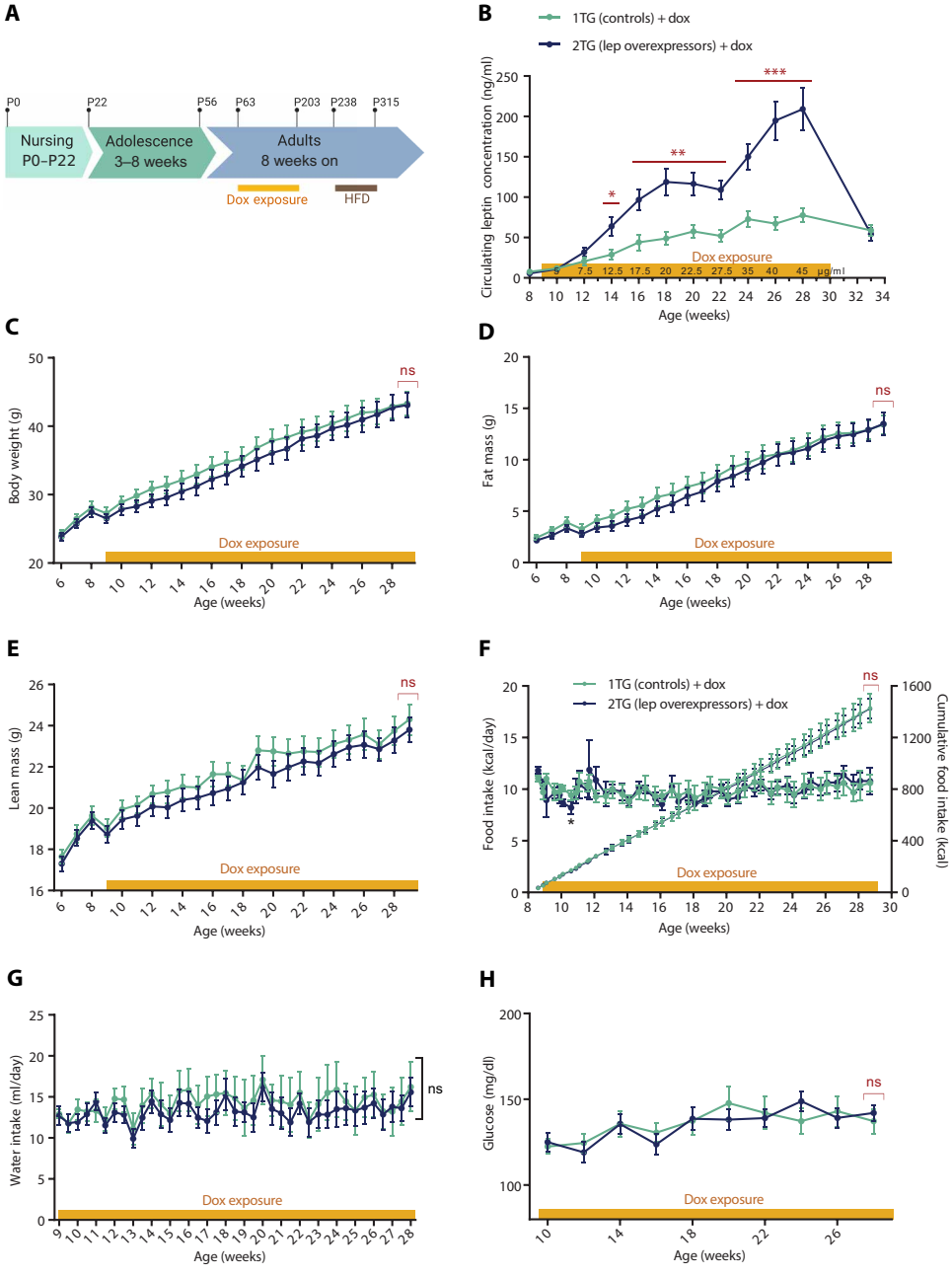

Fig. 2. Dox-induced chronic hyperleptinemia in adult mice (P63 to P203). (**A**) Schematic of the study timeline. (**B**) Plasma leptin concentrations (Student's *t* test at each dox dose). (**C**) Body weight (two-way RM ANOVA with Fisher's LSD post hoc analysis; genotype, $F_{1,32} = 0.34$, $P = 0.56$). ns, not significant. (**D**) Fat mass (two-way RM ANOVA with Fisher's LSD post hoc analysis; genotype, $F_{1,32} = 0.24$, $P = 0.63$). (**E**) Lean mass (two-way RM ANOVA with Fisher's LSD post hoc analysis; genotype, $F_{1,32} = 0.44$, $P = 0.51$). (**F**) Daily caloric food intake per mouse (left axis; two-way RM ANOVA with Fisher's LSD post hoc analysis; genotype, $F_{1,8} = 0.0036$, $P = 0.53$) and cumulative food intake (right axis). (**G**) Daily water intake per mouse (two-way RM ANOVA with Fisher's LSD post hoc analysis; genotype, $F_{1,8} = 0.18$, $P = 0.68$). (**H**) Venous whole blood glucose concentration (two-way RM ANOVA with Fisher's LSD post hoc analysis; genotype, $F_{1,32} = 0.016$, $P = 0.90$) in 1TG controls and 2TG dox-induced leptin-overexpressing mice given dox in 5% sucrose water during 20 weeks of escalating dox exposure. All values are means ± SEM. Red brackets, Student's *t* test of the final data point. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by Student's *t* test (red) or Fisher's LSD (black).

to the body fat (Fig. 2B). Leptin concentrations measured in 1TG and 2TG mice 5 weeks after dox cessation were indistinguishable (Fig. 2B). Body weight, composition, and food intake for 5 weeks after the cessation of hyperleptinemia were the same in both groups (Fig. 3). Mice were then fed a 60% HFD ad libitum to determine whether formerly hyperleptinemic mice were more responsive to highly palatable food. Mice were monitored for an additional 11 weeks but did not show any consistently significant differences in body weight, composition, or food intake (Fig. 3).

We repeated the experiment described above using a slightly different paradigm. Similar to the previous experiment, adult male 1TG controls and 2TG leptin-overexpressing mice were exposed to increasing concentrations of dox in water every 2 weeks for 20 weeks (fig. S4A). By the second dose of dox, the 2TG mice had gained less weight than the 1TG mice and had significantly lower body weight ($P < 0.01$), fat ($P < 0.01$), and lean mass ($P < 0.05$); the weights of 2TG mice recovered to those of controls within 4 weeks while still maintaining elevated circulating leptin concentrations (fig. S4, B to D). Seven weeks after the initiation of dox exposure, mice were given ad libitum access to 60% HFD concurrently with dox exposure. Both groups increased their caloric intake when exposed to HFD and gained a similar amount of weight (~18 g over 12 weeks; fig. S4, B and E). Body weight and composition of the HFD 1TG and 2TG animals did not differ for the remainder of the dox/HFD exposure period or after their cessation (figs. S4 and S5).

Hyperleptinemia in "adolescent" (P22 to P56) male mice

At weaning (P22), mice were separated by genotype, and three mice per cage were placed with ad libitum access to chow and given access to dox (50 μg/ml) in drinking water for 5 weeks (Fig. 4A). In response to dox, circulating leptin concentrations increased in 2TG mice by about 25-fold compared to 1TG controls. Leptin concentrations remained significantly elevated throughout the duration of the exposure ($P < 0.001$; Fig. 4B). Food intake was significantly reduced in 2TG mice compared to 1TG controls for 2 weeks after the switch to dox water ($P < 0.01$; Fig. 4C). Body weight, fat, and lean mass of 2TG mice were significantly lower than 1TG mice during dox exposure ($P < 0.001$; Fig. 4, D to F).

At 8 weeks of age, dox was discontinued, and plasma leptin concentrations measured 2 weeks after dox withdrawal had returned to fat mass proportional concentrations in the 2TG mice (Fig. 4B). After the sharp drop in circulating leptin, 2TG mice increased their

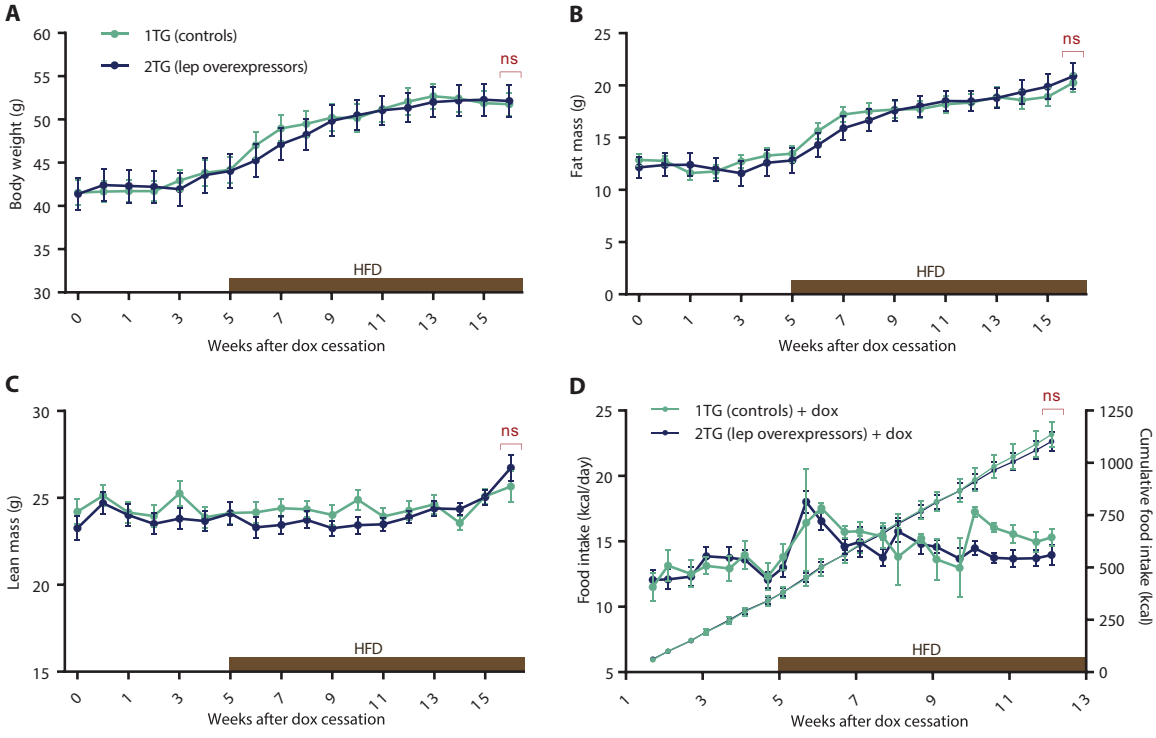

Fig. 3. Cessation of dox-induced chronic (P63 to P203) hyperleptinemia in adult mice. (**A**) Body weight (mixed-effects model with Fisher's LSD post hoc analysis; genotype, $F_{1,32} = 0.22$, $P = 0.88$). (**B**) Fat mass (mixed-effects model with Fisher's LSD post hoc analysis; genotype, $F_{1,32} = 0.012$, $P = 0.91$). (**C**) Lean mass (mixed-effects model with Fisher's LSD post hoc analysis; genotype, $F_{1,32} = 0.37$, $P = 0.55$). (**D**) Daily caloric food intake (left axis; two-way RM ANOVA with Fisher's LSD post hoc analysis; genotype, $F_{1,8} = 0.24$, $P = 0.63$) and cumulative food intake (right axis) per mouse after dox was discontinued in 1TG controls and 2TG dox-induced leptin-overexpressing mice. Five weeks after dox, cessation mice were switched from chow to 60% HFD (indicated by brown bar). All values are means ± SEM. Red brackets, Student's *t* test of the final data point.

food intake significantly for 1 week ($P < 0.01$; Fig. 4C) until their body weights increased to that of 1TG controls. Similarly, fat mass and lean mass were restored to those seen in 1TG mice within 2 weeks (Fig. 4, E and F). Once the 2TG mice caught up with controls, body weight, body composition, and food intake were not different from 1TG controls for the following 4 weeks, while mice were maintained on chow (Fig. 4, C to F).

Both 1TG and 2TG mice were switched from chow to 60% (calories as fat) HFD at 14 weeks (6 weeks after dox exposure). During the first 2 weeks, hyperphagia in response to the HFD was significantly greater in 2TG compared to 1TG mice ($P < 0.01$; Fig. 4C). During that time, 2TG mice gained significantly more weight than the 1TG controls ($P < 0.05$; Fig. 4G). After 4 weeks of HFD exposure, caloric intake remained identical in both groups until the end of the study. No statistically significant difference was detected in body weight between 1TG and 2TG mice after 8-week exposure to HFD ($P = 0.72$; Fig. 4D).

Hyperleptinemia in postnatal (P0 to P22) male and female mice

1TG dams were given dox in drinking water immediately after parturition until weaning of their progeny at P22 (Fig. 5A). Plasma leptin concentrations in 2TG leptin-overexpressing pups at P15 and P22 were elevated compared to 1TG control littermates (Fig. 5B) and were higher on P22 than on P15, presumably as a result of mice ingesting dox through drinking water directly at P22 as opposed to having dox transferred primarily through mother's milk at P15. Body composition (Fig. 5, C and D) and weight (Fig. 5E) were not significantly different ($P \geq 0.05$) between the 2TG leptin-overexpressing and 1TG control male mice during the period of hyperleptinemia (P0 to P22). At P22, pups were weaned, and dox exposure was stopped. One week after dox exposure, plasma leptin concentrations, body weight, and composition did not differ between 2TG and 1TG mice (Fig. 5B).

Until 10 weeks of age, mice were fed ad libitum chow, which resulted in no genotype-related differences in body weight, body composition, or food intake. Mice were then switched to 60% HFD. Both groups of mice increased food intake by about 50% in the first 3 days. There was no significant difference in caloric intake between the two groups ($P \geq 0.05$; Fig. 5F). However, the relative hyperphagia of 2TG mice (defined as caloric intake after the switch to HFD divided by caloric intake on the last week of regular chow before the switch) was significantly higher in the first 7 days of HFD feeding ($P < 0.05$; Fig. 5G). Within the first 3 days of HFD feeding, 2TG mice gained more weight than controls (2TG, 2.6 ± 0.2 g; 1TG, 1.6 ± 0.2 g; $P < 0.01$; Fig. 5H). 2TG mice continued to gain body weight at a greater rate than 1TG, and at the end of the study (38 weeks of age), the difference in body weight gain by genotype was 5.7 g ($P < 0.01$; Fig. 5H). The difference in absolute body weights reached significance 6 weeks after the start of HFD feeding ($P < 0.05$; Fig. 5E) and remained significant until the end of the study at 38 weeks of age (1TG, 49.9 ± 1.3 g; 2TG, 55.4 ± 1.5 g; $P < 0.05$; Fig. 5E). Throughout the study, lean mass did not differ by genotype (Fig. 5D). Fat mass was significantly higher in 2TG mice 4 weeks after the diet switch

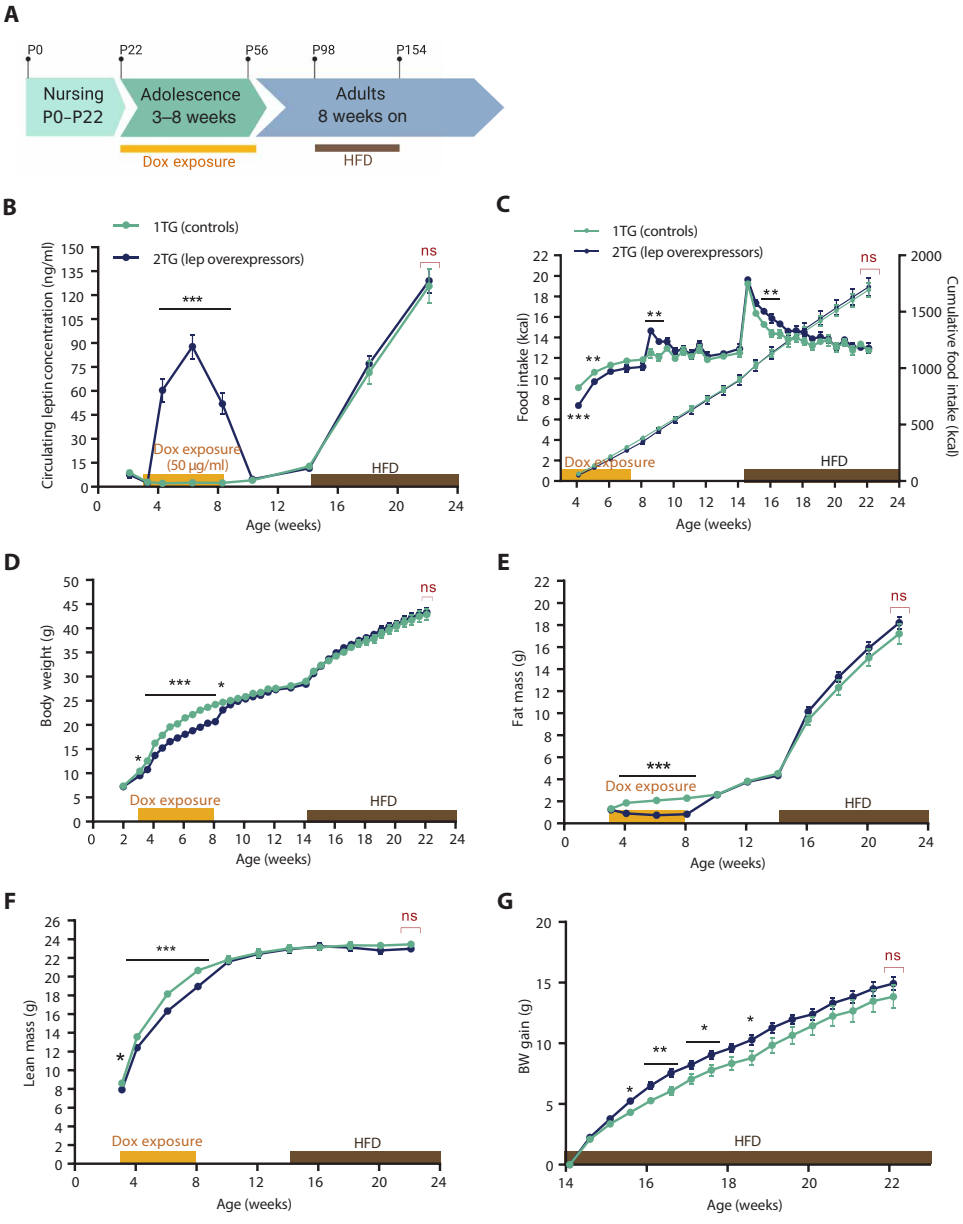

Fig. 4. Hyperleptinemia during adolescent period (P22 to P56), followed by 60% HFD ad libitum at 14 weeks. (**A**) Schematic of the study timeline. (**B**) Circulating leptin concentrations (mixed-effects model with Fisher's LSD post hoc analysis; genotype, $F_{1,34} = 38.6$, $P < 0.0001$). (**C**) Daily caloric food intake (left axis; two-way RM ANOVA with Fisher's LSD post hoc analysis; genotype, $F_{1,10} = 1.27$, $P = 0.29$) and cumulative food intake (right axis). (**D**) Body weight (two-way RM ANOVA with Fisher's LSD post hoc analysis; genotype, $F_{1,34} = 1.56$, $P = 0.22$). (**E**) Fat mass (two-way RM ANOVA with Fisher's LSD post hoc analysis; genotype, $F_{1,34} = 0.016$, $P = 0.90$). (**F**) Lean mass (two-way RM ANOVA with Fisher's LSD post hoc analysis; genotype, $F_{1,34} = 2.67$, $P = 0.11$). (**G**) Body weight gain (two-way RM ANOVA with Fisher's LSD post hoc analysis; genotype, $F_{1,34} = 3.01$, $P = 0.092$) of 1TG controls and 2TG dox-induced leptin-overexpressing mice throughout the study. All values are means ± SEM. Red brackets, Student's *t* test of the final data point. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by Student's *t* test (red) or post hoc Fisher's LSD (black). BW, body weight.

($P < 0.05$) and remained higher until the end of the study ($P < 0.001$; Fig. 5C). Circulating leptin concentrations were measured 4 and 18 weeks after the start of HFD exposure and were significantly higher in 2TG mice after 18 weeks of HFD feeding (1TG, 122.9 ± 8.0 ng/ml; 2TG, 172.6 ± 9.5 ng/ml; $P < 0.001$; Fig. 5B). Circulating leptin concentrations were proportional to fat mass in animals of both genotypes (Fig. 5I).

We repeated the experiment described above in female mice under similar conditions, with exposure to dox (200 μg/ml) in drinking water during the first 3 weeks of life, followed by HFD feeding at 10 weeks of age. The first measurements of plasma leptin concentrations were made at P9 (a week earlier than in the male cohort), P16, and P22. Plasma leptin concentrations were significantly elevated throughout the dox exposure ($P < 0.001$; Fig. 5J).

Fat mass was lower in 2TG leptin-overexpressing female mice compared to 1TG controls between P16 and P28. At this age, mice have very little body fat; nonetheless, the 2TG female mice had less fat than 1TG controls (Fig. 5L). In addition, lean mass and body weight were decreased at P22 in the 2TG versus 1TG group (Fig. 5, K and M). In male mice, we did not detect any differences in body weight or composition at this time.

Similar to the male cohort, female 2TG mice did not differ from the 1TG group in body weight, body composition, or food intake while being maintained on ad libitum chow. Once exposed to HFD, 1TG and 2TG female mice gained weight at a slower rate than male mice, and hyperphagia (relative to chow) in both 1TG and 2TG female mice was detected only during the first 3 days after the initiation of HFD exposure (Fig. 5N). However in male mice, HFD feeding induced hyperphagia for the duration of HFD exposure (Fig. 5F). There was no detectable difference in mean daily or cumulative food intake between the female 1TG and 2TG animals during the first 5 weeks of the HFD (Fig. 5N). After about 6 weeks of ad libitum HFD feeding, the female mice began to gain weight at a faster rate than the male mice, and 2TG female mice started to increase in body weight and fat mass compared to 1TG female mice, with genotype-related differences in body weight and fat mass reaching statistical significance at 10 and 11 weeks of HFD feeding, respectively (Fig. 5, J and K). In female mice, the final differences in body weight and fat mass at 28 weeks of age, by genotype, were 7.5 g ($P < 0.001$) and 6.9 g ($P < 0.001$), respectively (Fig. 5, J and K). Lean mass was not different by genotype at the end of the study (Fig. 5M).

After 16 weeks of HFD feeding, a subset of 16 females ($n = 8$ for each genotype) were housed in metabolic cages to assess energy expenditure (fig. S6). Absolute energy expenditure and energy expenditure per unit of lean mass were not significantly different

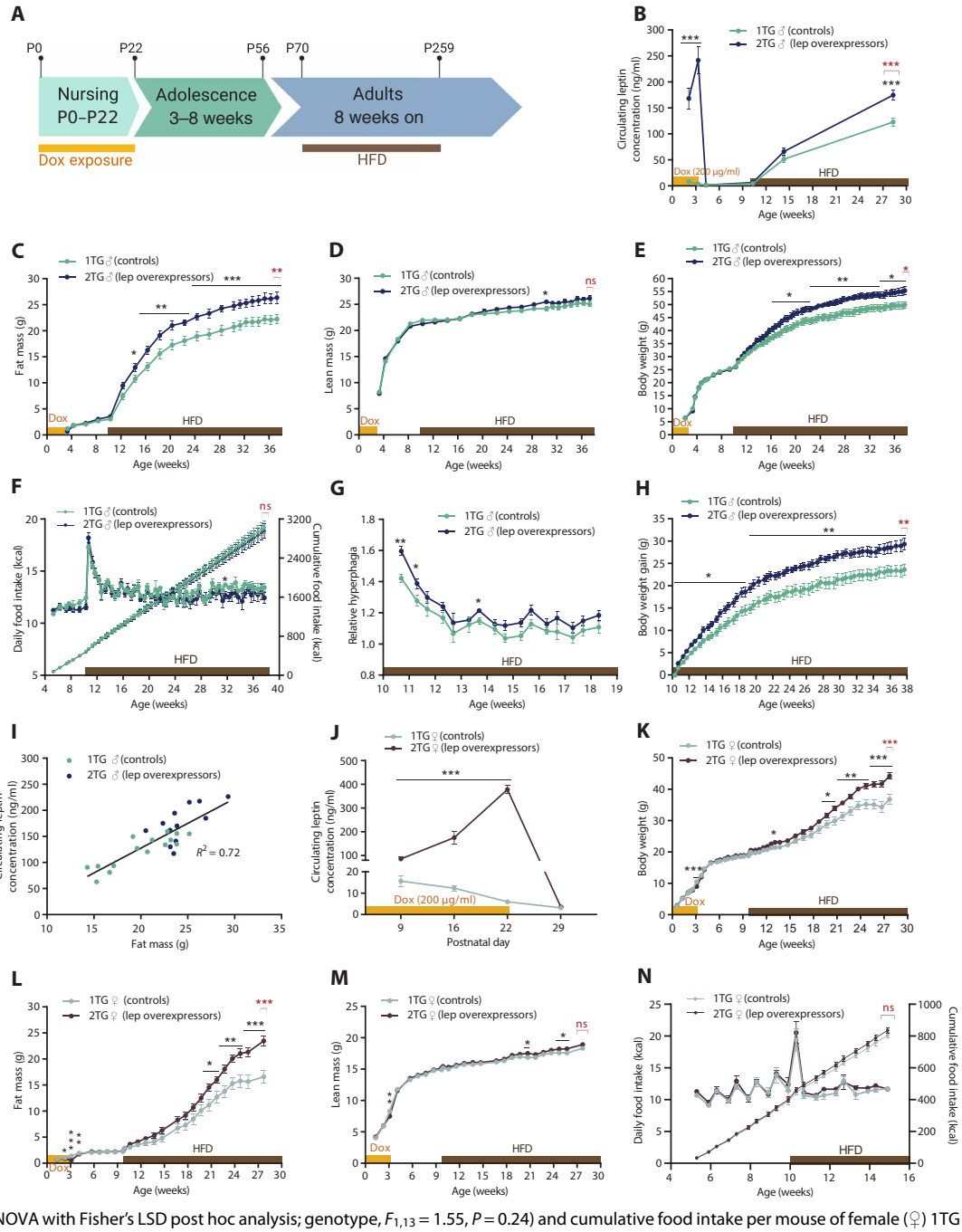

Fig. 5. Hyperleptinemia in male and female mice during postnatal period (P0 to P22), followed by switch from ad libitum chow to 60% HFD at 10 weeks. (**A**) Schematic of the study timeline. (**B**) Circulating leptin concentrations (mixed-effects model with Fisher's LSD post hoc analysis; genotype, $F_{1,25} = 67$, $P < 0.0001$). (**C**) Fat mass (mixed-effects model with Fisher's LSD post hoc analysis; genotype, $F_{1,25} = 9.4$, $P < 0.01$). (**D**) Lean mass (mixed-effects model with Fisher's LSD post hoc analysis; genotype, $F_{1,25} = 0.062$, $P = 0.44$). (**E**) Body weight (two-way RM ANOVA with Fisher's LSD post hoc analysis; genotype, $F_{1,25} = 7.0$, $P < 0.05$). (**F**) Daily caloric food intake (left axis; two-way RM ANOVA with Fisher's LSD post hoc analysis; genotype, $F_{1,8} = 0.62$, $P = 0.45$) and cumulative food intake (right axis) per mouse. (**G**) Fold increase in caloric intake after the initiation of HFD feeding (two-way RM ANOVA with Fisher's LSD post hoc analysis; genotype, $F_{1,8} = 5.0$, $P = 0.56$). (**H**) Body weight gain (two-way RM ANOVA with Fisher's LSD post hoc analysis; genotype, $F_{1,25} = 11.27$, $P < 0.01$) of 1TG controls and 2TG dox-induced leptin-overexpressing male (♂) mice throughout the study. (**I**) Regression of fat mass versus circulating leptin concentrations of male (♂) 1TG and 2TG mice at 28 weeks of age, after 18 weeks of HFD. (**J**) Plasma leptin concentrations (mixed-effects model with Fisher's LSD post hoc analysis; genotype, $F_{1,39} = 296.4$, $P < 0.0001$). (**K**) Body weight (two-way RM ANOVA with Fisher's LSD post hoc analysis; genotype, $F_{1,39} = 6.74$, $P < 0.013$). (**L**) Fat mass (mixed-effects model with Fisher's LSD post hoc analysis; genotype, $F_{1,39} = 6.90$, $P < 0.05$). (**M**) Lean mass (mixed-effects model with Fisher's LSD post hoc analysis; genotype, $F_{1,39} = 2.23$, $P = 0.14$). (**N**) Estimated daily caloric food intake (two-way RM ANOVA with Fisher's LSD post hoc analysis; genotype, $F_{1,13} = 1.55$, $P = 0.24$) and cumulative food intake per mouse of female (♀) 1TG controls and 2TG dox-induced leptin-overexpressing mice. All values are means ± SEM. Red brackets, Student's *t* test of the final data point. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by Student's *t* test (red) or post hoc Fisher's LSD (black).

between 1TG and 2TG female mice (fig. S6, A and B). Neither cumulative food intake over the 6-day measurement (fig. S6C) nor the respiratory exchange ratio (fig. S6D) differed significantly between the 1TG and 2TG groups.

DISCUSSION

Genetic changes cannot account for the rapid increase in obesity prevalence in the past four decades, and the relevant environmental factors are challenging to dissociate experimentally. Among potential factors of interest is exposure to hypernutrition in early infancy (*10*, *11*). We used transient elevation in circulating leptin as a surrogate for transient increases in adiposity to avoid the confounding effects of hyperphagia (*32*), diet composition (*19*, *31*), and weight change (*31*, *33*). Obese or HFD-fed animals have complex metabolic phenotypes that include increased circulating free fatty acids, elevated glucose, decreased insulin sensitivity, increased circulating insulin, and fatty liver. However, the transgenic mouse model used in this

study isolated hyperleptinemia from confounders of HFD feeding and maternal obesity. In addition, mice in the experimental and control groups shared dams and were therefore developed under shared embryonic and postnatal conditions with leptin concentrations being the only factor differing between the groups.

In the leptin/rtTA double transgenic mouse, leptin produced by the transgene is bioactive and emanates primarily from secretory cells in the gut. The transient weight loss of the animals observed in response to dox was proportional to circulating concentrations of leptin, consistent with the intact conventional leptin physiology in these animals.

We evaluated the effects of transient hyperleptinemia at three distinct developmental periods on body weight and subsequent responses to a highly palatable food. We found that inducing chronic hyperleptinemia in adult mice did not increase long-term body weight after hyperleptinemia was discontinued and did not affect hyperphagia in response to HFD. We then showed that hyperleptinemia during adolescence did not alter body weight after the cessation of dox exposure but did transiently increase the intake of an HFD without affecting weight gain or body composition after 8 weeks of being fed an HFD. Last, we found that transient elevation of circulating leptin in the immediate postnatal period increases the hyperphagic response to an HFD and renders animals more susceptible to obesity as adults 7 weeks after their transient hyperleptinemia. These results emphasize the importance of the timing of exposure to hyperleptinemia on the phenotypes of progeny. We identified the immediate postnatal period (P0 to P22) as a critical time window during which exposure to elevated leptin increases the body weight in adult offspring under conditions of ad libitum access to highly palatable food.

We found no effect on subsequent body weight of transient hyperleptinemia in adult mice. This result is consistent with an earlier study by our group (*21*). The absence of leptin's effect on body weight is consistent with leptin not providing a physiological potent defense against fat gain (*34*). It was shown that chronic (18 weeks) elevation of circulating leptin by administration of exogenous leptin via osmotic pumps did not trigger the physiological or behavioral mechanism in mice to maintain a higher body weight (*21*). One of the limitations of this study is the limited amount of exogenous leptin delivered via mini pump due to low leptin solubility at high concentrations. In addition, delivery efficiency of exogenous leptin via the mini pump was highly variable in individual mice, and the repeated surgeries necessary to replace mini pumps caused weight loss. These issues were avoided in the present study.

Inducing hyperleptinemia for 5 weeks in adolescent mice did not affect long-term body weight in response to HFD feeding ad libitum. Transgenic leptin expression induced by dox administration resulted in transiently reduced chow intake and slowed weight gain in 2TG versus 1TG controls only during active leptin overexpression. When challenged with HFD, 2TG mice increased food intake transiently and gained more weight than 1TG controls within the first 4 weeks of HFD exposure, but ultimately (at 8 weeks of HFD), there were no differences in body weight or body fat between 1TG and 2TG groups. Greater hyperphagia in response to highly palatable diet in the 2TG mice suggests that exposure to hyperleptinemia during P22 to P56 may have altered leptin-responsive neural circuitry.

In mice, a physiologic leptin surge (5- to 10-fold increase in circulating leptin concentrations) occurs between P8 and P12, independent of pup fat mass (*35*). This surge—the mechanism of which is unclear—is a major developmental signal that affects the outgrowth of neuronal projections from the ARH to PVH, DMH, and LHA involved in feeding circuits (*17*). Maternal HFD feeding during nursing causes the leptin surge in pups to initiate earlier with higher intensity and to last for a longer period of time; the pups have a higher body weight compared to the offspring of chow-fed dams (*29*). This surge is not due to leptin secreted in breast milk (*29*, *36*–*38*).

We found that isolated hyperleptinemia during the immediate postnatal period (P0 to P22) predisposed mice (both males and females) to hyperphagia and increased weight gain when exposed to highly palatable HFD at 10 weeks of age. At 3 weeks of age, mice have little body fat. Nonetheless, hyperleptinemic 2TG female mice had reduced body weight due to decreased somatic fat compared to 1TG mice. Exogenous leptin in mice does not suppress food intake during the first 2 weeks of life (*39*, *40*) but is an important neurotrophic factor affecting the development of ARH projections to downstream hypothalamic targets (*17*). It is possible that decreased fat mass in the hyperleptinemic 2TG female mice was caused by decreased energy intake of the 2TG pups and that effect may constitute a confounding factor with regard to the interpretation of possible leptin-specific effects in the CNS, especially because postnatal undernutrition in rodents can cause growth retardation and decreases body weight throughout life even when animals are subsequently exposed to HFD (*41*, *42*). The effect of this potential confounder would presumably be to reduce the magnitude of the increases in body weight and fat that we observed in adult HFD-fed animals after transient hyperleptinemia at P0 to P22. That is, the bias would be against our inference of a genotype-mediated difference in body weight.

One to 2 weeks after release from hyperleptinemia and ad libitum chow feeding, male and female 1TG and 2TG mice were indistinguishable in body weight, composition, and circulating leptin concentrations until 10 weeks of age when they were switched to 60% HFD. Immediately after HFD exposure, 2TG male and female mice gained more weight than 1TG littermates. In male mice, the difference in fat mass was about 4 g per mouse after 14 weeks of HFD, representing an increase of only 38 kcal of about 1300 kcal ingested over this period (*43*), or a positive energy balance of 3%. This difference is not detectable with current instrumentation for energy intake or expenditure. Note that we estimated energy intake on a per-cage basis, whereas all body weights, composition, and energy expenditure measurements were determined in individual animals. On the basis of these considerations, it appears most likely that the genotype-related differences in weight gain of the P0 to P22 animals fed an HFD were due primarily to subtle effects on food intake.

Transgenic mice that congenitally overexpress leptin have been described previously, and their body weight phenotypes are consistent with our results (*44*, *45*). These congenital leptin-overexpressing mice initially display reduced body weight and fat stores but, by 33 weeks of age, are indistinguishable in body mass and composition from wild-type (WT) littermates. Mice congenitally overexpressing leptin (off the adipocyte-specific *aP2* promoter) are more prone to HFD-induced obesity when exposed to 20 weeks of HFD initiated at 9 weeks of age (*46*). The congenital leptin-overexpressing mouse models suggest that the phenotype we report in mice that are postnatally hyperleptinemic (P0 to P22) is specifically a result of elevated leptin exposure as opposed to the withdrawal of transgenic leptin at the time of weaning. Although transgenic mice that constitutively overexpress leptin are useful in assessing the consequences of lifelong hyperleptinemia, congenital models do not permit the assessment of the effects of transient hyperleptinemia at specified developmental time points.

Three other groups have studied the physiological consequences for body weight of postnatal exogenous leptin supplementation in rats; these studies varied in the timing and route of leptin administration and have somewhat conflicting results. One study administered leptin orally to rats from P0 to P20. At weaning, rats were fed ad libitum HFD, and at 6 months of age, the leptin-fed rats had ~7% lower body weight than the controls (*47*). Plasma leptin concentrations in leptin-supplemented pups were not reported; therefore, it is unclear whether oral leptin elevated circulating leptin concentrations. Another study administered leptin to neonatal rats via intraperitoneal injection either during the first or last 10 days of nursing and showed that when fed chow ad libitum, by 16 weeks of age, leptin-injected rats had 11 and 10% higher body weights, respectively, than controls (*48*). When suckling rats were injected with intraperitoneal leptin from P3 to P13, the leptin-supplemented rats gained 10% more weight than controls after they were switched to HFD at weaning (*49*). The latter two studies are consistent with the results reported in this manuscript. In both studies, neonatal leptin treatment leads to transient decreases in weight gain of the pups (*48*, *49*).

Under normal physiological conditions, leptin is secreted in a diurnal pattern with highest plasma concentrations at night and lowest in the morning (*50*). The half-life of leptin is about 40 min with a sharp peak 30 min after intraperitoneal injection and a full clearance within 4 hours (*51*). Thus, in studies using daily injections to manipulate circulating leptin concentrations, large transient spikes of nonphysiological leptin concentrations are created, followed by a 20-hour period during which plasma concentrations are not elevated. Although the leptin-overexpressing mice reported here do not fully recapitulate normal circadian physiology either, they have ad libitum access to dox-supplemented water throughout the 24-hour period.

The mechanisms that might mediate the effects of transiently elevated circulating leptin concentrations during the postnatal period on subsequent body weight are unclear. Leptin has been shown to play a neurotrophic role in the development of feeding circuitry (*17*). In $Lep^{ob/ob}$ mice, ARH projections to hypothalamic regions (the PVH, DMH, and LHA) are greatly reduced, and the formation of these pathways is temporally delayed. For example, in WT mice, ARH neurons innervate the PVH on P12, whereas in $Lep^{ob/ob}$ littermates, no axons from the ARH are detected at this time (*17*). Administration of leptin to P4 to P12 $Lep^{ob/ob}$ mice restores the density of the innervation from the ARH to the PVH to the levels of a WT control. However, administering leptin to adult $Lep^{ob/ob}$ mice does not rescue the density of Agouti-related peptide (AgRP) and α–melanocyte-stimulating hormone (α-MSH) immunoreactive innervation in the PVH to levels seen in WT mice, indicating that there is a restricted neonatal time window in which ambient leptin affects the formation of ARH connections (*17*). It has been shown that the offspring of dams exposed to HFD during gestation and lactation gained more weight than that of dams fed regular chow and displayed decreased hypothalamic leptin sensitivity (reduced leptin-induced phosphorylated signal transducer and activator of transcription 3 in the ARH and VMH) at P30 and P90 (*29*). The density of AgRP immunoreactive fibers in the PVH was decreased in the offspring of HFD-fed dams compared to chow-fed dams (*29*). The authors hypothesized that early hyperleptinemia induces leptin resistance, thereby attenuating leptin signaling and impairing the development of hypothalamic projections (*29*). The most critical time for the effects of maternal HFD feeding on offspring body weight is during the immediate postnatal period (birth to weaning), and the fiber density of AgRP and α-MSH from ARH to three areas downstream of the hypothalamus (PVH, DMH, and LHA) is reduced in progeny of HFD-fed dams (*20*). Similar alterations in hypothalamic circuits are reported in $Lep^{ob/ob}$ mice (*17*). We found the preweaning period to be critical for the effects of hyperleptinemia on body weight and response to an HFD later in life. It is plausible that the neurobiological changes seen in the offspring of HFD-fed dams result, at least in part, from the hyperleptinemia associated with increased body fat of the pups. The normal plasticity of hypothalamic development during the second postnatal week renders this optimal time window for permanent structural impact of altered humoral signals such as leptin.

Other potential mechanisms linking hyperleptinemia during the postnatal period to developmental programming include epigenetic modifications, hypothalamic inflammation, and neuroanatomic changes in hedonic or reward circuits. Overnutrition of weanling rats (by decreasing litter size) during the perinatal period results in hypermethylation of the hypothalamic proopiomelanocortin (POMC) promoter, which is negatively correlated with hypothalamic POMC expression adjusted for circulating leptin concentrations (*52*).

Exposure to highly palatable food results in 1 to 2 weeks of hyperphagia in adult mice (*53*). This response is mediated, at least in part, by hedonic, reward-based mechanisms that can override homeostatic regulation during abundant food availability and drive consumption of highly palatable foods in the absence of hunger (*54*). The neuroanatomy of the hedonic circuits may also be affected by hypernutrition in the postnatal period. Because the increased weight gain in mice that were postnatally hyperleptinemic is evident only after exposure to a highly palatable diet, it is likely that hedonic pathways are affected in these mice (*55*).

Last, studies implicate hypothalamic inflammation as a mediator of diet-induced obesity. It has been demonstrated that within 1 to 3 days of HFD feeding, before substantial weight gain, mice develop hypothalamic inflammation characterized by activation of local microglia and astrocytes (gliosis) (*56*). In rodents, microglia cell number in the brain markedly increases in the first 2 weeks of postnatal life (*57*), coinciding with the leptin surge, and then starts declining in the third postnatal week until it reaches mature levels by 6 weeks of age (*57*). The proportion of microglia is lower in $Lep^{ob/ob}$ mice fed chow or HFD (*58*), suggesting that leptin is a partial mediator of microglial activation. Administration of leptin between P8 and P12 increases proliferation of astrocytes in the hypothalamus, whereas deletion of *LepRb* from astrocytes decreases astrogenesis (*59*). In addition, leptin-induced suppression of food intake is reduced in astrocyte-specific *LepRb* adult knockout mice (*60*). These data suggest that high ambient hypothalamic leptin during development may influence the susceptibility to HFD later in life via effects on proliferation and activation of astrocytes.

In humans, maternal circulating leptin is elevated throughout gestation, peaks at ~1.5- to 2-fold above the concentration accounted for by adipose tissue mass in the late second/early third trimester of pregnancy (*61*–*64*), decreases within 3 days postpartum to concentrations below those present pregravid (*65*, *66*), and then gradually increases to concentrations appropriate to fat mass by 6 months postpartum (*65*). In humans, umbilical vein leptin concentrations are positively correlated with neonatal fat mass and gestational size, suggesting that in humans, fetal adipose tissue is the primary source of circulating leptin (*67*, *68*).

The developmental timing of brain maturation differs greatly between mice and humans. In this regard, the immediate postnatal

period in mice is analogous to the third trimester of human (or primate) gestation (*69*). In rodents, the hypothalamic circuitry is immature until the third week of postnatal life, whereas in primates, this circuitry matures functionally in utero (*17*, *70*). From this perspective, studies investigating the effects of HFD feeding and obesity in postnatal rodents may be more relevant to the neurodevelopmental impact of maternal obesity and metabolic status during human late gestation.

Nutritional changes during critical developmental periods can have lasting effects on energy homeostasis. The hypothalamus is particularly sensitive to changes in the hormonal milieu. In mice during the perinatal period, changes in circulating leptin (*17*), insulin (*20*, *28*), and ghrelin alter the architecture of key hypothalamic centers involved in energy and glucose homeostasis; however, the mechanisms of these developmental effects remain unclear. Many physiological and molecular changes are triggered by obesity and HFD feeding; however, the direct mediators of phenotypes observed in offspring are largely unknown. In this study, we identified the immediate postnatal period in mice as a critical time window in which exposure to hyperleptinemia is associated with alterations in subsequent responses to highly palatable food. Further studies are needed to define the cell-molecular mechanism(s) by which hyperleptinemia during the postnatal period in mice mediates the developmental programming of body weight regulation. In humans, the development of energy homeostasis neurocircuits may be influenced by environmental cues, including leptin concentrations. The critical time window in humans that is analogous to the suckling period in mice (P0 to P22) likely occurs from the third trimester to early infancy (*69*).

There are several limitations of this work. In our study, hyperleptinemia in the immediate postnatal period transiently decreased fat mass, possibly as a consequence of reduced caloric intake. The difference in fat mass and body weight between 1TG and 2TG mice could potentially be larger than observed if the 2TG mice maintained the same fat mass as 1TG mice during the immediate postnatal period. There are also some limitations of the leptin transgenic mouse model. The 2TG mice had a wide range of responses of circulating leptin concentrations to the same concentrations of dox in the drinking water. Much of this variability was probably due to differences in water intake. However, bacterial DNA within transgenes has been reported to cause stochastic silencing of transgenes in mice (*71*), and it is possible that some of the variability in transgenic leptin expression seen in our LepTg/rtTA double transgenic mice was due to mosaic expression. At very high doses of dox, ectopic leptin production in the hypothalamus could contribute to the functional consequences of dox exposure. In some experimental circumstances, this effect could confound mechanistic inferences regarding the effects of transient leptin overexpression on subsequent ingestive behaviors. At doses of dox that produce circulating leptin concentrations that are within the physiological range, this potential hypothalamic effect is likely not substantial as the dose administered to the adolescent mice, and the maximal dose administered to the adult mice was sixfold lower than the dose that induced low amounts of leptin secretion in the hypothalamic incubates. Production of leptin in the gastrointestinal tract and the liver of dox-treated animals could also possibly influence vagal signaling by paracrine effects. Our dox-treated animals showed the anticipated behavioral responses to plasma concentrations of leptin that are comparable to those in animals administered exogenous leptin (*72*). Hence, we do not believe that local effects of ectopic leptin production are physiologically meaningful.

MATERIALS AND METHODS

Study design

The objective of the study was to determine the effects of elevated leptin in mice at different stages of life (nursing period, adolescence, and adulthood) on body weight later in life and the subsequent susceptibility of mice to gain weight when exposed to HFD. The study included a series of controlled laboratory experiments carried out in a transgenic mouse model. The transgenic mice used in the experiments were generated on a mixed genetic background of C57BL/6 and 129/Sv and backcrossed to C57BL/6J for at least four generations. All experiments were performed in mice from P0 up to 37 weeks of age. The transgenic experimental and control mice used in these experiments were born in the same litters at the approximate ratio of 1:1, and all of them were exposed to dox; therefore, no additional randomization was necessary. The investigators were not blinded while collecting and analyzing the data because the mice had to be assigned to appropriate cages per genotype. Sample sizes were based on previous experience with similar types of experiments. All mice in each cohort were born at the same time. Data collection was stopped early for mice with skin lesions (a common side effect of extended HFD feeding). The data for these mice were excluded from the analysis. Experiments investigating adult and postnatal (P0 to P22) hyperleptinemia were replicated in independent cohorts of mice. Additional method details are available in the Supplementary Materials.

Animals

Throughout the study, mice were maintained at room ambient 22° to 24°C with a 12-hour dark-light cycle (lights on at 0700 hour) in a pathogen-free barrier facility. Mice were maintained on 9% fat chow (Purina LabDiet; 22% calories from fat) or fed an HFD (Research Diets Inc.; 60% calories from fat; HFD) as indicated. The protocol was approved by the Columbia University Institutional Animal Care and Use Committee.

All experiments were carried out with dox-inducible leptin/rtTA double transgenic mice. Leptin/rtTA double transgenic mice were generated using a commercially available KH2 ES cell line (Mirimus Inc.; details on the transgenic mouse are provided in Supplementary Materials and Methods) (*73*).

Hyperleptinemia in adult (P63 to P203) male mice

Leptin-overexpressing (2TG, $n = 21$) and control (1TG, $n = 15$) mice shared dams and were born and raised in the same litters. After weaning, mice were group-housed three per cage by genotype with ad libitum access to chow and water. Dox exposure began at 9 weeks of age, and all mice were fed chow ad libitum during this period. Mice were exposed to increasing concentrations of dox in 5% sucrose water (for palatability) every 2 weeks. Dox water was changed twice weekly. Baseline blood was collected 1 week before dox exposure. Dox concentrations in water were 5, 7.5, 12.5, 17.5, 20, 22.5, 27.5, 35, 40, and 45 μg/ml and were increased stepwise every 2 weeks. Circulating leptin concentration was measured from plasma isolated from submandibular blood every 2 weeks, 1 week after each dox dose escalation. Glucose was measured in submandibular whole blood. Dox degrades over time, as was observed on week 22 when

circulating leptin concentrations were lower than the previous period despite higher nominal concentration of dox in the drinking water. A new batch of dox was purchased for the 24-week exposure.

Body weight and composition were measured weekly throughout the experiment. Food was placed on the wire cage tops, and food intake was measured twice a week for all mice (on a per-cage basis) throughout the study. During dox exposure, water intake was measured twice a week to ensure that there was no dox (taste) effect on the amount of water consumed. Dox-free water was provided after 20 weeks of dox exposure. Monitoring of body weight, composition, and food intake continued weekly after dox exposure was discontinued. Five weeks after release from dox exposure, mice were switched from chow to 60% HFD. Mice were euthanized 16 weeks after release from hyperleptinemia.

Two mice were excluded from the analysis: One died during magnetic resonance imaging assessment (1TG group, at 29 weeks of age, fed chow diet), and the other animal (2TG group, at 22 weeks of age, fed chow diet) was not gaining weight because of skin lesions. Exclusion of the first mouse decreased the mean body weight of 1TG group by 0.19% at 29 weeks of age, and that of the second mouse increased the mean body weight of 2TG group by 0.9% at 22 weeks of age.

Hyperleptinemia in adolescent (P22 to P56) male mice

1TG (n = 18) and 2TG (n = 18) littermates were weaned on P22. At weaning, pups were separated by genotype and group-housed three per cage with ad libitum access to chow. At the same time, mice began a 5-week exposure to dox (50 μg/ml) in drinking water. Blood was collected at P15 and P22 for baseline plasma leptin measurement and at weeks 4, 6, and 8 during dox exposure. At 8 weeks of age, mice were released from dox exposure and continued ad libitum access to chow. At 14 weeks (6 weeks after release from dox), both groups were switched to ad libitum 60% HFD. Throughout the experiment, body weight and food intake were recorded twice per week on a per-cage basis. Body composition was measured biweekly. Plasma leptin was measured every 4 weeks during the post-dox period.

Hyperleptinemia in postnatal (P0 to P22) male mice

1TG female mice homozygous for the TRE-*Lep* transgene but noncarriers for the R26-*rtTA* allele were crossed to 1TG male mice heterozygous for R26-*rtTA* but noncarriers for the TRE-*Lep* insert. This strategy resulted in all offspring carrying the TRE-*Lep*/$^{+}$ gene and about half segregating with *rtTA*/$^{+}$. Live offspring were born with the expected 1:1 ratio of 1TG and 2TG. At parturition, mothers were exposed to dox (200 μg/ml) in drinking water for 3 weeks with ad libitum access to chow. During the nursing period (P0 to P22), pups primarily drink mother's milk and not the dox-supplemented water; hence, the effective dox dose in postnatal pups is diluted in mother's breast milk. Plasma was collected on P15 and P22. At weaning, mice were separated by genotype (1TG, n = 15; 2TG, n = 14) into home cages (group-housed, two to three per cage) with ad libitum access to chow and dox-free water. At 10 weeks, mice were given ad libitum access to 60% HFD and maintained on this diet until the end of the study. Body weight and food intake per cage were collected twice per week. Body composition was measured biweekly throughout the experiment.

Two mice (genotypes = 2TG) in the postnatal hyperleptinemia experiment were euthanized before termination of the planned study period due to skin lesions (on weeks 10 and 24, respectively, of HFD feeding the two mice showed early signs of illness). These mice were excluded from the analyses. The first mouse was euthanized relatively early in the study, and its exclusion decreased the difference in body weight and fat mass between genotypes by 1 and 2%, respectively, in the first 10 weeks of HFD feeding. Exclusion of the second mouse reduced the difference in body weight and fat mass by 1% at the end of the study.

Hyperleptinemia in postnatal (P0 to P22) female mice

The breeding strategy used was identical to that described for the male cohort. Mice were exposed to dox (200 μg/ml) ad libitum at parturition for 3 weeks. Plasma was collected on P9, P15, P22, and P29. At weaning, mice were group-housed (two to three per cage) with ad libitum chow and dox-free water access. At 10 weeks, mice were switched from chow to 60% HFD ad libitum and maintained on this diet until the end of the study. Food intake was measured on a per-cage basis for 5 weeks after the switch to HFD. After 16 weeks of HFD feeding, a subset of 16 mice (8 of each genotype) was placed individually in metabolic chambers (LabMaster-CaloSys-Calorimetry System, TSE Systems) for 1 week to assess energy expenditure. Oxygen (O_2) and carbon dioxide (CO_2) concentrations were measured every 17 min during the 1-week assessment, during which mice had ad libitum HFD and water access. The first 24 hours was used as an acclimation period.

Statistical analysis

Data are expressed as means ± SEM. GraphPad Prism 8.0 software was used for statistical analyses. Two-tailed Student's t tests were used to compare 1TG and 2TG groups on the final data points for each phenotype. $P < 0.05$ was considered significant. Data were also analyzed using a two-way repeated-measures analysis of variance (RM ANOVA) or mixed-effects model (for datasets with any missing values), followed by a post hoc comparisons using a Fisher's least significant difference (LSD). GraphPad Prism 8.0 (www.graphpad.com/) offers a mixed-effects model that is designed to analyze the datasets instead of RM ANOVA when values are missing. This mixed model uses a compound symmetry covariance matrix and is fit using restricted maximum likelihood. When the mixed-effects model method is used on datasets without missing values, the P values and post hoc test results are the same as when analyzed with RM ANOVA. Red asterisks in figures indicate significance by Student's t test, whereas black asterisks denote significance by Fisher's LSD. Data were tested for normality before use of RM ANOVA, t tests, and mixed-effects models.

SUPPLEMENTARY MATERIALS

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Materials and Methods

Fig. S1. Validation of leptin-overexpressing ES cells and leptin-overexpressing mice.

Fig. S2. Bioactivity of leptin.

Fig. S3. Circulating leptin concentrations during postnatal dox exposure.

Fig. S4. Dox-induced chronic hyperleptinemia (P63 to P203) in adult mice with concurrent HFD feeding.

Fig. S5. Release of adult mice from dox-induced chronic (P63 to P203) hyperleptinemia and HFD feeding.

Fig. S6. Energy expenditure assessment (indirect calorimetry) at 26 weeks of age in postnatally (P0 to P22) hyperleptinemic female mice after 16 weeks of HFD feeding.

Data file S1. Primary data file used to generate main figures.

Data file S2. Primary data file used to generate supplementary figures.

Reference (*74*)

View/request a protocol for this paper from *Bio-protocol*.

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Physiological consequences of transient hyperleptinemia during discrete developmental periods on body weight in mice

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Prenatal priming of obesity

The hormone leptin suppresses food intake and regulates body weight, in part by acting on the hypothalamus. Here, Skowronski *et al*. studied the effects of elevated leptin in lean mice during discrete developmental time periods on subsequent weight gain. Hyperleptinemia during adolescence or adulthood did not affect weight gain upon later high-fat diet feeding. However, postnatal hyperleptinemia predisposed both male and female mice to diet-induced weight gain and adiposity as adults. Further studies should evaluate the mechanism by which elevated postnatal leptin affects future metabolism. If confirmed in humans, the authors' findings could have implications for the prevention of later-life obesity during early childhood development.