accumulation in cells that express NFAT4 (Fig. 3). The NFAT4 isoform therefore serves to integrate negative signals from JNK into the NFAT signaling pathway. Positive signals to calcium-stimulated NFAT4 derive, in part, from the Ras signaling pathway (Fig. 4). Together, these findings indicate that NFAT4 may mediate signal-specific responses in activated cells.

REFERENCES AND NOTES

1. B. Dérijard *et al.*, *Cell* **76**, 1025 (1994); J. M. Kyriakis *et al.*, *Nature* **369**, 156 (1994).
2. A. J. Whitmarsh and R. J. Davis, *J. Mol. Med.* **74**, 589 (1996).
3. H. K. Sluss, Z. Han, T. Barrett, R. J. Davis, T. Ip, *Genes Dev.* **10**, 2745 (1996); J. R. Riesgo-Escovar, M. Jenni, A. Fritz, E. Hafen, *ibid.*, p. 2759.
4. The two-hybrid screen (5.2×10^6 transformants) of a mouse embryo cDNA library was done with the yeast strain L40 [Z. Galcheva-Gargova *et al.*, *Science* **272**, 1797 (1996)]. The bait plasmid (pLexA-JNK1) was constructed by insertion of JNK1 in the polylinker of pBTM116 [M. Dickens *et al.*, *ibid.* **277**, 693 (1997)].
5. T. Hoey, Y.-L. Sun, K. Williamson, X. Xu, *Immunity* **2**, 461 (1995).
6. S. N. Ho, D. J. Thomas, L. A. Timmerman, U. Franke, G. R. Crabtree, *J. Biol. Chem.* **270**, 19898 (1995); E. S. Masuda *et al.*, *Mol. Cell. Biol.* **15**, 2697 (1995).
7. S. L. Schreiber and G. R. Crabtree, *Immunol. Today* **13**, 136 (1992); G. R. Crabtree and N. A. Clipstone, *Annu. Rev. Biochem.* **63**, 1045 (1994); A. Rao, *Immunol. Today* **15**, 274 (1994); *J. Leukocyte Biol.* **57**, 536 (1995).
8. J. Raingeaud *et al.*, *J. Biol. Chem.* **270**, 7420 (1995).
9. B. Su *et al.*, *Cell* **77**, 727 (1994).
10. C.-W. Chow and J. Cavanagh, unpublished data.
11. C. R. Beals, N. A. Clipstone, S. N. Ho, G. R. Crabtree, *Genes Dev.* **11**, 824 (1997); C. R. Beals, C. M. Sheridan, C. W. Turck, P. Gardner, G. R. Crabtree, *Science* **275**, 1930 (1997).
12. S. Gupta, D. Campbell, B. Dérijard, R. J. Davis, *Science* **267**, 389 (1995).
13. F. Shibasaki, E. R. Price, D. Lian, F. McKeon, *Nature* **382**, 370 (1996).
14. C. Tournier, A. J. Whitmarsh, J. Cavanagh, T. Barrett, R. J. Davis, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 7337 (1997).
15. D. Yang *et al.*, *ibid.*, p. 3004.
16. D. Cantrell, *Annu. Rev. Immunol.* **14**, 259 (1996); E. Genot, S. Cleverly, S. Henning, D. Cantrell, *EMBO J.* **15**, 3923 (1996).
17. S. Gupta *et al.*, *EMBO J.* **15**, 2760 (1996).
18. Expression vectors for activated calcineurin [Y. Watanabe, B. A. Perrino, B. H. Chang, T. R. Soderling, *J. Biol. Chem.* **270**, 456 (1995)], JNK (*17*), MKK7 (*14*), Ras61L (*1*), and dominant negative c-Jun [TAM67(Δ3-122)] [U. R. Rapp, J. Troppmair, T. Beck, M. J. Birrer, *Oncogene* **9**, 3493 (1994)] have been described. The human NFAT3 and NFAT4 cDNAs were cloned in the polylinker of the expression vector pCDNA3 (Invitrogen). The human NFATc and NFATp cDNAs cloned in the vector pREP4 were obtained from T. Hoey (*5*). The polymerase chain reaction (PCR) was used to create mutations. GAL4 and glutathione-S-transferase (GST) fusion proteins were constructed by subcloning PCR fragments in the polylinker of pSG424 and pGEX-5X-1 (*17*).
19. Electroporation (Jurkat T cells) and lipofectamine (COS-1 and BHK cells) were used for transfection studies (Gibco-BRL). Luciferase reporter gene expression was examined in cotransfection assays. The IL-2 NFAT (*5*) and GAL4 (*17*) luciferase reporter plasmids have been described. Transfection efficiency was monitored with a β-galactosidase expression vector. Luciferase and β-galactosidase activities were measured in cell lysates 48 hours after transfection (*17*). The data are presented as the relative luciferase activity [mean ± SD (n = 3) of the luciferase/β-galactosidase activity ratio].
20. BHK cells were grown on poly-D-lysine–coated cover slips, fixed with 4% paraformaldehyde, permeabilized with 0.25% Triton X-100, and processed for immunofluorescence microscopy. The primary antibody used for epitope-tagged proteins was the mouse monoclonal antibody (mAb) M2 (IBI-Kodak). NFATc and NFATp were detected by means of a mAb (Affinity Bioreagents) and a rabbit polyclonal antibody (Upstate Biotechnology), respectively. The secondary antibodies were Texas Red–labeled goat antibody to mouse immunoglobulin (Ig) and rhodamine-labeled goat antibody to rabbit Ig (Jackson Immunoresearch).

21. We thank M. Birrer, J. Cooper, T. Hoey, S. Hollenberg, and T. Soderling for reagents; S. Doxsey for assistance with microscopy; T. Barrett for DNA sequencing; and K. Gemme for secretarial assistance. Supported in part by National Cancer Institute grants CA58396 and CA65831. R.J.D. is an Investigator of the Howard Hughes Medical Institute.

30 January 1997; accepted 21 October 1997

Independent and Additive Effects of Central POMC and Leptin Pathways on Murine Obesity

Bruce A. Boston, Kathryn M. Blaydon, Jeffrey Varnerin, Roger D. Cone*

The *lethal yellow* (A^Y/a) mouse has a defect in proopiomelanocortin (POMC) signaling in the brain that leads to obesity, and is resistant to the anorexigenic effects of the hormone leptin. It has been proposed that the weight-reducing effects of leptin are thus transmitted primarily by way of POMC neurons. However, the central effects of defective POMC signaling, and the absence of leptin, on weight gain in double-mutant *lethal yellow* (A^Y/a) leptin-deficient (lep^{ob}/lep^{ob}) mice were shown to be independent and additive. Furthermore, deletion of the leptin gene restored leptin sensitivity to A^Y/a mice. This result implies that in the A^Y/a mouse, obesity is independent of leptin action, and resistance to leptin results from desensitization of leptin signaling.

Serum concentrations of the hormone leptin (*1*) correlate well with body mass index in both humans and rodents (*2*). The long form of the leptin receptor has been detected in multiple hypothalamic regions including the arcuate nucleus (*3*), where it completes a feedback loop that sends information on peripheral energy stores to the hypothalamus, altering both food intake and metabolic rate. Absence of leptin results in extreme obesity in the *obese* (lep^{ob}/lep^{ob}) mouse.

Obesity in another rodent model, the *lethal yellow* (A^Y/a) mouse (*4*), is caused by a dominantly inherited promotor rearrangement at the *agouti* locus that results in constitutive ectopic expression of the Agouti peptide (*5*). Agouti is a potent antagonist of the hypothalamic melanocortin-4 receptor (MC4-R) (*6*), and interruption of signaling at MC4-R increases feeding behavior in mice (*7–9*). Thus, desacetyl-α–melanocyte-stimulating hormone derived from arcuate nucleus POMC neurons, the primary source of ligand for MC4-R, appears to play a tonic inhibitory role in feeding and energy storage. Leptin levels in the A^Y/a mouse, as well as other rodent obesity models, are elevated (*2*), reflecting the increase in adipose tissue. The A^Y/a mouse is also resistant to leptin administered peripherally or intracerebroventricularly (*10*). Peripheral resistance to leptin occurs in other obese-rodent models (*2*, *11*), and it has been argued that both obesity in the A^Y/a mouse as well as common forms of human obesity may result from genetically determined resistance to leptin feedback (*10*, *12*)

To study the relation between leptin and POMC signaling pathways on weight homeostasis and the potential dependence of leptin action on POMC signaling, we generated a *lethal yellow obese* (A^Y/a lep^{ob}/lep^{ob}) double-mutant mouse. C57BL/6J mice with either the A^Y/a or $lep^{ob}/+$ genotypes were crossed to create A^Y/a $lep^{ob}/+$ breeders. These animals were bred to create the double-mutant A^Y/a lep^{ob}/lep^{ob} mice, which were identified by their yellow coat color and obese phenotype, and their genotype was confirmed by allele-specific oligonucleotide hybridization (*13*).

If defective POMC signaling in the A^Y/a animal causes obesity solely by blocking the anorexigenic leptin signal, then introduction of the A^Y allele into the leptin-deficient lep^{ob}/lep^{ob} background should have no added effect on weight gain or metabolism in this model. However, because of the extreme rate of weight gain that results from leptin deficiency, the modest effects of the A^Y allele on weight might be difficult to

B. A. Boston, and K. M. Blaydon, Department of Pediatrics, Oregon Health Sciences University, Portland, OR 97201, USA.
J. Varnerin, Department of Genetics and Molecular Biology, Merck Research Laboratories, Rahway, NJ 07065, USA.
R. D. Cone, Vollum Institute for Advanced Biomedical Research, Oregon Health Sciences University, Portland, OR 97201, USA.

*To whom correspondence should be addressed. E-mail: cone@ohsu.edu

detect in the intact *lep*^{ob}/*lep*^{ob} animal. Leptin deficiency in the *lep*^{ob}/*lep*^{ob} mouse markedly increases glucocorticoid levels, which indirectly are reponsible for a large percentage of the obesity phenotype in these animals. To examine the direct effects of leptin and POMC pathways in the brain, we first measured weight gain in animals adrenalectomized and then maintained on normal levels of corticosterone.

The weight gain of female mice from each genotype, fed normal Chow ad libitum, was measured over a period from 6 to 31 weeks after birth. Before the experiment, animals were adrenalectomized and placed on maintenance glucocorticoids supplied in drinking water (*14*). The presence of the A^{Y} allele increased weight gain to a similar extent in both the wild-type and leptin-deficient *lep*^{ob}/*lep*^{ob} backgrounds, indicating that the obesity-inducing actions of defective POMC signaling are leptin-independent (Fig. 1A). Increased linear growth, another phenotype induced by the A^{Y} allele (*15*), did not appear to explain the difference in weight, because the adrenalectomized A^{Y}/*a* , A^{Y}/*a lep*^{ob}/*lep*^{ob} , and *lep*^{ob}/*lep*^{ob} mice all demonstrated the same 10% increase in linear growth relative to control *a*/*a* mice (*16*).

Fasting serum insulin and glucose concentrations were determined monthly during the course of the experiment in the adrenalectomized female mice (*17*). A^{Y} alone produced a mild late-onset hyperinsulinemia (*18*, *19*), whereas leptin deficiency produced an early, more significant rise in serum insulin. The effects of defective POMC signaling and leptin deficiency on serum insulin also appeared to be additive (Fig. 1B). Fasting serum glucose concentrations in the A^{Y}/*a lep*^{ob}/*lep*^{ob} and *a*/*a lep*^{ob}/*lep*^{ob} mice, however, were not significantly different (*16*). When mice of similar weights were compared (Fig. 1C), the serum insulin concentrations in the A^{Y}/*a lep*^{ob}/*lep*^{ob} were increased compared with those in *a*/*a lep*^{ob}/*lep*^{ob} animals. Additionally, *a*/*a lep*^{ob}/*lep*^{ob} animals demonstrated a linear relation between weight and serum insulin, suggesting that the increase of insulin levels in the absence of leptin is partially a function of the increased adipose mass. In contrast, introduction of the A^{Y} allele into the *lep*^{ob}/*lep*^{ob} background removed any significant correlation between weight and insulin levels, suggesting that Agouti inhibition of the POMC signal causes a dysregulation of insulin by a second mechanism independent of adipose mass.

If the A^{Y} allele induces obesity in a leptin-independent manner, implying that MC4-R signaling is not required for the central anorexigenic actions of leptin, then why are A^{Y}/*a* mice resistant to an increase in either endogenous (*2*) or exogenous leptin (*10*)? To test the hypothesis that MC4-R signaling is not required for the weight-reducing action of leptin, we examined the effects of leptin administration in the four mouse genotypes. After a 4-day

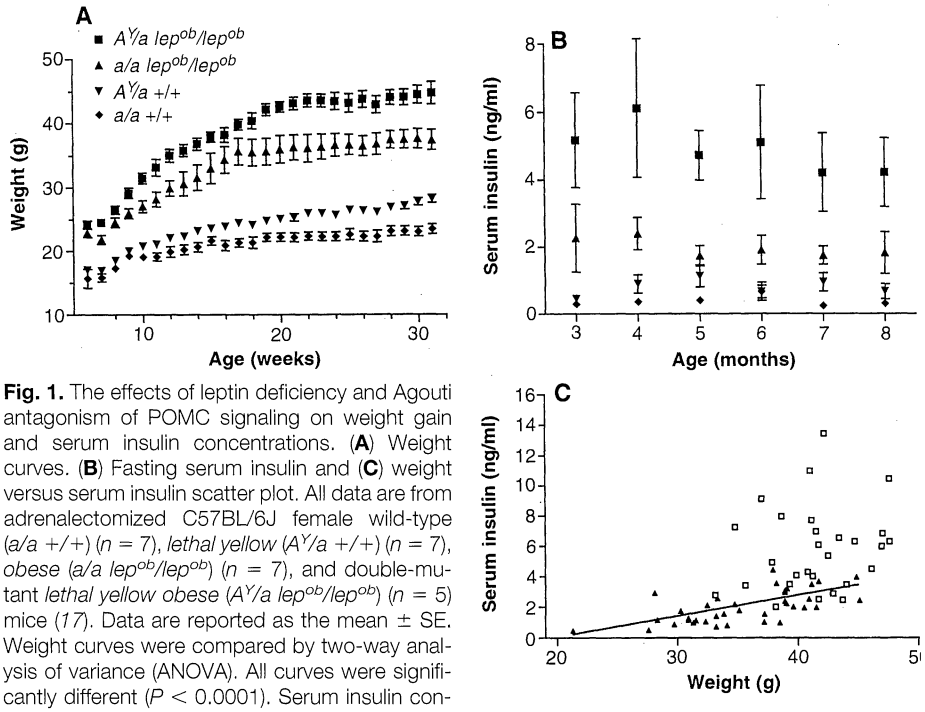

Fig. 1. The effects of leptin deficiency and Agouti antagonism of POMC signaling on weight gain and serum insulin concentrations. (**A**) Weight curves. (**B**) Fasting serum insulin and (**C**) weight versus serum insulin scatter plot. All data are from adrenalectomized C57BL/6J female wild-type (*a*/*a* +/+) (n = 7), *lethal yellow* (A^{Y}/*a* +/+) (n = 7), *obese* (*a*/*a lep*^{ob}/*lep*^{ob}) (n = 7), and double-mutant *lethal yellow obese* (A^{Y}/*a lep*^{ob}/*lep*^{ob}) (n = 5) mice (*17*). Data are reported as the mean ± SE. Weight curves were compared by two-way analysis of variance (ANOVA). All curves were significantly different ($P < 0.0001$). Serum insulin concentrations in *lethal yellow obese* mice were compared with those in *obese* mice by the two-tailed t test ($P < 0.05$ at 4 months and $P < 0.01$ at 5, 6, and 7 months). Linear regression of insulin versus weight in the *obese* mice was significant ($P = 0.02$) with $r = 0.46$. Linear regression of insulin versus weight in *lethal yellow obese* mice was not significant.

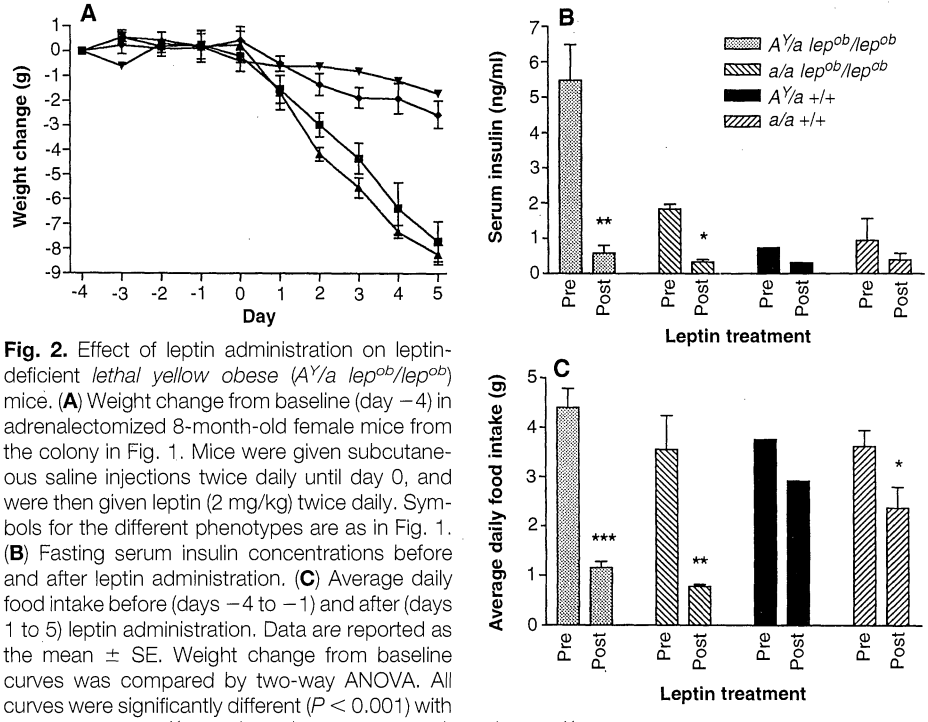

Fig. 2. Effect of leptin administration on leptin-deficient *lethal yellow obese* (A^{Y}/*a lep*^{ob}/*lep*^{ob}) mice. (**A**) Weight change from baseline (day −4) in adrenalectomized 8-month-old female mice from the colony in Fig. 1. Mice were given subcutaneous saline injections twice daily until day 0, and were then given leptin (2 mg/kg) twice daily. Symbols for the different phenotypes are as in Fig. 1. (**B**) Fasting serum insulin concentrations before and after leptin administration. (**C**) Average daily food intake before (days −4 to −1) and after (days 1 to 5) leptin administration. Data are reported as the mean ± SE. Weight change from baseline curves was compared by two-way ANOVA. All curves were significantly different ($P < 0.001$) with the exception of A^{Y}/*a lep*^{ob}/*lep*^{ob} versus *a*/*a lep*^{ob}/*lep*^{ob} and A^{Y}/*a* +/+ versus *a*/*a* +/+ ($P > 0.01$). The effect of leptin on serum insulin and food intake was analyzed by the one-tailed t test (pre- versus postleptin administration: *$P < 0.05$, **$P < 0.01$, ***$P \leq 0.001$). The number of mice used in these experiments is as follows: *a*/*a* +/+ (n = 3), A^{Y}/*a* +/+ (n = 1), *a*/*a lep*^{ob}/*lep*^{ob} (n = 3), and A^{Y}/*a lep*^{ob}/*lep*^{ob} (n = 3).

course of saline, the same adrenalectomized female mice used for analysis of weight gain were injected twice daily with human leptin (*20*). Wild-type and A^Y/a mice were resistant to leptin relative to the lep^{ob}/lep^{ob} mice, as measured by the inability of leptin to induce weight loss (Fig. 2A), lower serum insulin (Fig. 2B), and decrease food intake (Fig. 2C). In contrast, absence of the leptin gene restored full leptin sensitivity to the $A^Y/a\ lep^{ob}/lep^{ob}$ mice, as demonstrated by use of all three measures (Fig. 2, A to C).

To examine the effect of circulating glucocorticoids, gender, and age on leptin sensitivity in the $A^Y/a\ lep^{ob}/lep^{ob}$ mouse, we administered leptin to young nonadrenalectomized mice (Fig. 3A). Restoration of leptin responsiveness by deletion of the leptin gene in the A^Y/a mouse was independent of adrenal status. Whereas full leptin responsiveness was restored in adrenalectomized and normal female $A^Y/a\ lep^{ob}/lep^{ob}$ mice, leptin responsiveness was only partially restored in male $A^Y/a\ lep^{ob}/lep^{ob}$ animals. In agreement with previously reported data (*10*), both male and female A^Y/a mice exhibited greater leptin resistance than C57BL/6J controls.

Leptin administration also produced a reduction in serum insulin concentrations in $A^Y/a\ lep^{ob}/lep^{ob}$ and lep^{ob}/lep^{ob} young females by a factor of 15 (Fig. 3B) and in $A^Y/a\ lep^{ob}/lep^{ob}$ and lep^{ob}/lep^{ob} males by a factor of 5 and 15, respectively (Fig. 3C), indicating a significant restoration of leptin sensitivity in nonadrenalectomized $A^Y/a\ lep^{ob}/lep^{ob}$ mice. However, the serum insulin concentration in leptin-treated lep^{ob}/lep^{ob} males was significantly lower than that in leptin-treated $A^Y/a\ lep^{ob}/lep^{ob}$ males. This observation suggests there is a male-specific leptin-independent pathway for regulation of insulin by POMC neurons. Alternatively, the A^Y allele may cause a minor defect in this specific leptin response in the male only.

A^Y/a mice have normal concentrations of serum corticosterone (*19*, *21*), whereas lep^{ob}/lep^{ob} mice have increased basal corticosterone levels. The A^Y allele had no effect on basal serum corticosterone in the lep^{ob}/lep^{ob} background (Fig. 3D). Furthermore, leptin administration reduced corticosterone to normal levels in both the lep^{ob}/lep^{ob} and $A^Y/a\ lep^{ob}/lep^{ob}$ mice. Adrenalectomy revealed that increased glucocorticoids due to the absence of leptin are responsible for much of the hyperinsulinemia in the lep^{ob}/lep^{ob} mice (compare Fig. 1B to Fig. 3, B and C). The elevated glucocorticoid levels also increased fat deposition, as 3-month-old nonadrenalectomized lep^{ob}/lep^{ob} mice were ~60% heavier than adrenalectomized lep^{ob}/lep^{ob} mice (*16*).

These data demonstrate that the A^Y gene, and by inference disruption of central POMC signaling, does not cause obesity by acting as a genetic roadblock to the central anorexigenic action of leptin. The ability of Agouti to induce weight gain irrespective of the leptin state of the animal argues strongly that the POMC neurons can act independently of leptin in their actions on energy homeostasis. It remains likely that some aspect of leptin function not tested here is mediated by POMC neurons, given that mRNA encoding the long form of the receptor is expressed in some POMC-containing arcuate nucleus neurons (*22*). Furthermore, a recent report shows that, within a narrow dose range, an antagonist of the MC3 and MC4 receptors is able to block the acute inhibition of feeding by leptin in the rat (*23*). However, the results of the genetic studies shown here argue that normal POMC signaling is not required for the long-term ability of leptin to reduce weight, serum insulin, or serum corticosterone. The reduced sensitivity to leptin-induced weight loss and reduction in serum insulin in the male $A^Y/a\ lep^{ob}/lep^{ob}$ animal indicates the existence of a minor, sexually dimorphic defect in leptin signaling resulting from Agouti inhibition of MC4-R. Alternatively, the data could simply highlight a male-specific effect of POMC signaling downstream and independent of leptin action, reflected also by the observation that A^Y/a males gain weight faster and are more hyperinsulinemic than A^Y/a females (*19*).

Apparent leptin resistance is a hallmark of obesity in multiple species (*10*, *11*); however, our data suggest that it may be erroneous to assume that leptin resistance is indicative of genetic defects blocking leptin action. Rather, the data presented for the A^Y/a animal show that removal of leptin from this strain restores complete leptin sensitivity, strongly arguing that animals are

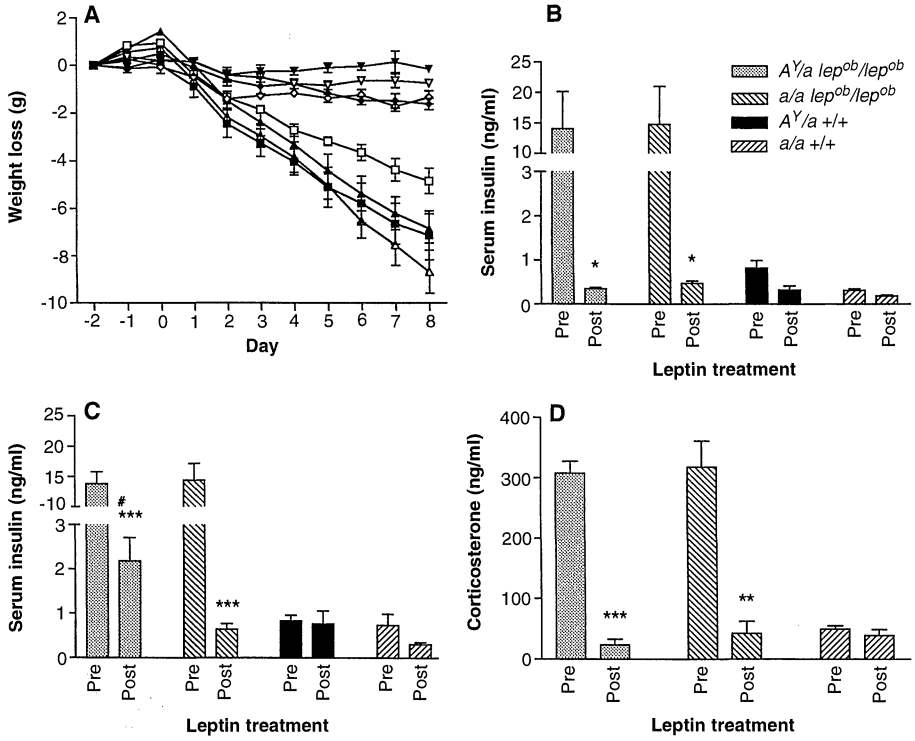

Fig. 3. Effect of leptin administration in young male and female nonadrenalectomized leptin-deficient *lethal yellow* mice ($A^Y/a\ lep^{ob}/lep^{ob}$). (**A**) Weight change from baseline (day −2) in 3-month-old male (open symbols) and female (closed symbols) mice. Symbols for the different phenotypes are as in Fig. 1. Mice were given subcutaneous saline injections twice daily until day 0, and were then given leptin (2 mg/kg) twice daily. (**B**) Fasting serum insulin concentrations in female mice before and after leptin administration. (**C**) Fasting serum insulin concentrations in male mice before and after leptin administration. (**D**) Serum corticosterone concentrations before and after leptin administration (*26*). Data are reported as the mean ± SE. Weight change from baseline curves was compared within gender by two-way ANOVA. All curves were significantly different ($P < 0.005$) with the exception of female $A^Y/a\ lep^{ob}/lep^{ob}$ versus $a/a\ lep^{ob}/lep^{ob}$ and male A^Y/a +/+ versus a/a +/+ curves ($P > 0.01$). The effect of leptin on serum insulin and corticosterone concentrations was analyzed by the one-tailed *t* test (pre- versus postleptin administration: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). Postleptin serum insulin concentrations in the male $A^Y/a\ lep^{ob}/lep^{ob}$ mice were compared with those in $a/a\ lep^{ob}/lep^{ob}$ mice by the two-tailed *t* test ($P < 0.05$). The number of mice used in all experiments except male serum insulin is as follows: a/a +/+ ($n = 3$), A^Y/a +/+ ($n = 3$), $a/a\ lep^{ob}/lep^{ob}$ ($n = 3$), and $A^Y/a\ lep^{ob}/lep^{ob}$ ($n = 3$). The number of mice used in the male serum insulin experiment is as follows: a/a +/+ ($n = 6$), A^Y/a +/+ ($n = 6$), $a/a\ lep^{ob}/lep^{ob}$ ($n = 6$), and $A^Y/a\ lep^{ob}/lep^{ob}$ ($n = 6$).

leptin resistant as a consequence of desensitization to further leptin action. The absence of leptin in the lep^{ob}/lep^{ob} animal is responsible for the deregulation of two genes expressed in the arcuate nucleus: neuropeptide Y (*24*) and Agouti-related transcript, a newly described brain homolog of Agouti (*8*, *9*). The expression levels of both these genes in the A^y/a animal (*9*, *25*) further indicates a normal sensing of leptin in the arcuate nucleus of these mice. Given the apparent independence of the POMC and leptin pathways with regard to energy and insulin homeostasis, additional work will be required to determine which peripheral or central signals are dependent on the POMC neurons for their integration into the energy homeostasis equation.

REFERENCES AND NOTES

1. Y. Zhang *et al.*, *Nature* **372**, 425 (1994).
2. M. Maffei *et al.*, *Nature Med.* **1**, 1155 (1995).
3. G. Lee *et al.*, *Nature* **379**, 632 (1996); L. A. Tartaglia *et al.*, *Cell* **83**, 1263 (1995).
4. M. M. Dickie, *J. Hered.* **53**, 4 (1962).
5. M. W. Miller *et al.*, *Genes Dev.* **7**, 454 (1993); S. J. Bultman, E. J. Michaud, R. P. Woychik, *Cell* **71**, 1195 (1992).
6. D. Lu *et al.*, *Nature* **371**, 799 (1994).
7. W. Fan, B. A. Boston, R. A. Kesterson, V. J. Hruby, R. D. Cone, *ibid.* **384**, 165 (1997); D. Huszar *et al.*, *Cell* **88**, 131 (1997).
8. M. Graham, J. Shutter, U. Sarmiento, I. Sarosi, K. L. Stark, *Nature Genet.* **17**, 273 (1997).
9. M. M. Ollman *et al.*, *Science* **278**, 135 (1997).
10. J. L. Halaas *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 8878 (1997).
11. M. Van Heek *et al.*, *J. Clin. Invest.* **99**, 385 (1997).
12. J. Friedman, *Nature* **385**, 119 (1997); T. Gura, *Science* **275**, 751 (1997).
13. C57BL/6J *lethal yellow* (A^y/a) and *obese* gene ($lep^{ob}/+$) breeder pairs (Jackson Labs, Bar Harbor, ME) were cross-bred to create *lethal yellow obese* breeders (A^y/a:$lep^{ob}/+$). *Obese* gene carriers were identified by polymerase chain reaction (PCR) with genomic DNA followed by allele-specific oligonucleotide hybridization to identify the presence or absence of the lep^{ob} mutation. Four phenotypes were used in experiments: *lethal yellow obese* (A^y/a:lep^{ob}/lep^{ob}), *obese* (a/a:$lep^{ob}/+$ or $+/+$), *lethal yellow* (A^y/a:$lep^{ob}/+$ or $+/+$), and wild-type (a/a:$lep^{ob}/+$ or $+/+$). PCR primers were 5′-ATGAATTCAGGAAAATGTGCTGGAGACCCCTGT and 3′-CAGTCGGTATCCGCCAAGCAG. PCR products were denatured at room temperature for 30 min in 0.2 N NaOH, 15 mM tris-HCl (pH 7.5), and 3.75 mM EDTA, then blotted onto nylon membranes (Bio-Rad, Hercules, CA). Membranes were prehybridized at 50°C for 30 min in 5× standard saline citrate (SSC) and 0.1% SDS, then hybridized with labeled oligonucleotide probes specific for the lep^{ob} mutation or wild-type sequence for 1 hour. Membranes were washed in 2× SSC and 0.1% SDS at room temperature for 10 min followed by 3 M tetramethyl ammonium chloride, 50 mM tris-HCl (pH 8), and 0.2% SDS at 63°C for 15 min, then exposed to a PhosphorImager screen overnight.
14. Female mice were adrenalectomized at 6 weeks of age with bilateral flank incisions under anesthesia. Mice were given stress doses of dexamethasone in decreasing doses twice daily for 2 days after adrenalectomy and were then maintained on normal saline drinking water supplemented with corticosterone (1 mg/ml). Complete adrenalectomy was confirmed by monthly corticosterone radioimmunoassay (ImmuChem Double Antibody Corticosterone 125-I RIA Kit; ICN Biomedicals, Costa Mesa, CA) after exposure of the animals to stress (handling and tail clip). Mice were maintained on normal Purina Rodent Chow, given ad libitum, and weighed weekly beginning at 6 weeks.
15. G. L. Wolff, *Genetics* **48**, 1041 (1963); T. A. Plocher and T. L. Powley, *Metabolism* **25**, 593 (1976).
16. B. A. Boston, data not shown.
17. Blood was obtained by tail clip each month, starting at 3 months. Mice were fasted 4 hours before an afternoon blood draw. Glucose concentrations were measured by glucose meter (One Touch Profile; Lifescan, Milpitas, CA), and serum corticosterone and insulin concentrations were measured by radioimmunoassay (Rat Insulin RIA Kit; Linco Research, St. Charles, MO).
18. M. L. Klebig, J. E. Wilkinson, J. G. Geisler, R. P. Woychik, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 4728 (1995).
19. T. T. Yen, A. M. Gill, L. G. Frigeri, G. S. Barsh, G. L. Wolff, *FASEB J.* **8**, 479 (1994).
20. Mice were individually housed. Daily food intake and mouse weights were recorded. Mice were given twice daily subcutaneous injections of saline for 3 to 4 days. Saline was then replaced with recombinant human leptin (2.0 mg/kg). Insulin and corticosterone concentrations were measured on the first and last days of the experiment. Two sets of mice were used: 8-month-old adrenalectomized females and 3-month-old nonadrenalectomized males and females. His-tagged recombinant human leptin was expressed in *Escherichia coli* and purified from inclusion bodies by nickel affinity chromatography. Large-scale refolding was obtained with an Amicon Spiral-wound cartridge. The histidine tag was removed by thrombin cleavage and the leptin was further purified by ion-exchange chromatography. Two independent batches were used for the experiments shown in Figs. 2 and 3. Both batches were greater than 99% pure as assayed by SDS–polyacrylamide gel electrophoresis and contained 0.357 U per milligram of leptin and 1.21 U per milligram of leptin of endotoxin, respectively.
21. H. Shimizu, N. S. Shargill, G. A. Bray, *Am. J. Physiol.* **256**, 494 (1989).
22. C. C. Cheung, D. K. Clifton, R. A. Steiner, *Endocrinology* **138**, 4489 (1997).
23. R. J. Seely *et al.*, *Nature*, in press.
24. T. Stephens *et al.*, *ibid.* **377**, 530 (1995).
25. R. Kesterson, D. Huszar, C. Lynch, R. Simerly, R. Cone, *Mol. Endocrinol.* **11**, 630 (1997).
26. Corticosterone concentrations in nonadrenalectomized mice were obtained within 1 min of handling mice that had been individually housed in covered cages to reduce the effects of stress on corticosterone level.

27. We thank L. Van der Ploeg and M. Tota for recombinant human leptin and helpful discussions. Supported by NIH grants to R.D.C. (DK/AR517330) and B.A.B. (DK02404 and HD33703).

4 August 1997; accepted 27 October 1997