Leptin action through hypothalamic nitric oxide synthase-1–expressing neurons controls energy balance

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Few effective measures exist to combat the worldwide obesity epidemic1, and the identification of potential therapeutic targets requires a deeper understanding of the mechanisms that control energy balance. Leptin, an adipocyte-derived hormone that signals the long-term status of bodily energy stores, acts through multiple types of leptin receptor long isoform (LepRb)-expressing neurons (called here LepRb neurons) in the brain to control feeding, energy expenditure and endocrine function2–4. The modest contributions to energy balance that are attributable to leptin action in many LepRb populations5–9 suggest that other previously unidentified hypothalamic LepRb neurons have key roles in energy balance. Here we examine the role of LepRb in neuronal nitric oxide synthase (NOS1)-expressing LepRb (LepRbNOS1) neurons that comprise approximately 20% of the total hypothalamic LepRb neurons. Nos1cre-mediated genetic ablation of LepRb (LeprNOS1KO) in mice produces hyperphagic obesity, decreased energy expenditure and hyperglycemia approaching that seen in whole-body LepRb-null mice. In contrast, the endocrine functions in LeprNOS1KO mice are only modestly affected by the genetic ablation of LepRb in these neurons. Thus, hypothalamic LepRbNOS1 neurons are a key site of action of the leptin-mediated control of systemic energy balance.

Commensurate with the diverse processes controlled by leptin, specialized types of LepRb neurons are located in multiple brain regions that are involved in the regulation of systemic energy balance, including the brainstem, midbrain and hypothalamus10–13. Knockdown or deletion of LepRb in the hindbrain interferes with satiety, although these alterations have only a slight affect on body adiposity8,9. Within the midbrain ventral tegmental area and substantia nigra, a subset of dopamine neurons contain LepRb; leptin action through these neurons contributes minimally to body weight control but does have a role in dopamine-mediated behaviors, including those that are linked to anxiety14–17. Midbrain serotonin neurons, although initially reported to have a key role in leptin action, neither express LepRb nor contribute to leptin action18,19. In contrast, genetic ablation of hypothalamic LepRb produces a profound metabolic phenotype, showing the key role of hypothalamic LepRb signaling in leptin action20.

Within the hypothalamus, the specific set(s) of LepRb neurons that are responsible for the control of energy balance by leptin are not completely defined. Direct leptin action through the proopiomelanocortin (Pomc)-expressing neurons (Pomc neurons) of the hypothalamic arcuate nucleus (ARC) and through the ARC agouti-related peptide (Agrp)-expressing neurons (Agrp neurons) and steroidogenic factor-1 (Sf-1)-expressing neurons of the ventromedial hypothalamic nucleus contributes only modestly to the overall energy balance5–7. LepRb neurons in the lateral hypothalamic area, including those that contain neurotensin, mediate the action of leptin on orexin neurons and the mesolimbic dopamine system, but genetic deletion of LepRb from these neurons only modestly increases adiposity21–23. Thus, the identity of the hypothalamic LepRb neurons that are responsible for the majority of leptin-mediated regulation of energy balance is unclear.

LepRbNOS1 neurons comprise a relatively small population of LepRb neurons and are primarily restricted to the hypothalamus, where they are distributed in areas in which they are poised to affect output from the paraventricular hypothalamic nucleus (signaling by this nucleus mediates much of the hypothalamic control of energy balance)24–26. To study LepRbNOS1 neurons, we inserted an internal ribosome entry site (IRES) plus the coding sequences for Cre recombinase into the 3′ untranslated region of Nos1 in mice to promote Nos1-restricted Cre expression (resulting in Nos1Cre mice) (Fig. 1a). We bred Nos1Cre mice to mice from the Cre-dependent ROSA26–enhanced GFP (eGFP) reporter line, generating Nos1GFP mice. The immunoreactivity of eGFP overlapped with the immunoreactivity of Nos1 in the soma of the Nos1GFP mice in all regions examined (data not shown).

We next examined the leptin-stimulated induction of phosphor -ylated signal transducer and activator-3 (pStat3), which reveals neurons containing functional LepRb (ref. 27), and its colocalization with eGFP immunoreactivity in Nos1GFP mice. We also evaluated the colocalization of Nos1 immunoreactivity with eGFP immunoreactivity in LepreGFP mice that express eGFP in their LepRb neurons (Supplementary Figs. 1 and 2). As has been previously suggested by the colocalization of NADPH diaphorase with leptin-stimulated

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Received 8 February; accepted 9 March; published online 22 April 2012; doi:10.1038/nm.2724
Fig. 2a, although Nos1 express in many brain areas alone, they are coexpressed predominantly in the hypothalamus: approximately 20% of hypothalamic LepRb neurons contain Nos1 expression. Most LepRbNOS1 neurons are located in the ventral premammillary nucleus (PMv), however, the dorsomedial hypothalamic nucleus (DMH) and ARC also contain a substantial number of these neurons; other areas contain few LepRbNOS1 neurons. Nos1-expressing cells in the ARC are distinct from POMc and Agrp neurons (Fig. 1b,c and Supplementary Fig. 3).

To determine the role of LepR-bNOS1 neurons in leptin action, we crossed Nos1Cre mice with Leprflox mice. Because Nos1 is expressed in gametes, germline Leprflox excision generated a LeprA allele and occurred in offspring having one Nos1Cre; Leprflox parent. We therefore interbred Nos1Cre; Leprflox and Leprflox/flox mice to generate Nos1Cre; Leprflox (LeprNOS1) mice in which LepRb was ablated specifically from the LepRbNOS1 neurons (Fig. 1a), as well as Nos1Cre; Leprflox and Leprflox littermates to use as the normal control groups. The persistence of Cre in the early embryos of some mice also led to the production of Nos1flox; LeprfloxA littermates that had inactivated LepRb in all tissues (LeprKO mice).

An analysis of the hypothalami of control, LeprNOS1KO and LeprKO mice (Supplementary Fig. 4a,b) showed an absence of pStat3 in the LeprKO mice. We also confirmed the ablation of leptin-stimulated pStat3 immunoreactivity from the PMvs of LeprNOS1KO mice, along with modestly lower pStat3 immunoreactivity in other areas (specifically, the ARC and DMH) that contained LepRbNOS1 neurons as compared to controls. Thus, functional LepRb was ablated from the LepRbNOS1 neurons of LeprNOS1KO mice. Consistent with the lack of overlap in the immunoreactivity between POMc and Nos1 neurons (Fig. 1b and Supplementary Fig. 3), treatment with leptin stimulated pStat3 immunoreactivity of ARC Nos1 neurons in similar amounts in control and LeprNOS1KO mice (Supplementary Fig. 4c).

Male LeprNOS1KO and LeprKO mice had similarly high body weight and food intake relative to controls (Fig. 2a,b). The maximal oxygen consumption (VO2) in male LeprNOS1KO mice was lower than in controls but was not different from the LeprKO mice; we observed similar trends for ambulatory activity (Fig. 2c,d and Supplementary Fig. 5). The excess weight in the male LeprNOS1KO and LeprKO mice was largely caused by higher adipose mass (Fig. 2e), which was reflected by higher leptin concentrations, relative to controls (Fig. 2f). We observed similar trends in female LeprNOS1KO mice (Supplementary Fig. 6), although the obesity and metabolic dysfunction in these mice was less severe than in female LeprKO mice. Thus, direct leptin action on LepRbNOS1 neurons is crucial for the regulation of feeding, activity and energy expenditure and, therefore, the control of body weight and adiposity.

Fig. 2 LepRbNOS1 neurons regulate energy balance and glucose homeostasis. (a,b) Body weight (a) and food intake (b) for male control (ctrl), LeprNOS1KO and LeprKO mice at 4–12 weeks of age. (c,d) Comprehensive Laboratory Animal Monitoring System analysis of 12- to 14-week-old mice showing ambulatory locomotor activity (c) and VO2 (d). Data are shown for the dark cycle (dark), the light cycle (light) and an average over 24 h (total). (e) Body composition analysis of 12- to 14-week-old mice. (f) Serum leptin concentrations for 12-week-old mice. (g) Biweekly blood glucose concentrations for male control, LeprNOS1KO and LeprKO mice at 4–12 weeks of age. (h) Serum insulin concentrations for 8-week-old mice. All data are means ± s.e.m. n ≥ 8 for all measurements. *P < 0.05 compared to control, **P < 0.01 compared to control, ***P < 0.001 compared to control, ∗P < 0.05. The P values were not significant for all other comparisons. Statistical significance was determined by analysis of variance (ANOVA).
To determine the potential role for leptin action through LepRb\textsuperscript{NOS1} neurons in glucose homeostasis, we examined circulating blood glucose and insulin concentrations in control, Lep\textsuperscript{Nos1}\textsuperscript{KO} and Lep\textsuperscript{KO} mice (Fig. 2g.h). As with body weight and adiposity, male Lep\textsuperscript{Nos1}\textsuperscript{KO} and Lep\textsuperscript{KO} mice had similarly high relative blood glucose concentrations relative to controls starting at weaning, despite having high insulin concentrations relative to controls. Thus, leptin action through LepRb\textsuperscript{NOS1} neurons is indispensable for the normal regulation of glucose homeostasis in addition to its role in energy balance, and LepRb\textsuperscript{NOS1} neurons are crucial regulators of metabolic leptin action.

To investigate the contribution of LepRb\textsuperscript{NOS1} neurons to the control of endocrine systems by leptin, we examined circulating hormone concentrations and parameters of reproductive function in control, Lep\textsuperscript{Nos1}\textsuperscript{KO} and Lep\textsuperscript{KO} mice (Fig. 3). Although the circulating T4 concentrations in male Lep\textsuperscript{Nos1}\textsuperscript{KO} mice were lower than in controls, they were higher than in male Lep\textsuperscript{KO} mice (Fig. 3a). Furthermore, although circulating corticosterone concentrations were higher in male Lep\textsuperscript{KO} mice than in male Lep\textsuperscript{Nos1}\textsuperscript{KO} and control mice, the circulating concentrations of corticosterone were not different between male Lep\textsuperscript{Nos1}\textsuperscript{KO} and control mice (Fig. 3b). The onset of vaginal estrus was delayed relative to controls in singly housed female Lep\textsuperscript{Nos1}\textsuperscript{KO} mice (Fig. 3c); however, after mating with male C57BL/6 mice, all female Lep\textsuperscript{Nos1}\textsuperscript{KO} mice delivered litters with similar timing as controls (control, 23 ± 1 d (mean ± s.e.m. for time from mating to delivery); Lep\textsuperscript{Nos1}\textsuperscript{KO}, 24 ± 1 d) and with similar litter sizes (control, 6.9 ± 1.1 pups per litter (mean ± s.e.m.); Lep\textsuperscript{Nos1}\textsuperscript{KO}, 7.1 ± 0.7 pups) (Fig. 3d). In contrast, the majority of the female Lep\textsuperscript{KO} mice failed to deliver pups within 6 weeks of mating.

Thus, although deletion of LepRb from LepRb\textsuperscript{Nos1} neurons promoted obesity and metabolic dysfunction to an extent similar to that observed in mice with LepRb deleted throughout the body, the control of the thyroid and adrenal axes remained relatively intact, and female reproductive function was partially affected. Hence, our findings reveal that leptin action through LepRb\textsuperscript{NOS1} neurons contributes modestly to the control of endocrine function but is indispensable for the proper control of energy balance and glucose homeostasis.

Relatively few ARC LepRb neurons express Nos1, and the deletion of LepRb from LepRb\textsuperscript{NOS1} neurons was not sufficient to detectably alter the expression of Socs3 in the ARCs (a surrogate for ARC LepRb signaling\textsuperscript{29}) of control, Lep\textsuperscript{Nos1}\textsuperscript{KO} and Lep\textsuperscript{KO} mice (Fig. 4a). We examined the expression of key neuropeptides that regulate food intake in these mice. Although the expressions of Npy and Agpr were the same in Lep\textsuperscript{Nos1}\textsuperscript{KO} mice as in controls, Pomc expression was lower in Lep\textsuperscript{Nos1}\textsuperscript{KO} mice than in controls (Fig. 4b–d), even though LepRb\textsuperscript{NOS1} neurons are distinct from Pomc neurons (Fig. 1b and Supplementary Figs. 3 and 4c). Thus, LepRb\textsuperscript{NOS1} neurons may indirectly mediate aspects of melanocortin action, which is consistent with recent findings revealing that leptin action through non-Pomc neurons controls key aspects of Pomc neuron function and the regulation of energy balance\textsuperscript{29,30}. Given the severity of the metabolic defects shown by Lep\textsuperscript{Nos1}\textsuperscript{KO} mice, LepRb\textsuperscript{NOS1} neurons may act by other mechanisms as well.

Thus, leptin action through LepRb\textsuperscript{NOS1} neurons is crucial for the control of energy balance and metabolism and for the regulation of Pomc expression in the ARC Pomc neurons. Although the frequency of Nos1\textsuperscript{cre}-mediated excision in gametes dictated that we study mice on the heterozygous Lep\textsuperscript{Aflo}\textsuperscript{b} background, Lep\textsuperscript{Aflo}\textsuperscript{b} mice express the wild-type receptor on all LepRb neurons and have no detectable phenotype; it is therefore unlikely that this genetic background contributed substantially to the notable phenotype of the Lep\textsuperscript{Nos1}\textsuperscript{KO} mice. Whereas Nos1 expression identifies LepRb\textsuperscript{NOS1} neurons that are crucial for metabolic control, Nos1 signaling probably does not mediate downstream leptin action; although leptin regulates the phosphorylation and, presumably, the activity of Nos1 in hypothalamic regions containing LepRb\textsuperscript{NOS1} neurons\textsuperscript{24}, nitric oxide is a retrograde transmitter\textsuperscript{31}, and mice null for Nos1 show no obvious primary metabolic phenotype\textsuperscript{32}. It is therefore probable that other transmitters in the LepRb\textsuperscript{NOS1} neurons have crucial roles in properly regulating energy balance and metabolism and will be the focus of future studies.

Recent data have suggested that LepRb neurons that produce γ-aminobutyric acid (GABAergic neurons, which also express vesicular GABA transporter (vGAT)), representing many of the LepRb neurons in the ARC, DMH and lateral hypothalamic area (and approximately 60–75% of the total hypothalamic LepRb neurons) are crucial for the proper control of energy balance. Approximately 20–30% of ARC and DMH LepRb\textsuperscript{NOS1} neurons contain Gad1 (which produces GABA), and a similar fraction of ARC and DMH LepRb\textsuperscript{NOS1}...
neurons are activated by leptin (Supplementary Fig. 7). By itself, direct leptin action on glutamatergic (vesicular glutamate transporter 2 (vGlut2) expressing) LepRb neurons, which comprise essentially all the LepRb neurons in the PMv and ventromedial hypothalamic nucleus, contributes only modestly to the control of energy balance by leptin. Leptin activates the vast majority of PMv LepRb neurons (most of which are LepRb-NOS1+ neurons)23,33, and a variety of data support a role for PMv LepRb-NOS1+ neurons (~70% of the total LepRb-NOS1+ neurons) in fertility, which is consistent with the delayed estrus observed in female LeprNOs1KO mice. Because the PMv is a sexually dimorphic nucleus that is rich in androgen receptor35, sex-specific differences in LepRb neurons in this nucleus could contribute to the sexual dimorphism observed in the LeprNOs1KO phenotype. The apparently minor role of glutamatergic LepRb neurons in energy balance suggests that PMv LepRb-NOS1+ neurons alone do not mediate the majority of the metabolic phenotype shown by the LeprNOs1KO mice, suggesting either that the modest number of LepRb-NOS1+ neurons elsewhere (for example, in the DMH and ARC) mediate this notable phenotype or that the PMv LepRb neurons reinforce the action of the DMH and ARC LepRb-NOS1+ neurons by supporting action on similar neural networks. It will be crucial to understand the detailed mechanisms of action of LepRb-NOS1+ neurons in the future, as this small group of cells controls energy balance and is thus a potential therapeutic target for obesity and related diseases.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

Note: Supplementary information is available on the Nature Medicine website.

ACKNOWLEDGMENTS

We thank Amylin Pharmaceuticals for the generous gift of leptin; S. Chua (Albert Einstein College of Medicine), B. Lowell (Beth Israel-Deaconess Medical Center) and G. Barsh (Stanford University) for the gift of Leprflox/flox, Agrpcre and Pomcre mice, respectively; and members of the Myers lab for helpful discussions and technical support. Core support (animal phenotyping, Cell and Molecular Biology (CMB); clinical, Microscopy and Image Analysis Core (MIAC)) was provided by the Michigan Diabetes Research and Training Center and Nutrition and Obesity Research Center. This work was supported by the Marilyn H. Vincent Foundation and grants from the American Diabetes Association (M.G.M.), the American Heart Association (M.G.M. and R.L.L.) and the US National Institutes of Health (NIDDK057768 to M.G.M.). M.G.-Y. is supported by NIH grant T32GM008322; C.M.P. was supported by NIH grant T32HL007853.

AUTHOR CONTRIBUTIONS

R.L.L., M.G.-Y. and C.M.P. carried out the experiments (with staff of the Core facilities 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. and R.L.L. and M.G.-Y. cowrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Materials. The leptin used in this study was a generous gift of Amylin Pharmaceuticals, Inc. (San Diego, CA).

Mice. We bred male and female mice in our colony in the Unit for Laboratory Animal Medicine at the University of Michigan. All mice and all procedures used were in accordance with the guidelines of and with the approval of the University of Michigan Committee on the Use and Care of Animals. We provided all mice ad libitum access to food and water. We purchased male C57BL/6 mice for breeding studies and Gr(ROSA)26Sor(eYFP)Cre(ROSA-eYFP) mice from Jackson Labs. We produced PomcYFP and AgrpYFP mice by crossing PomcCre and AgrpCre mice crossing PomcCre and AgrpCre mice by crossing PomcCre and AgrpCre mice by crossing PomcCre and AgrpCre mice by crossing PomcCre and AgrpCre mice by crossing PomcCre and AgrpCre mice by creating an AscI site 60 bp 3' of the stop codon for the introduction of the IRES-Cre-FrtNeo-Frt sequences to generate pCRNos1-IRES-Cre-Frt-Neo-Frt. Nos1 and NheI digestion excised the entire insert for subcloning into NotI- and XbaI-cut plasmid pPNT backbone to generate pPNT-Nos1-IRES-Cre-Frt-Neo-Frt. NotI and NheI digestion excised the entire insert for subcloning into NotI- and XbaI-cut plasmid pPNT backbone to generate pPNT-Nos1-IRES-Cre-Frt-Neo-Frt. To generate Nos1Cre mice, we used PCR amplification to produce a 6-kb fragment containing the mouse genomic Nos1 sequence centered on the stop codon in the final (3') exon for insertion into plasmid pCR2.1. We used PCR mutagenesis to create an Ascl site 60 bp 3' of the stop codon for the introduction of the IRES-Cre-FrtNeo-Frt sequences to generate pCRNos1-IRES-Cre-Frt-Neo-Frt. Nos1 and NheI digestion excised the entire insert for subcloning into NotI- and XbaI-cut plasmid pPNT backbone to generate pPNT-Nos1-IRES-Cre-Frt-Neo-Frt. We used TaqMan-based qPCR screening and XbaI-cut plasmid pPNT backbone to generate pPNT-Nos1-IRES-Cre-FrtNeo-Frt. We used TaqMan-based qPCR screening and XbaI-cut plasmid pPNT backbone to generate pPNT-Nos1-IRES-Cre-Frt-Neo-Frt. Experimenters used PCR mutagenesis to create an Ascl site 60 bp 3' of the stop codon for the introduction of the IRES-Cre-FrtNeo-Frt sequences to generate pCRNos1-IRES-Cre-Frt-Neo-Frt. We used TaqMan-based qPCR screening and XbaI-cut plasmid pPNT backbone to generate pPNT-Nos1-IRES-Cre-Frt-Neo-Frt. We used TaqMan-based qPCR screening and XbaI-cut plasmid pPNT backbone to generate pPNT-Nos1-IRES-Cre-Frt-Neo-Frt.

Perfusion and immunolabeling. We anesthetized the mice with an overdose of intraperitoneal pentobarbital and transcardially perfused them with 10% neutral buffered formalin. We sectioned the mouse brains coronally (30 μm) using a sliding microtome followed by immunofluorescent analysis. We visualized the antigens by immunofluorescence using species-specific Alexa Fluor 488 or 568 secondary antibodies (Invitrogen, A11039 and A10042; 1:200) and processed and imaged the sections as previously described. The antibodies used were antibodies to GFP (Abcam, chicken, ab13970; 1:1,000) and Nos1 (ImmunoStar, rabbit, 24287; 1:5,000). We purchased normal donkey serum and biotinylated donkey antibodies to rabbit (711-065-152, 1:2,000) from Jackson ImmunoResearch. We counted the cells using Adobe Photoshop software.

Phenotypic studies. We individually housed mice for study starting at the time of weaning at 21 d of age. Beginning at 28 d, we monitored the mice's body weight and weekly chow (Purina Lab Diet 5011) intake. We collected blood from the mice for serum and measured blood glucose concentrations with a glucometer biweekly. We monitored female mice for vaginal opening and then for vaginal estrus by cellular histology until 8 weeks of age. We collected all data between 1 p.m. and 4 p.m. We analyzed the mice for body fat and lean mass between 12 and 14 weeks of age using a nuclear magnetic resonance–based analyzer (Minispec LF90II, Bruker Optics). We also analyzed a subset of mice (13–16 weeks old) for VO2 and locomotor activity using the Comprehensive Laboratory Animal Monitoring System (Columbus Instruments). We analyzed the mice's serum for insulin and leptin concentrations using assays from Crystal Chem; we purchased assays for T4 and corticosterone concentrations from Siemens and Arbor Assays, respectively.

RNA extraction and qPCR. We prepared RNA from microdissected ARCs using TRIzol (Invitrogen) and converted 1 μg of the samples to complementary DNA using the Superscript First Strand Synthesis System for RT-PCR (Invitrogen). We analyzed these complementary DNAs in triplicate by qRT-PCR for Gapdh and Soc3 (Applied Biosystems) or Pomc, Npy and Agrp using an Applied Biosystems 7500. We calculated the relative mRNA expression values using the 2−ΔΔCt method with normalization of each sample ΔCt value to the average ΔCt value from the control mice.

Statistics. We used one-way ANOVA followed by a Bonferroni post hoc test for multiple comparisons using GraphPad Prism software. Differences were considered significant at P < 0.05.

Additional methods. Detailed methodology is described in the Supplementary Methods.

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