Supplementary Materials for

Manuscript Number: aax6629

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

The file includes:

Materials and Methods

Creation of the leptin transgenic embryonic stem (ES) cell line and mice

KH2 ES cells contain an FRT-hygro-pA "homing" cassette in the *Col1A1* locus and a reverse tet-transactivator (*M2rtTA*) located in the *Rosa26* locus (R26-*rtTA*; fig. S1A). The mouse leptin gene (*Lep*) was isolated using PCR with forward primer: 5' GGCGCGAATTCATGTGCTGGAGACCCCTGTGT and reverse primer 5' TCATCAAGACCATTGTCACCAGGAT (primers were designed to introduce EcoR1 sites flanking the gene). The PCR reaction was run using cDNA from a wild type C57BL6J mouse; the product was then cloned into the EcoRI site of pBS31tetOpgkATGfrt vector (source D. Egli, Columbia University, fig. S1A). Pre-engineered KH2 ES cells were co-electroporated with pBS31tetOpgkATGfrt-Lep vector and FlpE Recombinase-expressing vector (pCAGGS-FLPe, source D. Egli, Columbia University) to allow for site-directed recombination as described earlier (fig. S1A) (*30*). KH2 cells with an inserted *Lep* gene (KH2-*Lep*) were selected with hygromycin B (Sigma). The resulting KH2-*Lep* cell line contained the R26-*rtTA* and a leptin transgene downstream of the tetracycline-responsive elements (TRE) and the CMV promoter (located in the *Col1A1* locus, fig. S1A).

To confirm integration of the co-transfectants, leptin transgene expression in KH2-*Lep* cells was induced *in vitro* with 4 dox (Doxycycline hyclate, Sigma) concentrations: 50, 100, 200, and 400 ng/ml. Media was collected after 48 hours of dox exposure to measure leptin protein content (fig. S1A). Four dox-responsive clones were selected for karyotypic analysis (Cell Line Genetics, Inc). Cytogenetic analysis was performed on 20 G-banded metaphase cells for each clone. Three out of four clones were karyotypically abnormal; one had an inverted duplication on chromosome 4 from band D1 to band C4, and two had a large metacentric chromosome resulting

from fusion of the centromeres of chromosomes 1 and 13. The karyotypically normal clone was selected for mouse generation (fig. S1B). Chimeric mice were generated by injecting ES cells into non-agouti BDF2 blastocysts followed by transferring embryos into day 2.5 pseudopregnant albino ICR females. Pups were delivered by Caesarian section on embryonic day 19.5 and fostered to an ICR mother that had given birth within the previous 4 days. The transgenic pups were on a mixed genetic background of C57BL/6 and 129/Sv.

Genotypes in 1TG and 2TG mice were confirmed with the following genotyping primers:

Rosa26-rtTA locus:

Mutant Reverse 5' GCG AAG AGT TTG TCC TCA ACC

Common Forward 5' AAA GTC GCT CTG AGT TGT TAT

Wild type Reverse 5' GGA GCG GGA GAA ATG GAT ATG

Col1A1-TRE-*Lep* locus:

Mutant Forward 5' GCA GAA GCG CGG CCG TCT GG

Common Reverse 5' CCC TCC ATG TGT GAC CAA GG

Wild type Forward 5' GCA CAG CAT TGC GGA CAT GC

To secrete leptin in response to dox, mice must segregate for two transgenes (2TG): the dox-responsive leptin transgene (TRE-*Lep*), and the M2rtTA (driven by the endogenous *Rosa26* promoter; R26-*rtTA*, fig. S1B) (*75*). Mice that segregate for both transgenes are denominated: 2TG (lep-overexpressors). Single transgenic littermates (1TG) which only carry the TRE-*Lep* and do not produce leptin when exposed to dox were used as controls.

Dox dose response: Thirty 10-week-old male mice (12-1TG, 18-2TG) were exposed to increasing concentrations of dox in 5% sucrose water (provided *ad libitum*) every 2 weeks. Baseline blood was collected 1 week prior to dox exposure. Dox concentrations in sucrose water were 10, 15, 20 µg/ml.

Acute induction of leptin: Five 12-week-old male 2TG mice were treated with *ad libitum* access to 200 µg/ml of dox in drinking water for 24 hours to induce transgenic leptin expression. Blood was collected at baseline (prior to dox administration) and at 24 hours of dox exposure. Dox-free water was provided for the following 24 hours. This 24-hour on and off dox treatment was repeated. Blood was collected every morning.

Dox gavage: Five 8-week-old male 2TG mice were gavaged with 400 µg of dox. Blood was collected at baseline and at 2, 4, 6, 8, 10, 12, 14, and 24 hours using heparinized tubes.

Leptin secretion from ex-vivo tissues

14 2TG mice were treated with 300 µg/ml of dox in 5% sucrose water or with 5% sucrose water only (*ad libitum* access) for 48 hours to induce leptin expression. Mice were then sacrificed and tissues harvested for culture and RNA isolation: subcutaneous adipose tissue (SCAT), perigonadal adipose tissue (PGAT), brown adipose tissue (BAT), liver (LIV), kidney (KID), stomach (STO), duodenum (DUO), jejunum (JEJ), ileum (ILE), spleen (SPL), lung (LUNG), and hypothalamus (HYPO). Approximately 100 mg of each tissue (except for HYPO which was ~20 mg) was minced into 4-5 fragments and cultured for 4 h in 0.5 ml of M199 media with 7 nM insulin and 25 nM dexamethasone. Leptin secreted into the media was normalized by tissue mass. After 4 h in culture, media were collected for determination of leptin concentration.

RNA extraction, cDNA, qPCR: Total RNA was isolated using TRIzol reagent (Invitrogen) and was then DNase-treated with TURBO DNA-free kit (Ambion). One µg of RNA was reverse transcribed using Transcriptor First Strand cDNA Synthesis Kit (Roche) using both OligoDT and random hexamer primers. Lightcycler 480 SYBR Green I Master was used for quantitative PCR assays (Roche). Relative gene expression of *rtTA*, and *Lep* (total *Lep*, transgenic *Lep* only, and endogenous *Lep* only) in PGAT, SCAT, BAT, LIV, JEJ, and HYPO was calculated by Lightcycler 480 software (Roche) based on the Second Derivative Maximum method using a standard curve generated from a serially diluted pool of cDNA. Gene expression was normalized to the geometric mean of the housekeeping genes – *Actβ* and *36b4*. Primers used are listed below:

Actb Forward	5'-CGGGCTGTATTCCCCTCCAT
Actb Reverse	5'-GGGCCTCGTCACCCACATAG
36b4 Forward	5'-ACCTCCTTCTTCCAGGCTGG
36b4 Reverse	5'-CGAAGGAGAAGGGGGAGGTT
Total Lep Forward	5'- TGACACCAAAACCCTCATCA
Total Lep Reverse	5'- AGCCCAGGAATGAAGTCCA
Endogenous Lep Forward	5'- TTCTCCAAGAGCTGCTCCC
Endogenous Lep Reverse	5'- CCTGGTGGCCTTTGAAACTT
Transgenic Lep Forward	5'- GCCCCGAATTCATGTGCTG
Transgenic Lep Reverse	5'- CGACTGCGTGTGTGAAATGT

M2rtTA Forward 5'- GAAAATCAGCTCGCGTTCCT

M2rtTA Reverse 5'- GGGGCATAGAATCGGTGGTA

Bioactivity of leptin

Fourteen 10-week-old male mice (n=7 1TG, n=7 2TG) were housed 2-3 per cage and given *ad libitum* access to 200 µg/ml dox water for 10 days. Dox concentration was then increased by 5-fold to 1 mg/ml for another 4 days. Body weight and food intake (on a per cage basis) were measured daily. Body composition was measured using EchoMRI at baseline, 7, 10, and 14 days of exposure to dox. Venous blood was collected immediately after body composition measurement at baseline, 7, and 14 days of exposure to dox.

Plasma leptin concentrations during postnatal dox exposure

Nursing 1TG mothers were exposed to dox (200 µg/ml) or drug-free water from parturition until weaning. Mice were bred so that the expected offspring ratio was 1:1 1TG v. 2TG. At postnatal day 15 (P15) while the pups were still exclusively breastfeeding, pups (both males and females; drug-free water: 1TG, n=22 and 2TG, n=20; dox-supplemented water: 1TG, n=25 and 2TG, n=29) and mothers were bled for plasma leptin determination. Plasma leptin concentrations in 14 (of 29) 2TG pups exposed to dox were outside the standard curve in the leptin ELISA assay. The absolute values for leptin concentration are likely higher in these samples; these samples are designated with a dark gray triangle symbol within the dot plot of leptin concentration in P15 pups.

Hyperleptinemia with concurrent HFD feeding in adult male mice

This experiment was designed similarly to the experiment above. Mice were group-housed (3 per cage) with *ad libitum* access to chow and dox exposure started when mice were 9 weeks old. The concentration of dox in 5% sucrose water was increased every 2 weeks in the following order: 10, 15, 17.5, 20, 22.5, 25, 27.5, 30, 35, 45 ug/ml. The modification to this protocol was that after the initial 7 weeks of dox exposure mice were switched from regular chow diet to 60% HFD. Plasma leptin, glucose, body weight and composition, estimated food and water intake were measured at the same time points in the same way as the first experiment. Mice were released from hyperleptinemia after 20 weeks of dox exposure; at the same time they were switched back to regular chow diet. Post release, body weight, composition and food intake (on a per cage basis, 3 mice per cage) were monitored weekly. Mice were sacrificed 12 weeks after release from hyperleptinemia. Leptin concentrations in response to increasing dox dose prior to HFD exposure (dox dose 0 to 20 ug/ml) from this cohort of mice is reported in the Fig. 1A showing leptin dox dose responses in individual animals.

Hyperleptinemia in postnatal (P0-P22) female mice

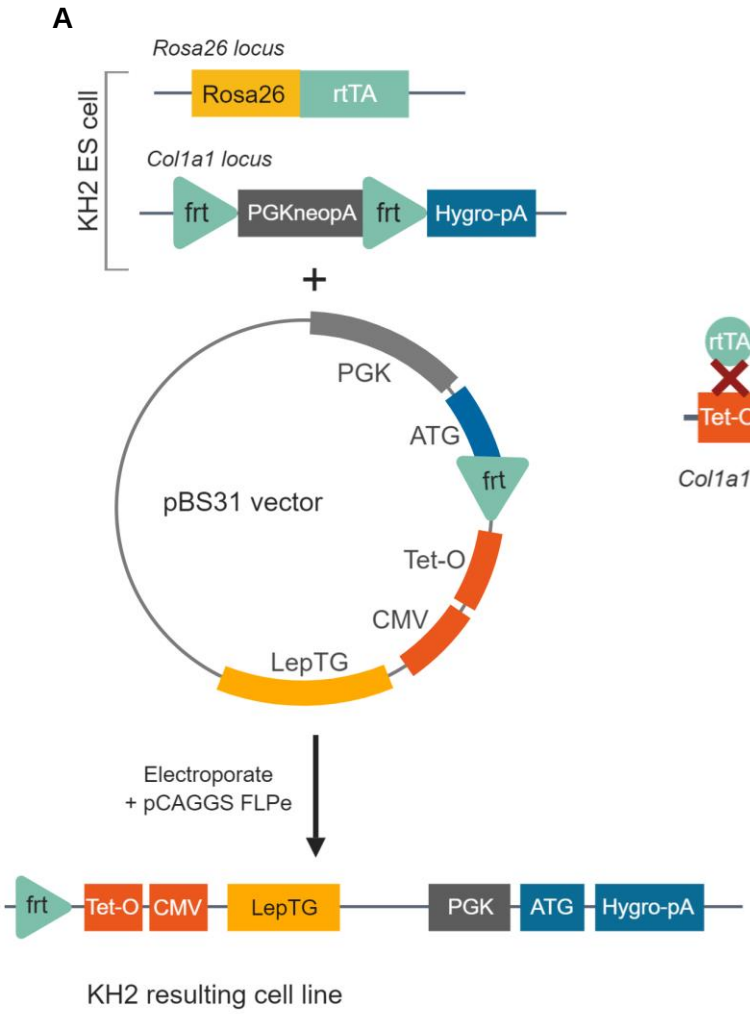
Breeding strategy was identical to that described for the male cohort (*Hyperleptinemia in postnatal [P0-P22] male mice, Methods*). Mice were exposed to 200 µg/ml of dox *ad libitum* at parturition for 3 weeks. Plasma was collected on P9, P15, P22, and P29. At weaning, mice were group-housed (2-3 per 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

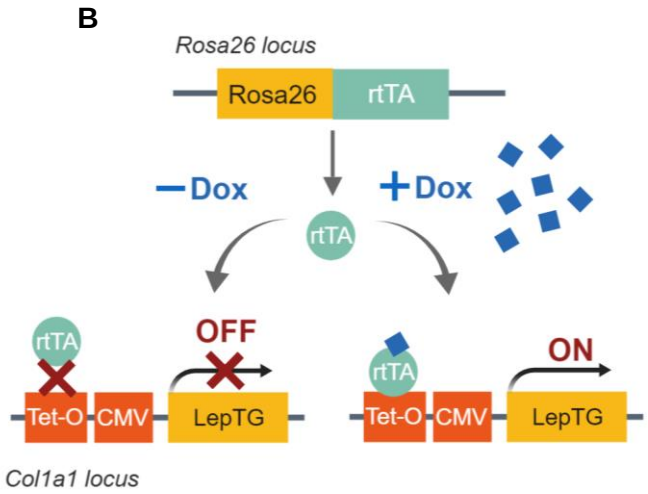
every 17 minutes during the 1-week assessment while mice had *ad libitum* HFD and water access. The first 24h hours was used as an acclimation period.

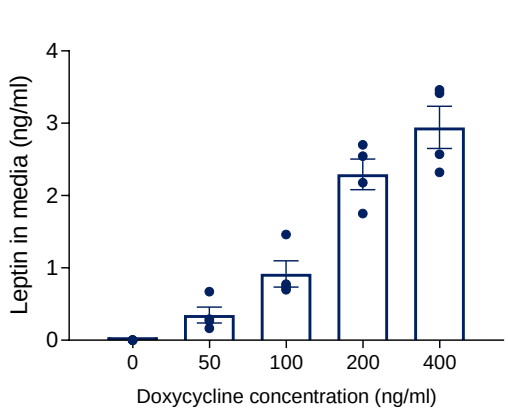
Plasma collection and assays

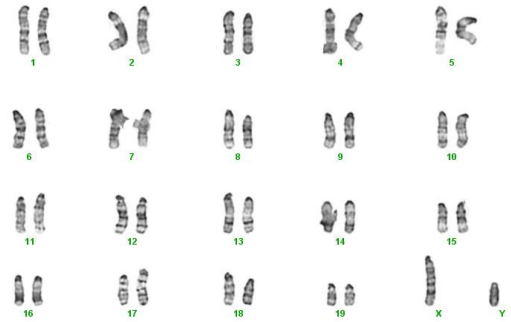
Blood was obtained by submandibular bleed at 9am from mice in a fed state. Plasma was collected on ice using heparinized tubes (Fisherbrand) and isolated by centrifugation for 20 min at 2,000 x g at 4°C, aliquoted, and frozen at −80°C until time of assay. Plasma leptin concentration was measured using mouse leptin ELISA (R&D). Glucose was measured in submandibular whole blood with a Freestyle Lite glucose meter (Abbott) at the same time as blood collection for leptin assay. Body composition was measured with an EchoMRI Body Composition Analyzer. The instrument was calibrated with canola oil on the day of each measurement.

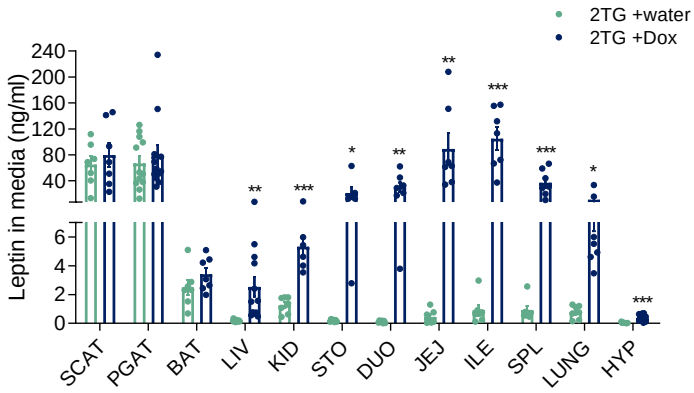
Supplementary Figures











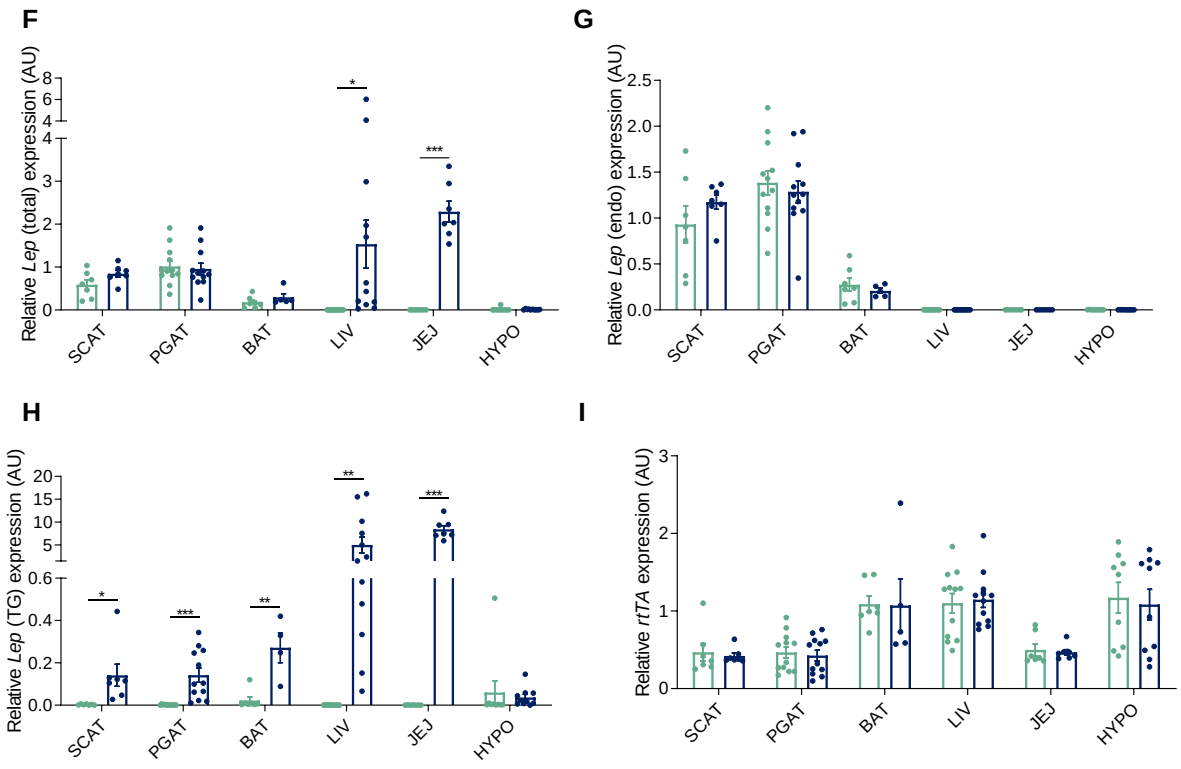
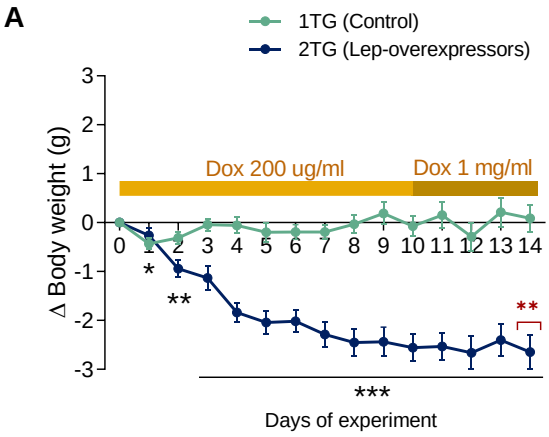
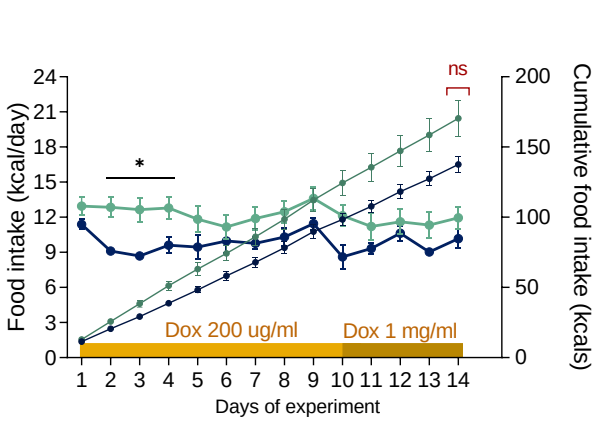


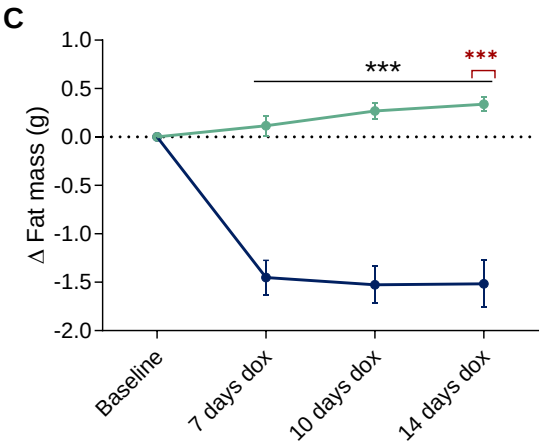
Fig. S1. Validation of leptin-overexpressing ES cells and leptin-overexpressing mice.

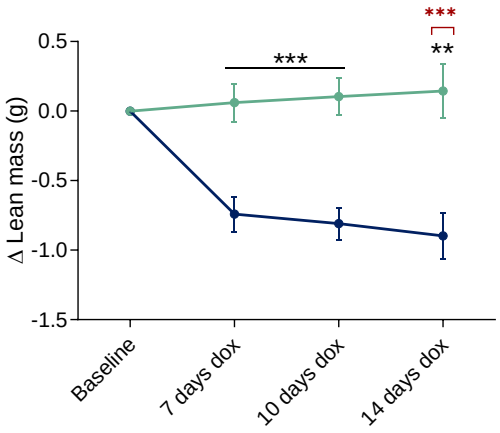
(**A**) Construct schematic [adapted from Beard *et al.* (*30*)]: KH2 embryonic stem cells containing a reverse tet-transactivator (rtTA) located in the Rosa26 locus and an FRT-hygro-pA "homing" cassette in the Col1A1 locus were electroporated with pBS31tetOpgkATGfrt-Lep vector and FlpE Recombinase (pCAGGS-FLPe) to generate the dox-inducible leptin-overexpressing mouse. (**B**) Tet-ON schematic: In the absence of dox the rtTA present in the cell cannot bind to the Tet-O responsive elements (TRE), thus transgenic leptin is not expressed; dox binds to rtTA changing its conformation and allowing it to bind to the TRE which in turn activates the CMV promoter and drives the expression of the leptin transgene. (**C**) Dose-dependent dox-induced secretion of leptin in the leptin transgenic mouse embryonic stem cell line, KH2-Lep, into culture media. (**D**) Cytogenetic analysis of G-banded metaphase cells from KH2-Lep. The ES cell clone selected for blastocyst injection was karyotypically normal. (**E**) secretion of leptin into culture

media from various ex-vivo tissues (SCAT-subcutaneous adipose tissue, PGAT-perigonadal adipose tissue, BAT-brown adipose tissue, LIV-liver, KID-kidney, STO-stomach, DUO-duodenum, JEJ-jejunum, ILE-ileum, SPL-spleen, LUNG, HYPO-hypothalamus), and relative expression of (**F**) Total *Lep*,(**G**) Endogenous (Endo) *Lep*, (**H**) Transgenic (TG) *Lep* and (**I**) *rtTA* in cultured tissues from 2TG leptin-overexpressing mice exposed to 5% sucrose (n=7) or 300 µg/ml of dox in 5% sucrose water (n=7) for 48h prior to sacrifice. Harvested tissues were minced into 4-5 fragments (~100 mg of each tissue, except for the hypothalamus which was ~20 mg) and cultured for 4h in media supplemented with insulin and dexamethasone. Culture media was collected for leptin protein content and RNA was extracted from tissues for expression data. Gene expression was normalized to housekeeping genes *Actb* and *36b4*. All values are means ± SEM. Student's t-test, ***p<0.001. AU, arbitrary units.


A
1TG (Control)
2TG (Lep-overexpressors)
Δ Body weight (g)
Dox 200 ug/ml
Dox 1 mg/ml
Days of experiment


B
Food intake (kcal/day)
Cumulative food intake (kcals)
ns
Dox 200 ug/ml
Dox 1 mg/ml
Days of experiment


C
Δ Fat mass (g)
Baseline
7 days dox
10 days dox
14 days dox


D
Δ Lean mass (g)
Baseline
7 days dox
10 days dox
14 days dox

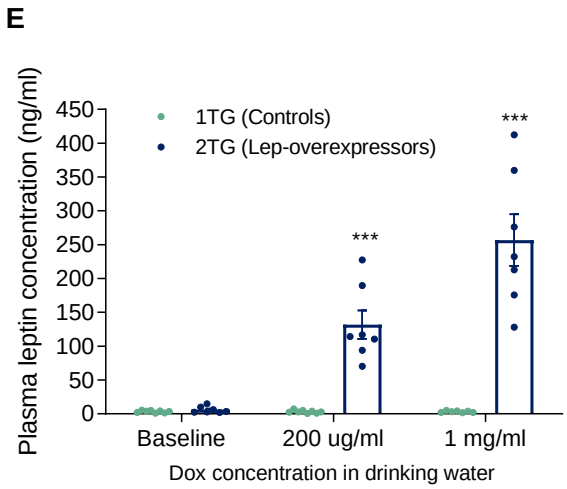

E
Plasma leptin concentration (ng/ml)
1TG (Controls)
2TG (Lep-overexpressors)
Baseline
200 ug/ml
1 mg/ml
Dox concentration in drinking water

Fig. S2. Bioactivity of leptin.

1TG control (n=7) and 2TG leptin-overexpressing (n=7) mice were housed 2-3 per cage and given *ad libitum* access to 200 µg/ml dox water for 10 days. Dox concentration in water was then increased by 5-fold to 1 mg/ml for another 4 days. (**A**) Daily body weight (two-way RM ANOVA with Fisher's LSD *post hoc* analysis, genotype: $F(1,12) = 42.7$, $p < 0.0001$), (**B**) Estimated daily chow intake (on a per cage basis); two-way RM ANOVA with Fisher's LSD *post hoc* analysis, genotype: $F(1,5) = 4.25$, $p = 0.09$), (**C**) Change in fat mass (two-way RM ANOVA with Fisher's LSD *post hoc* analysis, genotype: $F(1,12) = 67.5$, $p < 0.0001$), (**D**) Change in lean mass (two-way RM ANOVA with Fisher's LSD *post hoc* analysis, genotype: $F(1,12) = 23.4$, $p = 0.0004$), and (**E**) Circulating leptin concentrations at baseline (before mice exposed to any dox in drinking water), and at 7 and 10 days of 200 µg/ml dox exposure; also 4 additional days of exposure (from day 10 to 14) to 1 mg/ml (a total of 14 days receiving dox) in 1TG control and 2TG leptin-overexpressing mice. Plasma leptin concentration at 200 µg/ml of dox was measured on day 7 of exposure. All values are means ± SEM. Red brackets indicate a comparison by Student's t-test of the final datapoint. Student's t-test (in red) or *post hoc* Fisher's LSD (in black), *p<0.05, **p<0.01, ***p<0.001.

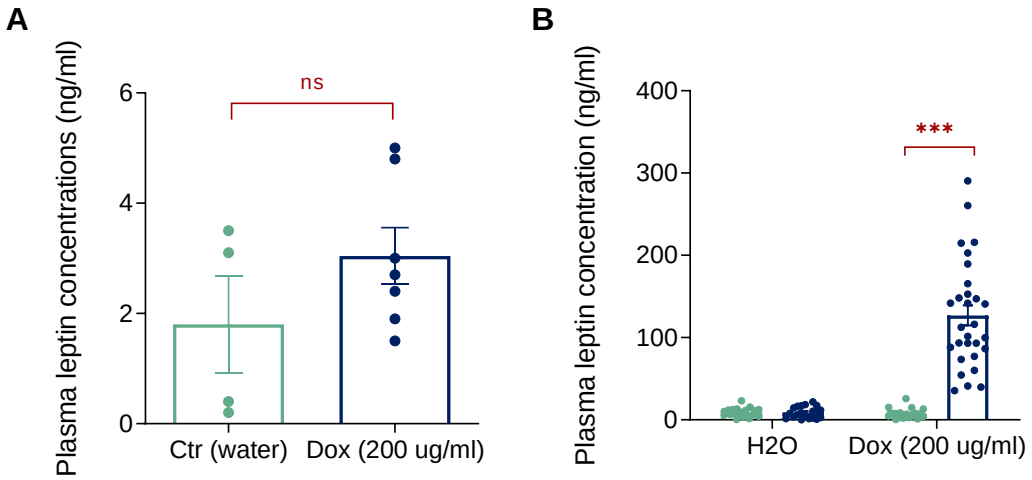


Fig. S3. Circulating leptin concentrations during postnatal dox exposure.

Nursing 1TG mothers were exposed to 200 µg/ml of dox in drinking water (n=7) or drug-free water (n=4) from parturition until weaning. At postnatal day 15 (P15) while the pups were primarily breastfeeding, pups (both males and females; drug-free water: 1TG, n=22 and 2TG, n=20; dox-supplemented water: 1TG, n=25 and 2TG, n=29) and mothers were bled for plasma leptin determination. Plasma leptin concentrations in (**A**) Nursing 1TG females, and (**B**) 1TG control and 2TG leptin-overexpressing suckling pups on day P15 during postnatal exposure of dam to ±dox water. All values are means ± SEM. Student's t-test, ** $p<0.01$, *** $p<0.001$. Plasma leptin concentrations in 14 (of 29) 2TG pups exposed to dox were above the standard curve in the leptin ELISA assay. The values reported were extrapolated from the standard curve but the absolute values for leptin concentration are likely higher in these samples; these samples are designated with a gray triangle symbol within the dot plot of leptin concentration in P15 pups.

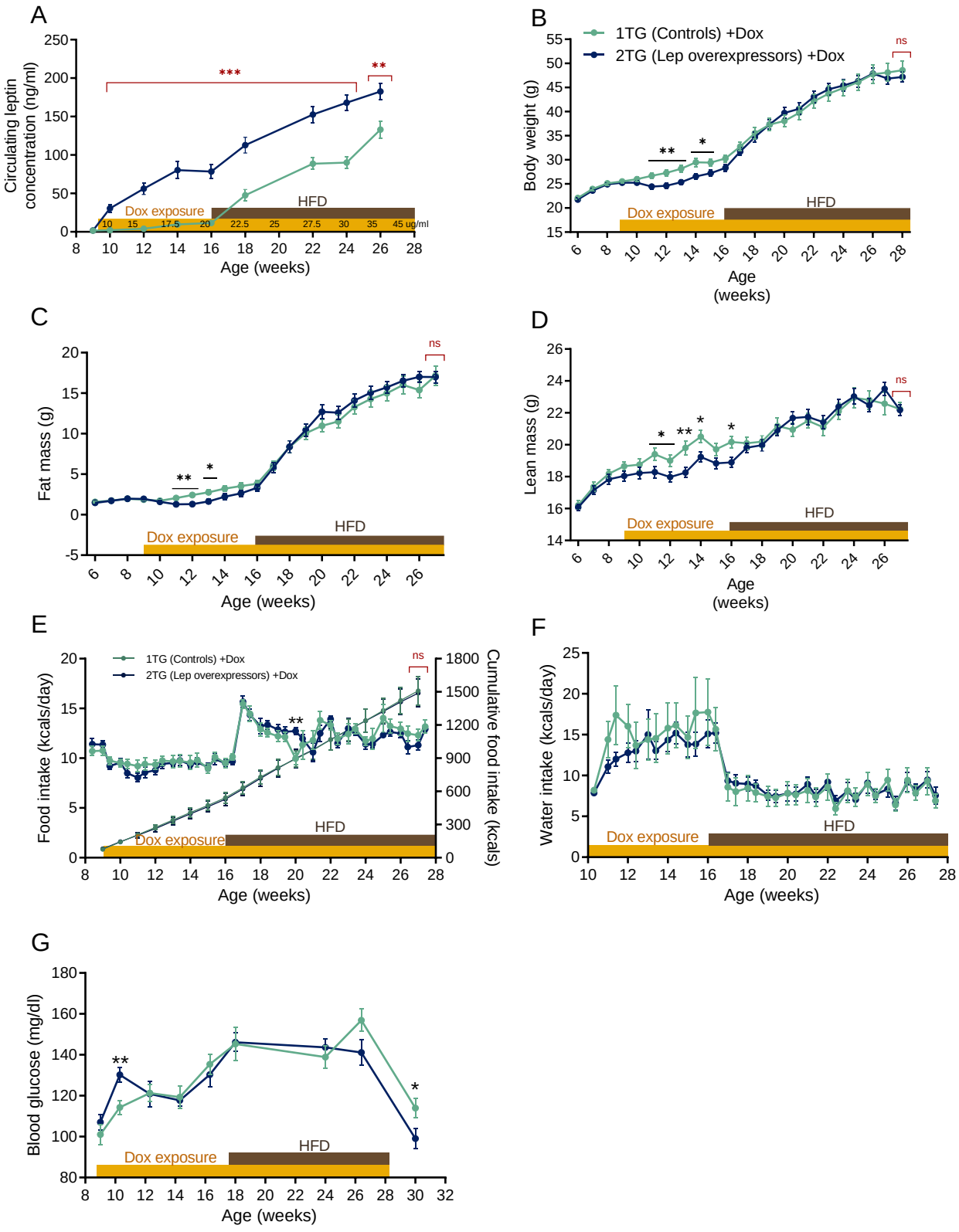

A
Circulating leptin concentration (ng/ml)
***
**
Dox exposure
HFD
10 15 17.5 20 22.5 25 27.5 30 35 45 ug/ml
Age (weeks)
B
1TG (Controls) +Dox
2TG (Lep overexpressors) +Dox
ns
Body weight (g)
**
*
Dox exposure
HFD
Age (weeks)
C
Fat mass (g)
ns
**
*
Dox exposure
HFD
Age (weeks)
D
Lean mass (g)
ns
*
**
*
*
Dox exposure
HFD
Age (weeks)
E
1TG (Controls) +Dox
2TG (Lep overexpressors) +Dox
ns
Food intake (kcals/day)
Cumulative food intake (kcals)
**
Dox exposure
HFD
Age (weeks)
F
Water intake (kcals/day)
Dox exposure
HFD
Age (weeks)
G
Blood glucose (mg/dl)
**
*
Dox exposure
HFD
Age (weeks)

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

(**A**) Circulating leptin concentrations (Student's t-test at each dox dose), (**B**) Body weight (two-way RM ANOVA with Fisher's LSD *post hoc* analysis, genotype: $F(1,28) = 0.34$, $p = 0.56$), (**C**) Fat mass (two-way RM ANOVA with Fisher's LSD *post hoc* analysis, genotype: $F(1,28) = 0.013$, $p = 0.91$), (**D**) Lean mass (two-way RM ANOVA with Fisher's LSD *post hoc* analysis, genotype: $F(1,28) = 0.48$, $p = 0.50$), (**E**) Estimated daily food intake in kcals (left axis; two-way RM ANOVA with Fisher's LSD *post hoc* analysis, genotype: $F(1,8) = 0.079$, $p = 0.79$) and cumulative food intake (right axis) per mouse, (**F**) Daily water intake per mouse (two-way RM ANOVA with Fisher's LSD *post hoc* analysis, genotype: $F(1,8) = 0.090$, $p = 0.77$), and (**G**) Submandibular whole blood glucose (two-way RM ANOVA with Fisher's LSD *post hoc* analysis, genotype: $F(1,28) = 0.053$, $p = 0.82$) in 1TG controls and 2TG leptin overexpressors given dox in 5% sucrose during 20 weeks of escalating dose of dox exposure. All values are means ± SEM. Red brackets indicate a comparison by Student's t-test of the final datapoints. Student's t-test (in red) or *post hoc* Fisher's LSD (in black), *p<0.05, **p<0.01, **p<0.001.

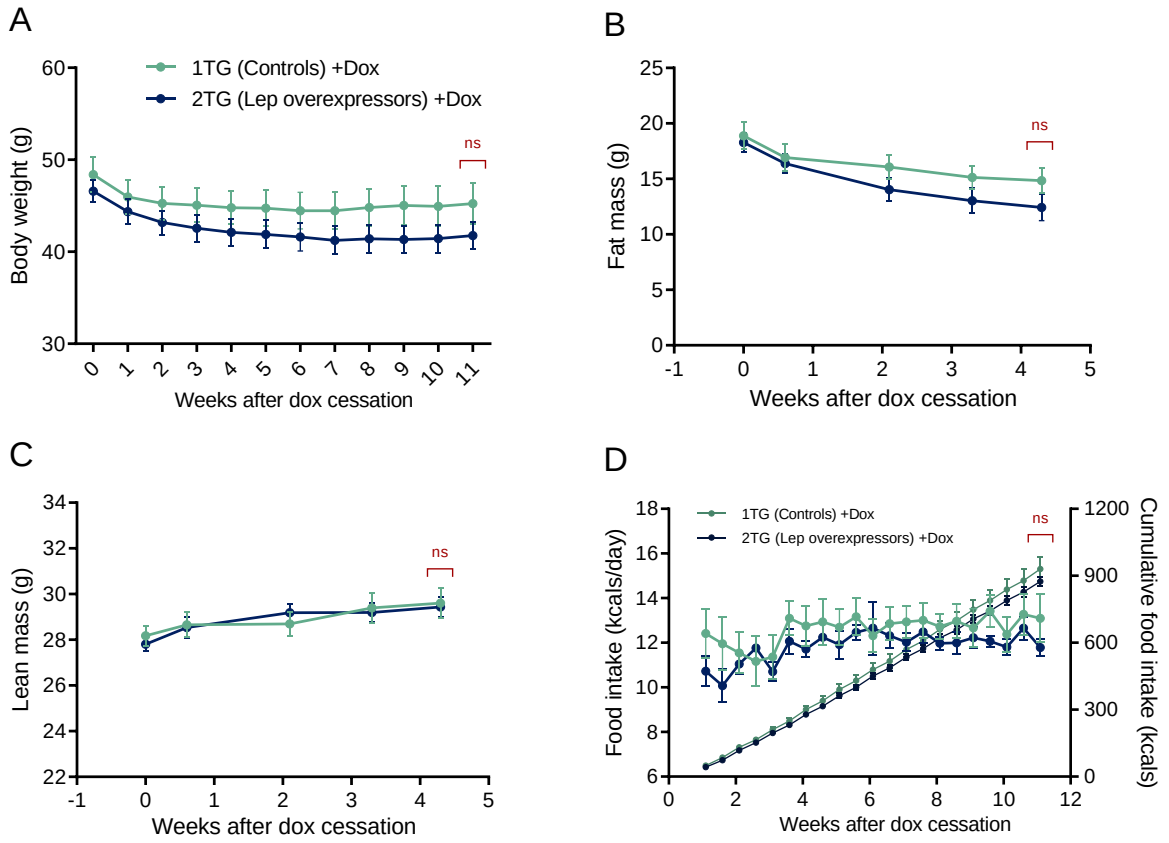


Fig. S5. Release of adult mice from dox-induced chronic (P63-P203) hyperleptinemia and HFD feeding.

(**A**) Body weight (two-way RM ANOVA with Fisher's LSD *post hoc* analysis, genotype: $F(1,28) = 1.44$, $p = 0.24$), (**B**) Fat mass (two-way RM ANOVA with Fisher's LSD *post hoc* analysis, genotype: $F(1,28) = 1.05$, $p = 0.31$), (**C**) Lean mass (two-way RM ANOVA with Fisher's LSD *post hoc* analysis, genotype: $F(1,28) = 0.0126$, $p = 0.91$), and (**D**) Estimated daily caloric food intake (left axis; two-way RM ANOVA with Fisher's LSD *post hoc* analysis, genotype: $F(1,9) = 1.01$, $p = 0.34$) and cumulative food intake (right axis) per mouse after mice were released from hyperleptinemia and HFD feeding (switched back to regular chow). All values are means ± SEM. Red brackets indicate a comparison by Student's t-test of the final datapoints.

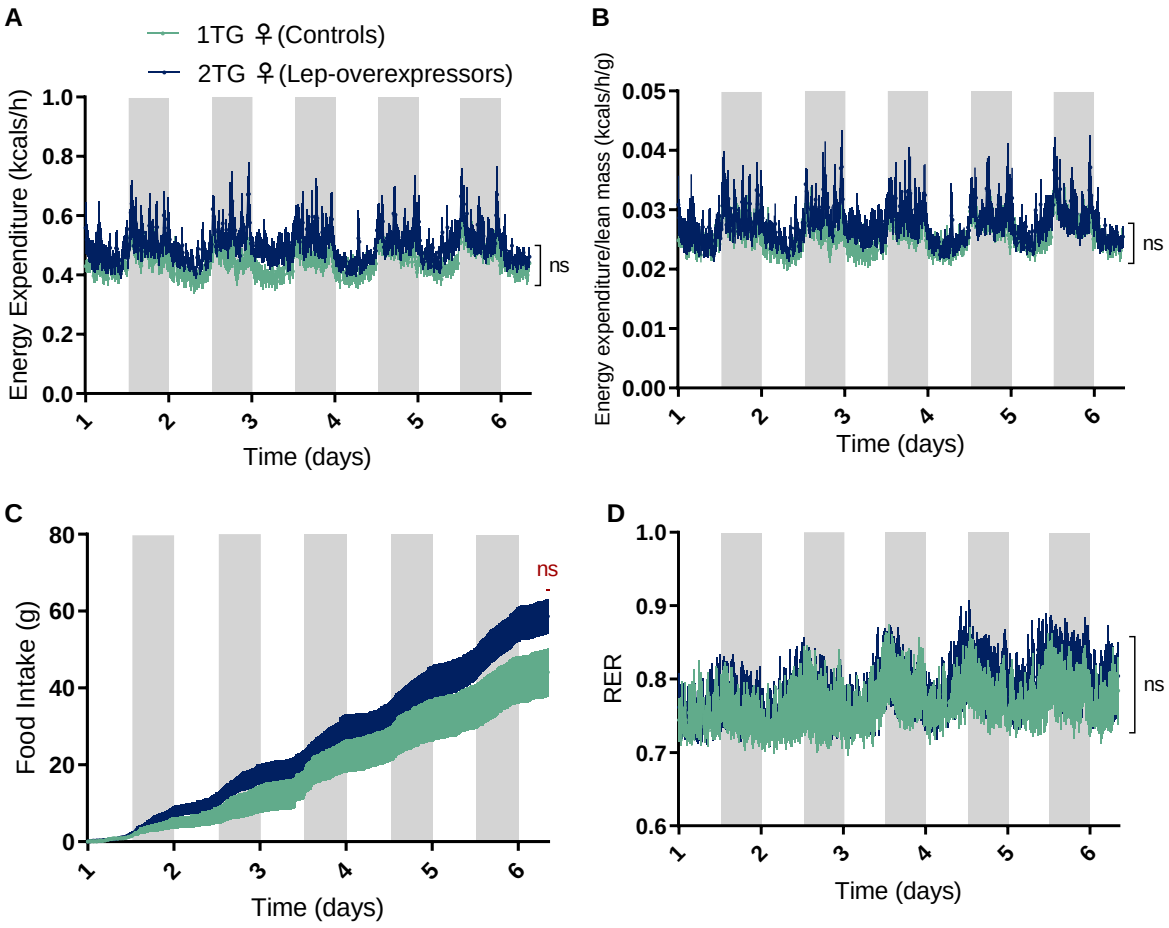


Fig. S6. Energy expenditure assessment (indirect calorimetry) at 26 weeks of age in postnatally (P0-P22) hyperleptinemic female mice after 16 weeks of HFD feeding.

(**A**) Absolute energy expenditure (kcal/h, two-way RM ANOVA with Fisher's LSD *post hoc* analysis, genotype: $F(1,14) = 1.89$, $p = 0.19$), (**B**) Energy expenditure normalized to lean mass (two-way RM ANOVA with Fisher's LSD *post hoc* analysis, genotype: $F(1,14) = 0.67$, $p = 0.43$), (**C**) Cumulative food intake (kcals), and (**D**) Respiratory exchange ratio (two-way RM ANOVA with Fisher's LSD *post hoc* analysis, genotype: $F(1,14) = 3.60$, $p = 0.079$) measured at 16 weeks of HFD feeding by indirect calorimetry (TSE LabMaster system) in 1TG controls and 2TG dox-induced leptin-overexpressing mice. All values are means ± SEM. Red brackets indicate a comparison by Student's t-test of the final datapoint.

Supplementary Excel Data file S1 [Primary data file used to generate main figures].

Supplementary Excel Data file S2 [Primary data file used to generate supplementary figures].