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Leptin Directly Activates SF1 Neurons in the VMH, and This Action by Leptin Is Required for Normal Body-Weight Homeostasis

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Summary

Leptin, an adipocyte-derived hormone, acts directly on the brain to control food intake and energy expenditure. An important question is the identity of first-order neurons initiating leptin's anti-obesity effects. A widely held view is that most, if not all, of leptin's effects are mediated by neurons located in the arcuate nucleus of the hypothalamus. However, leptin receptors (LEPRs) are expressed in other sites as well, including the ventromedial hypothalamus (VMH). The possible role of leptin acting in "nonarcuate" sites has largely been ignored. In the present study, we show that leptin depolarizes and increases the firing rate of steroidogenic factor-1 (SF1)-positive neurons in the VMH. We also show, by generating mice that lack LEPRs on SF1-positive neurons, that leptin action at this site plays an important role in reducing body weight and, of note, in resisting diet-induced obesity. These results reveal a critical role for leptin action on VMH neurons.

Introduction

Defects in leptin signaling cause severe obesity in rodents and humans. Mice lacking leptin (*Lep*$^{ob/ob}$ mice) or its receptor (*Lepr*$^{db/db}$ mice) are obese and diabetic (Bray and York, 1979; Coleman, 1978; Lee et al., 1996; Tartaglia et al., 1995; Zhang et al., 1994). It is well established that leptin acts via the brain to cause its weight reducing actions (Halaas et al., 1997). Central nervous system (CNS)-specific deletion of LEPRs results in marked obesity (Cohen et al., 2001), whereas transgenic, brain-specific reconstitution of LEPRs in leptin-receptor-deficient *Lepr*$^{db/db}$ mice ameliorates obesity (Kowalski et al., 2001). Leptin receptors (LEPRs) are abundantly expressed in several different sites within the hypothalamus, an area known to be important in regulating body weight. These sites include the arcuate (ARC), the dorsomedial nucleus of the hypothalamus (DMH), the lateral hypothalamic area (LHA), and the ventromedial hypothalamic nucleus (VMH) (Elmquist et al., 1998; Elmquist et al., 1997; Fei et al., 1997; Mercer et al., 1996; Schwartz et al., 1996). The relative importance of leptin action at these different sites is unknown.

Most models of leptin action in the brain give primary importance to neurons in the ARC. The ARC contains two populations of first-order, leptin-responsive neurons, specifically NPY/AGRP and POMC/CART neurons (Schwartz and Porte, 2005). NPY/AGRP neurons promote weight gain and are inhibited by leptin (Cowley et al., 2001; van den Top et al., 2004), whereas POMC/CART neurons promote weight loss and are activated by leptin (Cowley et al., 2001; Elias et al., 1999). The widely held view of the importance of the ARC is based largely on indirect evidence, the essence of which is summarized here: (1) NPY/AGRP neurons and POMC/CART neurons express LEPRs; (2) neuropeptides that can affect energy homeostasis are released by these neurons (orexigenic neuropeptides, NPY and AGRP, from NPY/AGRP neurons and the anorexigenic neuropeptide, αMSH, from POMC/CART neurons); (3) deficiency of leptin or LEPRs causes changes in neuropeptide gene expression consistent with observed effects on body weight; and (4) leptin has acute electrophysiologic effects on these neurons consistent with its effects on body weight (Bjorbaek and Kahn, 2004; Jobst et al., 2004; Kalra et al., 1999; Schwartz and Porte, 2005). Such compelling, indirect evidence supporting a role for leptin action in the ARC, in the relative absence of evidence implicating a role for leptin action in other sites, has led to the assumption that most, if not all, of leptin's important effects on energy homeostasis are mediated by actions on NPY/AGRP neurons and POMC/CART neurons in the arcuate nucleus of the hypothalamus.

Recently, we used Cre/lox conditional gene-knockout technology to generate mice lacking LEPRs on POMC neurons (Balthasar et al., 2004). Deletion of LEPRs on POMC neurons resulted in mild obesity. This is in stark contrast to the massive obesity observed in *Lepr*$^{db/db}$ mice, which lack LEPRs everywhere. From these results, we speculate that neurons in locations other than the ARC also contribute to the anti-obesity actions of leptin. One potentially important site for leptin action is the VMH. Evidence implicating this region includes the following: (1) destructive lesions of the VMH produce massive obesity (Hetherington and Ranson, 1940), although the specificity of such lesions has been called into question (Gold, 1973); (2) gene-knockout mice deficient in steroidogenic factor-1 (SF1), a transcription factor expressed in the VMH, have abnormal VMH development

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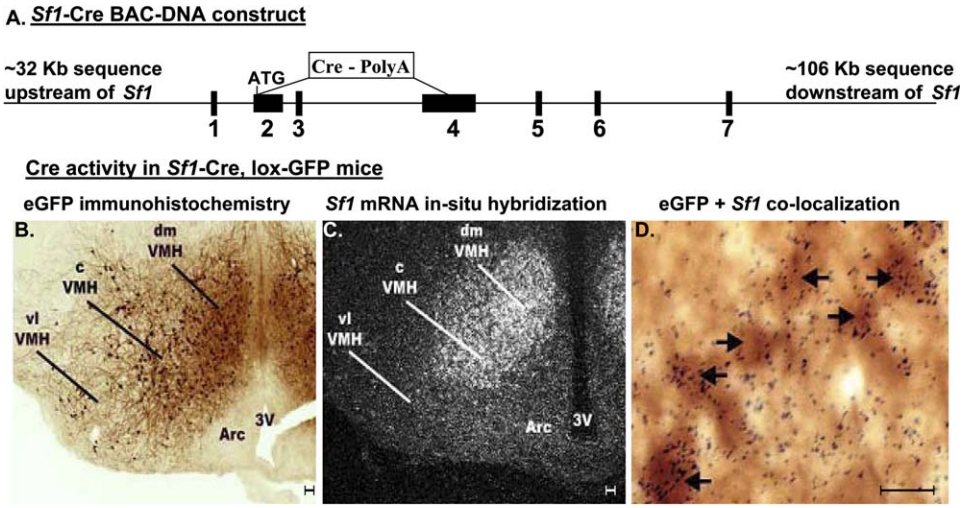


Figure 1. Generation and Validation of *Sf1-Cre* BAC-Transgenic Mice

(A) Mice expressing Cre recombinase under the control of the *Sf1* promoter were generated by inserting a Cre recombinase expression cassette into a BAC genomic clone containing *Sf1*.

(B) Immunohistochemical staining for GFP in an *Sf1-Cre*, lox-GFP mouse (*Sf1-Cre* mouse X Z/EG reporter mouse). Orange-brown stain represents Cre activity. Scale bar = 100 μm.

(C) In situ hybridization for endogenous *Sf1*-mRNA in a wild-type mouse with a ^{35}S-labeled riboprobe. Scale bar = 100 μm.

(D) Colocalization of Cre activity (as indicated by eGFP-IR [orange-brown stain]) with *Sf-1* mRNA (as indicated by overlying black silver granules) in an *Sf1-Cre*, lox-GFP reporter mouse. Arrows represent double labeled cells; scale bar = 10 μm.

(Dellovade et al., 2000) and are obese (Majdic et al., 2002); (3) the expression of brain-derived neurotrophic factor (BDNF), the deficiency of which causes obesity (Rios et al., 2001), is nutritionally regulated in the VMH (Xu et al., 2003); and (4) LEPRs are abundantly expressed in the VMH (Elmquist et al., 1998), and these LEPRs are readily engaged by peripheral leptin, as evidenced by induction of c-*fos* immunoreactivity and SOCS-3 mRNA (Bjorbaek et al., 1999; Elmquist et al., 1997). These findings raise the possibility that leptin action on VMH neurons plays an important role in regulating energy homeostasis. To test the hypothesis that LEPRs on SF1 neurons mediate important actions of leptin, we generated mice lacking LEPRs exclusively on SF1 neurons. Phenotypic and electrophysiologic analyses of these mice permit us to directly test the importance of leptin action on SF1 neurons.

Results

Generation and Characterization of *Sf1-Cre* Mice

Sf1-Cre transgenic mice expressing Cre recombinase (Cre) in SF1 neurons were generated by engineering an *Sf1* (approved mouse gene name, *Nr5a1*) bacterial artificial chromosome (BAC) such that Cre expression is driven by *Sf1* regulatory elements (Figure 1A). Several lines of *Sf1-Cre* transgenic mice were generated. To assess whether functional Cre protein was restricted to areas known to express SF1, we crossed all lines of *Sf1-Cre* mice with Z/EG Cre reporter mice (Tg [ACTBBgeo/GFP] 21Lbe stock# 003920; http://www.jaxmice.jax.org/). Z/EG Cre reporter mice express enhanced green fluorescent protein (eGFP) after Cre-mediated excision of a loxP-flanked *lacZ* gene (Novak et al., 2000). Lines of double-transgenic mice (henceforth referred to as *Sf1-Cre*, lox-GFP mice) were examined for eGFP expression in the CNS by immunohistochemical detection of GFP protein. Two lines (line 2 and 7) expressed Cre activity in a pattern mimicking that of *Sf1* mRNA expression (Figures 1B and 1C). In the case of line 2, Cre activity was also noted in the cerebral cortex and in a few scattered cells in the caudal brainstem. Notably, LEPRs are not expressed in these neurons; therefore, Cre expression in these sites will be without effect. Cre activity was also observed in the pituitary, gonads and adrenals, tissues known to express SF1 (data not shown).

Colocalization for Cre activity (immunohistochemical detection of GFP) and *Sf1 mRNA* expression (in situ hybridization) was performed to test whether Cre recombinase activity was restricted to SF1 neurons in the VMH (Figure 1D). A neuron was considered positive for Cre activity if the cell body and nucleus was clearly DAB labeled (orange-brown stain in Figure 1D). A GFP-positive neuron was considered "SF1 positive" if its cell body was decorated with at least ten black silver grains (3× above background). We identified 1000 VMH neurons that were GFP positive and scored these as either "SF1 positive" or "SF1 negative" in two series of brain sections from two *Sf1-Cre*, lox-GFP mice. Of these 1000 GFP-positive neurons, greater than 99% were SF1 positive. Thus, essentially all VMH neurons expressing Cre activity are SF1-positive neurons.

Generation of Mice Lacking Leptin Receptors in SF1 Neurons

We generated mice lacking LEPRs on SF1 neurons by first crossing mice homozygous for a loxP-modified *Lepr* allele (*Lepr*$^{flox/flox}$ mice) (Balthasar et al., 2004; McMinn et al., 2004) with *Sf1-Cre* mice. *Lepr*$^{flox/wt}$ offspring from this cross were then bred with *Sf1-Cre, Lepr*$^{flox/wt}$ littermates to generate *Sf1-Cre*, *Lepr*$^{flox/flox}$

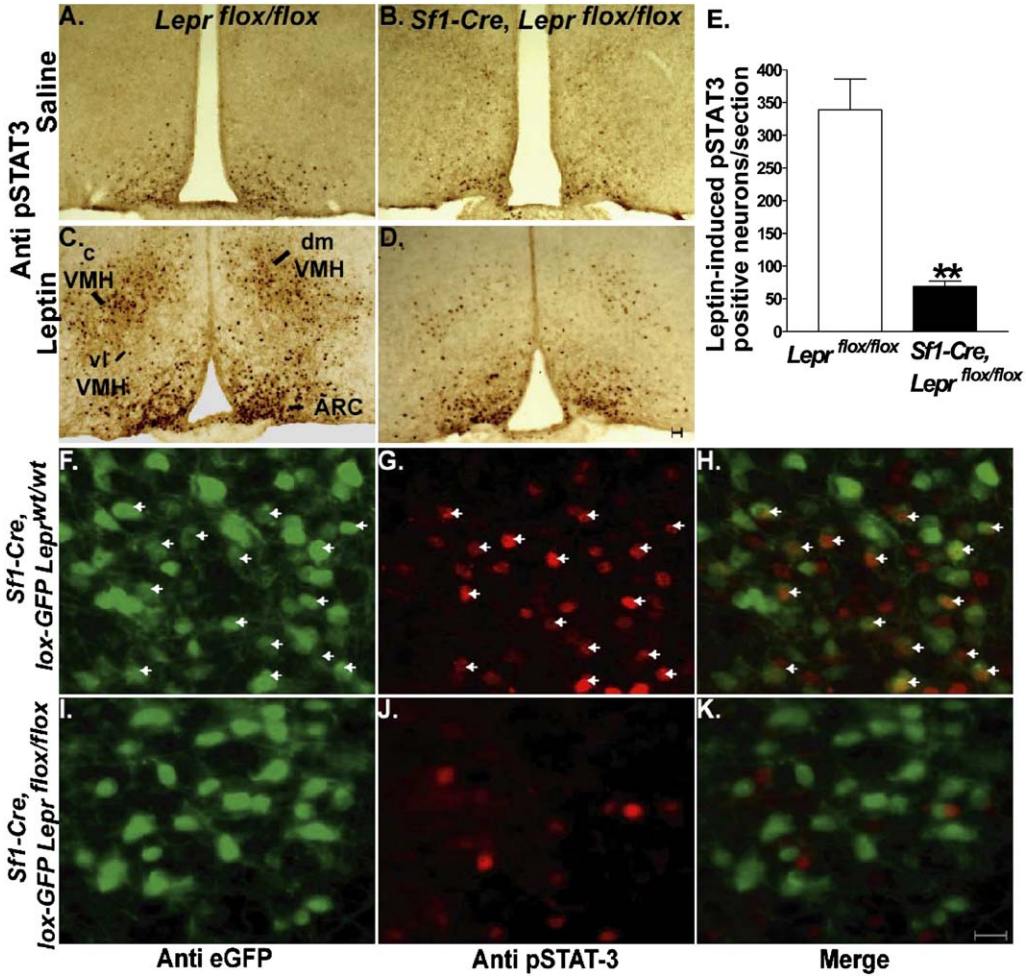


Figure 2. Leptin-Induced pSTAT3 Activation in $Lepr^{flox/flox}$ and *Sf1-Cre*, $Lepr^{flox/flox}$ Mice

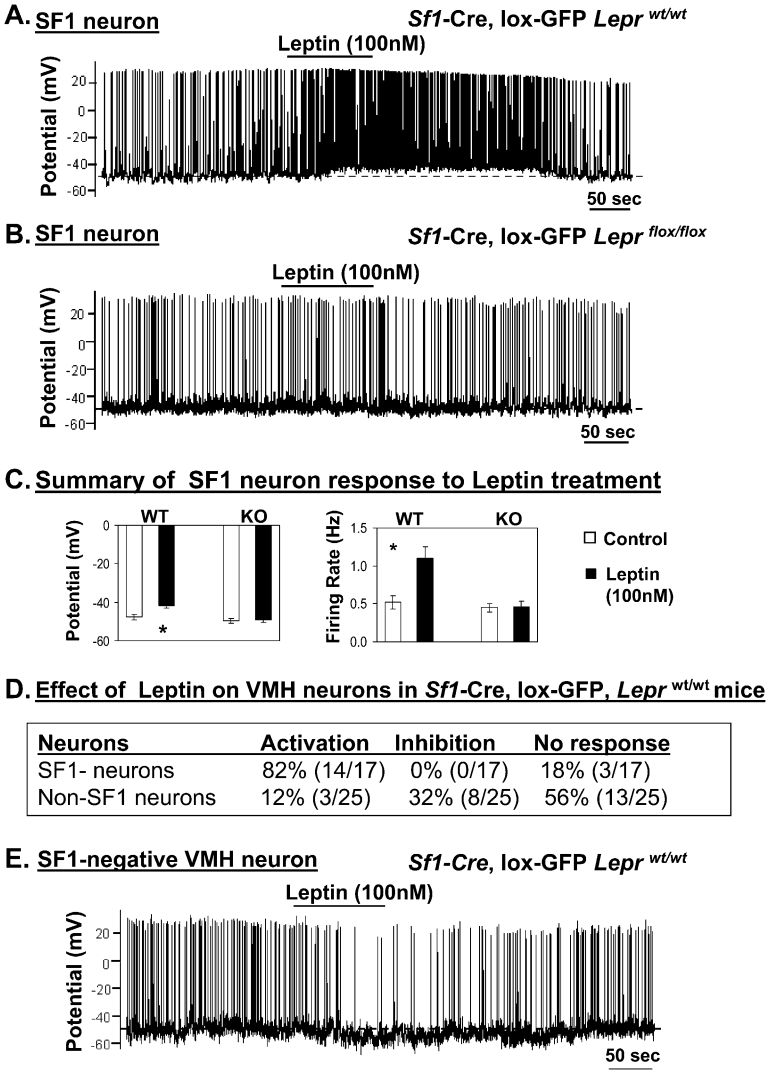
(A–D) pSTAT3 immunohistochemistry from representative saline-injected $Lepr^{flox/flox}$ and *Sf1-Cre*, $Lepr^{flox/flox}$ mice (A and B, respectively) and representative leptin-injected $Lepr^{flox/flox}$ and *Sf1-Cre*, $Lepr^{flox/flox}$ mice (C and D, respectively). Scale bar = 100 μm.

(E) The graph represents the number of pSTAT3-positive neurons in a single series of sections through the VMH. n = 3 per group; 2–4 VMH-containing sections were counted bilaterally for each animal. Data are represented as the average number of leptin-induced pSTAT3 positive cells per section. Double asterisk = $p < 0.01$ versus $Lepr^{flox/flox}$.

(F–K) Colocalization of leptin-induced pSTAT3 and GFP in the dorsomedial VMH (dmVMH) in an *Sf1-Cre*, lox-GFP $Lepr^{wt/wt}$ control and *Sf1-Cre*, lox-GFP $Lepr^{flox/flox}$ mouse. (F and I) SF1-positive neurons in the dmVMH in an *Sf1-Cre*, lox-GFP $Lepr^{wt/wt}$ control (F) and an *Sf1-Cre*, lox-GFP $Lepr^{flox/flox}$ mouse (I). (G and J) Leptin-induced pSTAT3-positive neurons in the dmVMH in an *Sf1-Cre*, lox-GFP $Lepr^{wt/wt}$ control (G) and an *Sf1-Cre*, lox-GFP $Lepr^{flox/flox}$ mouse (G). (H and K) Colocalization of leptin-induced pSTAT3 and GFP in the dmVMH in an *Sf1-Cre*, lox-GFP $Lepr^{wt/wt}$ control (H) and *Sf1-Cre*, lox-GFP $Lepr^{flox/flox}$ mouse (K). Scale bar = 20 μm. Green = GFP-positive neuron; red = pSTAT3-positive neuron; orange = GFP + pSTAT3 double-labeled neuron. Arrows in (F), (G), and (H) indicate the neurons, through all three views, that are doubly positive for both GFP and pSTAT3.

mice. The loxP modified *Lepr* allele was constructed such that loxP sequences flank exon 17 and 17′ of the *Lepr* allele. In the presence of Cre recombinase, a truncated nonfunctional LEPR-b protein is generated ($Lepr^{\Delta 17}$ allele). Indeed, mice homozygous for the deleted $Lepr^{\Delta 17}$ allele are phenotypically identical to $Lepr^{db/db}$ mice (Balthasar et al., 2004; McMinn et al., 2004).

To validate the loss of functional LEPRs exclusively on SF1 neurons in mice that are *Sf1-Cre* transgenic and homozygous for the loxP-modified *Lepr* allele (*Sf1-Cre*, $Lepr^{flox/flox}$ mice), we acutely injected leptin and performed immunohistochemistry (IHCC) for phosphorylated signal transducer and activator of transcription-3 (pSTAT3). Previous studies have shown that leptin binding to its receptor leads to induction of pSTAT3 in areas in the brain where LEPRs are present such as the hypothalamus and brainstem (Munzberg et al., 2003). Therefore, pSTAT3 induction can be used as a functional assay for leptin activated cells. A single intraperitonal injection of leptin induced the expected pattern of pSTAT3 activation in $Lepr^{flox/flox}$ mice, including prominent induction in the ARC, DMH, and the VMH (Figure 2C). Notably, leptin's ability to induce pSTAT3 in VMH neurons of *Sf1-Cre*, $Lepr^{flox/flox}$ mice was markedly reduced (Figure 2D). Quantification of VMH pSTAT3 leptin-responsive cells in $Lepr^{flox/flox}$ and *Sf1-Cre*, $Lepr^{flox/flox}$ mice revealed an ~80% reduction in the number of leptin-responsive cells in *Sf1-Cre*, $Lepr^{flox/flox}$ mice (Figure 2E).



D. Effect of Leptin on VMH neurons in *Sf1*-Cre, lox-GFP, *Lepr* wt/wt mice

Neurons	Activation	Inhibition	No response
SF1- neurons	82% (14/17)	0% (0/17)	18% (3/17)
Non-SF1 neurons	12% (3/25)	32% (8/25)	56% (13/25)

Figure 3. Electrophysiologic Recordings of VMH Neurons

(A) Leptin depolarized SF1 neurons and increased the frequency of action potentials in *Sf1-Cre*, lox-GFP, *Lepr*$^{wt/wt}$ mice. (A) shows a whole-cell recording trace representative of 14 out of 17 total recordings made on GFP-positive neurons from *Sf1-Cre*, lox-GFP, *Lepr*$^{wt/wt}$ mice. The bar above the trace indicates drug exposure time. The dashed line indicates the baseline membrane potential.

(B) Leptin did not alter membrane potential or firing rate of SF1 neurons from *Sf1-Cre*, lox-GFP, *Lepr*$^{flox/flox}$ mice. (B) shows a representative trace of 16 out of 16 total recordings made on GFP-positive neurons from *Sf1-Cre*, lox GFP, *Lepr*$^{flox/flox}$ mice.

(C) Statistical summary of leptin effects on *Sf1-Cre*, lox-GFP, *Lepr*$^{wt/wt}$ (wt) and *Sf1-Cre*, lox-GFP, *Lepr*$^{flox/flox}$ (KO) SF1 neurons. Data are represented as mean ± SEM. Leptin significantly induced membrane depolarization in wt (left, asterisk = $p < 0.0003$, n = 17), whereas no change was seen in KO neurons (n = 16). In addition, wt neurons had increased firing rate versus no change in KO cells (right, asterisk = $p < 0.003$, n = 17, wt, n = 16 KO).

(D) Table summarizing responses of SF1 and non-SF1 neurons to leptin in *Sf1-Cre*, lox-GFP, *Lepr*$^{wt/wt}$ mice. See Experimental Procedures for criteria used to define "Activation," "Inhibition," and "No response."

(E) Leptin hyperpolarized some GFP-negative VMH neurons. (E) shows a whole-cell recording trace representative of eight recordings made on GFP-negative neurons from *Sf1-Cre*, lox-GFP, *Lepr*$^{wt/wt}$ mice.

pSTAT3 induction by leptin persisted in some VMH neurons of *Sf1-Cre*, *Lepr*$^{flox/flox}$ mice. The majority of these neurons were located in the central subdivision of the VMH (cVMH) (Figure 2D) (Canteras et al., 1994). This is in contrast with the dorsomedial subdivision of the VMH (dmVMH), where nearly all pSTAT induction was lost in *Sf1-Cre*, *Lepr*$^{flox/flox}$ mice (Figure 2D). The persistence of pSTAT3 induction in some VMH neurons of *Sf1-Cre*, *Lepr*$^{flox/flox}$ mice could be explained by either incomplete inactivation of LEPRs on SF1-positive neurons or, alternatively, by expression of LEPRs on SF1-negative neurons. Unfortunately, antibodies suitable for SF1-protein detection in brain sections are unavailable. As an alternative approach toward addressing this issue, we performed double IHCC studies for Cre-dependent eGFP (used to indirectly detect SF1 neurons) and leptin-induced pSTAT3 in *Sf1-Cre*, lox-GFP, *Lepr*$^{wt/wt}$ mice and in *Sf1-Cre*, lox-GFP, *Lepr*$^{flox/flox}$ mice. As can be seen in "control" *Sf1-Cre*, lox-GFP, *Lepr*$^{wt/wt}$ mice (Figures 2F, 2G, and 2H), the majority of pSTAT3-positive neurons in the dmVMH express GFP (presumed to be SF1-positive neurons). On the other hand, in "LEPR-deleted" *Sf1-Cre*, lox-GFP, *Lepr*$^{flox/flox}$ mice (Figures 2I, 2J, and 2K), pSTAT3 induction in the dmVMH is greatly reduced, and the pSTAT3 that persists is located exclusively in neurons negative for GFP (presumed to be SF1-negative neurons). As in the dmVMH, persistent pSTAT3 in the cVMH of "LEPR-deleted" *Sf1-Cre*, lox-GFP, *Lepr*$^{flox/flox}$ mice was also found to be located exclusively in neurons negative for GFP (presumed to be SF1-negative neurons) (data not shown). These findings suggest that persistent pSTAT3 activity in *Sf1-Cre*, lox-GFP *Lepr*$^{flox/flox}$ mice is the result of leptin action on SF1-negative neurons. This interpretation of LEPRs being present on both SF1-positive and SF1-negative neurons in the VMH is strongly supported by our electrophysiological data (next section and Figure 3D), which demonstrate that most GFP-positive neurons (presumed to be SF1 positive) are activated by leptin (and never inhibited by leptin), whereas a significant proportion of GFP-negative neurons (presumed to be SF1 negative) are inhibited by leptin (32%).

Leptin Depolarizes and Increases the Firing Rate of SF1 Neurons

It has previously been shown in the ARC, that leptin activates POMC/CART neurons (depolarizes membrane potential and increases firing rate) and inhibits AGRP/

NPY neurons (hyperpolarizes membrane potential and decreases firing rate) (Cowley et al., 2001; van den Top et al., 2004). To assess the effects of leptin on SF1 neurons, we performed electrophysiologic recordings in the context of brain slices on GFP-labeled SF1 neurons from the dorsomedial subdivision of the VMH (dmVMH) of *Sf1-Cre*, lox-GFP, $Lepr^{wt/wt}$ mice. These GFP-labeled SF1 neurons are henceforth referred to as "SF1-positive" neurons. In whole-cell recordings from these neurons, we observed a resting membrane potential of −40 to −50 mV along with frequent and spontaneous action potentials. These SF1-positive neurons displayed membrane depolarization and increased frequency of action potentials in the presence of leptin (representative tracing shown in Figure 3A). This response was observed in 14 of 17 SF1-positive neurons. We compared the leptin responsiveness of SF1-positive neurons to that of POMC-positive neurons from POMC-GFP transgenic mice generated in our laboratory. We found that the responses of SF1-positive neurons to leptin were similar to those of POMC-positive neurons, i.e., both types of neurons were depolarized, and both had an increased firing rate when exposed to leptin (data for POMC-positive neurons not shown). Thus, leptin activates both SF1-positive and POMC-positive neurons in the hypothalamus.

We next assessed the electrophysiologic properties of SF1 neurons lacking LEPRs from *Sf1-Cre*, lox-GFP, $Lepr^{flox/flox}$ mice. Basal activity of these neurons was similar to that of SF1-positive neurons of control mice (resting membrane potential: −47.7 ± 1.4 mV, *Sf1-Cre*, lox-GFP, $Lepr^{wt/wt}$ versus −49.6 ± 1.3 mV *Sf1-Cre*, lox-GFP, $Lepr^{flox/flox}$; basal firing rate: 0.52 ± 0.09 Hz, *Sf1-Cre*, lox-GFP, $Lepr^{wt/wt}$ versus 0.45 ± 0.06 Hz, *Sf1-Cre*, lox-GFP, $Lepr^{flox/flox}$). In contrast to SF1-positive neurons from control mice, leptin had no effect on either membrane potential or firing rate of SF1 neurons from *Sf1-Cre*, lox-GFP, $Lepr^{flox/flox}$ mice (Figure 3B). In order to ensure that these neurons were viable, glutamate, an excitatory neurotransmitter, was applied to SF1-positive neurons of *Sf1-Cre*, lox-GFP, $Lepr^{flox/flox}$ mice. In all cases, glutamate depolarized and increased the firing rate of these SF1 neurons lacking LEPRs (data not shown). Leptin's effects on electrophysiologic properties of SF1 neurons with or without LEPRs are summarized in Figure 3C. Leptin depolarized (6.1 ± 1.0 mV) and increased the firing rate (2.12-fold ± 0.26-fold) of SF1 neurons in *Sf1-Cre*, lox-GFP, $Lepr^{wt/wt}$ mice. These responses were not observed in SF1 neurons from *Sf1-Cre*, lox-GFP, $Lepr^{flox/flox}$ mice, suggesting that SF1 neurons in the VMH are activated by leptin and that this activation is direct because it does not occur when LEPRs are deleted from SF1 neurons.

We next determined if neurons in the VMH, both SF1-positive and SF1-negative neurons, respond homogeneously to leptin. To accomplish this, using the same protocol as for SF1-positive neurons, we recorded from SF1-negative (i.e., GFP-negative) neurons in the same dorsomedial subdivision of the VMH (dmVMH) of *Sf1-Cre*, lox-GFP, $Lepr^{wt/wt}$ mice and compared these responses to those obtained from SF1-positive neurons. The SF1-negative neurons chosen for recording were those located in close proximity to SF1-positive neurons. In results summarized in Figure 3D, we observed that 56% of SF1-negative neurons were unresponsive to leptin (compare with 18% of SF1-positive neurons), 12% were activated by leptin (compare with 82% of SF1-positive neurons), and 32% were inhibited by leptin (compare with 0% of SF1-positive neurons). A representative trace showing inhibition by leptin is shown in Figure 3E. Thus, as is true for neurons in the ARC, neurons in the VMH are heterogeneous in their responses to leptin with SF1 neurons being activated by leptin and at least one population of SF1-negative neurons being inhibited by leptin.

Leptin Signaling in SF1 Neurons Is Required for Normal Energy Homeostasis

To determine the functional importance of LEPRs on SF1 neurons in body-weight regulation and to compare their relative importance to that of LEPRs on POMC neurons, we generated mice lacking LEPRs on SF1 neurons (*Sf1-Cre*, $Lepr^{flox/flox}$ mice) and mice lacking LEPRs on POMC neurons (*Pomc-Cre*, $Lepr^{flox/flox}$ mice). To rule out any potential nonspecific effects of the *Sf1-Cre* transgene by itself, or the insertion of loxP sites by themselves on body weight, we compared the body weights of *Sf1-Cre* mice and $Lepr^{flox/flox}$ mice to those of wild-type littermates. The body weights of the three control groups, i.e., the *Sf1-Cre,* $Lepr^{wt/wt}$ mice, $Lepr^{flox/flox}$ mice and wild-type mice were similar in males (Figure 4A). Further, two control groups of female mice, namely *Sf1-Cre,* $Lepr^{wt/wt}$ mice and $Lepr^{flox/flox}$ mice, were also similar (Figure 4B). Thus, neither the *Sf1-Cre* transgene nor the lox-modified LEPR allele, by themselves, have any effect on body weight.

Male and female *Sf1-Cre*, $Lepr^{flox/flox}$ mice had significantly increased body weights compared to $Lepr^{flox/flox}$ littermate controls (Figures 4A and 4B). At 20 weeks of age, *Sf1-Cre*, $Lepr^{flox/flox}$ mice had a 15% increase in body weight over that seen in $Lepr^{flox/flox}$ mice. This increase in body weight in *Sf1-Cre*, $Lepr^{flox/flox}$ mice was similar to that observed in *Pomc-Cre*, $Lepr^{flox/flox}$ mice (Figures 4A and 4B). The degree of obesity is approximately 20% of that seen in $Lepr^{\Delta/\Delta}$ mice (on a similar genetic background), which have complete deletion of LEPRs in all neurons (Balthasar et al., 2004; McMinn et al., 2004). To ensure that the obese phenotype in *Sf1-Cre*, $Lepr^{flox/flox}$ mice is specific to the loss of LEPRs on SF1 neurons and is unrelated to any effect caused by transgene insertion, we crossed a second line of *Sf1-Cre* mice (line 7) with $Lepr^{flox/flox}$ mice. A similar increase in body weight was observed in this second line of *Sf1-Cre*, $Lepr^{flox/flox}$ mice (Figure 4C). Thus, we conclude that LEPRs on SF1 neurons are required for normal body weight homeostasis and that leptin action on these neurons functions to restrain body weight gain.

To determine whether deficiency of LEPRs on these two sites (SF1 and POMC neurons) has nonadditive, additive, or synergistic effects on body weight gain, we further generated mice lacking LEPRs on both SF1 and POMC neurons (*Sf1-Cre*, *Pomc-Cre*, $Lepr^{flox/flox}$ mice). These mice were generated by crossing *Sf1-Cre*, $Lepr^{flox/flox}$ with *Pomc*-Cre, $Lepr^{flox/flox}$ mice. Littermates resulting from these crosses were used in studies described here. The double null mice, i.e., the *Sf1-Cre*, *Pomc-Cre*, $Lepr^{flox/flox}$ mice, weighed significantly more than either of the single-transgenic $Lepr^{flox/flox}$

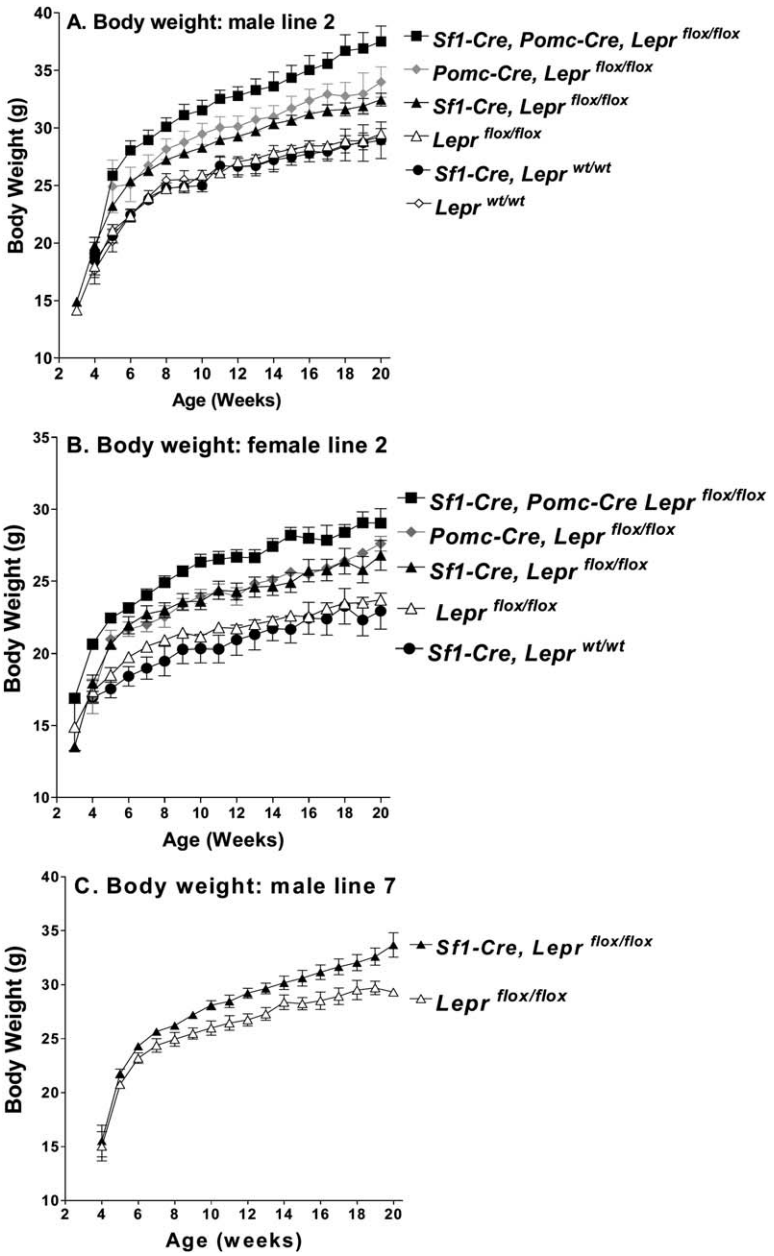


Figure 4. Effect of SF1 Neuron-Specific Deletion of LEPR on Body Weight

(A) Body weight from male mice, *SF1-Cre* (line 2).

(B) Body weight from female mice, *SF1-Cre* (line 2).

(C) Body weight from male mice, *SF1-Cre* (line 7) (data are represented as mean ± SEM, n = 10–12 per group).

mice. Loss of LEPRs on both SF1 and POMC neurons resulted in an additive effect on body weight (Figures 4A and 4B). The increased body weight with SF1 neuron-, POMC neuron-, or both SF1 and POMC neuron-specific deletion of LEPRS was not associated with any measurable increase in food intake (data not shown). This suggests that food intake is unaffected, or alternatively, that the effect is too small to be detected by the methods employed in this study.

Loss of Leptin Signaling in SF1 Neurons Results in Increased Fat Stores

To understand the effects of leptin receptor deletion in SF1 neurons on body composition, we performed dual-energy X-ray absorptiometry (DEXA) at 20 weeks of age. We also dissected and weighed individual fat pads in *Lepr*$^{flox/flox}$ and *Sf1-Cre*, *Lepr*$^{flox/flox}$ mice. As shown in Figures 5A and 5B, *Sf1-Cre*, *Lepr*$^{flox/flox}$ mice have an increase in fat mass. We did not observe any difference in lean mass across groups (data not shown). Dissection of three distinct fat pads (perigonadal, perirenal, and mesenteric) confirmed that the size of these individual fat stores had been increased in comparison to *Lepr*$^{flox/flox}$ control mice. The degree of adiposity in *Sf1-Cre*, *Lepr*$^{flox/flox}$ mice and *Pomc-Cre*, *Lepr*$^{flox/flox}$ mice were roughly similar. Consistent with the increased weight of the fat pads, serum leptin levels were also increased (Figure 5C).

Sf1-Cre, *Lepr*$^{flox/flox}$ Mice Have Normal Bone Mass

LEPRs in the VMH have previously been implicated in regulation of bone metabolism by leptin (Takeda et al., 2002). Thus, we assessed various bone parameters in *Sf1-Cre*, *Lepr*$^{flox/flox}$ mice. We did not observe any

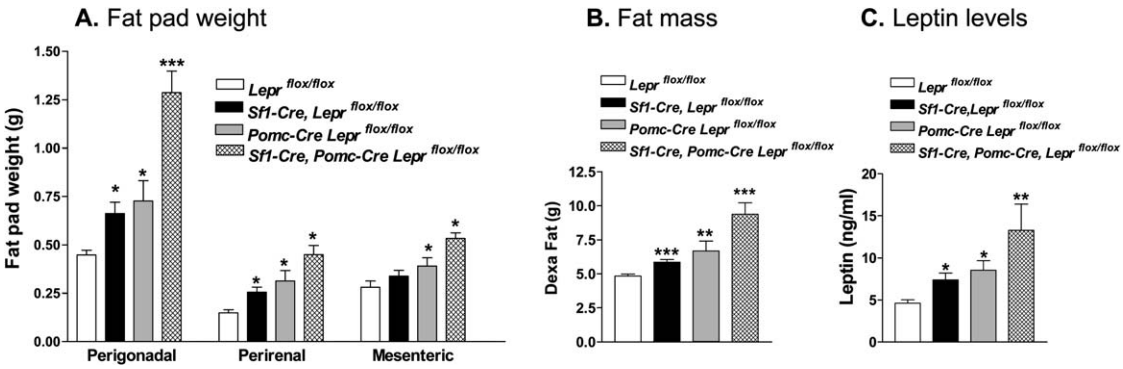


Figure 5. Effect of SF1 Neuron-Specific Deletion of LEPR on Fat Stores and Serum Leptin

(A) Fat pad weight.

(B) Body fat composition by DEXA analysis.

(C) Serum leptin levels. All data are represented as mean ± SEM, male mice, 20 weeks of age, n = 5–8 per group. Asterisk = $p < 0.05$ versus *Lepr*$^{flox/flox}$ controls; double asterisk = $p < 0.01$ versus *Lepr*$^{flox/flox}$ controls; triple asterisk = $p < 0.001$ versus *Lepr*$^{flox/flox}$ controls.

difference in either bone density, bone mineral content, total bone area, femur length, or whole-body length in mice lacking LEPRs on SF1 neurons or in mice lacking LEPRs on both SF1 and POMC neurons (Table 1). This indicates that neither SF1 neurons nor POMC neurons are involved in mediating the actions of leptin on bone mass.

Adrenal, Gonadal, and Gonadotroph Function Is Normal in *Sf1-Cre*, *Lepr*$^{flox/flox}$ Mice

In addition to the VMH, SF1 is expressed in the adrenal cortex, gonads, and gonadotrophs of the anterior pituitary (Stallings et al., 2002). To determine if gonadal or gonadotroph functions were adversely affected in *Sf1-Cre, Lepr*$^{flox/flox}$ mice, we systematically assessed fertility in male as well as in female mice. Male and female mice were housed together over extended periods as breeding pairs, with one member of the pair having the genotype of *Sf1-Cre*, *Lepr*$^{flox/flox}$ and the other member having the genotype of *Lepr*$^{flox/flox}$. The average time between litters was 22.28 ± 1.2 days for male *Sf1-Cre*, *Lepr*$^{flox/flox}$ mice and 22.00 ± 1.2 days for female *Sf1-Cre*, *Lepr*$^{flox/flox}$ mice. Because the gestation period for mice is 21 days, this indicates that male and female *Sf1-Cre*, *Lepr*$^{flox/flox}$ mice are able to produce pregnancy without any delay. Thus, their fertility is unimpaired. We also assessed seminal vesicle weights (an indicator of testosterone levels) in male *Sf1-Cre*, *Lepr*$^{flox/flox}$ mice and found no differences from control mice (Table 1). Thus, the body weight phenotype observed in *Sf1-Cre*, *Lepr*$^{flox/flox}$ mice is unlikely to be due to any abnormality in gonadal steroid function.

To assess the function of the hypothalamic-pituitary-adrenal axis (HPA axis), we measured serum corticosterone levels at two time points in *Lepr*$^{flox/flox}$ mice and *Sf1-Cre*, *Lepr*$^{flox/flox}$ mice. Corticosterone levels in *Sf1-Cre*, *Lepr*$^{flox/flox}$ mice exhibited normal diurnal variations and were not different from those observed in control mice (Table 1). These findings indicate that adrenal, gonadal, and gonadotroph function is unimpaired in *Sf1-Cre*, *Lepr*$^{flox/flox}$ mice. Therefore, the observed effects on body weight are due to deficiency of LEPRs on SF1 neurons in the VMH.

Leptin Signaling in SF1 Neurons Mediates Resistance to Diet-Induced Obesity

Rodents fed a high-fat diet become hyperleptinemic and modestly obese. To test the hypothesis that leptin action on SF1 neurons restrains further weight gain, we fed mice lacking LEPRs on SF1 neurons a high-fat, high-sucrose diet (HFD) from 4–20 weeks of age (Figure 6A). Control mice responded, as expected, by

Table 1. Corticosterone Levels, Gonad Weight, Bone Parameters in Male Mice

Parameter	*Lepr*$^{flox/flox}$	*Sf1-Cre*, *Lepr*$^{flox/flox}$	*Sf1-Cre*, *Pomc-Cre*, *Lepr*$^{flox/flox}$
Seminal vesicle weight (g) 5–8 weeks of age	0.1017 ± 0.02	0.1083 ± 0.015	0.1064 ± 0.013
Serum corticosterone 10–12 weeks of age (ng/ml)	16.9 ± 4.48 (AM) 109.8 ± 17.7 (PM)	11.50 ± 3.9 (AM) 114.50 ± 10.29 (PM)	16.7 ± 7.9 (AM) –
Bone-mineral density (20 weeks of age)	0.06152 ± 0.001	0.05926 ± 0.001	0.06194 ± 0.0015
Bone-mineral content (20 weeks of age)	0.0098 ± 0.0003	0.0096 ± 0.0002	0.0099 ± 0.0003
Total bone area (20 weeks of age)	0.158 ± 0.003	0.162 ± 0.002	0.160 ± 0.003
Femur length (20 weeks of age)	15.31 ± 0.08	15.43 ± 0.08	15.30 ± 0.15
Whole-body length (mm)	10.51 ± 0.069	10.58 ± 0.057	10.98 ± 0.025

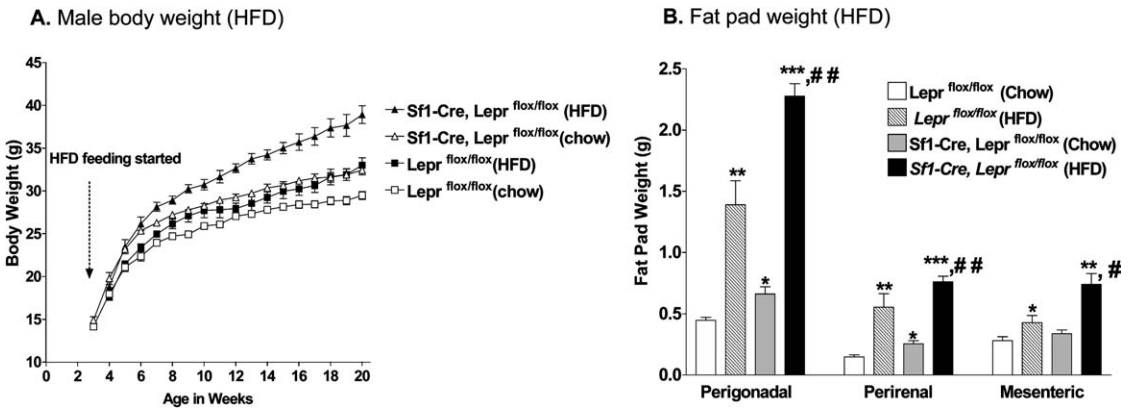


Figure 6. Effect of High-Fat Diet on Body Weight in Mice Lacking LEPRs on SF1 Neurons

(A) Effects of high-fat feeding on male mice lacking LEPRs in SF1 neurons.

(B) Fat pad weights. (Data are represented as mean ± SEM, male mice, 20 weeks of age, n = 6–7 per group, asterisk = $p < 0.05$ versus chow-fed *Lepr*$^{flox/flox}$ mice; double asterisk = $p < 0.01$ versus chow-fed *Lepr*$^{flox/flox}$ mice; triple asterisk = $p < 0.001$ versus chow-fed *Lepr*$^{flox/flox}$ mice; pound sign = $p < 0.05$ versus high-fat-diet [HFD]-fed *Lepr*$^{flox/flox}$ mice; double pound sign = $p < 0.01$ versus HFD-fed *Lepr*$^{flox/flox}$ mice).

gaining weight when fed the high-fat diet. The rate of weight gain, however, was small and decreased after sustained high-fat diet feeding. In contrast, *Sf1-Cre*, *Lepr*$^{flox/flox}$ mice rapidly gained weight when fed the high-fat diet and continued to do so throughout the study period. Furthermore, we observed that the combined effect of lack of LEPRs on SF1 neurons and high-fat diet feeding had a synergistic effect on body-weight gain in *Sf1-Cre*, *Lepr*$^{flox/flox}$ mice. The effect of high-fat feeding in mice lacking LEPRs on SF1 neurons was greater than the expected combined effect of either perturbation alone (Figure 6A). The weight gain by *Sf1-Cre*, *Lepr*$^{flox/flox}$ mice was reflected by increases in fat mass of all fat stores examined (Figure 6B).

To understand the mechanisms underlying the increased weight gain, we performed another study in which additional cohorts of mice were switched from chow to high-fat diet at 8 weeks of age. At this age (8 weeks old), there is an established difference in body weight between the control mice and the *Sf1-Cre*, *Lepr*$^{flox/flox}$ mice and, as mentioned earlier, we are unable to detect a difference in food intake. When mice were switched to high-fat diet, both *Lepr*$^{flox/flox}$ and *Sf1-Cre*, *Lepr*$^{flox/flox}$ mice responded by increasing body weight. However, *Sf1-Cre*, *Lepr*$^{flox/flox}$ mice gained substantially greater amounts of weight than did control mice (Figure 7A). When food intake was assessed, we observed that at the onset of high-fat diet, both control *Lepr*$^{flox/flox}$ mice and *Sf1-Cre*, *Lepr*$^{flox/flox}$ mice increased the amount of food consumed (Figure 7B). However, as the high-fat diet continued, food intake declined. Of note, the *Sf1-Cre*, *Lepr*$^{flox/flox}$ mice did not suppress food intake to the degree observed in *Lepr*$^{flox/flox}$ control mice. Thus, *Sf1-Cre*, *Lepr*$^{flox/flox}$ mice are deficient in normal adaptation to high-fat-diet feeding, which involves suppression of caloric intake.

Another feature of the adaptation to high-fat diet in rodents and mammals is an increase in energy

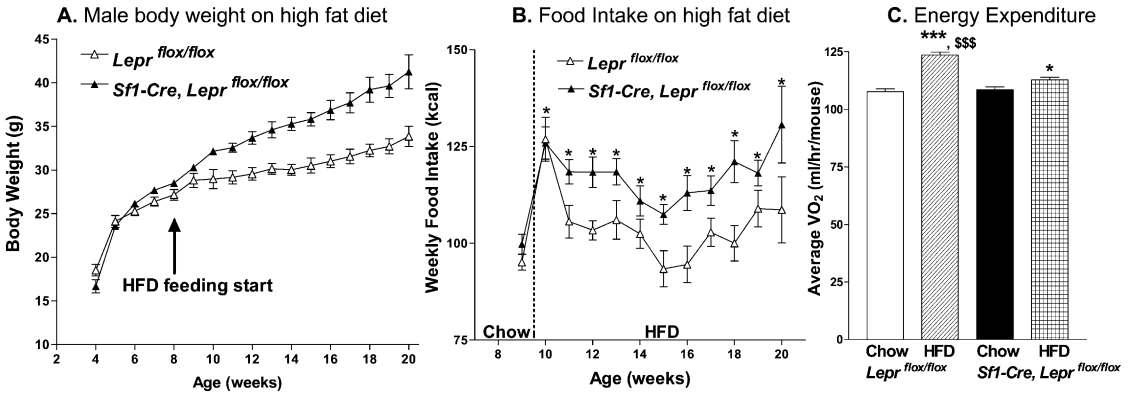


Figure 7. Impaired Response to High-Fat-Diet Feeding in Mice Lacking LEPRs in SF1 Neurons

(A) Effects of high-fat feeding on mice lacking LEPRs in SF1 neurons when switched to a high-fat diet (HFD) at 8 weeks of age.

(B) Effect of high-fat feeding on food intake in *Sf1-Cre, Lepr*$^{flox/flox}$ mice. (Data are represented as mean ± SEM, male mice, 20 weeks of age, n = 6–7 per group. Asterisk = $p < 0.05$ versus *Lepr*$^{flox/flox}$ HFD controls).

(C) Energy expenditure in *Sf1-Cre, Lepr*$^{flox/flox}$ mice on chow and HFD (data are represented as mean ± SEM male mice, n = 8 per group; triple asterisk = $p < 0.001$ versus chow-fed *Lepr*$^{flox/flox}$; triple dollar sign = $p < 0.001$ versus HFD-fed *Sf1-Cre, Lepr*$^{flox/flox}$; asterisk = $p < 0.05$ versus chow-fed *Sf1-Cre, Lepr*$^{flox/flox}$).

expenditure, a process referred to as diet-induced thermogenesis (Lowell and Spiegelman, 2000). To test whether the increased body weight in *Sf1-Cre*, *Lepr*$^{flox/flox}$ mice fed high-fat diet was associated with defective diet-induced thermogenesis, we assessed energy expenditure in mice for a period that spanned 3 days before and 3 days after the start of the high-fat feeding. Our data show that *Sf1-Cre*, *Lepr*$^{flox/flox}$ mice are markedly impaired in their ability to activate diet-induced thermogenesis in response to the high-fat diet (Figure 7C). Thus, diet-induced obesity in *Sf1-Cre*, *Lepr*$^{flox/flox}$ mice is caused by failure to adequately suppress food intake as well as failure to adequately stimulate energy expenditure. Lack of LEPRs on SF1 neurons impairs two important physiological adaptations to high-fat diet leading to a marked imbalance in energy homeostasis.

Discussion

Establishing the Functional Neuroanatomy of Body-Weight Control

In recent years, a number of proteins have been identified, such as leptin, the leptin receptor (LEPR), and the melancortin-4 receptor, that play key roles in the central regulation of energy homeostasis (Ahima et al., 2000; Cone, 2005; Cone et al., 2001; Elmquist, 2000; Heisler et al., 2003). Extensive neuroanatomical and electrophysiology studies have outlined neurocircuits that are hypothesized to be important in controlling energy homeostasis (Cone, 2005; Horvath, 2005; Schwartz and Porte, 2005; Seeley and Schwartz, 1999). The validity of these models, however, is largely untested, and this has been a major limitation for the field. To directly test the physiological significance of these models, we and others are now using cre/lox-mediated, neuron-specific gene manipulation. This methodology makes it possible to selectively perturb specific proposed neurocircuits and then assess consequences in awake, unrestrained mice. Approaches such as this, in conjunction with neuroanatomical techniques, will provide the means of establishing functional neurocircuitry responsible for maintaining normal body weight homeostasis.

It is well established that deficiency of leptin or its receptor, results in positive energy balance with concomitant obesity (Maffei et al., 1995; Zhang et al., 1994). Although it is known that the anti-obesity actions of leptin are mediated by the brain, the precise neural substrate responsible for this is unknown. Neuroanatomical studies have suggested that the majority of leptin's anti-obesity effects are mediated by LEPRs in the arcuate nucleus of the hypothalamus (Saper et al., 2002; Schwartz and Porte, 2005; Spiegelman and Flier, 2001). Although the arguments supporting this view are strong, they are based almost exclusively on indirect evidence reviewed in the introduction. As a direct test of the role of LEPRs on POMC neurons in the ARC, we recently generated mice that lack LEPRs on POMC neurons (Balthasar et al., 2004). These mice developed obesity; however, the magnitude of obesity was much less than that seen in mice lacking LEPRs everywhere, raising the possibility that LEPRs in other sites of the brain may also play an important role in controlling body weight.

Functional Importance of Leptin Action on SF1 Neurons in the VMH

One site of particular interest outside the ARC is the ventromedial hypothalamus (VMH). It was recently shown that steroidogenic factor-1 (SF1) gene knockout mice have abnormal VMH development and are obese, suggesting that SF1 neurons in the VMH play an important role in controlling body weight (Balthasar et al., 2004; Majdic et al., 2002). To directly test the hypothesis that LEPRs on VMH neurons contribute to the anti-obesity actions of leptin, we used cre/lox technology to generate mice lacking LEPRs on SF1 neurons (*Sf1-Cre*, *Lepr*$^{flox/flox}$ mice). Of note, SF1 neurons are located exclusively in the VMH (Stallings et al., 2002). *Sf1-Cre*, *Lepr*$^{flox/flox}$ mice develop mild obesity, which is similar in magnitude to mice lacking LEPRs on POMC neurons (*Pomc-Cre*, *Lepr*$^{flox/flox}$ mice). Importantly, mice lacking LEPRs on SF1 neurons are markedly sensitive to high-fat-diet-induced obesity. Taken together, these results demonstrate that leptin action on SF1 neurons in the VMH plays an important role in controlling body weight.

Interestingly, the degree of obesity seen in *Sf1-Cre*, *Lepr*$^{flox/flox}$ mice is similar to that seen in *Pomc-Cre*, *Lepr*$^{flox/flox}$ mice. Further, mice lacking LEPRs in both SF1 and POMC neurons have a body-weight phenotype that is approximately the sum of that observed with loss of LEPRs in ether neuronal group alone. Of note, the mutant mice investigated in these studies also exhibit increased leptin levels, which in turn would result in reduction of body weight through leptin-driven circuits that remain intact. Furthermore, it is also possible that compensatory changes could occur in feeding circuitry, permitting leptin signaling at some other site to bypass loss of LEPRs on either POMC or SF1 neurons. For these reasons, the relative importance of leptin signaling on a given population of neurons, as assessed by the degree of obesity observed in mice with POMC- or SF1-specific deletion of LEPRs, is likely to be an underestimate. Nevertheless, observation of a similar degree of obesity in the two models (*Pomc-Cre*, *Lepr*$^{flox/flox}$ mice and *Sf1-Cre*, *Lepr*$^{flox/flox}$ mice) suggests that leptin action on these two groups of neurons plays a quantitatively similar role in regulating body weight. The fact that mice with combined deficiency of LEPRs on both SF1 and POMC neurons are less obese than mice lacking LEPRs everywhere indicates that sites other than these are also important for leptin action in body-weight regulation. Possibilities include NPY/AGRP neurons in the ARC or LEPR-positive neurons in the DMH or brainstem (Elmquist, 2000). In total, these findings suggest that the ability of leptin to restrain body weight is distributed to a number of different sites in the brain.

Leptin Action on SF1 Neurons Promotes Resistance to Diet-Induced Obesity

It has recently been reported that high-fat feeding causes leptin resistance in neurons of the ARC but not in neurons outside of the ARC, including the VMH (Munzberg et al., 2004). If this were true, then it is predicted that leptin signaling in "non-ARC" sites would play an increasingly important role in restraining further weight gain during high-fat feeding. In our studies, we observed that the combined effects of LEPR deficiency on SF1 neurons and high-fat feeding are greater than

expected from the individual components added together. The magnitude of obesity seen in *Sf1-Cre*, *Lepr*$^{flox/flox}$ mice fed a high-fat diet is far greater than that seen in wild-type mice. This synergistic interaction strongly suggests that leptin action on SF1 neurons plays a particularly important role in resisting high-fat-diet-induced obesity. Of note, high-fat feeding of wild-type mice resulted in suppression of appetite and stimulation of energy expenditure. These adaptive responses were impaired in *Sf1-Cre*, *Lepr*$^{flox/flox}$ mice. Based upon these findings, we hypothesize that during high-fat feeding, increased leptin levels feed back on SF1 neurons, suppressing appetite and increasing energy expenditure, thus restricting further weight gain.

Leptin's Mechanism of Action in SF1 Neurons

The leptin-mediated, anti-obesity actions of SF1 neurons could be the result of long-term effects on gene expression secondary to activation of STAT3, rapid effects on membrane potential and firing rates with augmented release of neurotransmitters and/or neuropeptides, or the combined effects of both. Regarding the acute effects of leptin on membrane potential, we observed that acute addition of leptin to hypothalamic slices rapidly depolarized and increased the firing rate of SF1 neurons (82% activated by leptin, 0% inhibited by leptin, and 18% unresponsive to leptin). This effect was direct, i.e., mediated by LEPRs on SF1 neurons because it was absent in mice lacking LEPRs on these same neurons. Thus, SF1 neurons are similar to POMC neurons in that they are both activated by leptin and are distinguished from AGRP/NPY neurons in the ARC, which are inhibited by leptin (Cowley et al., 2001; van den Top et al., 2004). Of note, this activation response in SF1 neurons is not a general characteristic of VMH neurons but instead, is a relatively unique response of SF1-expressing cells. SF1-negative VMH neurons, by comparison, are heterogeneous in their response to leptin (12% activated by leptin, 32% inhibited by leptin, and 56% unresponsive to leptin).

The relevant anti-obesity signal emanating from SF1 neurons is presently unknown. Recent studies have raised the possibility of an important role for BDNF in the VMH (Xu et al., 2003). We suspect that BDNF is not the relevant mediator in SF1 neurons because mice lacking BDNF in SF1 neurons (*Sf1-Cre*, *Bdnf*$^{flox/flox}$ mice) failed to develop obesity (data not shown). This, of course, does not rule out a possible role for BDNF in "SF1-negative" neurons in the VMH, some of which may respond directly to leptin. Another candidate mediator, which could play an important role in SF1 neurons, is the excitatory neurotransmitter, glutamate. Based upon the heavy expression of VGLUT2 (glutamate synaptic vesicle transport protein) in the VMH and the relative absence of other neurotransmitter synaptic vesicle transport proteins in the VMH (Collin et al., 2003; Ziegler et al., 2002), it is likely that the majority of VMH neurons are glutamatergic. Of relevance, by using laser scanning photostimulation, it was recently shown that POMC neurons in the arcuate nucleus receive strong excitatory input from the medial VMH, a region likely to express SF1 (Sternson et al., 2005). To evaluate the anti-obesity role of glutamate release from SF1 neurons, we are presently generating mice that lack VGLUT2 in SF1 neurons (*Sf1-Cre*, v*Glut2*$^{flox/flox}$ mice). SF1 neurons from these mice will be unable to release glutamate.

In summary, this study demonstrates that SF1 neurons in the VMH are first-order, leptin-responsive neurons that mediate important anti-obesity effects of leptin. We argue that current models of leptin action will need to include an important role for SF1 neurons in the VMH, in addition to neurons in the arcuate nucleus. SF1 neurons are directly activated by leptin and this plays a particularly important role in resisting diet-induced obesity. Future work will be required to identify the relevant signaling factors released by SF1 neurons (glutamate versus neuropeptides) as well as the downstream neurons whose activity is controlled by leptin action on SF1 neurons.

Experimental Procedures

Animal Care

Care of all animals and procedures were approved by the Beth Israel Deaconess Medical Center Institutional animal care and use committee. Mice were housed at 22ºC–24ºC with a 14 hr light/10 hr dark cycle with standard mouse chow (Teklad F6 Rodent Diet 8664, 4.05 kcal/g, 3.3 kcal/g metabolizable energy, 12.5% kcal from fat, Harlan Teklad, Madison, WI) and water provided ad libitum. Body weight was measured once a week. HFD male mice were housed individually for all food intake measurements. All diets were provided as pellets, and chow was weighed once every 7 days. High-fat, high-sucrose diet (Research Diets, D-12331, 58% kcal from fat, 26% from sucrose, 5.557 kcal/gm) was refilled and weighed twice per week in order to reduce spillage. Mice were euthanized by CO_2 narcosis. Blood was obtained by cardiac puncture, and tissues were collected and stored at −80ºC until used for analysis.

Generation of *Sf1-Cre* BAC Transgenic Mice

SF1-Cre BAC transgenic mice were generated by engineering a BAC genomic clone containing Sf1 and inserting the Cre recombinase sequence into the translational site of SF1. For details see Supplemental Data section.

Generation of *Sf1-Cre*, *Lepr*$^{flox/flox}$ and *Sf1-Cre*, *Pomc-Cre*, *Lepr*$^{flox/flox}$ Mice

Sf1-Cre mice were mated with *Lepr*$^{flox/flox}$ (129-C57Bl6/J FVB N2) mice (supplied by S. Chua, Jr., Columbia University, New York), and a breeding colony was maintained by mating *Lepr*$^{flox/wt}$ and *Sf1-Cre*, *Lepr*$^{flox/wt}$ mice. Only littermates were used as study subjects. *Lepr*$^{flox/flox}$ animals were genotyped by PCR with primers spanning the loxP site: 5′-AAT GAA AAA GTT GTT TTG GGA CGA-3′, and 5′-CAG GCT TGA GAA CAT GAA CAC AAC AAC-3′. *Sf1-Cre*, *Pomc-Cre*, *Lepr*$^{flox/flox}$ were generated by mating *Sf1-Cre*, *Lepr*$^{flox/flox}$ mice with *Pomc-Cre, Lepr*$^{flox/flox}$ mice previously generated in our laboratory. Mice were genotyped for *Pomc-Cre* as described earlier (Balthasar et al. 2004). *Pomc-Cre*, *Lepr*$^{flox/flox}$ mice used in studies described in this paper were generated from the above described cross.

Electrophysiological Studies

Slice Preparation

Young adult mice (4–7 weeks old) were deeply anesthetized with Isoflurane prior to decapitation and removal of the entire brain, as previously described (Cowley et al., 2001; van den Top et al., 2004). Briefly, brains were removed and immediately submerged in ice-cold, carbogen-saturated (95% O_2 / 5% CO_2) aCSF (in mM: 2.5 NaCl, 126 KCl, 1.2 $MgCl_2$, 2.4 $CaCl_2$, 1.2 NaH_2PO_4, 21.4 $NaHCO_3$, 11.1 glucose). A brain block containing the hypothalamus was made. Coronal sections (200 μM) were cut with a Leica VT1000S Vibratome and then incubated in oxygenated aCSF at room temperature for at least 1 hr before recording.

Electrophysiological Recordings

Slices were transferred to the recording chamber and allowed to equilibrate for 10–20 min prior to recording. The slices were bathed in oxygenated aCSF heated to approximately 34ºC at a flow rate of approximately 2 ml/min. Under ultraviolet (450–480 nm) excitation, SF-1 neurons were clearly distinguished from adjacent, nonfluorescent neurons with an Axioskop FS2 Plus microscope (Zeiss) equipped with a fixed stage and a C2741-60 CCD camera (Hamamatsu). Whole-cell recordings were then made on identified neurons under IR-DIC optics by using a MultiClamp 700B Amplifier (Axon Instrument) with the software pClamp 9.0 (Axon Instrument). Recording electrodes had resistances of 2.5–4 MΩ when filled with the K-gluconate internal solution, which contained (in mM) K-gluconate 128, HEPEs 10, EGTA 1, KCl 10, $MgCl_2$ 1, $CaCl_2$ 0.3, Mg-ATP 5, and Na-GTP 0.3 (pH 7.35 with KOH). Leptin or other agents were applied to bath solution through perfusion. After acquisition of stable whole-cell recording for 2–5 min, aCSF solution containing 100 nM leptin was perfused for 60 to 150 s. This was followed by plain aCSF application for 5 to 15 min or until significant recovery in membrane activity was observed. In Figure 3, the bar above the recording trace indicates the duration of leptin application.

Baseline membrane potential and firing rate recorded by Clampex 9.2 were analyzed with MiniAnalysis Program (Synaptosoft, Inc.). For each recording, the firing rate (or baseline membrane potential) averaged from every 10 s was taken as one data point, 12 to 18 data points that represent stable activity levels were taken to calculate mean and standard deviation before and after application of leptin respectively. A neuron was considered activated or inhibited if the change (in membrane potential or firing rate) induced by leptin was ±3 times the standard deviation prior to leptin addition.

Tissue Collection for Histology Studies

Animals were deeply anesthetized with intraperitoneal injection of chloral hydrate (500 mg/kg for mice) and subsequently perfused transcardially with diethylpyrocarbonate (DEPC)-treated 0.9% phosphate buffered saline (PBS) followed by 10% neutral buffered formalin. Brains were removed, stored in the same fixative for 4–6 hr at 4ºC, immersed in 20% sucrose in DEPC-treated PBS (pH 7.0) at 4ºC overnight, and cut into 25 μm sections on a freezing microtome coronally into five equal series. The sections were placed at −20ºC in an antifreeze solution (Balthasar et al., 2004; Simmons et al., 1989) until further histological processing. A single series of sections per animal was used the histological studies.

Generation of *SF1* Riboprobe and SF1 In Situ Hybridization Histochemistry

An SF1 riboprobe was generated by PCR amplification of a 408 bp fragment of DNA complementary to a portion of exon 4 of mouse SF-1 from mouse brain cDNA (Clontech, Palo Alto, CA). This amplicon was subcloned into pcR4-TOPO vector (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol. The sequences and directionalities of the inserts were confirmed by DNA sequencing. To generate an antisense ^{35}S-labeled cRNA probe, the plasmid was linearized by restriction digestion and then subjected to in vitro transcription with either T3 or T7 RNA polymerases according to the manufacturer's protocol (Ambion, Inc., Austin, TX). Control sense riboprobes were similarly generated.

Single-label in situ hybridization histochemistry (ISHH) was performed as reported previously (Kishi et al., 2003; Marcus et al., 2001).

Data Analysis and Production of Photomicrographs for ISHH

ISHH patterns were visualized first on autoradiographic film and then by observing slides dipped in photographic emulsion for direct, cellular visualization. Histological detail was noted by examining adjacent brain sections stained for thionin. Brain sections were viewed with both a Zeiss Axioskop and a Zeiss Stemi 2000-C dissecting microscope with both brightfield and darkfield optics. Photomicrographs were produced with a Zeiss digital camera attached to the microscopes and a Dell desktop computer. Qualitative estimates of SF1 mRNA expression in specific brain sites were made by considering both signal strength and the number of labeled cells. An image editing software program, Adobe Photoshop 7.0, was used to combine the photomicrographs into plates.

Single-Label Immunohistochemistry for GFP

IHC was begun after first washing the ISHH-processed sections in PBS (pH 7.4). Sections were pretreated with 0.3% hydrogen peroxide in PBS (pH 7.4) for 30 min at room temperature and then were incubated in 3% normal donkey serum (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) with 0.25% Triton X-100 in PBS (PBT-azide) for 2 hr. Next, the slides were incubated overnight at room temperature in a rabbit anti-GFP primary antiserum (Molecular Probes, Cat # a-6455; 1:20,000 in PBT-azide). After washing in PBS, sections were incubated in either biotinylated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories; 1:1,000) for 2 hr at room temperature, followed by incubation for 1 hr in a solution of avidin-biotin complex (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA; 1:500) diluted in PBS. The sections next were washed in PBS and incubated in a solution of 0.04% diaminobenzidine tetrahydrochloride (DAB; Sigma) and 0.01% hydrogen peroxide in PBS. Sections were mounted onto SuperFrost slides and then visualized with Zeiss Axioskop as described above.

Dual-Label ISHH/Immunohistochemistry for SF1 and GFP

Free-floating sections of mouse brains were processed sequentially by ISHH and IHC by a protocol reported previously (Elias et al., 1998; Liu et al., 2003). Two series of different *Sf1-Cre*, lox-GFP mouse brains were processed for SF1 coexpression with GFP as described in the section above. A neuron was considered GFP-positive if the cell body and nucleus was clearly DAB labeled (orange-brown stain in Figure 1D). A GFP-positive neuron was considered Sf1 positive if it was decorated with at least ten silver grains (which is 3× above background). 1000 GFP-positive VMH neurons were identified and scored as either SF1 positive or SF1 negative based on the criteria set forth above.

Single Immunohistochemistry for pSTAT3

Fed male 4–6 week old $Lepr^{flox/flox}$ and *Sf1-Cre,* $Lepr^{flox/flox}$ mice were injected (n = 3 per genotype) intraperitoneally with 100 μg recombinant mouse leptin (A.F. Parlow, National Hormone and Peptide Program) or saline and perfused with 10% formalin 45 min later. Brain tissue was processed as described in section of tissues processing for histological studies earlier. p-STAT3 IHC was performed as described earlier (Munzberg et al., 2003) for further details see Supplemental Data.

Dual-Label Immunohistochemistry for pSTAT3 and GFP

Single IHCC for pSTAT3 was performed as stated above followed by IHCC for GFP. For details please see Supplemental Data.

Body and Blood Composition

Ad libitum fed 20 week old mice were anesthetized for dual-energy X-ray absorptiometry (MEC LunarCorp. Minster, OH). Blood was collected by cardiac puncture. Serum was assayed with a commercially available kit for leptin (Crystal Chem. Inc., Downers Grove, IL). Mesenteric, perigonadal, and perirenal fat pads were harvested from these mice and weighed. Femur length, bone mineral content, bone mineral density, and bone area was measured by DEXA. Corticosterone was measured with a commercially available kit at two time points in mice 10–12 weeks old (ICN Biomedicals, Inc., Costa Mesa, CA). Animals were sacrificed within 1 min of handling to obtain serum used for corticosterone analysis. Seminal vesicle weight was assessed in another set of animals, male 5–8 weeks of age.

Energy Expenditure

Oxygen consumption was measured by indirect calorimetry. Male mice, 6–8 weeks of age, were placed at room temperature (22ºC–24ºC) in chambers in an OXYMAX system (Columbus Instruments, Columbus, OH) with a settling time of 120 s and a measuring time of 60 s with room air as the reference. Food and water were provided ad libitum. Mice were acclimatized in the chambers for 48 hr prior to data collection. Oxygen consumption was measured for 72 hr on chow diet followed by 72 hr on HFD in the same set of animals. Data is presented as average VO_2 ml/hr/mouse.

Data Analysis

All values are reported as mean ± SEM. Data analysis was performed with GraphPad PRISM software (GraphPad, San Diego,

CA). Statistical significance was determined by one-way analysis of variance or two-tailed unpaired Student's t tests; $p < 0.05$ was considered significant.

Supplemental Data

The Supplemental Data for this article can be found online at http://www.neuron.org/cgi/content/full/49/2/191/DC1/.

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