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The hypothalamic arcuate nucleus: A key site for mediating leptin's effects on glucose homeostasis and locomotor activity

Roberto Coppari,[1,6] Masumi Ichinose,[1,5,6] Charlotte E. Lee,[1] Abigail E. Pullen,[1] Christopher D. Kenny,[1] Robert A. McGovern,[1] Vinsee Tang,[1] Shun M. Liu,[3] Thomas Ludwig,[4] Streamson C. Chua, Jr.,[3,7] Bradford B. Lowell,[1,7,*] and Joel K. Elmquist[1,2,7,*]

[1] Department of Medicine, Division of Endocrinology
[2] Department of Neurology, Program in Neuroscience, Beth Israel Deaconess Medical Center, Harvard Medical School, 99 Brookline Avenue, Boston, Massachusetts 02215
[3] Department of Pediatrics
[4] Department of Anatomy and Cell Biology, Columbia University, New York, New York 10032
[5] Department of Anatomy, Shiga University of Medical Science, Seta, Otsu, Shiga 520-2192, Japan
[6] These authors contributed equally to this work.
[7] These authors contributed equally to this work.
*Correspondence: blowell@bidmc.harvard.edu (B.B.L.); jelmquis@bidmc.harvard.edu (J.K.E.)

Summary

Leptin is required for normal energy and glucose homeostasis. The hypothalamic arcuate nucleus (ARH) has been proposed as an important site of leptin action. To assess the physiological significance of leptin signaling in the ARH, we used mice homozygous for a FLPe-reactivatable, leptin receptor null allele (*Lepr^{neo/neo}* mice). Similar to *Lepr^{db/db}* mice, these mice are obese, hyperglycemic, hyperinsulinemic, infertile, and hypoactive. To selectively restore leptin signaling in the ARH, we generated an adeno-associated virus expressing FLPe-recombinase, which was delivered unilaterally into the hypothalamus using stereotaxic injections. We found that unilateral restoration of leptin signaling in the ARH of *Lepr^{neo/neo}* mice leads to a modest decrease in body weight and food intake. In contrast, unilateral reactivation markedly improved hyperinsulinemia and normalized blood glucose levels and locomotor activity. These data demonstrate that leptin signaling in the ARH is sufficient for mediating leptin's effects on glucose homeostasis and locomotor activity.

Introduction

Leptin is secreted by adipocytes and signals to the brain the status of the body's energy content (Spiegelman and Flier, 2001; Friedman, 2004). Mice lacking leptin (*Lep^{ob/ob}* mice) or leptin receptor signaling (*Lepr^{db/db}* mice) are obese, diabetic, infertile, and hypoactive (Chen et al., 1996; Chua et al., 1996; Lee et al., 1996; Tartaglia et al., 1995; Zhang et al., 1994; Coleman, 1978). Recently, it has also been shown that leptin plays a critical role in neuronal plasticity (Pinto et al., 2004; Bouret et al., 2004). Substantial evidence suggests that the brain mediates the majority of leptin's action on energy homeostasis. For example, deletion of leptin receptors (LEPRs) in neurons induces obesity (Cohen et al., 2001), whereas expression of LEPRs in neurons of *Lepr^{db/db}* mice leads to an amelioration of their obesity (Kowalski et al., 2001). Moreover, intracerebroventricular (icv) administration of leptin in *Lep^{ob/ob}* mice causes reduction of body weight and food intake (Campfield et al., 1995).

Among the five splice variants described in mice (Lee et al., 1996), the long form of the leptin receptor (LEPR-B) is required for normal body weight homeostasis (Chen et al., 1996; Lee et al., 1996). Within the brain, abundant expression of LEPR-B has been found in several sites including hypothalamic groups such as the arcuate (ARH), the ventromedial (VMH), the dorsomedial (DMH), and the ventral premammillary (PMV) nuclei (Mercer et al., 1996; Elmquist et al., 1998; Fei et al., 1997; Schwartz et al., 1996; Thornton et al., 1997). Prominent among

these, the ARH has been proposed as an important site for mediating leptin's effect on energy homeostasis (Cowley et al., 2003; Schwartz et al., 2003; Zigman and Elmquist, 2003). Indeed, several reports support this view: Takeda et al. (2002) demonstrated that icv leptin infusion failed to reduce body weight in ARH-lesioned *Lep^{ob/ob}* mice. Moreover, ARH-specific LEPR-B gene therapy in rats lacking functional leptin receptor results in an amelioration of the obese phenotype (Morton et al., 2003). The ARH contains two populations of first-order, leptin-responsive neurons: The orexigenic NPY/AGRP and the anorexigenic CART/POMC neurons (Spiegelman and Flier, 2001; Saper et al., 2002). NPY/AGRP neurons are directly inhibited by leptin (van den Top et al., 2004), whereas CART/POMC neurons are directly activated by leptin (Cowley et al., 2001; Elias et al., 1999). Consistent with this, *Lep^{ob/ob}* mice have increased hypothalamic *Agrp* and *Npy* mRNA levels (Mizuno and Mobbs, 1999; Schwartz et al., 1996; Ahima et al., 1996; Stephens et al., 1995) and reduced *Pomc* mRNA levels (Schwartz et al., 1997; Thornton et al., 1997). Further supporting the importance of the ARH in controlling leptin actions on energy homeostasis, we have recently shown that mice lacking LEPRs only in POMC neurons are mildly obese (Balthasar et al., 2004).

In addition to the well-documented effects on body weight, leptin signaling is required for normal glucose homeostasis as demonstrated by the fact that both *Lep^{ob/ob}* and *Lepr^{db/db}*

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mice have impaired insulin/glucose homeostasis (Spiegelman
and Flier, 2001; Pelleymounter et al., 1995; Coleman, 1978).
However, it is still unclear whether leptin regulates glucose ho-
meostasis directly or indirectly through its action on body
weight. Several studies support the view that leptin does have
an effect on glucose homeostasis independent of its effect on
body weight regulation. For example, Pelleymounter et al.
(1995) reported that daily leptin administration in Lep^ob/ob
mice, at doses that did not have an effect on body weight,
normalized serum glucose level. Moreover, Schwartz et al.
(1996) showed that leptin-treated Lep^ob/ob mice had 40%
greater reduction in glucose level compared with pair-fed
Lep^ob/ob control mice. Furthermore, Shimomura et al. (1999)
described that adipose-deficient, leptin-deficient, lipodystro-
phic mice are insulin resistant and that insulin sensitivity can
be restored in these mice by leptin infusion. This effect was
also independent of leptin's action on body weight. To date, it
is unclear whether leptin's effects on insulin-target tissues are
mediated indirectly by the brain or directly by LEPRs in these
tissues (Kamohara et al., 1997; Minokoshi et al., 2002).

As previously stated, leptin directly acts on NPY/AGRP and
CART/POMC neurons. Thus, the NPY pathway and the mela-
nocortin pathway might be involved in leptin-mediated control
of glucose homeostasis. Indeed, Lep^ob/ob mice lacking the Npy
gene (Lep^ob/ob; Npy^−/− mice) have almost normal serum glu-
cose levels and 50% reduced insulinemia compared to
Lep^ob/ob mice (Erickson et al., 1996). Leptin activates the mela-
nocortin pathway by stimulating melanocortinergic POMC neu-
rons and by inhibiting AGRP neurons (AGRP is the natural an-
tagonist at the melanocortin receptors) (Cowley et al., 2001;
Elias et al., 1999; Elmquist et al., 1999; Schwartz et al., 1996;
Roseberry et al., 2004). Recently, it has also been shown that
central melanocortin signaling regulates insulin action. For ex-
ample, icv infusion of either the natural agonist (α-melanocyte
stimulating hormone [α-MSH]) or the synthetic antagonist
(SHU9119) of the melanocortin receptors 3 and 4 (MC3R and
MC4R) in rats has opposite effects. α-MSH-treated rats have
enhanced insulin action, whereas SHU9119-treated rats have
diminished insulin action (Obici et al., 2001).

Leptin signaling has also been shown to be a critical regula-
tor of reproductive function. Indeed both Lep^ob/ob and Lepr^db/db
mice are infertile (Spiegelman and Flier, 2001; Bates et al.,
2003; Coleman, 1978). The NPY pathway has been proposed
to mediate leptin's effect on reproductive function. Consistent
with this hypothesis, Lep^ob/ob; Npy^−/− mice have improved fer-
tility (Erickson et al., 1996). Also, in agreement with this, mice
lacking leptin-mediated STAT3 activation, which are obese but
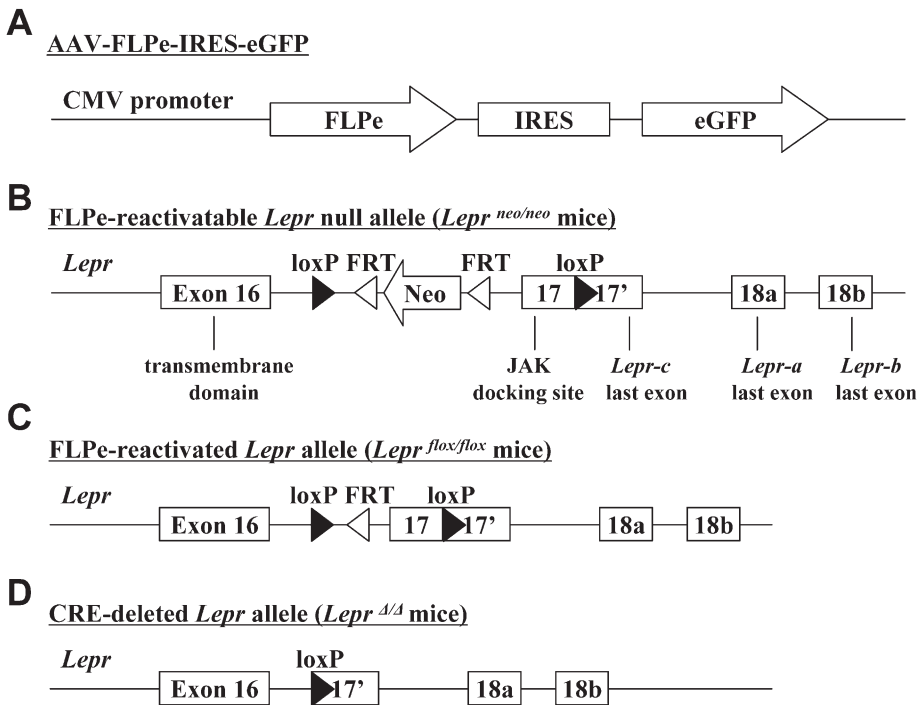have normal hypothalamic Npy gene expression, are fertile
(Bates et al., 2003).

Finally, leptin exerts also a positive action on locomotor ac-
tivity as suggested by the fact that Lep^ob/ob mice are hypo-
active and that their locomotor activity can be normalized by
leptin treatment (Pelleymounter et al., 1995). To examine whether
leptin signaling only in ARH neurons is sufficient to prevent obe-
sity, diabetes, infertility, and hypoactivity, we re-expressed LEPRs
under the control of the endogenous leptin receptor promoter in
neurons in the ARH of mice otherwise deficient in leptin recep-
tor expression.

Results

Restoring leptin receptor expression
in the arcuate nucleus

In order to selectively express LEPRs in ARH neurons, we em-
ployed Lepr^neo/neo mice (McMinn et al., 2004). These mice are
homozygous for a FLPe-reactivatable, Lepr-null allele (Figure
1B) and, as a result, are similar to Lepr^db/db mice (McMinn et
al., 2004). FLPe-mediated deletion of the FRT-flanked Neo cas-
sette creates a normally functioning Lepr allele. Indeed, mice
homozygous for the FLPe-reactivated, Lepr allele (Lepr^flox/flox
mice, Figure 1C) had body weights indistinguishable from wild-
type littermates (McMinn et al., 2004; Balthasar et al., 2004).
Site-specific reactivation of the Lepr allele was achieved by
stereotaxic delivery of FLPe-recombinase in Lepr^neo/neo male
mice. Due to the unavailability of antibodies for FLPe-recombi-
nase and in order to visualize FLPe-expressing cells in stereo-
taxically injected Lepr^neo/neo mice, we engineered an adeno-
associated viral vector that would also express enhanced
green fluorescent protein (eGFP) (Figure 1A). Therefore, we
were able to visualize FLPe-expressing cells by performing
immunohistochemistry for eGFP.

The AAV-FLPe-IRES-eGFP vector was stereotaxically in-
jected into the hypothalamus with coordinates focused on the
ARH of Lepr^neo/neo mice. This procedure inherently resulted in
a high percentage of injection sites that were centered outside
of the ARH as well as several injections that were centered in
the ARH. However, these missed injections served as impor-
tant anatomic controls. Thus, we categorized the injections as
either ARH-misses (control group) or ARH-hits first by neuro-
anatomic inspection. In addition, as described in Experimental
procedures, we also categorized the injections by counting
eGFP-positive cells within the hypothalamus. Briefly, brains
that had >100 eGFP-positive cells in the ARH (25 μm sections
containing the ARH in a 1:5 series) were scored as ARH-hit.
Those cases with <100 eGFP-positive cells in the ARH were
grouped as ARH-missed. Importantly, we also obtained cases
that had >100 eGFP-positive cells in the ARH plus in other
nuclei which are known to contain Lepr-b mRNA (e.g., the DMH
and the VMH). These cases with ARH rescue plus the addi-
tional sites were not categorized in the ARH-hit group. Figures
2A and 2B show representative photomicrographs of im-
munohistochemistry for eGFP in ARH-missed and ARH-hit
Lepr^neo/neo mice, respectively. The eGFP immunohistochem-
istry was used as an index to categorize the center of the injec-
tion sites and, thus, the site containing the majority of FLPe-
expressing cells. We also determined whether the delivery of
FLPe successfully restored leptin signaling in transduced neu-
rons. To accomplish this, we assessed the rapid phosphoryla-
tion and nuclear translocation of signal transducer and activa-
tor of transcription 3 (STAT3) in response to leptin (Munzberg
et al., 2003; Li and Friedman, 1999; Baumann et al., 1996;
Hosoi et al., 2002). Therefore, 45 min before perfusion, mice
were injected intraperitoneally with leptin, and phospho-STAT3
(P-STAT3) immunohistochemistry was performed. As can be
seen in Figure 2C, ARH-missed cases displayed very modest
leptin-induced P-STAT3 immunoreactivity in the ARH. In con-
trast, prominent P-STAT3 immunoreactivity was characteristi-
cally observed in mice with ARH-specific restoration of leptin
receptor signaling (Figure 2D). The hypothalamic distribution
of leptin-induced P-STAT3-positive neurons in these mice is

A

AAV-FLPe-IRES-eGFP

B

FLPe-reactivatable *Lepr* null allele (*Lepr* ^neo/neo^ mice)

C

FLPe-reactivated *Lepr* allele (*Lepr* ^flox/flox^ mice)

D

CRE-deleted *Lepr* allele (*Lepr* ^Δ/Δ^ mice)

Figure 1. Schematic representation of adeno-associated viral (AAV) vector and modified *Lepr* alleles

A) AAV-FLPe-IRES-eGFP was obtained by cloning the FLPe gene into a transfer vector (pAAV-M2-IGFP) such that FLPe and eGFP are driven by CMV regulatory elements.

B) The FRT-Neo-FRT cassette is located upstream of exon 17 of the *Lepr* allele. This allele was bred to homozygosity to generate *Lepr*^neo/neo^ mice.

C) The FLPe-mediated removal of the FRT-Neo-FRT cassette produces the FRT-modified and loxP-flanked *Lepr* allele which functions as a wild-type allele. *Lepr*^flox/flox^ mice are homozygous for this allele.

D) The CRE-deleted *Lepr* allele lacks exon 17 and is a null allele. *Lepr*^Δ/Δ^ mice are homozygous for this allele.

outlined in Table 1. These data demonstrate that leptin signaling was established in ARH-neurons of *Lepr*^neo/neo^ mice.

We also attempted to rule out the possibility that the viral injections and/or the expression of FLPe and eGFP by neurons in the ARH per se had effects on body weight and glucose homeostasis in obese and diabetic mice. We performed the following experiment. The AAV-FLPe-IRES-eGFP vector was stereotaxically injected into the hypothalamus with coordinates focused on the ARH of mice homozygous for a *Lepr*-null allele that cannot be reactivated by FLPe (*Lepr*^Δ/Δ^ mice, Figure 2D). *Lepr*^Δ/Δ^ mice were categorized as ARH-hit and ARH-missed as described above and in Experimental procedures. Figures 3A and 3B show representative photomicrographs of immunohistochemistry for eGFP in ARH-missed and ARH-hit *Lepr*^Δ/Δ^ mice, respectively. Both, ARH-missed and ARH-hit *Lepr*^Δ/Δ^ mice displayed no leptin-induced P-STAT3 immunoreactivity (Figures 3C and 3D, respectively). Importantly, ARH-missed and ARH-hit *Lepr*^Δ/Δ^ mice had indistinguishable body weight (12-week-old *Lepr*^Δ/Δ^ mice: ARH-missed = 50.02 [g] ± 1.85 [n = 6]; ARH-hit = 51.12 [g] ± 1.6 [n = 3], Figure 4C). These mice also had indistinguishable serum insulin (12-week-old *Lepr*^Δ/Δ^ mice: ARH-missed = 89.22 ng/ml ± 18.21 [n = 4]; ARH-hit = 80.88 ng/ml ± 27.58 [n = 3], Figure 5B) and glucose levels (12-week-old *Lepr*^Δ/Δ^ mice: ARH-missed = 492.5 mg/dl ± 34.17 [n = 4]; ARH-hit = 562 mg/dl ± 81.6 [n = 3], Figure 5D). These data demonstrate that the viral injections and/or the expression of FLPe and eGFP in neurons in the ARH had no effects on body weight and glucose homeostasis in obese and diabetic mice.

**Leptin action in the arcuate nucleus reduces body weight**
Mice lacking leptin signaling have excessive body and fat mass, increased food intake, and reduced energy expenditure (Spiegelman and Flier, 2001; Friedman, 2004). Thus, we investi-

gated the possibility that re-establishment, unilaterally, of leptin signaling in ARH neurons would be sufficient to restore normal energy homeostasis in *Lepr*^neo/neo^ mice. As shown in Figure 4A, ARH-hit *Lepr*^neo/neo^ mice had significantly reduced body weight (starting at 7 weeks of age) when compared to ARH-missed *Lepr*^neo/neo^ mice. We found that the difference in body weight between 12-week-old ARH-hit *Lepr*^neo/neo^ mice and ARH-missed *Lepr*^neo/neo^ mice was 5.2 g. This represented approximately 22% of the total body weight difference between mice with normal leptin signaling (not surgically treated *Lepr*^+/+^ mice) and ARH-missed *Lepr*^neo/neo^ mice (Figure 4B). Body composition analysis revealed that the reduction in body weight observed in ARH-hit *Lepr*^neo/neo^ mice was due to a reduction in fat mass (Figure 4D). Since ARH-hit *Lepr*^neo/neo^ mice had improved energy homeostasis, they must have either reduced food intake or increased energy expenditure or both. ARH-hit *Lepr*^neo/neo^ mice had reduced cumulative food intake (Figure 4E). However, energy expenditure was not elevated in these mice (Figure 4F). These data suggest that leptin signaling in one side of the ARH is sufficient to mediate ∼20% of leptin's action on body weight homeostasis.

Restoration of leptin receptors in the arcuate nucleus dramatically improves glucose homeostasis
As noted, leptin signaling is required for normal blood glucose and insulin levels. In fact, *Lep*^ob/ob^ mice and *Lepr*^db/db^ mice develop overt diabetes (Spiegelman and Flier, 2001; Coleman, 1978). We found that unilateral restoration of leptin signaling in the ARH was sufficient to remarkably improve glucose homeostasis in *Lepr*^neo/neo^ mice. Indeed, insulinemia was greatly reduced at both 4 and 8 weeks after FLPe-recombinase was delivered into the ARH of *Lepr*^neo/neo^ mice (Figure 5A). Notably, 4 weeks after surgery, the blood glucose levels in ARH-hit
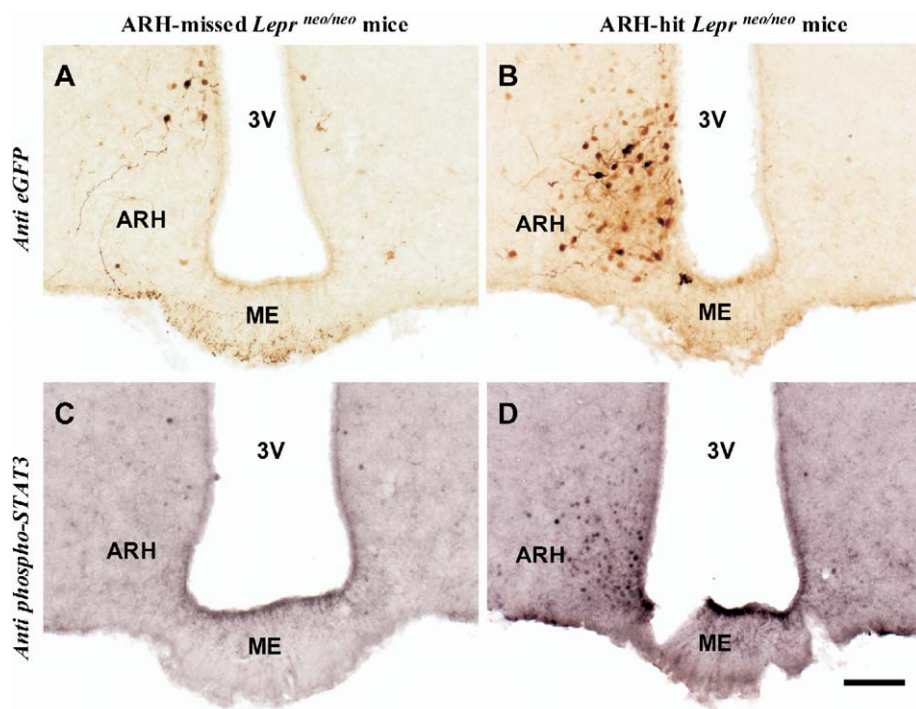
Figure 2. Unilateral *Lepr* gene reactivation in the ARH of *Lepr^neo/neo^* mice

Photomicrograph of immunohistochemistry for eGFP in ARH-missed (**A**; Case 17 in Table 1) and ARH-hit (**B**; Case 9 in Table 1) *Lepr^neo/neo^* mice. Photomicrograph of immunohistochemistry for leptin-induced phospho-STAT3 in ARH-missed (**C**; Case 17) and ARH-hit (**D**; Case 9) *Lepr^neo/neo^* mice. Median eminence (ME), third ventricle (3V), hypothalamic arcuate nucleus (ARH). Scale bar = 100 μm.

*Lepr^neo/neo^* mice were not statistically reduced compared to that of ARH-missed *Lepr^neo/neo^* mice (although a trend toward lower glycemia was seen). However, at 8 weeks after surgery, blood glucose levels were normalized. Indeed, we found that 12-week-old ARH-hit *Lepr^neo/neo^* mice had blood glucose levels indistinguishable to age-matched *Lepr^+/+^* mice (Figure 5C). These data strongly suggest that insulin action was greatly improved in ARH-hit *Lepr^neo/neo^* mice.

Arcuate nucleus leptin receptors and reproduction
Leptin is also known to affect fertility. In fact, mice lacking leptin signaling are infertile (Spiegelman and Flier, 2001; Coleman, 1978; Bates et al., 2003). Therefore, we tested whether ARH-hit *Lepr^neo/neo^* male mice were able to produce offspring by breeding these male mice for one week with adult females. The one-week breeding period was chosen because in similar housing conditions, adult wild-type male mice were able to impregnate adult wild-type female mice in this window of time. All male mice (n = 4) that were bred with female mice for 6–7 days generated offspring. In contrast, both ARH-hit and ARH-missed *Lepr^neo/neo^* mice were unable to produce offspring. Indeed, none of the females were found to be pregnant after the one-week breeding period. These data indicate that unilateral leptin signaling in the ARH is insufficient to restore the capacity of *Lepr^neo/neo^* male mice to generate offspring.

Leptin action in the arcuate nucleus and locomotor activity
Leptin positively regulates locomotor activity as supported by the fact that *Lep^ob/ob^* mice are hypoactive and their total activity can be normalized by leptin administration (Pelleymounter et al., 1995). To date, it is unknown which sites in the brain mediate this effect of leptin. In order to assess if ARH neurons

are able to mediate leptin's effect on locomotor activity, we recorded ambulatory movements of ARH-hit *Lepr^neo/neo^* and ARH-missed *Lepr^neo/neo^* mice. As shown in Figure 6A, ARH-hit *Lepr^neo/neo^* mice had significantly increased 24 hr locomotor activity when compared to ARH-missed *Lepr^neo/neo^* mice. Notably, the majority of the increase in activity occurred during the dark cycle (Figures 6B and 6C). Since the ambulatory activity of ARH-hit *Lepr^neo/neo^* mice was not statistically different to that of wild-type control mice (Figure 6A), we conclude that leptin-sensitive, ARH neurons are sufficient for mediating the majority, if not all, of leptin's action on locomotor activity.

Discussion

The incidence of obesity and diabetes continues to rise in industrialized countries (Flier, 2004; Friedman, 2004; Barsh et al., 2000). In order to prevent and/or treat these conditions it is critical to understand the cellular and neuroanatomic pathways that control energy and glucose homeostasis. The hormone leptin is required for normal body weight and glucose homeostasis and is key in governing these biological programs (Flier, 2004; Friedman, 2004; Barsh et al., 2000). During the last decade it has become evident that leptin's primary site of action is the central nervous system (CNS). However, leptin receptors are expressed in several CNS sites and relatively little is known about which neuronal groups mediate each of the specific actions of leptin. The arcuate nucleus in the hypothalamus has been proposed as one important site for mediating leptin's effect on energy homeostasis (Cowley et al., 2003; Schwartz et al., 2003; Zigman and Elmquist, 2003). Supporting this view, we have shown that deletion of LEPRs only in POMC cells leads to mild obesity (Balthasar et al., 2004). In addition, Morton et al. (2003) performed ARH-specific *Lepr-b* gene ther-

Table 1. Hypothalamic distribution of leptin-induced P-STAT3 positive neurons in ARH-missed and ARH-hit $Lepr^{neo/neo}$ mice

	ARH		RCA		VMH		DMH		LH		Sch	
	Lt	Rt	Lt	Rt	Lt	Rt	Lt	Rt	Lt	Rt	Lt	Rt
ARH-hit $Lepr^{neo/neo}$ mice												
1	531	44	16	9	14	0	1	0	0	0	0	0
2	525	96	20	9	8	0	152	0	25	0	0	0
3	213	25	4	2	0	0	0	0	0	0	0	0
4	364	43	0	0	0	0	0	2	0	0	0	0
5	606	7	23	0	41	0	75	0	0	0	0	0
6	411	77	15	16	7	2	0	0	0	0	4	0
7	432	136	26	11	3	0	0	0	0	0	0	0
8	325	31	7	3	2	0	0	0	0	0	0	0
9	360	63	10	0	23	0	11	0	1	0	0	0
ARH-missed $Lepr^{neo/neo}$ mice												
10	25	18	22	23	1	1	0	0	0	0	0	3
11	16	4	0	0	0	0	0	0	0	0	0	0
12	6	2	1	0	0	0	0	0	0	0	0	0
13	62	32	17	14	0	0	0	0	0	0	3	12
14	74	7	19	2	12	0	17	0	0	0	0	0
15	7	4	0	0	0	0	0	0	0	0	0	0
16	1	6	0	0	0	0	0	0	0	0	0	0
17	81	51	17	11	7	0	2	0	0	0	0	0
18	2	5	11	12	0	0	0	0	0	0	0	4

P-STAT3-positive neurons were estimated in hypothalamic nuclei using a camera lucida device (all sections; 1:5 series). Arcuate nucleus (ARH); retrochiasmatic area (RCA); ventromedial nucleus (VMH); dorsomedial nucleus (DMH); lateral hypothalamic area (LH); suprachiasmatic nucleus (Sch). Lt = left side; Rt = right side.

apy in leptin receptor-deficient rats and showed that this ameliorated obesity. This study used adenoviral vectors to transgenically express LEPR-B under the control of CMV regulatory elements. In contrast, our approach used the delivery of FLPe-recombinase in young FLPe-reactivatalbe, *Lepr*-null mice (*Lepr^neo/neo* mice) (McMinn et al., 2004). This approach allowed us to express endogenous LEPRs at physiological levels only in neurons that would normally express LEPRs.

Collectively, our data suggest that restoring physiological leptin signaling in ARH neurons is sufficient to prevent the full obesity syndrome seen in leptin receptor-deficient mice. However, the reduction in body weight is relatively modest (~20%)

ARH-missed *Lepr* $^{Δ/Δ}$ **mice**   **ARH-hit** *Lepr* $^{Δ/Δ}$ **mice**

Anti eGFP

Anti phospho-STAT3
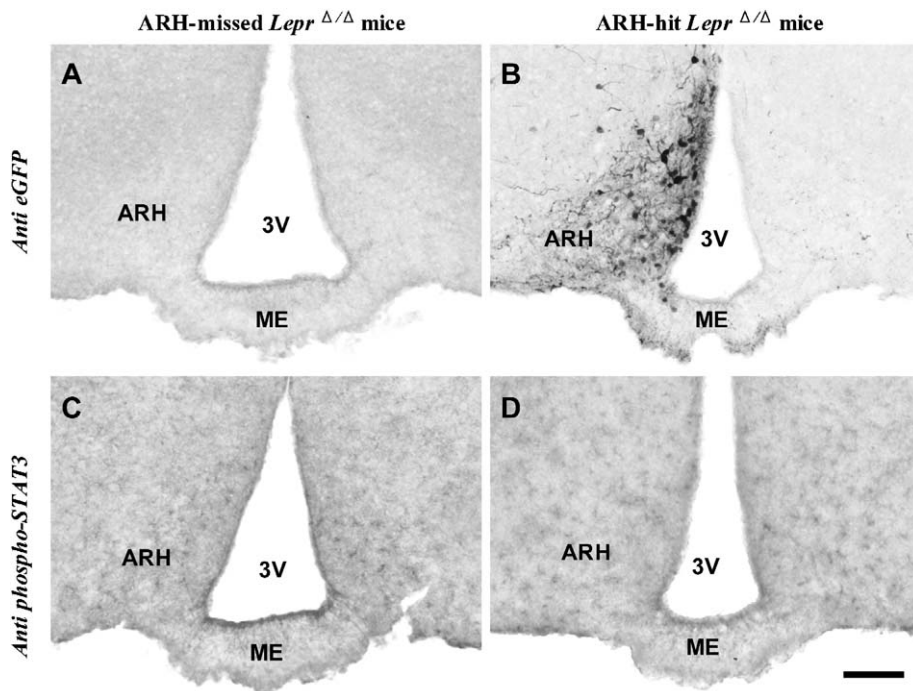
Figure 3. Delivery of AAV-FLPe-IRES-eGFP in the ARH of $Lepr^{Δ/Δ}$ mice does not lead to *Lepr* gene reactivation

Photomicrograph of immunohistochemistry for eGFP in ARH-missed (**A**) and ARH-hit (**B**) $Lepr^{Δ/Δ}$ mice. Photomicrograph of immunohistochemistry for leptin-induced phospho-STAT3 in ARH-missed (**C**) and ARH-hit (**D**) $Lepr^{Δ/Δ}$ mice. Median eminence (ME), third ventricle (3V), hypothalamic arcuate nucleus (ARH). Scale bar = 100 μm.
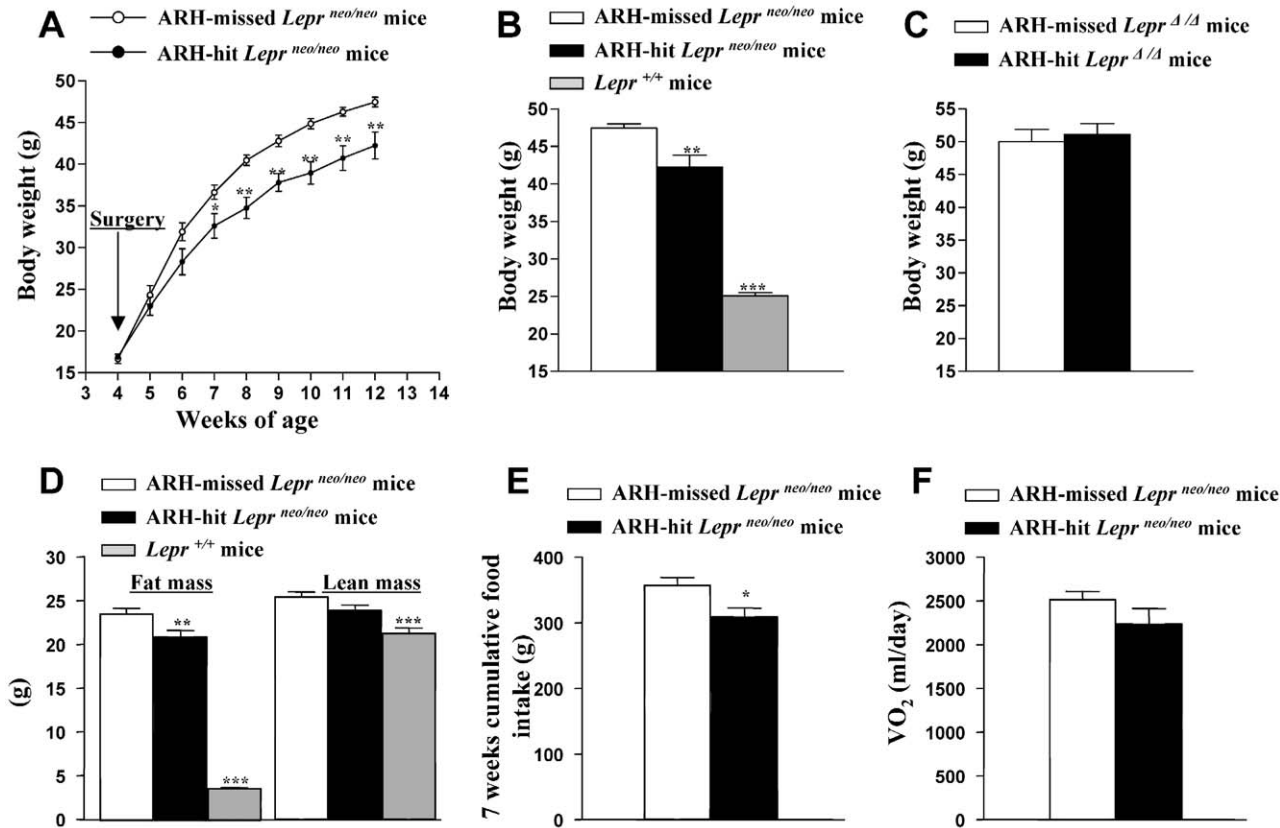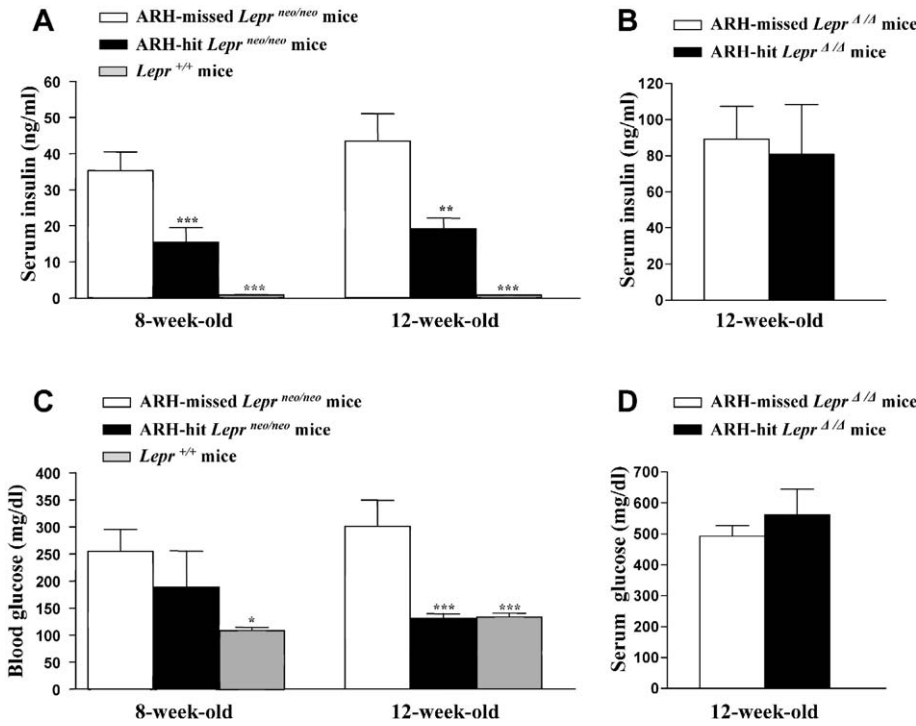
Figure 4. Unilateral reactivation of the *Lepr* gene in the hypothalamic arcuate nucleus of *Lepr^neo/neo* mice leads to reduced body weight, fat mass, and food intake

A) Body weight curves of ARH-missed (n = 9) and ARH-hit (n = 9) *Lepr^neo/neo* mice.

B) Body weight of 12-week-old ARH-missed (n = 9), ARH-hit (n = 9) *Lepr^neo/neo* and *Lepr^+/+* (n = 10) mice.

C) Body weight of 12-week-old ARH-missed (n = 6), ARH-hit (n = 3) *Lepr^Δ/Δ* mice. Please note that the body weights are displayed separately from the mice in (**B**) since the mice have dissimilar genetic backgrounds (C57Bl6/J and 129 in the case of *Lepr^neo/neo* mice and C57Bl6/J, 129 and FVB in the case of *Lepr^Δ/Δ* mice).

D) Fat and lean mass in 12-week-old ARH-missed (n = 9), ARH-hit (n = 6) *Lepr^neo/neo* and *Lepr^+/+* (n = 4) mice were measured by DEXA.

E) Cumulative food intake in ARH-missed (n = 9) and ARH-hit (n = 9) *Lepr^neo/neo* mice was collected between ages 5 and 12 weeks.

F) Oxygen consumption was measured with CLAMS in 13-week-old ARH-missed (n = 9) and ARH-hit (n = 5) *Lepr^neo/neo* mice. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ versus ARH-missed *Lepr^neo/neo* mice.

compared to mice with normal leptin signaling. Given the fact that leptin signaling was restored only in one side of the ARH, it remains unknown whether bilateral reactivation would lead to normal body weight homeostasis. However, it is notable that hypothalamic lesions (including the ARH) in rodents cause significant obesity only when performed bilaterally (Elmquist et al., 1999). The fact that unilateral lesions fail to produce obesity suggests that unilateral ARH function should be sufficient for normal body weight regulation.

Interestingly, despite the modest reduction in body weight, unilateral reactivation markedly improved hyperinsulinemia and normalized blood glucose levels. Although our study demonstrates that LEPRs expression by ARH neurons is sufficient to mediate leptin's effects on glucose homeostasis, the downstream pathways mediating these effects are still unknown. We propose that melanocortin receptor- and NPY receptor-expressing neurons are downstream targets of leptin-responsive ARH neurons in the leptin-mediated control of glucose homeostasis. Consistent with this hypothesis, it has been shown that activation of the central melanocortin pathway leads to increased insulin action (Obici et al., 2001) and decreased insulin levels (Fan et

al., 2000). Since the ARH contains first-order, leptin-responsive neurons that secrete either the agonist (α-MSH) or the antagonist (AGRP) of the melanocortin receptors (Schwartz et al., 1996, 1997; Cowley et al., 2001; van den Top et al., 2004), mice with restored leptin signaling in ARH neurons would be expected to have improved melanocortin signaling and perhaps insulin action. Moreover, it has been shown that NPY is required for the development of the full diabetes syndrome in leptin-deficient mice (Erickson et al., 1996), suggesting that elevated NPY (as found in *Lep^ob/ob*) positively contributes to their diabetes. Since leptin inhibits NPY-secreting ARH neurons (van den Top et al., 2004; Roseberry et al., 2004), restoration of leptin signaling in the ARH is expected to reduce NPY and therefore to ameliorate glucose homeostasis. Alternatively, it is possible that the improvement of glucose/insulin homeostasis is secondary to the reduction in body weight. However, it is unlikely that this is the sole mechanism underlying the improvement in glucose homeostasis. First, blood glucose levels, while normalized at 8 weeks after FLPe-recombinase was delivered into the ARH of *Lepr^neo/neo* mice, were not normalized at 4
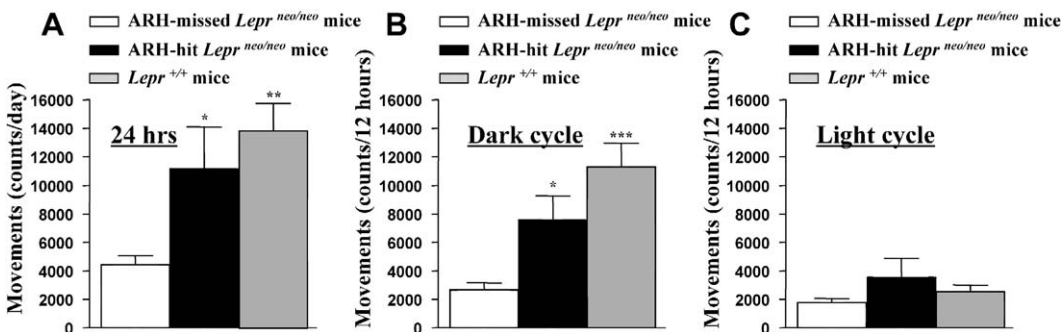
Figure 5. Unilateral reactivation of the *Lepr* gene in the hypothalamic arcuate nucleus of *Lepr^neo/neo* mice leads to improved glucose homeostasis

(**A**) Serum insulin and (**C**) blood glucose levels in fed ARH-missed (n = 9), ARH-hit (n = 6) *Lepr^neo/neo* and *Lepr^+/+* (n = 3–9) mice. Note that the AAV injections do not affect glucose homeostasis. Also note that panels (**B**) and (**D**) are separated from (**A**) and (**C**) since the mice have dissimilar genetic backgrounds (as noted in the legend for Figure 4). (**B**) Serum insulin and (**D**) serum glucose levels in fed ARH-missed (n = 4), ARH-hit (n = 3) *Lepr^Δ/Δ* mice. *p < 0.05; **p < 0.01; ***p < 0.001 versus ARH-missed *Lepr^neo/neo* mice.

weeks after surgery. Since body weight was similarly reduced at both the 4 and 8 week points, it is unlikely to be the cause of the normal glycemia at the 8 week point. Moreover, food restriction experiments in obese mice, which led to a similar reduction of body weight as that seen in ARH-hit *Lepr^neo/neo* mice, were not able to normalize blood glucose levels (Schwartz et al., 1996; Yamamoto et al., 2000). In addition, as detailed below, reactivation of LEPRs in both the VMH and the lateral hypothalamic area (LH) of *Lepr^neo/neo* mice resulted in a reduction in body weight similar to ARH-hit *Lepr^neo/neo* mice. However, this body weight reduction was not associated with improved glucose homeostasis. Thus, we conclude that the improvements in glucose homeostasis are likely to be independent of a body weight reduction. This suggests that leptin signaling in ARH neurons exerts direct control over insulin/glucose

homeostasis. This concept would be consistent with other claims that hypothalamic neurons regulate glucose homeostasis (Obici et al., 2001; Fan et al., 2000).

Leptin also exerts a positive action on locomotor activity as suggested by the fact that *Lep^ob/ob* mice are hypoactive and that their locomotor activity can be normalized by leptin treatment (Pelleymounter et al., 1995). However, the neuronal groups that mediate this effect of leptin are unknown. Our study shows that restoring leptin signaling in ARH neurons is sufficient to normalize locomotor activity. Therefore, this finding establishes the ARH as a key site for mediating leptin's effect on locomotor activity. We suggest the existence of a novel, yet undefined, neuronal pathway connecting ARH neurons to cortical and/or subcortical areas regulating voluntary locomotion. Additional studies will be required to reveal the impor-

Figure 6. Unilateral reactivation of the *Lepr* gene in the hypothalamic arcuate nucleus of *Lepr^neo/neo* mice leads to increased locomotor activity

(**A**) 24 hr (**B**) nocturnal and (**C**) diurnal locomotor activity were measured with CLAMS in 13-week-old ARH-missed (n = 9), ARH-hit (n = 5) *Lepr^neo/neo* and *Lepr^+/+* (n = 5) mice. *p < 0.05; **p < 0.01; ***p < 0.001 versus ARH-missed *Lepr^neo/neo* mice.

tance of this hypothesized neurocircuit mediating leptin's effect on ambulatory movements.

Further supporting our conclusions that leptin signaling specifically in the ARH is a major feeding-independent regulator of glucose homeostasis and locomotor activity were physiological data on four $Lepr^{neo/neo}$ mice that had brains with >100 eGFP-positive cells in both the LH and the VMH and <100 eGFP-positive cells in any other nucleus known to contain $Lepr$-b mRNA. Like ARH-hit $Lepr^{neo/neo}$ mice, these LH + VMH-hit $Lepr^{neo/neo}$ mice had ~20% reduction in body weight (12-week-old $Lepr^{neo/neo}$ mice: ARH-missed = 47.44 [g] ± 0.56 [n = 9]; ARH-hit = **42.23 [g] ± 1.6 [n = 9]; LH + VMH-hit = *43.74 [g] ± 2.09 [n = 4]; *p < 0.05, **p < 0.01 versus ARH-missed). Importantly, despite the reduced body weight, blood glucose levels and locomotor activity were not normalized in LH + VMH-hit $Lepr^{neo/neo}$ mice (blood glucose levels in 12-week-old $Lepr^{neo/neo}$ mice: ARH-missed = 300 [mg/dl] ± 48 [9]; LH + VMH-hit = 231 [mg/dl] ± 76 [n = 4]; locomotor activity in 12-week-old $Lepr^{neo/neo}$ mice: ARH-missed = 4447 [counts/day] ± 631 [n=9]; LH+VMH-hit = 4646 [counts/day] ± 923 [n = 4]). Further analysis, which is in progress in our laboratories, is needed to definitely assess the physiological importance of leptin signaling specifically in the LH or the VMH. However, we predict that leptin's effects on glucose homeostasis and locomotor activity are not mediated by LEPRs on neurons contained within these two hypothalamic areas.

In summary, restoration of leptin signaling in ARH neurons leads to improved, but not normal energy homeostasis. Thus, other leptin-responsive neuronal groups are likely also to be important in mediating leptin's effect on food intake and body weight. In contrast, LEPRs expression by ARH neurons is sufficient to mediate the majority of leptin's effects on glucose homeostasis and locomotor activity. Delivery of FLPe in a neuron-specific fashion by the generation of neuron-specific FLPe-transgenic $Lepr^{neo/neo}$ mice (for example, Npy-FLPe, $Pomc$-FLPe, or $Agrp$-FLPe transgenic mice) will be critical to reveal the relative contribution of leptin signaling in specific populations on neurons in the ARH in mediating the varied effects of leptin.

Experimental procedures

Animal care
Care of all mice was within the Institutional Animal Care and Use Committee (IACUC) guidelines, and all the procedures were approved by the Beth Israel Deaconess Medical Center IACUC. Mice were housed individually at 22°C–24°C using a 14 hr light/10 hr dark cycle with chow food (Tekland F6 Rodent Diet8664, Harlan Tekland, Madison, Wisconsisn) and water provided ad libitum.

Experimental mice
$Lepr^{neo/+}$ mice were provided by Dr. S. Chua, Jr. (McMinn et al., 2004) . The genetic background of $Lepr^{neo/+}$ mice is an admixture of C57Bl6/J and 129. $Lepr^{neo/neo}$ and $Lepr^{+/+}$ male mice were obtained by mating $Lepr^{neo/+}$ mice with $Lepr^{neo/+}$ mice. Mice were genotyped by PCR with primers (1 and 2) across the loxP site: 1, 5′-AAT GAA AAA GTT GTT TTG GGA CGA-3′ and 2, 5′-CAG GCT TGA GAA CAT GAA CAC AAC AAC-3′. $Lepr^{\Delta/\Delta}$ mice are homozygous for a null LEPR allele lacking exon 17 and were generated as described previously (Balthasar et al., 2004). The genetic background of $Lepr^{\Delta/\Delta}$ mice is an admixture of C57Bl6/J, 129, and FVB.

AAV-FLPe-IRES-eGFP generation and microinjection
The FLPe gene was cloned into the transfer vector pAAV-M2-IGFP such that the AAV vector plasmid called here pAAV-FLPe-M2-IGFP was generated. The virus was generated by tripartite transfection (AAV-rep/cap expression plasmid, adenovirus miniplasmid, and pAAV-FLPe-M2-IGFP) into

293A cells and purified by heparin column. The eluted virus was dialyzed against PBS and the titer was assessed by dot blot hybridization. All these procedures were performed by the Harvard Gene Therapy Initiative core facility (http://hgti.med.harvard.edu).

Four-week-old $Lepr^{neo/neo}$ and $Lepr^{\Delta/\Delta}$ male mice were stereotaxically injected with AAV-FLPe-IRES-eGFP into the ARH with a glass micropipette and air pressure injector system (Chamberlin et al., 1998).

Body and blood composition
Tail vein blood was collected at noon ± 2 hr from fed 8- and 12-week-old mice. Blood was assayed for glucose level (Fisher Scientific, Morrison Plains, New Jersey) and successively serum was collected by centrifugation and assayed for insulin levels using commercially available kits (Crystal Chem. Inc., Downers Grove, Illinois). Serum was assayed for glucose levels using an enzymatic glucose oxidase method (Thermo Electron, Victoria, Australia). After blood was collected, mice were ketamine anesthetized for dual-energy X-ray absorptiometry (MEC Lunar Corp., Minster, Ohio) analysis.

Oxygen consumption, locomotor activity, and fertility test
Metabolic rate and physical activity were measured in 13-week-old mice using a comprehensive lab animal monitoring system (CLAMS, Columbia Instruments, Columbus, Ohio). Mice were acclimated in the monitoring chambers for 2 days then data were collected for 3 days. Data analysis was performed only in mice that did not lose weight during the experiment. After CLAMS analysis, every mouse was housed with two 7- to 12-week-old FVB female mice for a period of 6–7 days. Female mice were monitored for the following 4 weeks and the presence of the offspring was counted as event of pregnancy. Female control mice were tested for their fertility by breeding with wild-type male mice. All female mice had events of pregnancy when bred with wild-type male mice. Food and water were provided ad libitum during the entire period.

eGFP and P-STAT3 immunohistochemistry
Fed 14-week-old male mice were injected intraperitoneally with 100 μg of recombinant mouse leptin (A.F. Parlow, National Hormone and Peptide Program) and perfused with 10% formalin 45 min later. Either eGFP or P-STAT3 immunohistochemistry was performed on microtome cut 25 μm brain sections as described earlier (Liu et al., 2003; Elias et al., 1999; Munzberg et al. 2003).

Categorization of ARH-hit and ARH-missed $Lepr^{neo/neo}$ and $Lepr^{\Delta/\Delta}$ mice
Brain sections stained against eGFP by immunohistochemistry were used for this categorization. The borders of the nuclei containing eGFP-positive cells were drawn on a paper sheet using a camera lucida device using darkfield optics and an atlas of the mouse brain (Franklin and Paxinos, 1997). The eGFP-positive cells were plotted and counted such that the ARH-hit and the ARH-missed were grouped as follows. Brains that had >100 eGFP-positive cells in the arcuate nucleus of the hypothalamus (all sections in a 1:5 series) and <100 eGFP-positive cells in any other nucleus known to contain $Lepr$-b mRNA (all sections in a 1:5 series) were scored as ARH-hit. Brains that had <100 eGFP-positive cells in any nucleus known to contain $Lepr$-b mRNA (all sections in a 1:5 series) were scored as ARH-missed. These criteria were established a priori. Also, an individual blinded to the neuroanatomic categorizations and physiological responses of each case performed this analysis.

Statistical analysis
Data sets were analyzed for statistical significance using PRISM 3.0 (GraphPad, San Diego, California) for a two-tailed unpaired Student's t test. Statistical comparisons shown in Figures 4B, 4D, 5A, 5C, 6A, 6B, and 6C were made by using one-way ANOVA (Turkey's post test). All parameters are expressed as mean ± SEM.

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