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Leptin action via hypothalamic nitric oxide synthase-1 neurons controls energy balance

Rebecca L. Leshan[1,2,*,**], **Megan Greenwald-Yarnell**[1,3,**], **Christa M. Patterson**[1], **Ian E. Gonzalez**[1], and **Martin G. Myers Jr.**[1,2,3,***]

[1]Department of Internal Medicine, University of Michigan, Ann Arbor, MI 48105

[2]Department of Molecular and Integrative Physiology, University of Michigan, Ann Arbor, MI 48105

[3]Neuroscience Graduate Program, University of Michigan, Ann Arbor, MI 48105

Summary

Few effective measures exist to combat the worldwide obesity epidemic[1], and the identification of potential therapeutic targets requires a deeper understanding of the mechanisms that control energy balance. Leptin, an adipocyte hormone that signals the status of cellular energy stores, acts via multiple types of leptin receptor (LepR-b)-expressing neurons in the brain to control feeding, energy expenditure and endocrine function[2–4]. The modest contributions to energy balance attributable to leptin action via many previously-studied LepR-b populations[5–9] suggest that other, heretofore unidentified, hypothalamic LepR-b neurons play important roles. Here, we examine the role of LepR-b in neuronal nitric oxide synthase (NOS1)-expressing (LepR-b^{NOS1}) neurons that comprise approximately 20% of hypothalamic LepR-b neurons. *Nos1^{cre}*-mediated ablation of LepR-b (*Lepr^{NOS1KO}* mice) produces hyperphagic obesity, decreased energy expenditure and hyperglycemia approaching that of LepR-b-null mice. In contrast, endocrine functions in *Lepr^{NOS1KO}* mice are relatively spared. Thus, hypothalamic LepR-b^{NOS1} neurons are essential for the control of energy balance by leptin.

Commensurate with the diverse processes controlled by leptin, specialized types of LepR-b neurons lie in multiple brain regions involved in energy balance, including the brainstem, midbrain, and hypothalamus[10–13]. LepR-b knockdown or deletion in the hindbrain interferes with satiation, although these manipulations only slightly alter body adiposity[8,9]. Within the midbrain ventral tegmental area (VTA) and substantia nigra (SN), a subset of dopamine (DA) neurons contain LepR-b; leptin action via these neurons contributes minimally to body

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***Correspondence: Martin G. Myers, Jr., M.D., Ph.D., Division of Metabolism, Endocrinology and Diabetes, Department of Internal Medicine, University of Michigan Medical School, 6317 Brehm Tower, 1000 Wall St., Ann Arbor, MI 48105, PH: 734-647-9515, Fax: 734-232-8175, mgmyers@umich.edu.

*Present Address: Laboratory of Neurobiology and Behavior, Rockefeller University, 1230 York Ave, New York, NY 10065

**Equal contribution

Contributions:

R.L.L., M.G.Y., C.M.P., and I.E.G. carried out the experiments (with Core and other technical assistance). R.L.L., M.G.Y. and C.M.P. analyzed and prepared data for publication. M.G.M. guided the overall approach in collaboration with R.L.L. and M.G.Y.; M.G.M., R.L.L. and M.G.Y. co-wrote the manuscript.

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weight control, but plays a role in DA-mediated behaviors, including those linked to anxiety[14–17]. Midbrain serotonin neurons, while initially reported to play an important role in leptin action, neither express LepR-b nor contribute to leptin action[18,19]. In contrast, ablation of hypothalamic LepR-b produces a profound metabolic phenotype, demonstrating the importance of hypothalamic LepR-b signaling for leptin action[20].

Within the hypothalamus, the specific set(s) of LepR-b neurons responsible for the control of energy balance by leptin remain incompletely defined. Direct leptin action via proopiomelanocortin (Pomc) neurons of the hypothalamic arcuate nucleus (ARC), ARC Agouti-related peptide (Agrp) neurons, and steroidogenic factor-1 (Sf-1) neurons in the ventromedial hypothalamic nucleus (VMH) contributes only modestly to overall energy balance[5–7]. LepR-b neurons in the lateral hypothalamic area (LHA), including those that contain neurotensin, mediate leptin action on orexin neurons and the mesolimbic DA system, but deletion of LepR-b from these neurons only modestly increases adiposity[21–23]. Thus, the identity of the hypothalamic LepR-b neurons responsible for the majority of leptin action on energy balance has remained unclear.

Nos1-expressing LepR-b (LepR-b^{NOS1}) neurons represent a relatively small population of LepR-b neurons mainly restricted to the hypothalamus, where they are distributed in areas poised to impact output from the paraventricular hypothalamic nucleus (signaling by which mediates much of the hypothalamic control of energy balance)[24–26]. To study LepR-b^{NOS1} neurons, we inserted an internal ribosome entry site (IRES) plus the coding sequences for cre recombinase into the 3'-UTR of *Nos1* in mice to promote Nos1-restricted cre expression (*Nos1^{cre}*)(Figure 1a). We bred *Nos1^{cre}* mice to the cre-dependent ROSA26-EGFP reporter line, generating *Nos1^{EGFP}* mice. EGFP-IR overlapped with Nos1-IR soma in all regions examined (data not shown).

We examined the leptin-stimulated induction of phosphorylated signal transducer and activator-3 (pStat3; which reveals neurons containing functional LepR-b[27]) and its colocalization with EGFP-immunoreactivity (−IR) in *Nos1^{EGFP}* mice, as well as evaluating the colocalization of Nos1-IR with EGFP-IR in *Lepr^{EGFP}* mice[12] that express EGFP in LepR-b neurons (Supplemental Figures 1–2). As suggested by NADPH-diaphorase colocalization with leptin-stimulated pStat3-IR[24], our analysis revealed that although Nos1 and LepR-b are each expressed in many brain areas, they are co-expressed predominantly in the hypothalamus; approximately 20% of hypothalamic LepR-b neurons contain *Nos1*. Most LepR-b^{NOS1} neurons lie within the ventral premammillary nucleus (PMv); the DMH and ARC also contain a substantial number; other areas contain few LepR-b^{NOS1} neurons. ARC Nos1-expressing cells are distinct from Pomc and Agrp neurons (Figure 1b, c; Supplemental Figure 3).

To determine the role of LepR-b^{NOS1} neurons in leptin action, we crossed *Nos1^{cre}* mice with *Lepr^{fl}* animals[28]. Since *Nos1* is expressed in gametes, germline *Lepr^{fl}* excision occurred in the offspring of *Nos1^{cre};Lepr$^{fl/+}$* parents, generating *Lepr^{Δ}*. We therefore interbred *Nos1^{cre};Lepr$^{Δ/+}$* to *Lepr$^{fl/fl}$* animals to generate *Nos1^{cre};Lepr$^{Δ/fl}$* (*Lepr^{NOS1}KO*) animals in which LepR-b was ablated specifically from LepR-b^{NOS1} neurons (Figure 1a), along with *Nos1^{cre};Lepr$^{Δ/+}$* and *Lepr$^{Δ/fl}$* (control) littermates. The persistence of cre in the early embryo

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of some animals also produced *Nos1*^{cre};*Lepr*$^{\Delta/\Delta}$ littermates with LepR-b inactivated in all tissues (*LeprKO*).

Analysis of the hypothalamus of control, *Lepr*^{NOS1}*KO*, and *LeprKO* animals (Supplemental Figure 4a, b) demonstrated the absence of pStat3 in *LeprKO* animals. We also confirmed the ablation of leptin-stimulated pStat3-IR from the PMv in *Lepr*^{NOS1}*KO* animals, along with modestly lower pStat3-IR from other areas (ARC and DMH) containing LepR-b^{NOS1} neurons. Thus, functional LepR-b was ablated from LepR-b^{NOS1} neurons in *Lepr*^{NOS1}*KO* mice. Consistent with the lack of overlap between Pomc and Nos1 neurons (Figure 1b; Supplemental Figure 3), leptin treatment stimulated pStat3-IR in similar proportions of ARC Pomc neurons in control and *Lepr*^{NOS1}*KO* mice (Supplemental Figure 4c).

Lepr^{NOS1}*KO* and *LeprKO* males exhibited similarly high body weight and food intake relative to controls (Figure 2a, b). VO_2 for *Lepr*^{NOS1}*KO* mice was lower than controls and not different from *LeprKO* animals; similar trends were observed for ambulatory activity (Figure 2c, d; Supplemental Figure 5). The excess weight in *Lepr*^{NOS1}*KO* and *LeprKO* mice was largely due to increased adipose mass (Figure 2e), which was reflected by high leptin concentrations relative to controls (Figure 2f). We observed similar trends in *Lepr*^{NOS1}*KO* females (Supplemental Figure 6), although the obesity and metabolic dysfunction in *Lepr*^{NOS1}*KO* females was less severe relative to *LeprKO* females. Thus, direct leptin action on LepR-b^{NOS1} neurons is crucial for the regulation of feeding, activity, and energy expenditure, and therefore for the control of body weight and adiposity.

To determine the potential role for leptin action via LepR-b^{NOS1} neurons in glucose homeostasis, we examined circulating blood glucose and insulin concentrations in control, *Lepr*^{NOS1}*KO* and *LeprKO* mice (Figure 2g, h). As with body weight and adiposity, male *Lepr*^{NOS1}*KO* and *LeprKO* mice exhibited similarly high blood glucose concentrations relative to controls from an early age, despite high insulin concentrations relative to controls. Thus, leptin action via LepR-b^{NOS1} neurons is indispensible for the regulation of glucose homeostasis, in addition to energy balance, and LepR-b^{NOS1} neurons represent crucial regulators of metabolic leptin action.

To interrogate the contribution of LepR-b^{NOS1} neurons to the control of endocrine systems by leptin, we examined circulating hormone concentrations along with parameters of reproductive function in *Lepr*^{NOS1}*KO* mice (Figure 3). While circulating T4 concentrations in *Lepr*^{NOS1}*KO* males were lower than for controls, they remained higher than in *LeprKO* mice (Figure 3a). Furthermore, while circulating corticosterone was higher in *LeprKO* males than in *Lepr*^{NOS1}*KO* and control animals, corticosterone concentrations were not different between *Lepr*^{NOS1}*KO* and controls (Figure 3b). The onset of vaginal estrus was delayed relative to controls in singly-housed *Lepr*^{NOS1}*KO* females (Figure 3c); upon mating with C57Bl/6 males, all *Lepr*^{Nos1}*KO* females delivered litters with similar timing as controls (Control = 23 ± 1 d; *Lepr*^{Nos1}*KO* = 24 ± 1 d), and with similar litter sizes (Control = 6.9 ± 1.1; *Lepr*^{Nos1}*KO* = 7.1 ± 0.7 pups litter^{-1}), however (Figure 3d). In contrast, the majority of *LeprKO* females failed to deliver pups within 6 weeks of mating.

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Thus, while deletion of LepR-b from LepR-b^{NOS1} neurons promoted obesity and metabolic dysfunction to an extent similar to that observed in animals with *Lepr^{fl}* deleted throughout the body, the control of the thyroid and adrenal axes remained relatively intact, and female reproductive function was partially impacted. Hence, our present findings reveal that leptin action via LepR-b^{NOS1} neurons contributes modestly to the control of endocrine function, but is indispensible for the control of energy balance and glucose homeostasis.

Relatively few ARC LepR-b neurons express Nos1, and the deletion of LepR-b from LepR-b^{NOS1} neurons was insufficient to detectably alter ARC *Socs3* expression (a surrogate for LepR-b signaling in the ARC[29])(Figure 4a). Altered ARC physiology may contribute to the phenotype of *Lepr^{NOS1}KO* mice, however; while the expression of *Npy* and *Agrp* were unaltered in *Lepr^{NOS1}KO* mice relative to controls, *Pomc* expression was lower in *Lepr^{NOS1}KO* mice than in controls (Figure 4b–d), even though LepR-b^{NOS1} neurons are distinct from Pomc neurons (Figures 1b; Supplemental Figures 3, 4c). Thus, LepR-b^{NOS1} cells may indirectly mediate aspects of melanocortin action, consistent with recent findings revealing that leptin action via non-Pomc cells controls important aspects of Pomc neuron function and the regulation of energy balance[5,30]. Given the severity of the metabolic defects exhibited by *Lepr^{NOS1}KO* mice, LepR-b^{NOS1} neurons may act by other mechanisms, as well.

Thus, leptin action via LepR-b^{NOS1} neurons is crucial for the control of energy balance and metabolism and for the regulation of ARC Pomc neurons. While the frequency of *Nos1^{cre}*-mediated excision in gametes dictated that we study mice on the heterozygous *Lepr$^{\Delta/fl}$* background, *Lepr$^{\Delta/fl}$* mice express the wild-type receptor on all LepR-b neurons and have no detectable phenotype; it is therefore unlikely that this genetic background contributed substantially to the dramatic phenotype of *Lepr^{NOS1}KO* mice. While *Nos1* expression identifies LepR-b^{NOS1} neurons important for metabolic control, Nos1 signaling is unlikley to mediate downstream leptin action; although leptin regulates the phosphorylation and presumably the activity of Nos1 in hypothalamic regions containing LepR-b^{NOS1} neurons[24], NO is a retrograde transmitter[31] and animals null for *Nos1* exhibit no obvious primary metabolic phenotype[32]. It is therefore likely that other transmitters in the LepR-b^{NOS1} neurons play crucial roles.

Recent data suggest that GABAergic (vGat-expressing) LepR-b neurons (which represent many ARC, DMH, and LHA LepR-b neurons- approximately 60–75% of total hypothalamic LepR-b neurons) are important for the control of energy balance. Approximately 20–30% of ARC and DMH LepR-b^{NOS1} neurons contain Gad1 (which produces GABA), and a similar fraction of ARC/DMH LepR-b^{NOS1} neurons are activated by leptin (Supplemental Figure 7). By itself, direct leptin action on glutamatergic (vGlut2-expressing) LepR-b neurons (essentially all PMv and VMH LepR-b neurons) contributes only modestly to the control of energy balance by leptin[30]. Leptin activates the vast majority of PMv LepR-b neurons (most of which are LepR-b^{NOS1} neurons) [25,33], and a variety of data support a role for PMv LepR-b^{NOS1} neurons (~70% of total LepR-b^{NOS1} neurons) in fertility, consistent with the delayed estrus observed in *Lepr^{Nos1}KO* females[34]. Since the PMv represents a sexually-dimorphic nucleus rich in androgen receptor [35], sex-specific differences in LepR-b neurons in this nucleus could contribute to the sexual dimorphism observed in the *Lepr^{Nos1}KO* phenotype.

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The apparently minor role played by glutamatergic LepR-b neurons in energy balance suggests that PMv LepR-b^{NOS1} neurons alone do not mediate the majority of the metabolic phenotype displayed by *Lepr^{Nos1}KO* mice, suggesting either that the modest number of LepR-b^{NOS1} neurons elsewhere (e.g., in the DMH and ARC) mediate this dramatic phenotype, or that the PMv LepR-b neurons may reinforce the action of the DMH and ARC LepR-b^{NOS1} neurons via supporting action on similar neural networks. It will be important to understand the detailed mechanisms of action for LepR-b^{NOS1} neurons in the future, since this small group of cells controls energy balance and thus represents a potential therapeutic target for obesity and related diseases.

Methods

Materials

Leptin was the generous gift of Amylin Pharmaceuticals, Inc. (San Diego, CA).

Animals

We bred all mice in our colony in the Unit for Laboratory Animal Medicine at the University of Michigan. All animals and procedures used were in accordance with the guidelines and with the approval of the University Committee on the Use and Care of Animals. We provided all animals *ad libitum* access to food and water. We purchased male C57Bl/6J animals for breeding studies and *Gt(ROSA)26Sor$^{tm1(EYFP)Cos}$* (*ROSA^{EYFP}*) mice from Jackson Labs. We produced *Pomc^{EYFP}* and *Agrp^{EYFP}* animals by crossing *Pomc^{Cre}* and *Agrp^{Cre}* animals[5,36] onto the *ROSA^{EYFP}* background.

To generate *Nos1^{cre}*, PCR amplification produced a 6 kb fragment containing the mouse genomic *Nos1* sequence centered on the STOP codon in the final (3') exon for insertion into pCR2.1. PCR mutagenesis created an AscI site 60 bp 3' to the STOP codon, for the introduction of the IRES-Cre-Frt-Neo-Frt sequences to generate pCRNos1-IRES-Cre-Frt-Neo-Frt. NotI/NheI digestion excised the entire insert for subcloning into NotI/XbaI-cut pPNT backbone to generate pPNT-Nos1-IRES-Cre for targeting. NotI digestion linearized the vector for electroporation into R1 ES cells. We used Taqman-based qPCR screening to initially identify correctly targeted clones[37], followed by Southern blotting for final confirmation. We injected correctly targeted ES cells into blastocysts to generate chimeras, which we bred to C57Bl/6 animals to establish germline transmission.

We bred *Nos1^{cre}* mice with *ROSA^{EGFP}* mice to generate *Nos1^{cre};ROSA^{EGFP}* (*Nos1^{EGFP}*) animals for the analysis of *Nos1^{cre}* expression. We also bred *Nos1^{cre}* animals with *Lepr$^{fl/fl}$* mice. Due to the periodic expression of *Nos1^{cre}* during gametogenesis, we bred *Nos1^{cre};lepr$^{\Delta/+}$* to *Lepr$^{fl/fl}$* mice in order to obtain littermate *Nos1^{cre};Lepr$^{\Delta/fl}$* (*Lepr^{NOS1}KO*), *Nos1^{cre}*; *Lepr$^{\Delta/+}$* and *Lepr$^{\Delta/fl}$* (control) and *Nos1^{cre};Lepr$^{\Delta/\Delta}$* (*LeprKO*) animals for study. We genotyped the offspring by PCR.

Perfusion and immunolabeling

We anesthetized the mice with an overdose of intraperitoneal (IP) pentobarbital and transcardially perfused them with 10% neutral buffered formalin. We sectioned the brains

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coronally (30 μm) using a sliding microtome followed by immunofluoresent analysis. We visualized the antigens via immunofluorescence using species-specific AlexaFluor-488 or -568 secondary antibodies (Invitrogen, Cat# A11039 and A10042; 1:200), and processed and imaged the sections as previously described[38]. Antibodies used were GFP (Abcam, chicken, Cat# ab13970; 1:1000) and Nos1 (ImmunoStar, rabbit, Cat# 24287; 1:5000). We purchased normal donkey serum and biotinylated donkey anti-rabbit (Cat# 711-065-152) from Jackson ImmunoResearch. We counted cells using Adobe Photoshop software.

Phenotypic studies

We individually housed mice for study from the time of weaning at 21 d. Beginning at 28 d, we monitored body weight and chow (Purina Lab Diet #5001) intake weekly. We collected blood for serum and measured blood glucose with a glucometer biweekly. We monitored female mice for vaginal opening and then for vaginal estrus by cellular histology until eight weeks of age. We collected all data between 13:00 and 16:00. We analyzed mice for body fat and lean mass between 12–14 weeks of age using an NMR-based analyzer (Minispec LF90II, Bruker Optics). We also analyzed a subset of mice (13–16 weeks old) for oxygen consumption (VO_2) and locomotor activity using the Comprehensive Laboratory Animal Monitoring System (CLAMS, Columbus Instruments). We analyzed serum for insulin and leptin using assays from Crystal Chem; we purchased assays for T4 and corticosterone from Siemens and Arbor Assays, respectively.

RNA extraction and qPCR

We prepared RNA from microdissected ARC using Trizol (Invitrogen) and converted 1 μg samples to cDNA using the Superscript First Strand Synthesis System for RT-PCR (Invitrogen). We analyzed these cDNAs in triplicate via quantitative RT-PCR for *Gapdh* and *Socs3* (Applied Biosystems) or *Pomc*, *Npy*, and *Agrp*[39] using an Applied Biosystems 7500. We calculated relative mRNA expression values by the $2^{-\Delta\Delta Ct}$ method, with normalization of each sample ΔCt value to the average ΔCt from Ctrl mice.

Statistics

We used one-way ANOVA followed by Bonferroni *post hoc* test for multiple comparisons using Graphpad Prism software for PC. Differences were considered significant for $p < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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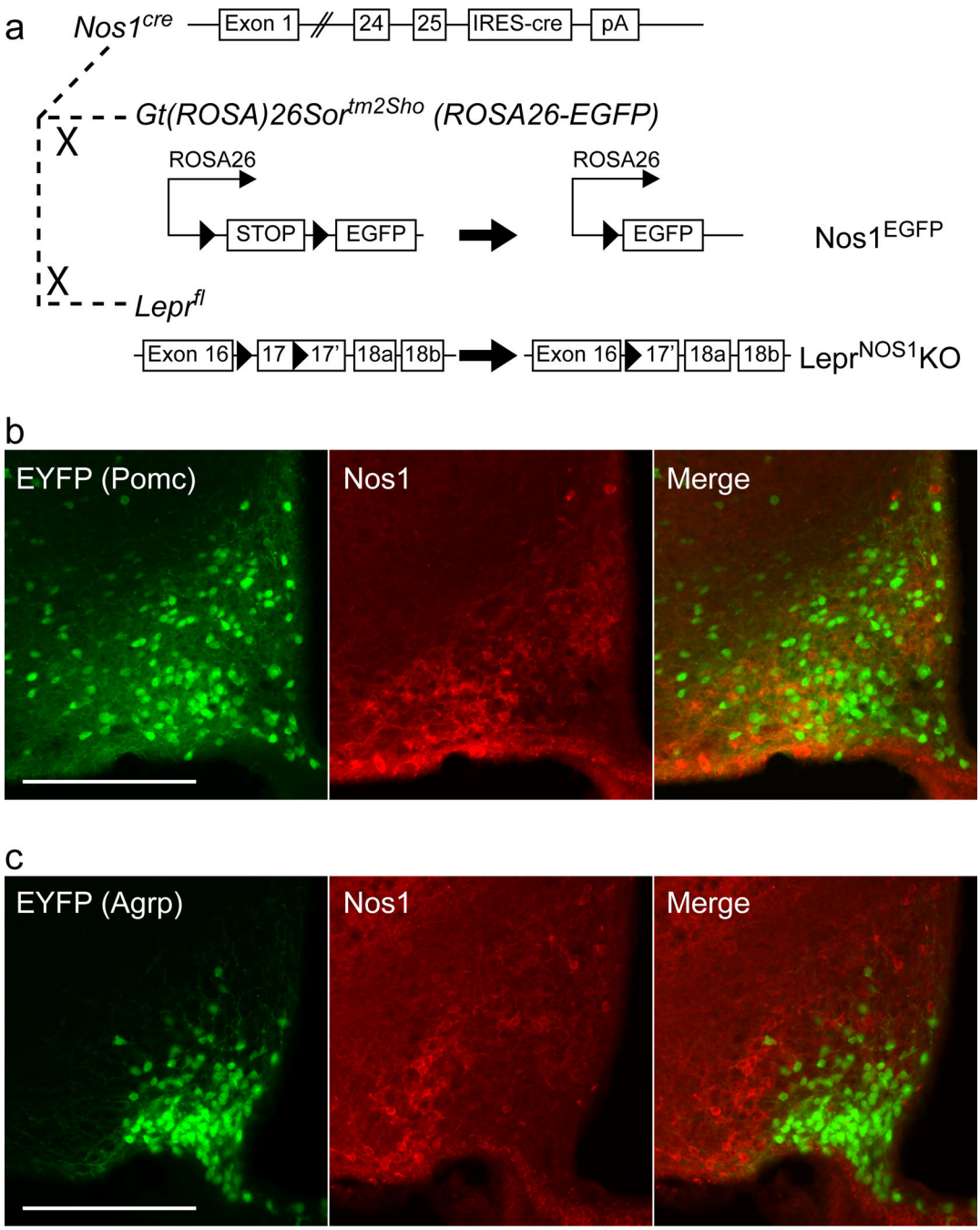

Figure 1. Generation of *Nos1*^{cre} and lack of Nos1 in ARC Pomc and Agrp neurons

(a) Schematic diagram depicting *Nos1*^{cre} and its use to generate *Nos1*^{EGFP} and *Lepr*^{NOS1}*KO* mice. (b) Representative images from the ARC of *Pomc*^{EYFP} mice demonstrating EYFP (green, left panel), Nos1-IR (red, middle panel), and merged images (right panel). (c) Representative images from the ARC of *Agrp*^{EYFP} mice demonstrating EYFP (green, left panel), Nos1-IR (red, middle panel), and merged (right panel). Scale bars = 200 μm.

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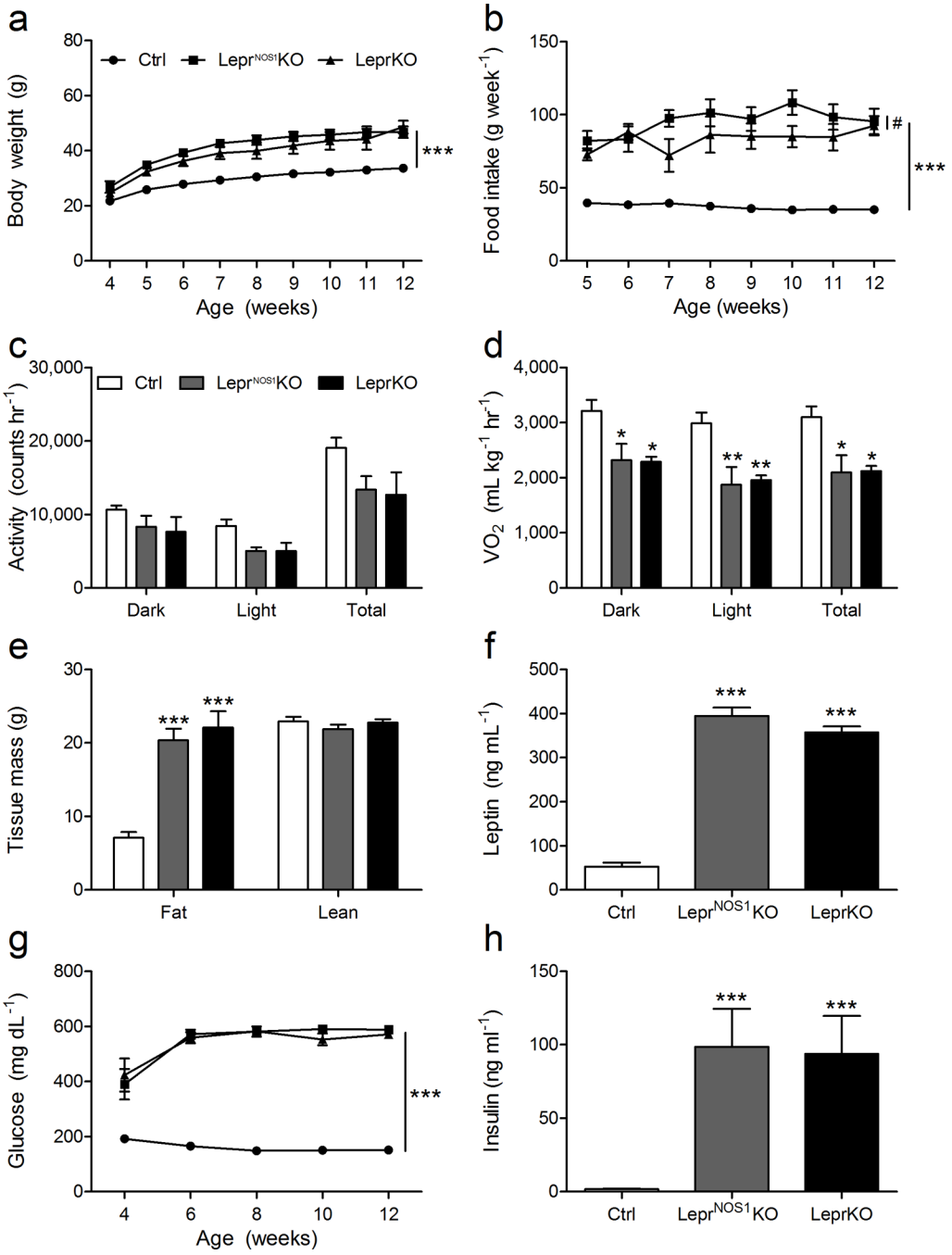

Figure 2. LepR-b^{NOS1} neurons regulate energy balance and glucose homeostasis

(a) Body weight and (b) food intake from 4–12 weeks of age for male control (Ctrl), *Lepr^{NOS1}KO* and *LeprKO* mice. CLAMS analysis of 12–14 week old male mice determined (c) ambulatory locomotor activity and (d) VO_2. Data are shown for dark cycle (Dark), light cycle (Light) and averaged over 24 h (Total). (e) Body composition analysis of 12–14 week old animals. (f) Serum leptin concentrations for 12 week old animals. (g) Biweekly blood glucose concentrations from 4–12 weeks of age for male Ctrl, *Lepr^{NOS1}KO* and *LeprKO* mice. (h) Serum insulin concentrations for 8 week old animals. Graphed data represent

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average values ± SEM; $n \geq 8$ for all measurements. ANOVA: *, $p < 0.05$ vs Ctrl; **, $p < 0.01$ vs Ctrl; ***, $p < 0.001$ vs Ctrl; $^{\#}$, $p < 0.05$; all other comparisons, p = NS.

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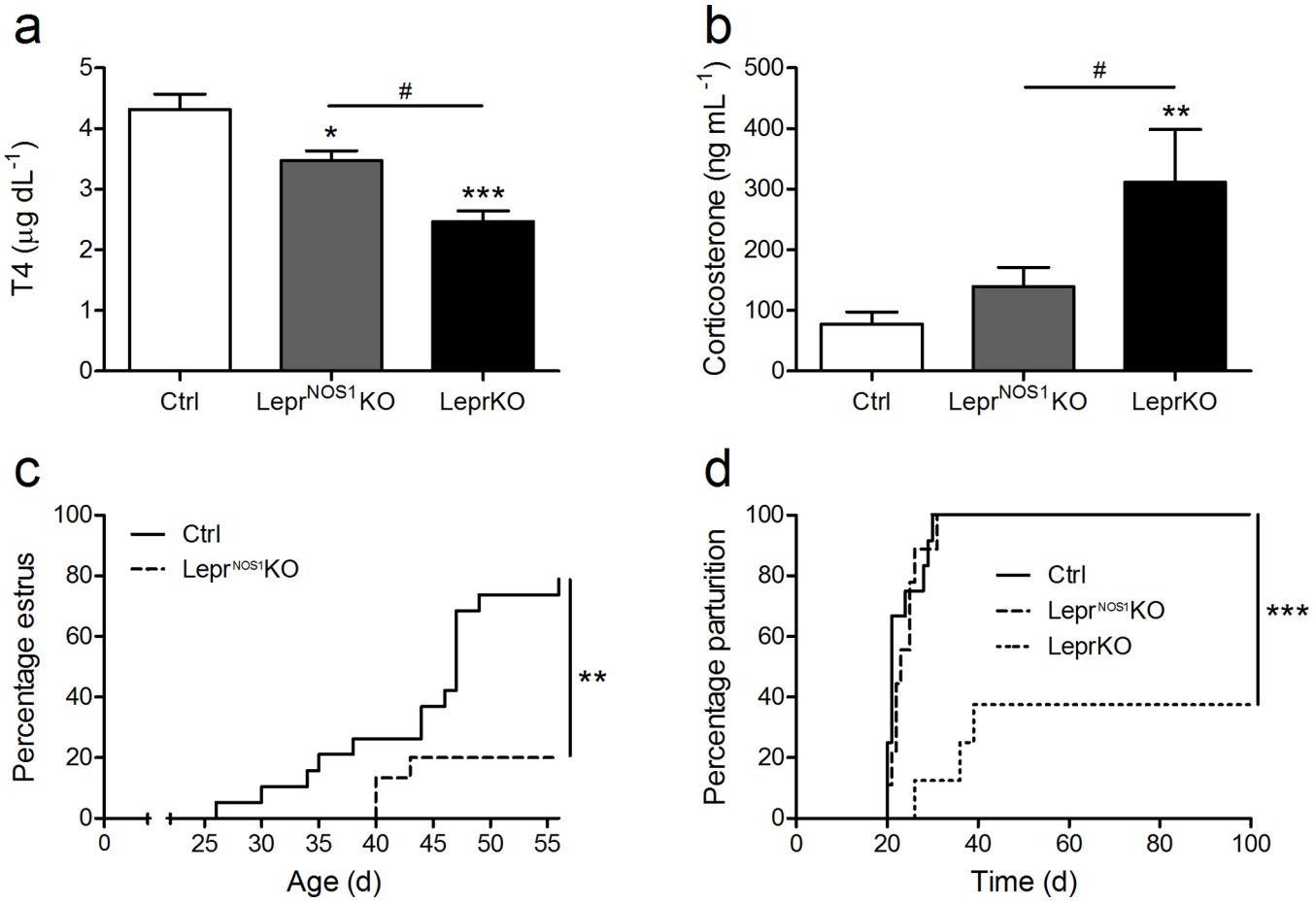

Figure 3. LepR-b^{NOS1} neurons contribute modestly to endocrine functions

Serum T4 (a) and corticosterone (b) concentrations from 7–8 week old male control (Ctrl), *Lepr^{NOS1}KO* and *LeprKO* mice. All measurements, $n \geq 5$. Graphed data represent average values ± SEM. (c) Analysis of time to first estrus for female Ctrl and *Lepr^{NOS1}KO* mice; percent exhibiting vaginal estrus is plotted by age. All measurements, $n \geq 9$. (d) Analysis of reproductive competence in female Ctrl, *Lepr^{NOS1}KO* and *LeprKO* mice. Experimental animals were mated with C57Bl/6 males and the delivery of pups was monitored daily. Percent delivering pups by days post-mating is plotted. All measurements, $n \geq 8$. ANOVA: *, $p < 0.05$ vs Ctrl; **, $p < 0.01$ vs Ctrl; ***, $p < 0.001$ vs Ctrl; $^{\#}$, $p < 0.05$; all other comparisons, p = NS.

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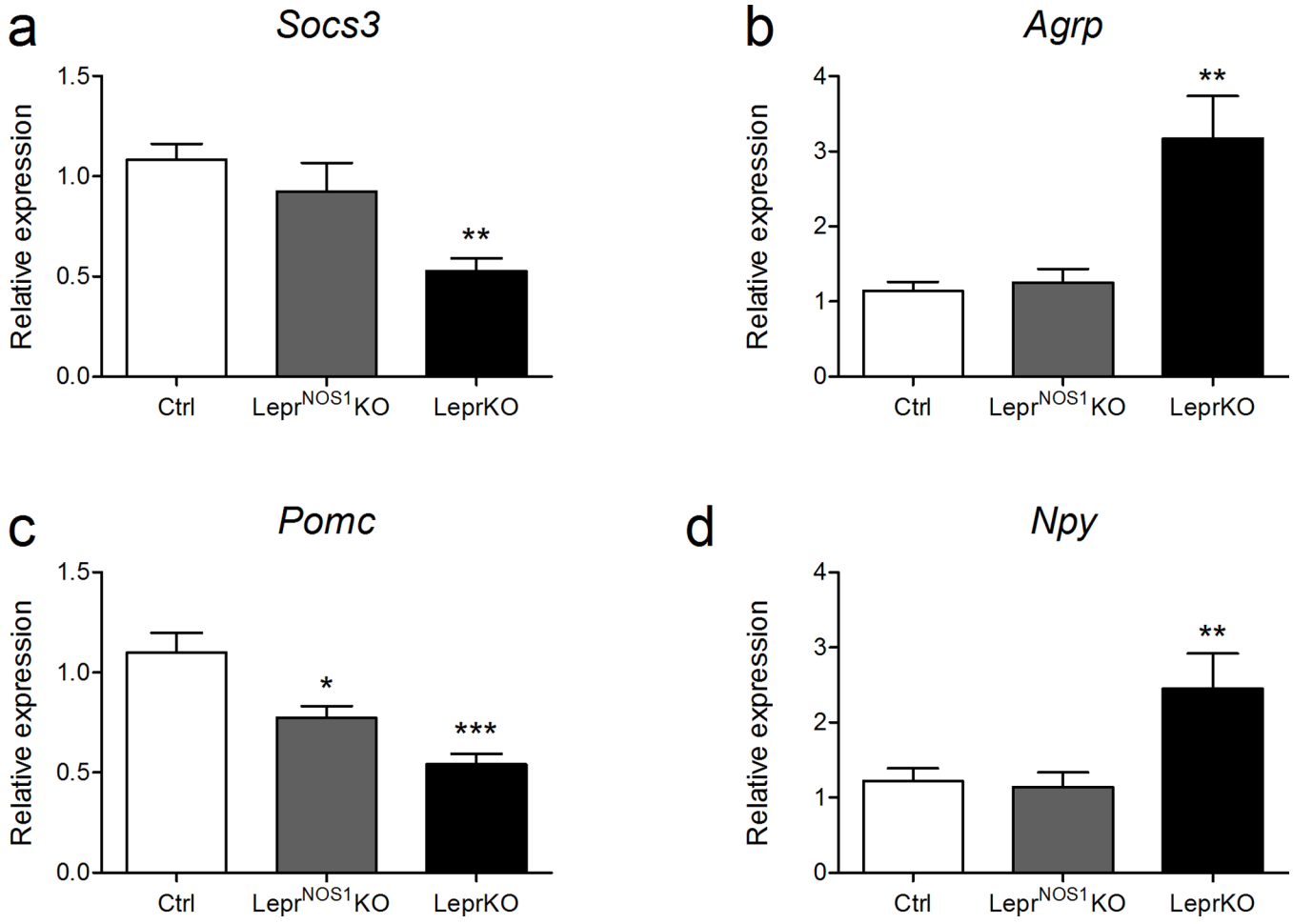

Figure 4. ARC gene expression in *Lepr^{NOS1}KO* animals

(a) *Socs3*, (b) *Agrp*, (c) *Pomc*, and (d) *Npy* mRNA expression by qPCR of microdissected ARC samples from *ad libitum*-fed male and female 7–8 week old control (Ctrl), *Lepr^{NOS1}KO* and *LeprKO* mice. All measurements $n \geq 14$. Graphed data represent average values ± SEM. ANOVA: *, $p < 0.05$ vs Ctrl; **, $p < 0.01$ vs Ctrl; ***, $p < 0.001$ vs Ctrl; all other comparisons, p = NS.