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Leptin Receptor Signaling in POMC Neurons Is Required for Normal Body Weight Homeostasis

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Summary

Neuroanatomical and electrophysiological studies have shown that hypothalamic POMC neurons are targets of the adipostatic hormone leptin. However, the physiological relevance of leptin signaling in these neurons has not yet been directly tested. Here, using the Cre/loxP system, we critically test the functional importance of leptin action on POMC neurons by deleting leptin receptors specifically from these cells in mice. Mice lacking leptin signaling in POMC neurons are mildly obese, hyperleptinemic, and have altered expression of hypothalamic neuropeptides. In summary, leptin receptors on POMC neurons are required but not solely responsible for leptin's regulation of body weight homeostasis.

Introduction

Mice carrying mutations either in the adipocyte-derived hormone leptin (*Lep*$^{ob/ob}$) or the leptin receptor (*Lepr*$^{db/db}$) genes have an array of abnormalities, including obesity, diabetes, infertility, impaired growth, high bone mass, and hypercorticosteronemia (Chen et al., 1996; Chua et al., 1996; Lee et al., 1996; Takeda et al., 2002; Tartaglia et al., 1995; Zhang et al., 1994). Leptin is secreted by adipocytes and is thought to act mainly on the central nervous system (CNS) (Spiegelman and Flier, 2001). Indeed, the deletion of all forms of the leptin receptor (LEPR) specifically in neurons leads to an obese phenotype (Cohen et al., 2001). Furthermore, transgenic complementation of the LEPR in neurons of *Lepr*$^{db/db}$ mice results in an amelioration of the obese phenotype (Kowalski et al., 2001). Additionally, central leptin administration into the cerebral ventricles (icv) reduces body weight and food intake in *Lep*$^{ob/ob}$ and normal mice (Campfield et al., 1995). In the brain, the signaling form of the leptin receptor (*Lepr-b*) is expressed in several sites. This includes dense expression in the arcuate nucleus (ARC) of the hypothalamus, which has been proposed as an important site for the regulation of energy balance (Cowley et al., 2003; Schwartz et al., 2003; Zigman and Elmquist, 2003). Indeed, ARC-specific lesions performed in rodents produce a profound obese phenotype (Bergen et al., 1998; Meister et al., 1989). Moreover, the ARC is required for leptin-induced anorexigenic responses, as icv leptin infusion in ARC-lesioned *Lep*$^{ob/ob}$ mice do not cause body weight reduction (Takeda et al., 2002).

The ARC contains orexigenic NPY/AGRP and anorexigenic CART/POMC neurons, which are direct leptin targets (Cowley et al., 2001; Elias et al., 1999; Elmquist et al., 1999). Leptin activates CART/POMC-expressing neurons, as demonstrated by electrophysiological recordings that show that leptin depolarizes (i.e., activates) POMC neurons (Cowley et al., 2001). Furthermore, fasted rodents (a condition of reduced leptinemia) and *Lep*$^{ob/ob}$ mice both have decreased hypothalamic *Pomc* mRNA content which can be normalized by exogenous leptin administration (Mizuno et al., 1998; Schwartz et al., 1997; Thornton et al., 1997). In contrast, leptin inhibits NPY/AGRP-expressing neurons; *Lep*$^{ob/ob}$ mice as well as fasted rodents have increased hypothalamic *Agrp* and *Npy* mRNA levels which can be reduced by exogenous leptin administration (Ahima et al., 1996; Schwartz et al., 1996; Stephens et al., 1995). However, the physiological relevance of leptin's direct action on CART/POMC or NPY/AGRP neurons has not yet been tested.

Although the importance of central and in particular ARC leptin signaling has been demonstrated, it is not clear which neuronal cell type or neurocircuits might be the principal mediators of leptin actions. Given that ARC lesions cause obesity, not leanness, and considering the well-established role of α-MSH (one of POMC's endoproteolytic products) and its downstream receptors in energy balance (Butler et al., 2001; Chen et al., 2000; Huszar et al., 1997; Michaud et al., 1994; Yaswen et al., 1999), POMC neurons have been proposed as the primary cell type for mediating leptin's anorexigenic effect (Cowley et al., 2001; Schwartz et al., 2003; Seeley et al., 1997). However, others argue that leptin's anorexigenic effects are not dependent on its regulation of α-MSH signaling (Boston et al., 1997; Challis et al., 2004; Marsh et al., 1999). In order to definitively test the hypothesis that LEPRs on POMC neurons are required to prevent excessive weight gain, we generated mice lacking LEPRs specifically on these cells. This animal model presents a unique opportunity to evaluate the specific role of leptin signaling in POMC neurons.

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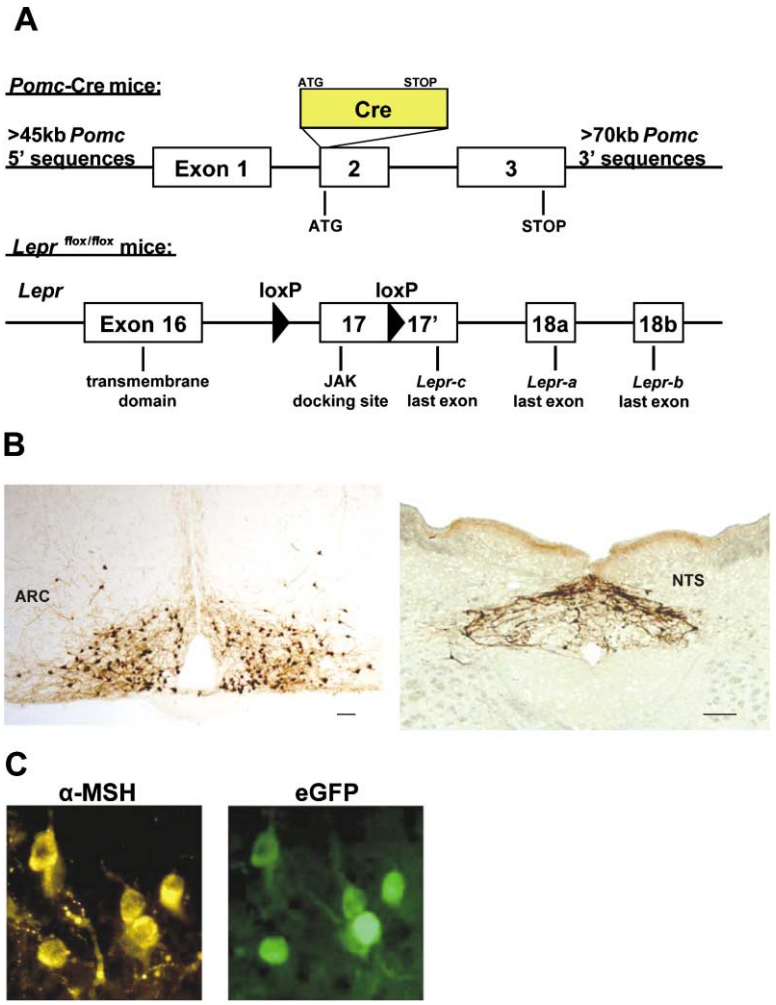

Figure 1. Generation of Mice Lacking Leptin Receptors on POMC Neurons

(A) Mice expressing Cre recombinase (Cre) under *Pomc* promoter control were generated by engineering a *Pomc* bacterial artificial chromosome. *Lepr*$^{flox/flox}$ mice are homozygous for a loxP-flanked exon 17, i.e., JAK docking site, of the leptin receptor allele.

(B) *Pomc*-Cre mice were mated with Z/EG reporter mice and immunohistochemistry for eGFP was performed in double transgenic mice. Scale bar, 50 μm.

(C) Double immunohistochemistry for α-MSH and eGFP was performed in *Pomc*-Cre, Z/EG mice.

Results

Transgenic mice expressing Cre recombinase (Cre) in POMC neurons were generated by engineering a *Pomc* bacterial artificial chromosome (BAC) such that Cre is driven by *Pomc* regulatory elements (Figure 1A). To assess whether functional Cre protein was restricted to areas known to contain POMC neurons, we crossed *Pomc*-Cre mice with Z/EG reporter mice [Tg(ACTB-Bgeo/GFP)21Lbe stock# 003920; www.jaxmice.jax.org], which express enhanced green fluorescent protein (eGFP) after Cre-mediated deletion of a loxP-flanked *lacZ* gene (Novak et al., 2000). Double transgenic mice expressed eGFP in the ARC and the nucleus of the solitary tract (NTS) in the hindbrain (Figure 1B). *Pomc* expression is reported to be limited to these two sites in the rodent brain (Bronstein et al., 1992; Elias et al., 1999). Scattered Cre activity was also noted in the dentate gyrus of the hippocampus (data not shown). In order to ascertain that all ARC POMC neurons expressed Cre recombinase, double immunohistochemistry analysis of α-MSH and eGFP were performed in double transgenic *Pomc*-Cre, Z/EG mice (Figure 1C). Quantification analysis indicated that >90% of α-MSH-positive neurons expressed eGFP, suggesting that the vast majority of POMC neurons in the ARC expressed functional Cre recombinase.

In order to generate mice lacking LEPR only on POMC neurons, mice homozygous for a loxP-modified *Lepr* allele (*Lepr*$^{flox/flox}$, Figure 1A) were used. Cre-mediated deletion of the loxP-modified *Lepr* allele is expected to recapitulate the spontaneous *db* mutation. *Lepr*$^{db/db}$ mice carry a point mutation which inserts a premature STOP codon five amino acids downstream of the *Lepr* exon 18b splice site. The resulting truncated LEPR-b protein lacks the STAT3 motif required for leptin receptor signaling (Bates et al., 2003). The Cre-deleted *Lepr* allele (*Lepr*$^{\Delta}$) is expected to also generate a truncated LEPR-b protein lacking both the STAT3 and also the JAK (encoded by the loxP-flanked exon 17, Figure 1A) motifs. Loss of the STAT3 motif is expected because, if exon 16 were to splice to exon 18b, exon 18b would be out of frame. Thus, the *Lepr*$^{\Delta}$ allele is expected to be null for *Lepr-b*. To assess whether the *Lepr*$^{\Delta}$ allele is indeed null for *Lepr-b*, we first generated mice that are homozygous for the Cre-deleted *Lepr* allele (*Lepr*$^{\Delta/\Delta}$ mice). This was achieved by taking advantage of Cre being sporadically expressed in the germline of *Pomc*-Cre, *Lepr*$^{flox/+}$ mice before the first meiotic division, thereby creating non-Cre-transgenic gametes bearing

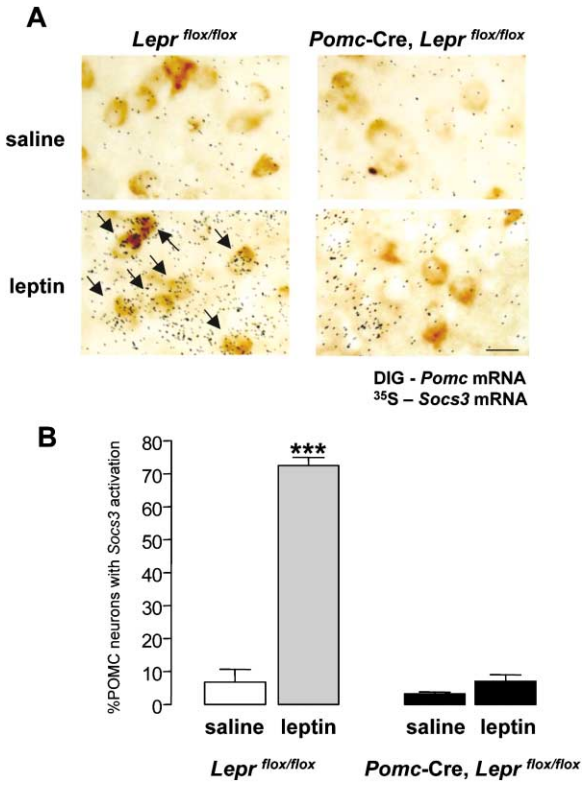

Figure 2. Leptin-Induced *Socs3* Activation Is Absent in POMC Neurons of *Pomc*-Cre, *Lepr*$^{flox/flox}$ Mice

(A) Representative in situ hybridization for *Pomc* (DIG, brown stain) and *Socs3* (^{35}S, silver grains) mRNA. Scale bar, 10 μm.

(B) *Socs3* mRNA induction was quantified by counting silver grains decorating DIG-labeled POMC neurons (n = 3–5, ***$p < 0.001$). Arrows denote neurons expressing both *Pomc* and *Socs3* mRNA.

a *Lepr*$^{\Delta}$ allele. This *Lepr*$^{\Delta}$ allele was then bred to homozygosity. *Lepr*$^{\Delta/\Delta}$ mice were markedly obese, and their body weight was identical to that of *Lepr*$^{db/db}$ mice on a similar genetic background (Figure 3C). Thus, because the phenotype of *Lepr*$^{\Delta/\Delta}$ mice is indistinguishable from that of *Lepr*$^{db/db}$ mice, the Cre-deleted *Lepr* allele must be null for *Lepr-b*.

Mice lacking leptin receptors only on POMC neurons were obtained by crossing *Pomc*-Cre mice with *Lepr*$^{flox/flox}$ mice. To validate the loss of functional leptin receptors exclusively on POMC neurons in mice that are *Pomc*-Cre transgenic and homozygous for the loxP-modified *Lepr* allele (*Pomc*-Cre, *Lepr*$^{flox/flox}$ mice), we performed double in situ hybridization of *Pomc* mRNA and leptin-induced *Socs3* mRNA. It has previously been shown that leptin binding to its receptor leads to induction of *Socs3* mRNA (Bjorbaek et al., 1998; Elias et al., 1999). Leptin induced *Socs3* mRNA in 70% of hypothalamic POMC neurons in *Lepr*$^{flox/flox}$ mice (Figure 2). However, leptin was unable to induce *Socs3* mRNA in hypothalamic POMC neurons of *Pomc*-Cre, *Lepr*$^{flox/flox}$ mice (Figure 2). In addition to POMC neurons, the ARC also contains NPY/AGRP and other leptin-responsive neurons. Importantly, leptin induced *Socs3* mRNA in POMC-negative neurons in both *Lepr*$^{flox/flox}$ and *Pomc*-Cre, *Lepr*$^{flox/flox}$ mice. Thus, *Pomc*-Cre, *Lepr*$^{flox/flox}$ mice lack functional leptin signaling only in POMC neurons.

Since leptin receptors have been shown to be critically involved in energy homeostasis, we explored the importance of LEPRs on POMC neurons in energy balance. As important negative controls, we noted that *Pomc*-Cre mice and *Lepr*$^{flox/flox}$ mice have body weights identical to wild-type littermates (data not shown). However, *Pomc*-Cre, *Lepr*$^{flox/flox}$ mice had significantly increased body weights (Figures 3A and 3B). We compared the weights of 10-week-old *Lepr*$^{flox/flox}$, *Lepr*$^{\Delta/\Delta}$ and *Pomc*-Cre, *Lepr*$^{flox/flox}$ mice and demonstrated that the increase in body weight in *Pomc*-Cre, *Lepr*$^{flox/flox}$ mice is 18.2% of that in *Lepr*$^{\Delta/\Delta}$ mice (Figure 3C). To rule out that the obesity phenotype in *Pomc*-Cre, *Lepr*$^{flox/flox}$ mice may have been the result of insertion site effects of the *Pomc*-Cre transgene, a second line of *Pomc*-Cre mice was crossed with *Lepr*$^{flox/flox}$ mice. A similar increase in body weight was observed in this second line of *Pomc*-Cre, *Lepr*$^{flox/flox}$ mice as was demonstrated for the first line (Figure 3D).

To determine the origin of increased body weight in *Pomc*-Cre, *Lepr*$^{flox/flox}$ mice, body composition was assessed using dual-energy X-ray absorptiometry (DEXA). As shown in Figure 4A, the increased body weight in mice lacking leptin signaling in POMC neurons arises mainly from an increase in fat mass. Indeed, dissection of distinct fat pads confirmed that these were at least twice as large as in *Lepr*$^{flox/flox}$ control mice (Figure 4B). Consistent with the increased fat pads, serum leptin levels were also increased (Figure 4C). These data demonstrate that the absence of leptin signaling in POMC neurons causes an impairment in energy balance which leads to an increase in body fat. *Lep*$^{ob/ob}$ and *Lepr*$^{db/db}$ mice have increased food intake and decreased energy expenditure (Pelleymounter et al., 1995). However, neither of these parameters were significantly altered in *Pomc*-Cre, *Lepr*$^{flox/flox}$ mice (Figures 4D and 4E).

Leptin action has been shown to alter hypothalamic neuropeptide expression levels. For example, both *Lep*$^{ob/ob}$ and *Lepr*$^{db/db}$ mice have decreased *Pomc* and increased *Agrp* and *Npy* mRNA (Ahima et al., 1996; Mizuno et al., 1998; Schwartz et al., 1996, 1997; Stephens et al., 1995; Thornton et al., 1997). Consistent with a direct action of LEPR signaling on *Pomc* gene expression, mice lacking LEPRs on POMC neurons had reduced hypothalamic *Pomc* mRNA content (Figure 5). In addition, hypothalamic *Agrp* mRNA was also significantly decreased, while *Npy* showed a similar trend as *Agrp* mRNA in adult *Pomc*-Cre, *Lepr*$^{flox/flox}$ mice (Figure 5). At first pass, a change in *Npy/Agrp* mRNA levels may be unexpected since NPY/AGRP neurons in *Pomc*-Cre, *Lepr*$^{flox/flox}$ mice still have functional LEPRs. Indeed, leptin was able to induce *Socs3* mRNA in ARC non-POMC neurons, which presumably included NPY/AGRP neurons. As mentioned previously, *Pomc*-Cre, *Lepr*$^{flox/flox}$ mice are significantly hyperleptinemic. We thus hypothesize that the increased adiposity in *Pomc*-Cre, *Lepr*$^{flox/flox}$ mice activates compensatory mechanisms, which may include increased inhibitory leptin action on NPY/AGRP neurons, thereby reducing *Npy/Agrp* expression. In support of this, hypothalamic neuropeptide levels were measured before the onset of obesity, and *Agrp* mRNA content was found to be normal (measured in 4-week-old females; *Lepr*$^{flox/flox}$ = 2.28 ± 0.44 *Agrp*/18S; *Pomc*-Cre, *Lepr*$^{flox/flox}$ = 2.42 ± 0.30 *Agrp*/18S; n =

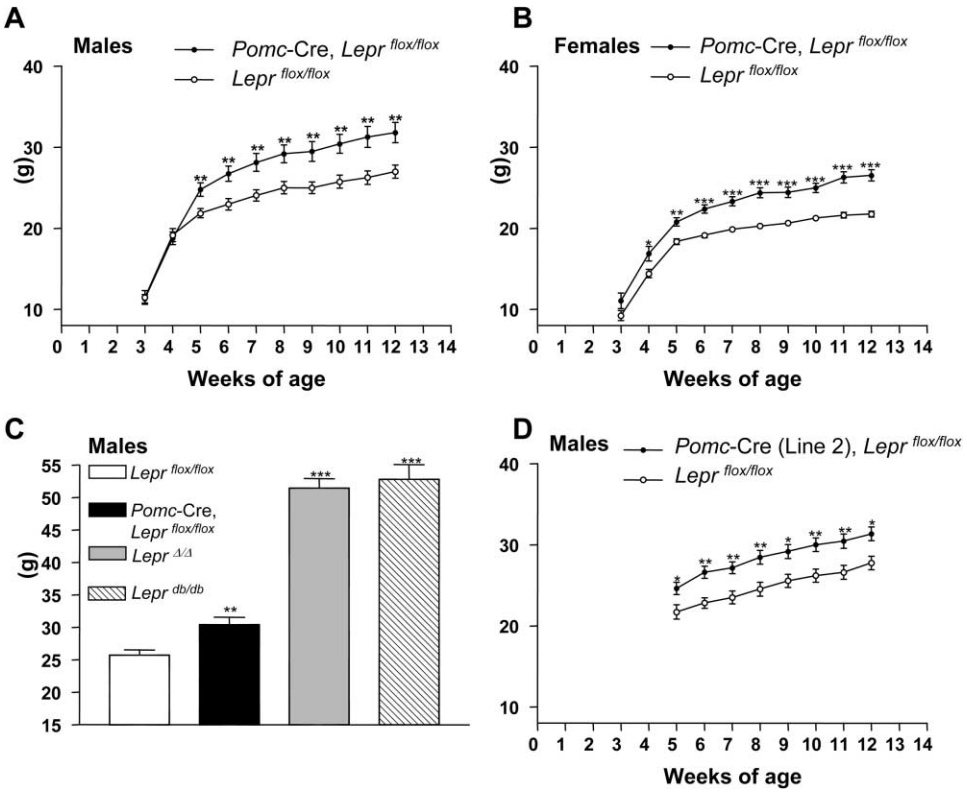

Figure 3. Body Weight of Mice Fed a Normal-Fat Diet: 12.5% Kcal from Fat

(A) Body weight curve of male *Lepr*$^{flox/flox}$ (○, n = 13) and *Pomc*-Cre, *Lepr*$^{flox/flox}$ (●, n = 11) mice from line 1.

(B) Body weight curve of female *Lepr*$^{flox/flox}$ (○, n = 13) and *Pomc*-Cre, *Lepr*$^{flox/flox}$ (●, n = 16) mice from line 1.

(C) Body weight of male *Lepr*$^{flox/flox}$ (n = 13), *Pomc*-Cre, *Lepr*$^{flox/flox}$ (n = 11), *Lepr*$^{\Delta/\Delta}$(n = 8), *Lepr*$^{db/db}$ (genetic background C57Bl6/J × FVB N2, n = 5) mice at 10 weeks of age from line 1.

(D) Body weight curve of male *Lepr*$^{flox/flox}$ (○, n = 8) and *Pomc*-Cre, *Lepr*$^{flox/flox}$ (●, n = 9) mice from line 2. (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ versus *Lepr*$^{flox/flox}$).

6). Importantly, *Pomc* mRNA was significanlty reduced even before the onset of obesity (measured in 4-week-old females; *Lepr*$^{flox/flox}$ = 2.85 ± 0.39 *Pomc*/18S; *Pomc*-Cre, *Lepr*$^{flox/flox}$ = 1.86 ± 0.15 *Pomc*/18S; n = 6, $p <$ 0.05). The reduced *Npy/Agrp* expression levels in adult *Pomc*-Cre, *Lepr*$^{flox/flox}$ mice could ultimately be limiting the degree of obesity.

In addition to regulating energy balance, leptin is also

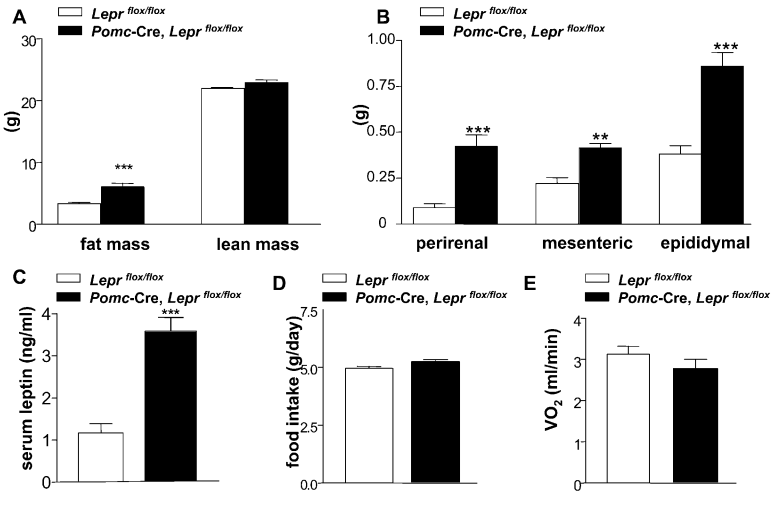

Figure 4. Body Composition, Serum Leptin Levels, Food Intake, and Oxygen Consumption in Mice Lacking LEPR on POMC Neurons

(A) Fat and lean mass in male 8- to 10-week-old *Lepr*$^{flox/flox}$ and *Pomc*-Cre, *Lepr*$^{flox/flox}$ mice were analyzed by DEXA measurement (n = 6–7).

(B) Distinct fat pads were dissected and weighed in male *Lepr*$^{flox/flox}$ and *Pomc*-Cre, *Lepr*$^{flox/flox}$ mice (n = 6–7).

(C) Serum leptin levels were assessed by ELISA in male 8- to 10-week-old *Lepr*$^{flox/flox}$ and *Pomc*-Cre, *Lepr*$^{flox/flox}$ mice (n = 6–7).

(D) Food intake was measured from week 3–14 in male *Lepr*$^{flox/flox}$ and *Pomc*-Cre, *Lepr*$^{flox/flox}$ mice (n = 10–12) and is presented here as food intake/day per mouse.

(E) Oxygen consumption was measured and averaged over a 48 hr time period in male 8- to 10-week-old *Lepr*$^{flox/flox}$ and *Pomc*-Cre, *Lepr*$^{flox/flox}$ mice (n = 8) using indirect calorimetry and is presented here as ml/min per mouse. (**$p < 0.01$; ***$p < 0.001$).

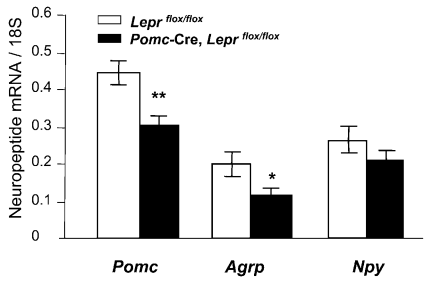

Figure 5. Hypothalamic Neuropeptide Expression Levels

Neuropeptide expression levels in female 8- to 10-week-old *Lepr*$^{flox/flox}$ and *Pomc*-Cre, *Lepr*$^{flox/flox}$ mice were measured in hypothalamic RNA extracts by TaqMan quantitative RT-PCR and normalized to 18S ribosomal RNA content. ($n = 8$, $^{**}p < 0.01$, $^{*}p < 0.05$).

known to affect fertility, bone mineral density, body length, glucose, insulin, corticosterone, and thyroxine (T4) serum levels (Ahima et al., 1996; Coleman, 1978, 1982; Takeda et al., 2002). Of note, none of these parameters were altered in *Pomc*-Cre, *Lepr*$^{flox/flox}$ mice. Glucose and insulin levels were normal, as shown in Table 1. Furthermore, *Pomc*-Cre, *Lepr*$^{flox/flox}$ are fertile and able to lactate. Bone mineral density is unaltered (measured by DEXA in 10-week-old males; *Lepr*$^{flox/flox}$ = 0.0513 ± 0.0006 g/cm^2; *Pomc*-Cre, *Lepr*$^{flox/flox}$ = 0.0506 ± 0.0014 g/cm^2; n = 6–7). *Pomc*-Cre, *Lepr*$^{flox/flox}$ mice have the same body length as littermate controls (Table 1). Serum corticosterone and T4 levels were also normal (Table 1). These data demonstrate that LEPRs on POMC neurons are not required for the aforementioned effects of leptin and that these must be regulated by LEPRs on other cells.

Discussion

Leptin is secreted by adipocytes, and its level in the blood reflects the status of fat stores. Leptin's primary site of action is the brain, where it promotes decreased food intake and increased energy expenditure, hence reducing adiposity (Spiegelman and Flier, 2001). A key question in the field is the identity of first-order, leptin-responsive neurons that relay this leptin signal from the circulation to downstream neural circuits regulating energy balance. Numerous lines of evidence have led to a model in which NPY/AGRP neurons and CART/POMC neurons, both located in the arcuate nucleus of the hypothalamus, are critical first-order, leptin-responsive neurons (Cowley et al., 2003; Schwartz et al., 2003; Zigman and Elmquist, 2003). While supported by indirect evidence, the importance of these first-order neurons has not yet been directly tested. To determine whether LEPRs on POMC neurons are critical mediators of leptin's effects, we used the Cre/loxP system to delete *Lepr* from these neurons in mice.

Our data demonstrate that LEPRs on POMC neurons, a subpopulation of only ~3000 neurons in mice (Cowley et al., 2001), are required for normal body weight homeostasis. However, while an increase in body weight and body fat is clearly seen in *Pomc*-Cre, *Lepr*$^{flox/flox}$ mice, it is markedly smaller than that caused by complete deficiency of LEPRs (i.e., body weight increase of only 18% of that observed in mice with complete deficiency of LEPRs). This demonstrates that LEPRs on POMC neurons are not solely responsible for leptin's regulation of body weight homeostasis and that LEPRs on other neurons are also important. Indeed, as will be discussed later, leptin-sensitive neurons have been demonstrated in numerous hypothalamic (Elmquist et al., 1999) and extrahypothalamic CNS sites (Elmquist et al., 1998; Grill et al., 2002).

Given the established role of leptin in controlling POMC neuron activity and neuropeptide expression, as well as the important role of α-MSH and its downstream receptors in energy homeostasis, the degree of disregulation of energy balance seen in *Pomc*-Cre, *Lepr*$^{flox/flox}$ mice is less than expected. It is formally possible that the absence of a more dramatic obesity phenotype in these mice may be due to incomplete deletion of *Lepr* on POMC neurons. Thus, it is critical to demonstrate Cre expression in all POMC neurons and more importantly to validate the lack of functional LEPRs on all POMC neurons in *Pomc*-Cre, *Lepr*$^{flox/flox}$ mice. Double immuno-

Table 1. Body Length and Blood Composition of Mice Lacking LEPRs on POMC Neurons

	Lepr$^{flox/flox}$	*Pomc-Cre, Lepr*$^{flox/flox}$
Nose/anus length (cm)		
Males	10.12 ± 0.04 (12)	10.16 ± 0.07 (10)
Females	9.69 ± 0.08 (8)	9.86 ± 0.06 (9)
Femur length (mm)		
Males	14.3 ± 0.1 (10)	14.7 ± 0.2 (6)
Females	14.0 ± 0.5 (6)	14.2 ± 0.2 (10)
Insulin (ng/ml)		
Males	0.74 ± 0.15 (9)	1.49 ± 0.47 (8)
Females	0.69 ± 0.15 (5)	0.98 ± 0.18 (6)
Glucose (mg/dl)		
Males	165.21 ± 3.83 (14)	173.42 ± 12.06 (12)
Females	151.00 ± 4.82 (10)	148.12 ± 4.35 (16)
Corticosterone (ng/ml)		
Males	9.06 ± 1.77 (7)	6.86 ± 1.33 (6)
T4 (ng/dl)		
Males	2.08 ± 0.18 (6)	2.24 ± 0.07 (5)
Females	2.11 ± 0.26 (6)	2.07 ± 0.14 (6)

Mice at 8–10 weeks of age. All data represent the mean ± SEM. The number of mice per group is shown in parentheses.

histochemistry analysis of α-MSH and eGFP in double transgenic *Pomc*-Cre, Z/EG mice showed that >90% of ARC POMC neurons expressed eGFP, indicating that Cre was active in most if not all POMC neurons. To date, antibodies suitable for LEPR detection are not available, and we were unable to generate an in situ hybridization probe capable of detecting the "floxed" exon 17 sequence. Thus, to demonstrate the absence of functional LEPRs on POMC neurons, a well-characterized assay for testing direct leptin signaling, i.e., leptin's ability to induce *Socs3* mRNA, was chosen (Bjorbaek et al., 1998; Elias et al., 1999). Indeed, we demonstrated absence of LEPR signaling in POMC neurons of *Pomc*-Cre, *Lepr*$^{flox/flox}$ mice by showing that leptin was unable to significantly induce *Socs3* mRNA specifically in these neurons. However, although not statistically different from *Socs3* mRNA activation in saline control animals, we did observe 7% of *Socs3*-positive POMC neurons in *Pomc*-Cre, *Lepr*$^{flox/flox}$ mice. Whether these very few potentially leptin-responsive POMC neurons, if they do indeed exist, would be able to prevent a more drastic obese phenotype in *Pomc*-Cre, *Lepr*$^{flox/flox}$ mice is unknown but seems unlikely.

Alternatively, we suggest that the smaller than expected degree of obesity observed in mice lacking LEPRs on POMC neurons is related to one of the following two possibilities. Either leptin regulation of POMC neurons can occur indirectly, for example, through LEPRs on NPY/AGRP neurons and their collateral projections to POMC neurons, or leptin regulation of POMC neurons, either directly or indirectly, plays only a small role in mediating leptin's effects on energy homeostasis. With regard to indirect regulation of POMC neurons by leptin, it has been shown that NPY/AGRP neurons, which are themselves inhibited by leptin, synapse onto hypothalamic POMC neurons, inhibiting these POMC neurons by release of GABA and NPY (Cowley et al., 2001; Roseberry et al., 2004). With this in mind, it is of interest that adult obese *Pomc*-Cre, *Lepr*$^{flox/flox}$ mice have reduced *Npy*/*Agrp* mRNA content. This suggests that NPY/AGRP neurons in adult obese mice are receiving increased inhibitory signals, thus leading to disinhibition of POMC neurons.

As suggested above, it is also possible that leptin regulation of POMC neurons, either directly or indirectly, plays only a small role in controlling body weight. In contrast to this view, Seeley et al. (1997) suggested that MC4R signaling is important in mediating leptin's acute effects on food intake and body weight. However, others have shown that leptin's anorexigenic effects are independent of melanocortin receptor signaling (Boston et al., 1997; Challis et al., 2004; Marsh et al., 1999). These melanocortin receptor-independent actions of leptin could be mediated by additional factors released by POMC neurons, for example, the neuropeptide CART (Elias et al., 1998a) or the neurotransmitter glutamate (Collin et al., 2003), or could be mediated by other first-order, leptin-responsive neurons. Our study, in which we deleted leptin receptors from POMC neurons, strongly supports the latter possibility. These non-POMC, leptin-responsive neurons could be within the arcuate nucleus (NPY/AGRP neurons, for example) or could be located in a number of other hypothalamic nuclei, such as the dorsomedial, ventral medial, and premammilary nuclei. In addition, LEPRs are found in extrahypothalamic sites, including the brain stem (Elmquist et al., 1998). With respect to the latter, the nucleus of the solitary tract (NTS) has been suggested to be an important site of leptin's anorexigenic action (Grill et al., 2002). Additional studies will be required to evaluate the importance of these other candidate, first-order, leptin-responsive neurons.

As reviewed above, our study strongly suggests the existence of other first-order, leptin-responsive neurons that play an important role in controlling energy balance. Given this, the body weight curves of *Pomc*-Cre, *Lepr*$^{flox/flox}$ mice are worthy of further comment. The majority of the increase in body weight, in comparison to controls, occurs between the age of 4 and 6 weeks. After that, the rate of increase in weight, in comparison to controls, is greatly reduced. This pattern of weight gain is uncommon and suggests that the *Pomc*-Cre, *Lepr*$^{flox/flox}$ mice, after the age of 6 weeks, have reached a new set point which they are then able to defend. It is interesting to speculate whether this attenuation in weight gain after the age of 6 weeks is the result of compensatory mechanisms activated by the increased adiposity and their action on neurons, for example NPY/AGRP neurons, as supported by the neuropeptide mRNA data. These compensatory mechanisms may include hyperleptinemia.

The increased weight in *Pomc*-Cre, *Lepr*$^{flox/flox}$ mice is mainly due to increased fat mass, suggesting that LEPRs on POMC neurons are important regulators of body fat content. Indeed the melanocortins have been proposed as important regulators in lipid metabolism (Albarado et al., 2004; Richter and Schwandt, 1987). Neither food intake nor energy expenditure is statistically significantly affected by the loss of LEPRs on POMC neurons. Since the body weight increase in *Pomc*-Cre, *Lepr*$^{flox/flox}$ mice is small, any differences in food intake or energy expenditure would also be expected to be small and may thus be difficult to detect.

As mentioned earlier, leptin is known to control glucose homeostasis and reproductive function. *Pomc*-Cre, *Lepr*$^{flox/flox}$ mice are fertile and not diabetic. Consistent with this finding, both reproductive function and glucose homeostasis have previously been suggested to be regulated through NPY and not POMC pathways (Hohmann et al., 2000). For example improved diabetes and reproductive function were noted in *Lep*$^{ob/ob}$ mice lacking the *Npy* gene, and NPY infusion in rodents leads to reduced reproductive function (Catzeflis et al., 1993; Erickson et al., 1996). Furthermore, improved reproductive function and glycemic control were also observed in mice lacking leptin-mediated STAT3 activation, which has been attributed to their normal *Npy* expression levels (Bates et al., 2003), even in the presence of reduced hypothalamic *Pomc* mRNA contents. The generation of mice lacking LEPRs specifically on NPY neurons will critically test the hypothesis that reproductive function and glucose homeostasis are regulated through leptin-NPY pathways.

Leptin signaling has been shown to be a critical regulator of growth and bone formation (Ducy et al., 2000). Our data suggest that leptin signaling in POMC neurons is not involved in this process. Indeed, Takeda et al. (2002) suggested that melanocortin agonists are not ma-

jor regulators of bone formation, which is also consistent with arcuate MSG-induced damage not having an effect on leptin's antiosteogenic function (Takeda et al., 2002).

In summary, lack of leptin signaling only in POMC neurons leads to impaired energy homeostasis. However, given the established role of leptin in controlling POMC neurons, as well as the important role of α-MSH and its downstream receptors in energy homeostasis, the small increase in adiposity is less than expected. Thus, other sites of leptin action must also be important in leptin's regulation of energy homeostasis. Delivery of Cre either by stereotaxic injections of AAV-Cre or by transgenesis, using neuron-specific promoters in *Lepr*$^{flox/flox}$ mice, will be powerful tools for surveying the physiologically important neurocircuits mediating leptin's effects.

Experimental Procedures

Animal Care

Care of all mice was within institutional Institutional Animal Care and Use Committee (IACUC) guidelines, and all procedures were approved by the Beth Israel Deaconess Medical Center IACUCC. Mice were housed in groups of two to four at 22°C–24°C using a 14 hr light/10 hr dark cycle with chow food (Teklad F6 Rodent Diet 8664, 4.05 kcal/g, 3.3 kcal/g metabolizable energy, 12.5% kcal from fat, Harlan Teklad, Madison, WI, www.harlan.com) and water provided ad libitum. Body weight was measured once a week. For food intake studies, male mice were housed individually. Large, intact pellets of food were provided every 7 days in order to reduce spillage, and cages were changed every time that food weight was measured. Mice were killed by CO_2 narcosis.

Generation of *Pomc*-Cre BAC Transgenic Mice

The FRT-Kan-FRT cassette from the plasmid pSV-FLP (a generous gift from Dr. F. Stewart, Heidelberg, Germany) was amplified by PCR and cloned into pGEM-T-Easy (Promega, Madison, WI), generating the vector here called pGEM-FRT-Kan-FRT. The Cre gene was amplified by PCR from the plasmid pMC-Cre (a generous gift from Dr. K. Rajewsky, Boston, MA) using the following primer set: N09 5′-ATCGGGCCCATGCCCAAGAAGAAGAGGAAG-3′ and N10 5′-ATC GTCGACTCGACAATTCCAACCTTACCCA-3′. The Cre amplicon was then cloned into pTOPO (Invitrogen, Carlsbad, CA), and the vector here called pTOPO-Cre was obtained. pTOPO-Cre was cut with ApaI, the Cre-containing fragment was then cloned into the ApaI site of pGEM-FRT-Kan-FRT, and the vector here called pGEM-Cre-FRT-Kan-FRT was generated. A mouse genomic bacterial artificial chromosome DNA library was screened for the *Pomc* gene (Invitrogen). DNA from the BAC clone containing at least 45 kb of 5′ and 70 kb of 3′ *Pomc* flanking sequences was transformed into the recombinogenic EL250 bacteria cells (Lee et al., 2001), and homologous recombination was performed as described by Lee et. al. The Cre-FRT-Kan-FRT cassette from the plasmid pGEM-Cre-FRT-Kan-FRT was amplified by PCR using the following primer set: N15 5′-GGCACTGGCTGCTCTCCAGGCACCAGCTCCACACATCTA TGGAGGTCTGAAGCAGGAGGGCCAGCAACAGGCGGTGGCGGC CGCTTAGTT-3′ and N18 5′-TCTGCTCCTTGCAGGGGTCCCTCCAA TCTTGTTTGCCTCTGCAGAGACTAGGCCTGACACGTGGAAGATG CCCAAGAAGAAGAGGAAGGTGTC-3′. Use of these primers inserts the Cre ATG exactly into the *Pomc* ATG and deletes the first 30 bp of the *Pomc* gene. *Pomc* BAC host EL250 cells were made electrocompetent, and the homologous recombinases were induced according to published protocols (Lee et al., 2001). The Cre-FRT-Kan-FRT cassette was then transformed into the *Pomc* BAC host EL250 cells and recombined. *Pomc*-Cre-FRT-Kan-FRT BAC host EL250 clones were identified by PCR screening. The FRT-Kan-FRT cassette was removed according to published protocols (Lee et al., 2001), and a *Pomc*-Cre BAC host EL250 clone without mutation in the Cre coding sequence was obtained. The loxP site present in the vector sequence of the *Pomc*-Cre BAC was removed as described by Lee et al. The *Pomc*-Cre BAC DNA was prepared using a commercially available kit (Quiagen, Valencia, CA) and microinjected circular into pronuclei of fertilized one-cell stage embryos of FVB mice (Jackson Laboratories) using standard methods (Hogan et al., 1986). Ten founders were obtained. Genotyping of *Pomc*-Cre transgenic mice was performed by PCR. Endogenous *Pomc* sequences were amplified with two primers (1, 5′-TGG CTC AAT GTC CTT CCT GG; 2, 5′-CAC ATA AGC TGC ATC GTT AAG), while a third primer (3, 5′-GAG ATA TCT TTA ACC CTG ATC) in combination with (1) generated a transgene-specific amplicon.

Generation of *Pomc*-Cre, *Lepr*$^{flox/flox}$ Mice

Pomc-Cre mice were mated with *Lepr*$^{flox/flox}$ (129-C57Bl6/J × FVB N2) mice (supplied by S. Chua Jr., New York), and a breeding colony was maintained by mating *Lepr*$^{flox/flox}$ and *Pomc*-Cre, *Lepr*$^{flox/flox}$ mice. Only animals from the same mixed background strain generation were compared to each other. *Lepr*$^{flox/flox}$ animals were genotyped by PCR with primers crossing the loxP site: (4, 5′-AAT GAA AAA GTT GTT TTG GGA CGA, and 5, 5′-CAG GCT TGA GAA CAT GAA CAC AAC AAC).

Generation of *Lepr*$^{\Delta/\Delta}$ Mice

Pomc-Cre, *Lepr*$^{flox/+}$ mice were mated with *Lepr*$^{flox/+}$ mice, and *Lepr*$^{\Delta/+}$ mice were obtained sporadically through expression of Cre during gametogenesis, before the first meiotic division. *Lepr*$^{\Delta/+}$ were mated with *Lepr*$^{\Delta/+}$ and *Lepr*$^{\Delta/\Delta}$ were obtained. *Lepr*$^{\Delta/\Delta}$ were genotyped by PCR across the floxed exon 17 using primer 4 and 6, 5′-CTG ATT TGA TAG ATG GTC TTG AG).

Generation of *Lepr*$^{db/db}$ Mice

Lepr$^{db/db}$ (C57Bl6/J) mice were purchased from Jackson Laboratories (stock# 00697) and mated with FVB mice for two generations.

α-MSH and eGFP Immunohistochemistry

Pomc-Cre mice were mated with Z/EG reporter mice (Jackson Labs, stock# 003920 [Novak et al., 2000]). *Pomc*-Cre, Z/EG double transgenic mice were perfused with 10% formalin, the brains sectioned on a microtome, and eGFP immunohistochemistry was performed as previously described (Liu et al., 2003). *Pomc*-Cre, Z/EG double transgenic mice used for eGFP and α-MSH double staining were colchicine treated and immunohistochemistry performed as described previously (Elias et al., 1998b). eGFP and α-MSH double labeled neurons were counted in three arcuate nucleus sections per mouse (n = 2).

Socs3 Induction in POMC Neurons

Fed male 10-week-old *Lepr*$^{flox/flox}$ and *Pomc*-Cre, *Lepr*$^{flox/flox}$ mice were injected ip with 100 μg recombinant mouse leptin (A.F. Parlow, National Hormone and Peptide Program) and perfused with 10% formalin 45 min later. In situ hybridization for *Pomc* (DIG) and *Socs3* (^{35}S) mRNA was performed on microtome cut 25 μm brain sections as described earlier (Elias et al., 1999). As described previously, silver grains on POMC neurons were counted in three arcuate nucleus sections per animal (n = 3–5 per group), and neurons were deemed responsive if silver grain counts were two times above background (Elias et al., 1999).

Body and Blood Composition

Mice at 8–10 week of age and fed ad libitum were either ketamine anesthetized for dual-energy X-ray absorptiometry (MEC Lunar Corp., Minster, OH) analysis or sacrificed and exsanguinated. Serum was collected by centrifugation and assayed for leptin (Crystal Chem. Inc., Downers Grove, IL), insulin (Crystal Chem. Inc.), corticosterone (ICN Biomedicals, Inc., Costa Mesa, CA), and T4 (Diagnostic Products Corporation, Los Angeles, CA) levels using commercially available kits. Tail vein blood was assayed for glucose levels before the sacrifice (Fisher Scientific, Morris Plains, NJ). The femur length was measured using the DEXA image's printout.

Oxygen Consumption

Oxygen consumption was measured by indirect calorimetry. Mice were placed at room temperature (22°C–24°C) in 1.0 l chambers in an OXYMAX system 4.93 (Columbus Instruments, Columbus, OH) with a settling time of 100 s and a measuring time of 50 s with room

air as the reference. Food and water were provided ad libitum. Mice were acclimatized in the chambers for 48 hr. Then oxygen consumption was measured for 48 hr, and the average VO_2 is presented as ml/min.

Hypothalamic Neuropeptide Expression

Neuropeptide mRNA was analyzed using quantitative PCR. RNA was extracted from hypothalamic blocks using the Trizol Reagent (Invitrogen, Life Technologies, Carlsbad, CA). Hypothalamic RNA was reverse transcribed with RETROscript (Ambion, Inc., Austin, TX) and amplified using Stratagene Brilliant QPCR Core reactions (Stratagene, La Jolla, CA) with TaqMan probes and primers (Biosearch Technologies Inc., Novato, CA). Primer and probe sequences were as follows: POMC sense, 5′-GACACGTGGAAGATGCCGAG; antisense, 5′-CAGCGAGAGGTCGAGTTTGC; probe sequence, 5′-FAM-CAACCTGCTGGCTTGCATCCGG-TAMRA. AGRP sense, 5′-CTTTGGCGGAGGTGCTAGA; antisense, 5′-GGACTCGTGCAGCCTTACACA; probe sequence, 5′-FAM-TCCACAGAACCGCGAGTCTCGTTC-TAMRA. NPY sense, 5′-CACCAGACAGAGATATGGCAAGA; antisense, 5′-TTTCATTTCCCATCACCACATG; probe sequence, 5′-FAM-CAGAAAACGCCCCCAGAACAAGGC-TAMRA. Relative expression of neuropeptide mRNA was determined using standard curves based on hypothalamic cDNA, and samples were adjusted for total RNA content by 18S ribosomal RNA quantitative PCR (Applied Biosystems, Foster City, CA). Quantitative PCR was performed on an Mx4000 instrument (Stratagene). Assays were linear over five orders of magnitude.

Statistical Methods

Data sets were analyzed for statistical significance using PRISM (GraphPad, San Diego, CA) for a two-tailed unpaired Student's t test.

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