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Leptin-Dependent Control of Glucose Balance and Locomotor Activity by POMC Neurons

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SUMMARY

Leptin plays a pivotal role in regulation of energy balance. Via unknown central pathways, leptin also affects peripheral glucose homeostasis and locomotor activity. We hypothesized that, specifically, pro-opiomelanocortin (POMC) neurons mediate those actions. To examine this possibility, we applied *Cre-Lox* technology to express leptin receptors (ObRb) exclusively in POMC neurons of the morbidly obese, profoundly diabetic, and severely hypoactive leptin receptor-deficient $Lepr^{db/db}$ mice. Here, we show that expression of ObRb only in POMC neurons leads to a marked decrease in energy intake and a modest reduction in body weight in $Lepr^{db/db}$ mice. Remarkably, blood glucose levels are entirely normalized. This normalization occurs independently of changes in food intake and body weight. In addition, physical activity is greatly increased despite profound obesity. Our results suggest that leptin signaling exclusively in POMC neurons is sufficient to stimulate locomotion and prevent diabetes in the severely hypoactive and hyperglycemic obese $Lepr^{db/db}$ mice.

INTRODUCTION

Leptin, an adipocyte-derived hormone, acts on the central nervous system to regulate energy balance by activating the long form of the leptin receptor (ObRb or LEPR-B) (Friedman and Halaas, 1998). The absence of leptin or leptin receptors in $Lep^{ob/ob}$ mice or $Lepr^{db/db}$ mice, respectively, results in morbid obesity, hyperphagia, severe hyperglycemia and insulin resistance, and marked hypoactivity (Chen et al., 1996; Coleman, 1978; Lee et al., 1996; Pelleymounter et al., 1995; Tartaglia et al., 1995; Zhang et al., 1994). Among several leptin-responsive brain regions, the arcuate nucleus of the hypothalamus (ARC) is a key area for mediating leptin actions on energy homeostasis. Consistent with this, leptin receptor mRNA is densely expressed in the ARC of mice and rats (Elmquist et al., 1998; Mercer et al., 1996), and injection of leptin directly into the ARC is sufficient to acutely reduce food intake (Satoh et al., 1997). Moreover, unilateral restoration of leptin receptor expression in the ARC of leptin receptor-deficient $Lepr^{db/db}$ mice leads to long-term reduction of body weight and food intake (Coppari et al., 2005), and ARC-specific *Lepr* gene therapy is sufficient to attenuate the obesity phenotype of leptin receptor-deficient Koletsky fa^k/fa^k rats (Morton et al., 2003).

The ARC contains at least two subsets of leptin-responsive neurons, namely the anorexigenic pro-opiomelanocortin (POMC) neurons and the orexigenic Agouti-related peptide (AgRP) neurons. POMC neurons are depolarized by leptin, leading to release of α-melanocyte-stimulating hormone (α-MSH), a POMC-derived neuropeptide that mediates its anorexigenic effects through activation of melanocortin receptors (Cone, 2005; Cowley et al., 2001; Schwartz et al., 2000). AgRP is an endogenous melanocortin receptor antagonist that potently stimulates feeding (Ollmann et al., 1997). Consistent with this, AgRP neurons are inhibited by leptin (van den Top et al., 2004). Mice lacking leptin receptors only in POMC or AgRP neurons are mildly obese, demonstrating that both groups of cells are partly required for maintenance of body-weight homeostasis by leptin (Balthasar et al., 2004; van de Wall et al., 2007).

In addition to its role in energy homeostasis, leptin can regulate peripheral glucose and insulin homeostasis via the central nervous system. For example, leptin-deficient $Lep^{ob/ob}$ mice exhibit profound diabetes that can be fully prevented by low doses of leptin that do not affect body weight and food intake (Pelleymounter et al., 1995). In addition, intracerebroventricular leptin can acutely stimulate glucose uptake in skeletal muscle (Cusin et al., 1998; Haque et al., 1999; Kamohara et al., 1997; Minokoshi et al., 1999) and inhibit hepatic glucose production (Pocai et al., 2005; van den Hoek et al., 2008). Moreover, leptin dramatically improves insulin sensitivity in human lipodystrophy and in lipodystrophic mouse models, which are characterized by low serum leptin levels and by severe insulin resistance (Oral et al., 2002; Petersen et al., 2002; Shimomura et al., 1999). Genetic studies in mice suggest that the ARC plays a major role in mediating effects of leptin on glucose balance and on voluntary locomotor activity (Coppari et al., 2005); however, the specific arcuate neurons responsible remain unspecified.

Long-term impairment of central melanocortin receptor action in mice results in marked obesity, hyperinsulinemia, and late-onset hyperglycemia (Huszar et al., 1997). Insulin resistance is present before the onset of obesity in melanocortin-4-receptor-deficient mice (Fan et al., 2000). In addition, ventricular infusion of α-MSH into rats enhances acute insulin-stimulated peripheral glucose uptake and reduces hepatic glucose production, while a melanocortin receptor antagonist exerts opposite effects (Obici et al., 2001). Furthermore, loss of glucose sensing

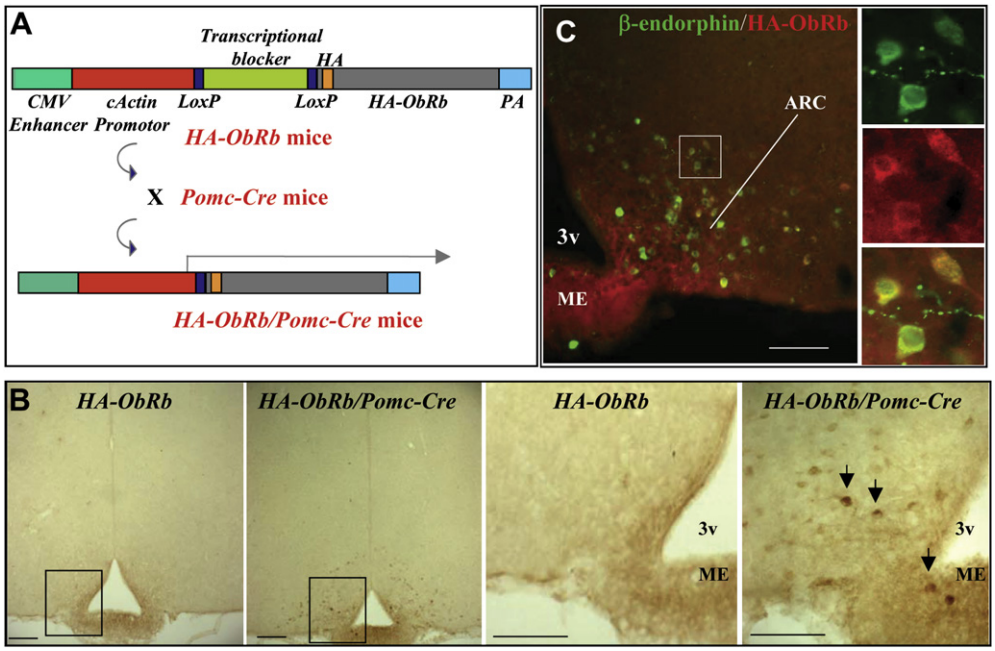

Figure 1. Generation of Mice Expressing HA-Tagged Leptin Receptors in POMC Neurons

(A) Strategy for generation of *HA-ObRb/Pomc-Cre* mice.

(B) HA-ObRb-expressing neurons of *HA-ObRb/Pomc-Cre* mice are located in the ARC, as shown by HA IHC. Right images: high magnification of areas marked in the left two photomicrographs. Arrows indicate examples of HA-positive neurons.

(C) HA-ObRb is expressed in POMC neurons of *HA-ObRb/Pomc-Cre* mice, as demonstrated by double-fluorescent IHC for HA (red) and β-endorphin (green). High-magnification images are shown on the right. ARC, arcuate nucleus; 3v, third ventricle; ME, median eminence. Scale bars, 50 μm.

by POMC neurons and of glucose-dependent α-MSH release leads to impaired whole-body glucose tolerance (Parton et al., 2007). Combined, these studies suggest a role of POMC neurons and the downstream melanocortin pathway in influencing peripheral insulin sensitivity and glucose balance. However, the extent to which POMC neuronal action can prevent severe hyperglycemia is unknown.

Given that arcuate POMC neurons express leptin receptors and that both leptin and the melanocortin system can influence glucose and insulin homeostasis, we hypothesized that POMC neurons mediate those leptin actions. In addition, yet-unspecified arcuate neurons can control locomotor activity by leptin. To that end, we generated mice expressing leptin receptors exclusively in POMC neurons of the severely diabetic and hypoactive *Lepr$^{db/db}$* mice.

RESULTS

Generation of Mice Expressing Leptin Receptors in POMC Neurons

Mice expressing HA-tagged ObRb in POMC neurons were generated by mating *HA-ObRb* transgenic mice with transgenic *Pomc-Cre* mice (Figure 1A). The HA tag at the N terminus of ObRb was introduced to facilitate detection of the leptin receptor protein. To first assess whether HA-ObRb was expressed in areas known to contain POMC neurons, we performed HA immunohistochemistry (IHC). As expected, HA immunoreactive (IR) cells were found in the ARC of *HA-ObRb/Pomc-Cre* mice (Figure 1B), but not in brains from *HA-ObRb* control mice (Figure 1B). Double HA and β-endorphin IHC analyses on brains from three *HA-ObRb/Pomc-Cre* mice showed that 44% (± 12%) of POMC neurons (3760 ± 440) in each animal expressed HA-ObRb. This percentage may be underestimated due to reduced sensitivity of the HA antibody combined with low HA-ObRb expression. Importantly, the vast majority (87% ± 9%) of detectable HA IR neurons were POMC neurons (Figure 1C).

The HA tag did not interfere with STAT3 activation by ObRb in transfected cells (not shown). To determine whether HA-tagged leptin receptors are also functional in vivo, we generated mice that express HA-ObRb in POMC neurons but lack endogenous long-form receptors (*HA-ObRb/Pomc-Cre/Lepr$^{db/db}$*) and measured leptin-dependent STAT3 phosphorylation in the brain. As predicted, leptin did not induce P-STAT3 in the hypothalamus of *HA-ObRb/Lepr$^{db/db}$* control mice (Figure 2A) or *Pomc-Cre/Lepr$^{db/db}$* control mice (not shown). In leptin-treated *Lepr$^{db/db}$* mice, we counted a baseline of 106 ± 39 P-STAT3 IR neurons in the entire ARC. In contrast, 1877 ± 612 P-STAT3-positive cells were found in the arcuate of leptin-treated *HA-ObRb/Pomc-Cre/Lepr$^{db/db}$* mice (Figure 2B) and 9540 ± 885 in wild-type mice (Figure 2C). Importantly, P-STAT3 was not detected in the ventromedial (VMH), the dorsomedial (DMH), or the premammillary (PMV) nuclei of *HA-ObRb/Pomc-Cre/Lepr$^{db/db}$* mice, regions known to express P-STAT3 in leptin-treated wild-type mice (Figure 2C) (Munzberg et al., 2004). A small group of POMC neurons exists in the nucleus of the solitary tract (NTS) of the caudal hindbrain (Palkovits and Eskay, 1987). Despite lack of detectable HA-positive neurons in this region (not shown), P-STAT3 IR cells were detected in the NTS of

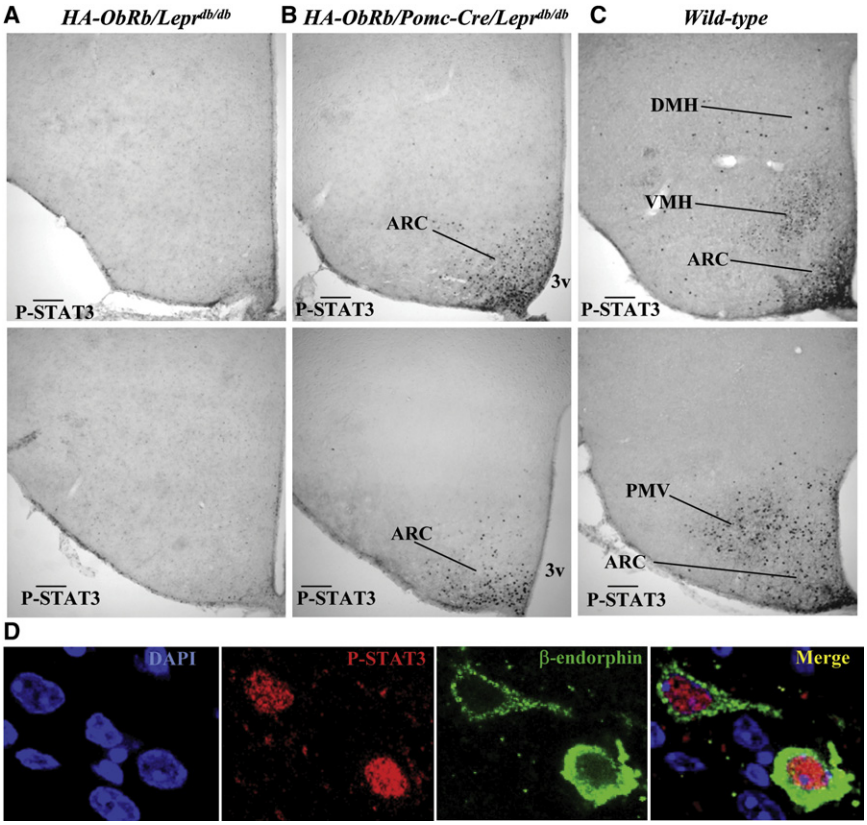

Figure 2. HA-Tagged Leptin Receptors in POMC Neurons Are Functional In Vivo

(A–C) Representative photomicrographs of P-STAT3 IHC in brains from leptin-treated (i.p. 4 mg/kg, 45 min) *HA-ObRb/Lepr$^{db/db}$* (A), *HA-ObRb/Pomc-Cre/Lepr$^{db/db}$* (B), and wild-type (C) mice. Matched medial (top row) and caudal (bottom row) coronal sections from the medio-basal hypothalamus are shown. ARC, arcuate nucleus; DMH, dorsomedial nucleus; VMH, ventromedial nucleus; PMV, ventral premammillary nucleus. Scale bars, 200 μm.

(D) Leptin activates nuclear P-STAT3 in two POMC neurons of *HA-ObRb/Pomc-Cre/Lepr$^{db/db}$* mice, as shown by confocal microscopy.

HA-ObRb/Pomc-Cre/Lepr$^{db/db}$ mice (not shown). The expression of HA-ObRb in the NTS is thus lower compared to the ARC, but still sufficient to activate STAT3.

Counts of β-endorphin and P-STAT3-positive neurons (Figure 2D) in leptin-treated *HA-ObRb/Pomc-Cre/Lepr$^{db/db}$* mice showed that 56% (± 24%) of POMC neurons expressed P-STAT3 and that 88% (± 8%) of the P-STAT3 IR cells were POMC neurons. Further, 60% (± 5%) of POMC neurons in leptin-treated wild-type mice and less than 2% of POMC neurons in *Lepr$^{db/db}$* mice expressed P-STAT3. To assess the expression level of functional HA-ObRb in POMC neurons of transgenic mice relative to endogenous leptin receptors in POMC neurons of wild-type mice, leptin-dependent STAT3 phosphorylation in individual POMC neurons was quantified. We found a 50% decreased level of P-STAT3 in *HA-ObRb/Pomc-Cre/Lepr$^{db/db}$* mice relative to control mice (Figure S1). These data, combined, demonstrate that HA-ObRb receptors in POMC neurons are functional in vivo and suggest that HA-ObRb levels in POMC neurons of transgenic *HA-ObRb/Pomc-Cre/Lepr$^{db/db}$* mice are modestly lower compared to wild-type mice.

Leptin Signaling in POMC Neurons of *Lepr$^{db/db}$* Mice Reduces Body Weight and Food Intake

Body weights of *HA-ObRb/Pomc-Cre/Lepr$^{db/db}$* mice were slightly decreased (10%–15%) as compared to *Lepr$^{db/db}$* mice but remained greatly increased (65%–90%) relative to wild-type mice (Figures 3A and 3B). Fat mass and circulating leptin levels tended toward a modest decrease in *HA-ObRb/Pomc-Cre/Lepr$^{db/db}$* mice versus *Lepr$^{db/db}$* mice (Figures 3E and 3F, Table 1). Body length (Table 1) and lean mass were not different among these groups (not shown). Food intake was markedly reduced (30%–40%) in *HA-ObRb/Pomc-Cre/Lepr$^{db/db}$* mice compared to *Lepr$^{db/db}$* control mice, but remained significantly higher than wild-type mice (Figures 3C and 3D). Whole-body energy expenditure as assessed by indirect calorimetry was not different between *HA-ObRb/Pomc-Cre/Lepr$^{db/db}$* and *Lepr$^{db/db}$* mice (Table 1). The respiratory exchange ratio was modestly increased in female but not male *HA-ObRb/Pomc-Cre/Lepr$^{db/db}$* mice relative to *Lepr$^{db/db}$* animals. Altogether, the data suggest that the decreased body weight of HA-ObRb/*Pomc*-Cre/*Lepr$^{db/db}$* mice is primarily due to reduced energy intake.

Normalization of Glucose by ObRb in POMC Neurons in *Lepr$^{db/db}$* Mice

Expression of HA-ObRb in POMC neurons of *Lepr$^{db/db}$* mice dramatically improves blood glucose levels. Remarkably, in 5-week-old *HA-ObRb/Pomc-Cre/Lepr$^{db/db}$* mice, blood glucose levels were fully normalized (Figures 4A–4D). This normalization was also observed in overnight-fasted female and male *HA-ObRb/Pomc-Cre/Lepr$^{db/db}$* mice (not shown). To examine whether this reduction in circulating glucose in *HA-ObRb/Pomc-Cre/Lepr$^{db/db}$* mice was independent of energy intake, 8-week-old female *Lepr$^{db/db}$* mice were pair-fed (~30% intake reduction) to ad libitum-fed *HA-ObRb/Pomc-Cre/Lepr$^{db/db}$* littermates for 3 weeks (Figure 4F). As shown in Figure 4E, this food restriction did not reduce hyperglycemia in *Lepr$^{db/db}$* mice. During the 3 weeks, the average body weight of *Lepr$^{db/db}$* mice fell 3.5 g (42.1 [± 1.9] to 38.6 [± 1.2]). In comparison, ad libitum-fed *Lepr$^{db/db}$* mice with the same age gained 8.6 g (40.4 [± 1.4] to 49.0 [± 1.2]) during 3 weeks (Figure 3A), altogether demonstrating that the pair-feeding paradigm greatly impacted the metabolic status of *Lepr$^{db/db}$* mice and that this alone was insufficient to attenuate hyperglycemia. Therefore, expression of leptin receptors in POMC neurons of *Lepr$^{db/db}$* mice is sufficient to correct hyperglycemia independently of changes in energy intake and body weight.

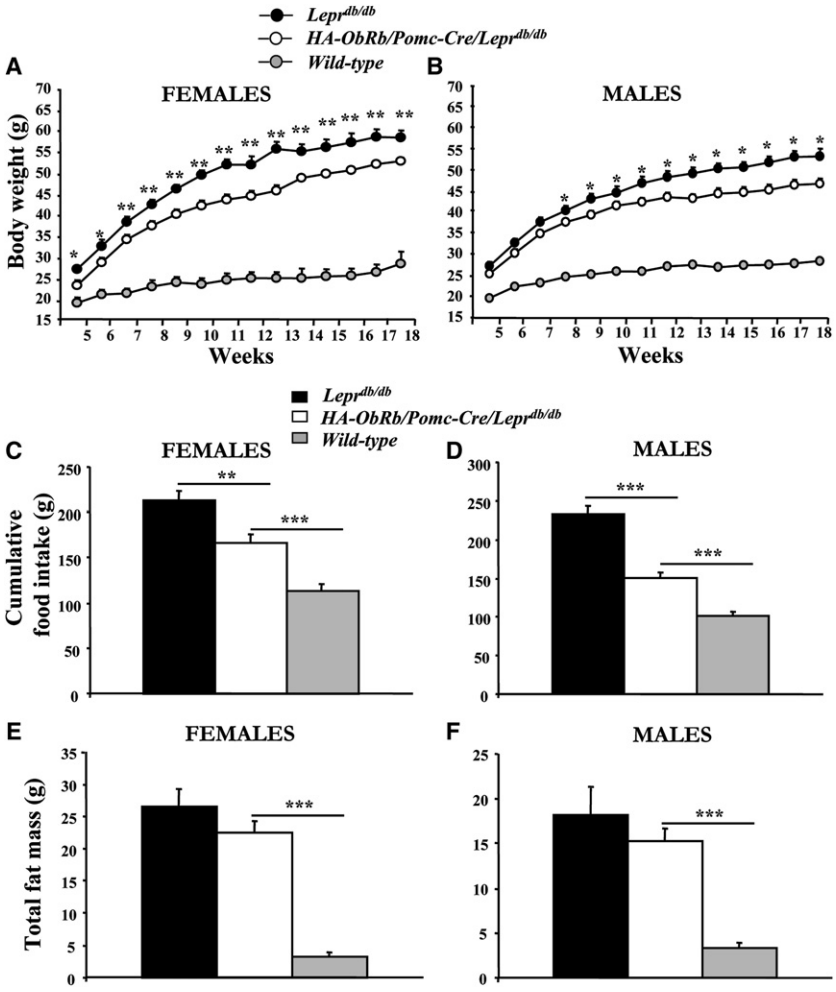

Figure 3. Expression of HA-ObRb in POMC Neurons of *Lepr*$^{db/db}$ Mice Reduces Body Weight and Food Intake

(A–D) Body-weight curves (A and B) and cumulative food intake (7–9 weeks) (C and D). Data were collected from 11 female and 14 male *Lepr*$^{db/db}$ mice, 11 female and 17 male *HA-ObRb/Pomc-Cre/Lepr*$^{db/db}$ mice, and 5 female and 9 male wild-type mice.

(E and F) Adipose mass (12 weeks of age). Fat mass was measured by Echo-MRI in 5 female and 4 male *Lepr*$^{db/db}$ mice, 5 female and 7 male *HA-ObRb/Pomc-Cre/Lepr*$^{db/db}$ mice, and 8 female and 6 male wild-type mice. All animals are littermates. *$p < 0.05$; **$p < 0.01$, ***$p < 0.001$. Error bars are shown as SEM.

Serum insulin levels were reduced by 60% in *HA-ObRb/Pomc-Cre/Lepr*$^{db/db}$ mice compared to *Lepr*$^{db/db}$ control mice, but elevated relative to control animals (Figure 4G). Insulin tolerance was significantly improved in *HA-ObRb/Pomc-Cre/Lepr*$^{db/db}$ mice relative to *Lepr*$^{db/db}$ mice over time ($p = 0.021$) and trended toward a decrease (0–30 min) compared to wild-type mice ($p = 0.097$) (Figure 4H). In 12-week-old fasted *HA-ObRb/Pomc-Cre/Lepr*$^{db/db}$ mice, blood glucose levels were markedly reduced as compared to *Lepr*$^{db/db}$ mice (Figure S2A), despite only a slight decrease in weight (Figure S2B). Fed glucose was similarly attenuated (not shown). To further assess whether the reduced glucose levels occurred independently of reduced body weight, we selected mice from the *Lepr*$^{db/db}$ and *HA-ObRb/Pomc-Cre/Lepr*$^{db/db}$ groups such that the average body weights of both subgroups were not different (Figure S2D). Despite equal body weights, glucose remained reduced in *HA-ObRb/Pomc-Cre/Lepr*$^{db/db}$ mice (Figure S2C), thus further supporting the notion that leptin signaling in POMC neurons of *Lepr*$^{db/db}$ mice is sufficient to reduce circulating glucose levels independent of body weight. Expression of HA-ObRb in POMC neurons of lean non-*Lepr*$^{db/db}$ mice did not affect body weight or glucose levels on the mixed (FVB/C57BLKSJ) genetic background (Figures S3A and S3B). Similarly, *HA-ObRb/Pomc-Cre* mice on a pure FVB background were indistinguishable from wild-type littermates with regard to body weight, food intake, circulating glucose and insulin, and glucose and insulin tolerance (Figures S4 and S5).

Leptin Receptors in POMC Neurons of *Lepr*$^{db/db}$ Mice Increase Islet Mass

Pancreatic islets in *Lepr*$^{db/db}$ mice have altered morphology and impaired function (Baetens et al., 1978; Leiter et al., 1983). To indirectly assess islet function in *HA-ObRb/Pomc-Cre/Lepr*$^{db/db}$ mice, we investigated islet morphology, islet size, and total endocrine mass (Figure S6). Total endocrine mass was increased by 2.5-fold in *HA-ObRb/Pomc-Cre/Lepr*$^{db/db}$ mice as compared to both *Lepr*$^{db/db}$ and wild-type mice and was caused by increased islet size. The total number of islets was similar among the three groups (not shown). Islets from *HA-ObRb/Pomc-Cre/Lepr*$^{db/db}$ mice appeared structurally intact. In contrast, *Lepr*$^{db/db}$ islets exhibited variable size and irregular morphology. The total islet mass did not correlate with circulating insulin levels between *Lepr*$^{db/db}$ and *HA-ObRb/Pomc-Cre/Lepr*$^{db/db}$ groups, indicating differential β cell regulation and/or functionality.

Leptin Receptors in POMC Neurons Increase Activity

Restoration of leptin receptors in arcuate neurons greatly increases voluntary locomotor activity in *Lepr*$^{db/db}$ mice (Coppari et al., 2005), but the specific neurons mediating this action remain unknown. We therefore measured ambulatory movements of *HA-ObRb/Pomc-Cre/Lepr*$^{db/db}$ mice and found a remarkable increase (~100%) compared to *Lepr*$^{db/db}$ mice (Figure 5). The activity of *HA-ObRb/Pomc-Cre/Lepr*$^{db/db}$ mice was not statistically different from lean wild-type mice, although there was a trend toward a reduction.

Effects of Leptin Signaling in POMC Neurons on Neuropeptides

HA-ObRb/Pomc-Cre/Lepr$^{db/db}$ mice had modestly increased hypothalamic POMC mRNA (Figure 6A), consistent with a role of leptin signaling to stimulate *Pomc* gene expression (Munzberg

Table 1. Body Length, O_2 Consumption, CO_2 Production, Respiratory Exchange Ratio, Leptin, and Triglycerides

	Lepr$^{db/db}$	*HA-ObRb/Pomc-Cre/Lepr*$^{db/db}$
Nose/anus length (cm)		
Males	10.6 ± 0.2 (5)	10.5 ± 0.2 (7)
Females	10.7 ± 0.1 (7)	10.7 ± 0.1 (8)
Leptin (ng/ml)		
Males	100.4 ± 8.5 (17)	91.7 ± 5.4 (12)
Females	106.8 ± 6.7 (11)	96.1 ± 6.9 (11)
Triglycerides (mg/dl)		
Males	223.1 ± 21.9 (7)	236.9 ± 33.3 (11)
Females	220.3 ± 19.2 (10)	180.1 ± 21.3 (8)
Respiratory Exchange Ratio (RER)		
Males	0.86 ± 0.03 (6)	0.91 ± 0.03 (4)
Females	0.80 ± 0.04 (7)	0.89 ± 0.03 (5) *
VO_2 (ml/day)		
Males	7639 ± 730 (6)	5844 ± 885 (4)
Females	5566 ± 552 (7)	5408 ± 691 (5)
VCO_2 (ml/day)		
Males	6613 ± 758 (6)	5335 ± 779 (4)
Females	4406 ± 728 (7)	4984 ± 521 (5)

Serum analyses and body length were measurements in 18-week-old mice. O_2 consumption, CO_2 production, and RER were measured at 13 weeks of age. The number of mice per group is shown in parentheses. *$p < 0.05$.

et al., 2003; Thornton et al., 1997). Moreover, total hypothalamic α-MSH peptide levels were 3.5-fold increased in *HA-ObRb/Pomc-Cre/Lepr*$^{db/db}$ mice relative to *Lepr*$^{db/db}$ mice and were similar to wild-type levels (Figure 6D). The increase in α-MSH was observed both in the arcuate and in POMC fibers innervating the DMH (Figure 6G). NPY and AgRP neuropeptide levels were unchanged despite slight changes in AgRP and NPY mRNA (Figures 6B, 6C, 6E, and 6F).

DISCUSSION

Through actions in the CNS, leptin affects a wide number of biological systems and processes, including feeding behavior, body weight balance, neuroendocrine function, insulin sensitivity, glucose homeostasis, and physical activity, demonstrating pleiotrophic properties of the hormone. However, little is known about which specific neuronal groups mediate each of leptin's actions. We show here that expression of ObRb only in POMC neurons is sufficient to correct diabetes and drastically stimulate locomotor activity of the leptin receptor-deficient *Lepr*$^{db/db}$ mice.

Restoration of leptin receptors in arcuate neurons normalizes blood glucose levels and voluntary locomotor activity in mice lacking all leptin receptors (Coppari et al., 2005). However, the specific arcuate neurons responsible remained unspecified. Here we extend those findings by identifying POMC neurons as the likely mediators of those effects of leptin. Interestingly, deletion of ObRb from only POMC neurons of lean mice does not lead to hyperglycemia (Balthasar et al., 2004) or hypoactivity (Shi et al., 2008), suggesting that leptin receptors in POMC neurons are not required for normal regulation of glucose balance and locomotion. In contrast, our studies demonstrate that leptin receptor signaling in POMC neurons is sufficient to exert remarkable control of serum glucose concentrations and locomotion. These data combined could be explained by the existence of several groups of leptin-responsive neurons that are each capable of independently regulating glucose homeostasis and physical activity.

The specific mechanism by which leptin signaling in POMC neurons leads to normalization of blood glucose is unknown, but likely involves neuroendocrine processes and/or autonomic actions. Our data suggest that the normalization is a direct consequence of leptin action in POMC neurons and is not secondary to differences in energy consumption or body weight. Consistent with this, central leptin administration can acutely increase muscle glucose uptake (Kamohara et al., 1997) and decrease hepatic glucose production (van den Hoek et al., 2008) through altered autonomic output (Haque et al., 1999). Furthermore, the central melanocortin pathway can increase sympathetic drive to peripheral tissues and enhance insulin-dependent glucose disposal (Brito et al., 2007; Heijboer et al., 2005; Rahmouni et al., 2003). Therefore, since *HA-ObRb/Pomc-Cre/Lepr*$^{db/db}$ mice have greatly elevated hypothalamic levels of α-MSH peptides, increased activity of the melanocortin system may contribute to the normoglycemic phenotype, which likely results in part from increased insulin sensitivity and enhanced islet function/capacity. Whether the increased islet mass is a direct result of leptin action via POMC neurons and CNS regulation (Imai et al., 2008) or an indirect consequence of altered metabolic state or incretin action (Karaca et al., 2009) remains to be elucidated. Moreover, detailed experiments are required to determine the specific role of the melanocortin system and the relative contribution of the pancreas and insulin-sensitive tissues, such as muscle and liver, in the normalization of serum glucose levels.

Exercise training increases insulin sensitivity and improves glucose homeostasis (Hespel et al., 1996; Ryder et al., 2001). It is therefore possible that insulin sensitivity and whole-body glucose balance of *HA-ObRb/Pomc-Cre/Lepr*$^{db/db}$ mice is enhanced by the increased physical activity. A role of skeletal muscle AMP-activated protein kinase (AMPK) in mediating exercise-dependent stimulation of insulin sensitivity and glucose uptake has been reported (Musi and Goodyear, 2003). In addition, central administration of melanocortin receptor agonists is sufficient to activate muscle AMPK phosphorylation in mice (Tanaka et al., 2007). The extent to which increased locomotion may contribute to the lowered circulating glucose concentration remains to be determined.

The marked increase in locomotion of mice expressing ObRb exclusively in POMC neurons could be due to their improved metabolic status or to a direct effect of leptin action via POMC neurons. In favor of the latter possibility, genetically obese rodents such as melanocortin-4-receptor knockout mice have reduced physical activity before they become obese (Ste Marie et al., 2000). In addition, AgRP-deficient mice are hyperactive (Wortley et al., 2005), and recombinant AgRP decreases locomotor activity in normal mice (Tang-Christensen et al., 2004), further suggesting a role of the melanocortin pathway. Moreover, deletion of leptin receptors from AgRP neurons results in a modest reduction in activity (van de Wall et al., 2007), and

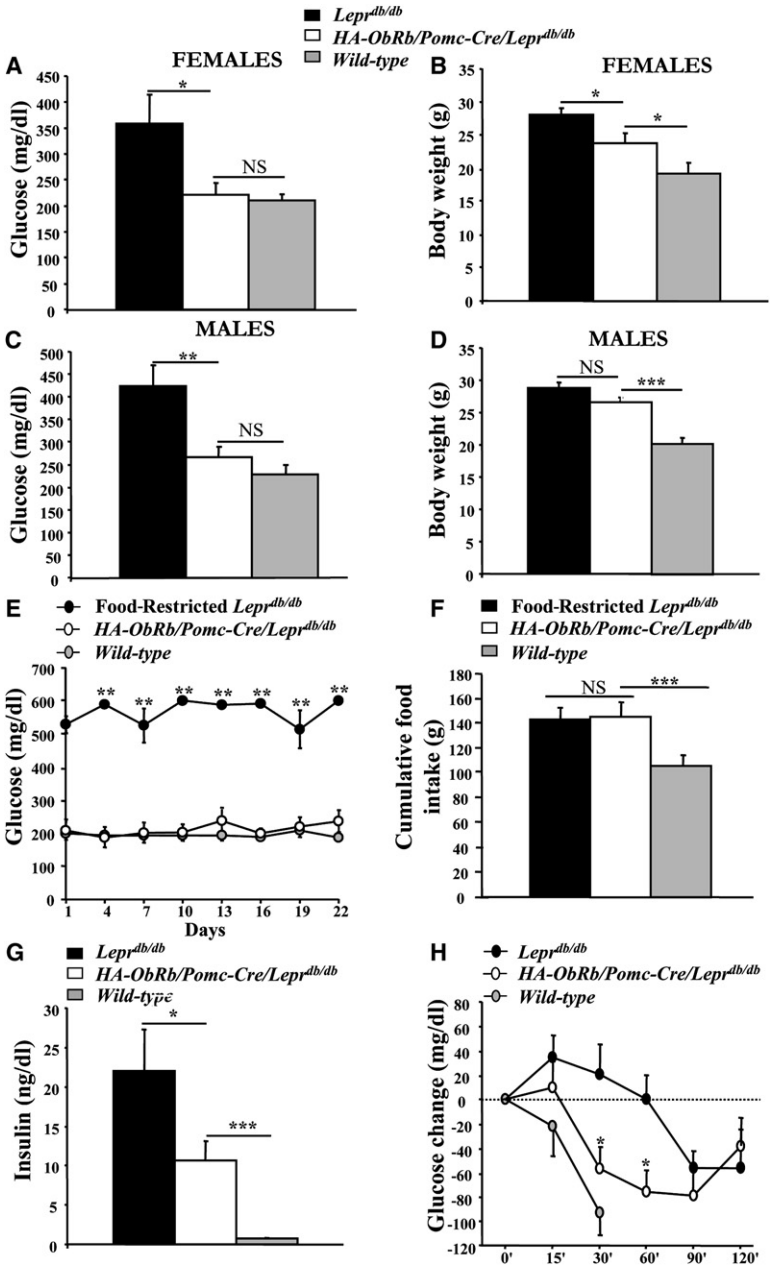

Figure 4. Expression of HA-ObRb in POMC Neurons of *Lepr*$^{db/db}$ Mice Normalizes Circulating Glucose Levels and Increases Insulin Sensitivity

(A and B) Fed glucose levels (A) and body weight (B) of 5-week-old female *Lepr*$^{db/db}$ mice (n = 7), *HA-ObRb/Pomc-Cre/Lepr*$^{db/db}$ mice (n = 8), and wild-type mice (n = 8).

(C and D) Fed glucose levels (C) and body weight (D) of 5-week-old male *Lepr*$^{db/db}$ mice (n = 11), *HA-ObRb/Pomc-Cre/Lepr*$^{db/db}$ mice (n = 12), and wild-type mice (n = 14).

(E) Glucose levels in food-restricted *Lepr*$^{db/db}$ mice. Over a period of 3 weeks, 8-week-old female *Lepr*$^{db/db}$ mice (n = 4) were pair-fed to ad libitum-fed *HA-ObRb/Pomc-Cre/Lepr*$^{db/db}$ littermates (n = 4). Wild-type littermates (n = 6) were fed ad libitum.

(F) Cumulative intake over the 3 weeks of pair-feeding.

(G) Fasted serum insulin levels of 12-week-old female *Lepr*$^{db/db}$ mice (n = 7), *HA-ObRb/Pomc-Cre/Lepr*$^{db/db}$ mice (n = 8), and wild-type mice (n = 8).

(H) Insulin tolerance tests in 13-week-old female *Lepr*$^{db/db}$ mice (n = 7), *HA-ObRb/Pomc-Cre/Lepr*$^{db/db}$ mice (n = 8), and 4 wild-type mice (*, *Lepr*$^{db/db}$ versus *HA-ObRb/Pomc-Cre/Lepr*$^{db/db}$). Glucose was administered to wild-type mice after 30 min to prevent severe hypoglycemia caused by the high insulin dose (1.5 U/kg). Shown are changes from baseline. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; NS, not significant. Error bars are shown as SEM.

constitutive STAT3 signaling specifically in AgRP neurons increases locomotion (Mesaros et al., 2008). We did not find altered levels of AgRP in *HA-ObRb/Pomc-Cre/Lepr*$^{db/db}$ mice, but the increase in hypothalamic α-MSH peptides supports the possibility of a role of the melanocortin pathway in stimulating locomotor activity in these mice.

In contrast to the striking effects of leptin signaling in POMC neurons on blood glucose and locomotor activity in *Lepr*$^{db/db}$ mice, regulation of body weight appears only partially mediated by POMC neurons. At 12 weeks, *HA-ObRb/Pomc-Cre/Lepr*$^{db/db}$ mice are ~10% lighter relative to mice lacking all leptin receptors. It is, however, possible that the difference in weight between *HA-ObRb/Pomc-Cre/Lepr*$^{db/db}$ and *Lepr*$^{db/db}$ mice would be greater if *Lepr*$^{db/db}$ mice had not been diabetic and therefore lost calories through the urine. The contribution of POMC neurons in leptin's regulation of body weight might thus be underestimated. However, our result showing a modest contribution of POMC neurons on body weight is consistent with data from nondiabetic 12-week-old mice, where deletion of leptin receptors from POMC neurons results in a 10% weight increase (Balthasar et al., 2004). Therefore, both deletion and restoration data show that leptin signaling elsewhere in the CNS is required for leptin's full effect on long-term body-weight regulation. In further support of this, deletion of ObRb in the VMH in 12-week-old mice increases body weight by ~10%, and a combined loss in POMC and VMH neurons increases weight by 25%, suggesting additive effects (Dhillon et al., 2006). Further, deletion of leptin receptors selectively from AgRP neurons results in a 16% increase in body weight in 8- to 12-week-old mice, and combined ObRb deletion from both AgRP and POMC neurons increases body weight by ~28% (van de Wall et al., 2007). Body weight regulation by leptin is therefore likely distributed between several brain nuclei and cell types, each having partial but additive roles.

We found a marked 30%–40% reduction in energy intake in mice expressing leptin receptors solely in POMC neurons relative to *Lepr*$^{db/db}$ mice, but did not detect changes in whole-body O_2 consumption. The greatly increased locomotor activity therefore does not appear to significantly impact whole-body energy expenditure, and the reduction in food intake likely accounts for the decrease in body weight of *HA-ObRb/Pomc-Cre/Lepr*$^{db/db}$ mice. This suggests that POMC neurons play a partial role in attenuating food intake by leptin, but have little

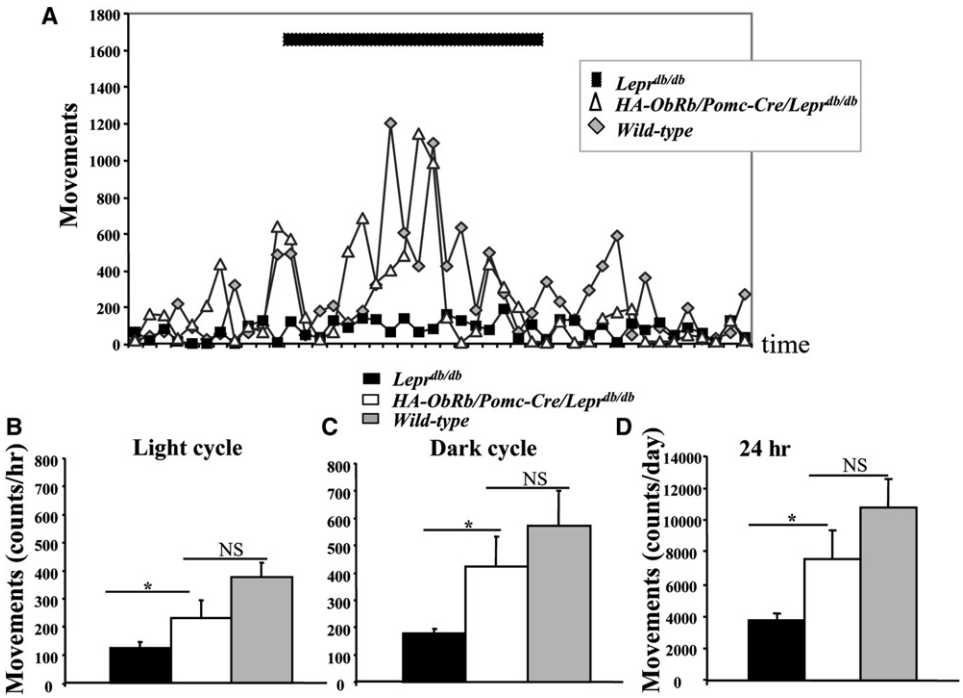

Figure 5. Increased Activity of *Lepr*$^{db/db}$ Mice Expressing HA-ObRb in POMC Neurons

(A) Examples of locomotor activity from 1 *Lepr*$^{db/db}$, 1 *HA-ObRb/Pomc-Cre/Lepr*$^{db/db}$, and 1 wild-type mouse. Black bar depicts lights-off period.

(B–D) Average nocturnal (B), diurnal (C), and 24 hr (D) locomotor activity in 13-week-old male *Lepr*$^{db/db}$ mice (n = 4), *HA-ObRb/Pomc-Cre/Lepr*$^{db/db}$ mice (n = 6), and wild-type mice (n = 5). *p < 0.05; NS, not significant. Error bars are shown as SEM.

if any independent role in leptin's action to increase energy expenditure (Hwa et al., 1997).

In addition to the hypothalamus, a small group of POMC neurons exists in the NTS of the caudal brainstem (Palkovits and Eskay, 1987). The *Cre-loxP* strategy was expected to induce expression of HA-ObRb both in arcuate and NTS POMC neurons. Indeed, STAT3 phosphorylation was detected in both sites of leptin-treated *HA-ObRb/Pomc-Cre/Lepr*$^{db/db}$ animals. However, in normal mice, NTS POMC neurons do not appear respond to leptin, as measured by lack of leptin-inducible STAT3 phosphorylation (Huo et al., 2006), although another group reported the opposite results using a similar strategy (Ellacott et al., 2006). The reason for the discrepancy between the two studies is yet unclear. Regardless, since leptin receptors are expressed in NTS neurons of *HA-ObRb/Pomc-Cre/Lepr*$^{db/db}$ mice, a possible role of these cells in mediating some of the phenotypic changes in *HA-ObRb/Pomc-Cre/Lepr*$^{db/db}$ animals cannot be excluded. However, restoration of endogenous leptin receptors solely in the ARC, accomplished by stereotaxic delivery of adenoviruses expressing FLPe-recombinase in otherwise leptin-receptor-deficient mice, also results in a partial reduction in body weight and food intake and a normalization of serum glucose levels and of locomotor activity (Coppari et al., 2005). Since these findings are directly comparable to the phenotype of our *HA-ObRb/Pomc-Cre/Lepr*$^{db/db}$ mice, we conclude that arcuate POMC neurons, but not NTS POMC neurons, are responsible for mediating those effects of leptin.

Approximately 50% of hypothalamic POMC neurons express functional HA-leptin receptors in the transgenic mice, even though the *Cre-loxP* strategy might have predicted a higher fraction. The reason for this is unclear. However, we found that in wild-type mice, only about 60% of POMC neurons respond to leptin, as measured by STAT3 phosphorylation. Furthermore, unilateral, not bilateral, restoration of endogenous leptin receptors in arcuate neurons of leptin-receptor-deficient mice (Coppari et al., 2005) results in a phenotype strikingly similar to that of our *HA-ObRb/Pomc-Cre/Lepr*$^{db/db}$ mice. These data combined therefore suggest that leptin signaling in ~50% of hypothalamic POMC neurons is sufficient to normalize blood glucose levels and stimulate physical activity.

In conclusion, our data show that leptin signaling specifically in hypothalamic POMC neurons is sufficient to correct diabetes and greatly increase locomotion in *Lepr*$^{db/db}$ mice, revealing a remarkable regulatory capacity of POMC neurons.

EXPERIMENTAL PROCEDURES

Animal Care

Animal care and procedures were approved by the Institutional Animal Care and Use Committee at Beth Israel Deaconess Medical Center. Mice were housed at 22°C–24°C using a 14 hr light/10 hr dark cycle with chow food (Teklad F6 Rodent Diet 8664, Harlan Teklad; Madison, WI).

Generation of *HA-ObRb* Transgenic Mice

A 6.8 kb *HA-ObRb STOP* transgene was generated, consisting of the following elements (5′ to 3′): (1) a 1.6 kb *CAG* promoter that combines the human cytomegalovirus (*CMV*) enhancer, a chicken β-*actin* promoter, and the first intron; (2) a *loxP* site; (3) a *STOP* sequence containing a false translational *stop* sequence and an SV40 splice donor/poly(A) site; (4) a *loxP* site; (5) an HA-tagged murine leptin receptor (*HA-ObRb*) cDNA sequence (the HA epitope

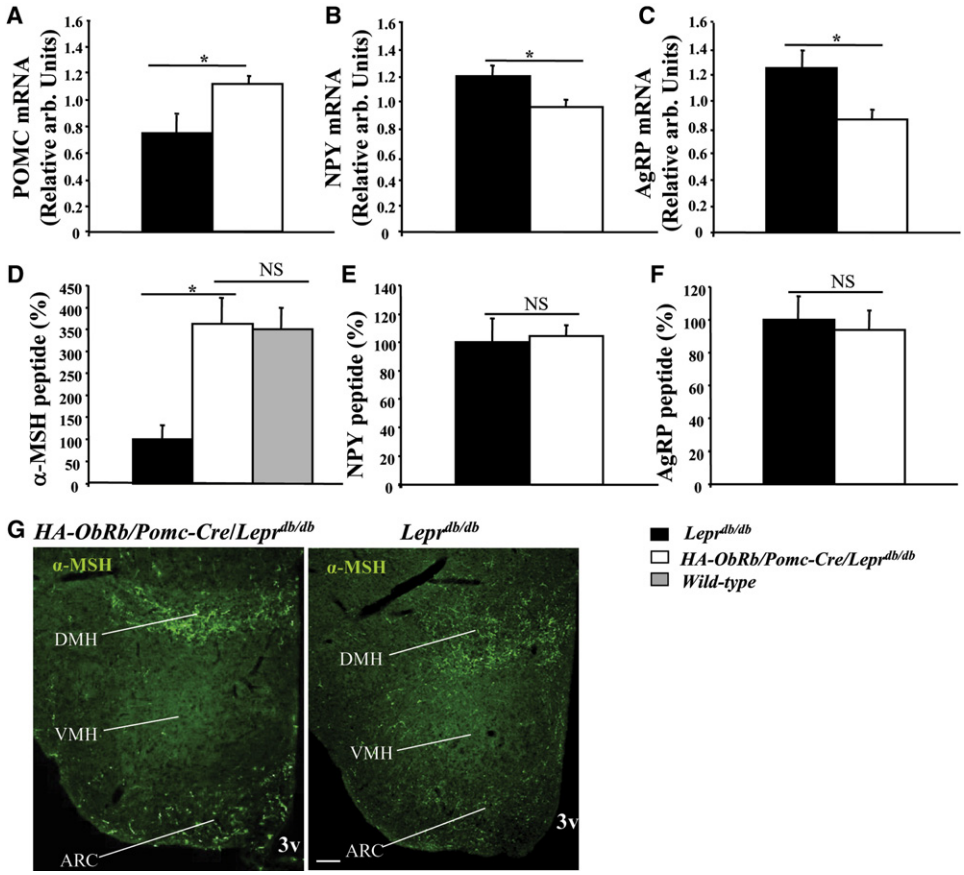

Figure 6. Hypothalamic POMC mRNA and α-MSH Peptide Levels Are Increased in *Lepr*$^{db/db}$ Mice Expressing Leptin Receptors Exclusively in POMC Neurons

(A–C) Arcuate POMC, NPY, and AgRP mRNA from 18-week-old control *Lepr*$^{db/db}$ mice (n = 7) and *HA-ObRb/Pomc-Cre/Lepr*$^{db/db}$ mice (n = 5).

(D–F) Hypothalamic α-MSH, NPY, and AgRP neuropeptide levels as measured by EIAs in 18-week-old *Lepr*$^{db/db}$ mice (n = 3), *HA-ObRb/Pomc-Cre/Lepr*$^{db/db}$ mice (n = 3), and wild-type mice (n = 3). Numbers are depicted as percent of *Lepr*$^{db/db}$ levels. *$p < 0.05$; **$p < 0.01$; NS, not significant.

(G) Representative images of hypothalamic α-MSH immunostaining in 18-week-old *HA-ObRb/Pomc-Cre/Lepr*$^{db/db}$ and *Lepr*$^{db/db}$ mice. Scale bar, 200 μm. Error bars are shown as SEM.

[TyrProTyrAspValProAspTyrAla] was inserted after residue 42 of the leptin receptor); and (6) a bovine growth hormone poly(A) sequence. Briefly, the 6.8 kb construct was produced by first cloning the *HA-ObRb* cDNA on a NotI/HindIII fragment from a plasmid generously given by Dr. J. Flier (Harvard Medical School; Boston) into pGEM-11Zf(+) (Promega; Madison, WI). The *PA* sequence with flanking HindIII and SfiI restriction sites was amplified from pcDNA3.1Zeo(−) (Invitrogen; Carlsbad, CA) using the following primer set: 5′-CCCAAGCTTAAGTTTAAACCGCTGATCAGC-3′ and 5′-CCCAAGCTTGGC CATGCAGGCCGCCATAGAGCCCACCGCATCC-3′. It was then cloned into a HindIII site downstream of the *HA-ObRb* cDNA. A SalI/NotI fragment encompassing the *loxP*-flanked *STOP* sequence from plasmid cAct-XstopXnz (a generous gift from Dr. Anderson, California Institute of Technology; Pasadena, CA) (Tsien et al., 1996) was then inserted upstream of the *HA-ObRb* cDNA. Finally, the *CAG* promoter isolated from pDRIVE-CAG (InvivoGen; San Diego, CA) was cloned into a SalI site located upstream of the *loxP*-flanked *STOP* sequence. Transgenic mice were generated by microinjecting the 6.8 kb SfiI *HA-ObRb STOP* DNA fragment into fertilized one-cell embryos (FVB strain) by the transgenic facility at Beth Israel Deaconess Medical Center; Boston.

Generation of *HA-ObRb/Pomc-Cre* Mice

HA-ObRb transgenic mice (FVB background) were mated with transgenic *Pomc-Cre* mice (FVB), which have been described earlier (Balthasar et al., 2004; van de Wall et al., 2007) and were kindly supplied by Dr. Lowell (BIDMC).

Generation of *HA-ObRb/Pomc-Cre/Lepr*$^{db/db}$ Mice

Lepr$^{db/+}$ (C57BLKSJ, stock #00642) mice were purchased from Jackson Laboratory (Bar Harbor, ME). *HA-ObRb/Pomc-Cre* mice (FVB) were then mated with *Lepr*$^{db/+}$ mice. *HA-ObRb/Pomc-Cre/Lepr*$^{db/db}$ mice were obtained by intermating of *HA-ObRb/Pomc-Cre/Lepr*$^{db/+}$ mice. Only littermates with the same mixed genetic background (FVB/C57BLKSJ) were compared, except for Figures S4 and S5, where pure FVB non-*Lepr*$^{db/db}$ mice were studied.

Materials

Murine leptin was purchased from Dr. E. Parlow (NIDDK; Torrance, CA), and human insulin was from Eli Lilly (Indianapolis, IN). Supplies for IHC were from Sigma-Aldrich (St. Louis) and the ABC VECTASTAIN Elite kit from Vector Laboratories (Burlingame, CA). The phospho-specific-(Y705)-STAT3 rabbit antibody was from New England Biolabs (Beverly, MA); the anti-HA mouse antibody from Covance, Inc. (Berkeley, CA); and the rabbit anti-β-endorphin antibody was a kind gift from Dr. Ronnekleiv (Oregon Health and Science University; Portland, OR) (Qiu et al., 2003). Sheep anti-α-MSH antiserum was from Chemicon International, Inc. (Temecula, CA). The biotinylated donkey anti-mouse antibody and the biotinylated donkey anti-rabbit antibody were from Jackson ImmunoResearch Laboratories (West Grove, PA). Fluorescent donkey anti-rabbit, fluorescent donkey anti-sheep, and fluorescent donkey anti-mouse immunoglobulin conjugates were from Molecular Probes (Eugene, OR), and donkey serum was from Invitrogen.

Immunohistochemistry

Twenty-five micrometer coronal brain sections were generated as described earlier (Huo et al., 2006). For nonfluorescent HA IHC, brain sections were pretreated with citrate buffer for 30 min at 80°C. Sections were incubated with anti-HA antibody (1:250) and biotinylated anti-mouse antibodies (1:1000), followed by avidin-biotin complex labeling, and developed with Nickel-diaminobenzidine (DAB). For double fluorescence HA and β-endorphin IHC, sections were incubated with anti-HA (1:250) and anti-β-endorphin (1:5000) and then with fluorescent-labeled (red or green) secondary antibodies. P-STAT3 IHC was performed as described earlier (Huo et al., 2006). For α-MSH fluorescence IHC, sections were incubated with anti-α-MSH (1:20,000) and fluorescent-labeled secondary antibodies generating green fluorescence. Results were visualized using fluorescent or bright-field light and captured with a digital camera (AxioCam, Carl Zeiss; Thornwood, NY) mounted on a Zeiss microscope (Axioscope 2). To visualize double-labeled cells, Adobe Photoshop software (Adobe; San Jose, CA) was used to merge fluorescence via RGB channels. Single- and double-labeled cells were counted bilaterally in every fifth section of one brain series and multiplied with five to obtain a total for the entire hypothalamus, as we have done earlier (Huo et al., 2006). Confocal laser scanning microscopy was performed using a Zeiss LSM510 system.

Quantification of Leptin-Induced STAT3 Phosphorylation

HA-ObRb/Pomc-Cre/Lepr$^{db/db}$ and wild-type littermates were injected i.p. with leptin (45 min). Brain sections were subjected in parallel to double IHC for P-STAT3 (DAB) and β-endorphin (fluorescent) (Huo et al., 2006). Digital images were obtained with a Zeiss Axioscope. Nuclear DAB staining in POMC neurons from three anatomically comparable brain sections from each mouse was quantified using ImageQuant software (GE Healthcare; Piscataway, NJ). In total, P-STAT3 was quantified in 204 random POMC neurons from wild-type mice (n = 3) and in 234 POMC neurons from transgenic mice (n = 3).

Blood Composition, Insulin Tolerance, and Glucose Tolerance Tests

Tail vein blood was collected at 12:00 p.m. ± 2 hr from either ad libitum-fed or fasted mice. Blood glucose was assayed with OneTouch Ultra Blood Glucose Monitoring System (Fisher Scientific; Morrison Plains, NJ). ELISAs were used to measure serum insulin and leptin (Crystal Chem Inc.; Downers Grove, IL) and triglycerides (Thermo Fisher Scientific, Inc.; Waltham, MA). For insulin tolerance tests, food was removed for 5 hr, and blood glucose concentrations were measured at 15, 30, 60, 90, and 120 min after i.p. injection of insulin (1.0 or 1.5 U/kg). For glucose tolerance tests, food was removed for 15 hr, and blood glucose concentrations were measured at 15, 30, 60, 90, and 120 min after i.p. injection of D-glucose (2 mg/g).

Pancreas Histology

Mice (11–20 weeks) were sacrificed by cervical dislocation. Pancreata were fixed in 10% phosphate-buffered formalin and paraffin embedded. Each pancreas was cut in serial 5 μm sections, and every 20th section was H&E stained and analyzed. Following digital image acquisition using a CCD camera on a Zeiss microscope, islet and exocrine areas (after exclusion of vessels and connective tissue) were measured in each section with Adobe Photoshop and AxioVision software. At 5× magnification, 1 pixel equals 2.126 square μm. For each pancreas, the total number of islets, total islet (endocrine) area, and total exocrine areas were determined. The relative islet mass ([total islet area]/[total islet area + exocrine area] × 100) and the mean islet size (total islet area/number of islets) were then calculated.

Oxygen Consumption and Locomotor Activity

Oxygen consumption, CO_2 production, and physical activity were measured in 13-week-old mice using a comprehensive lab animal monitoring system (CLAMS, Columbia Instruments; Columbus, OH). Mice were acclimated in the monitoring chambers for 2 days followed by data collection for 3 days.

Hypothalamic Neuropeptide mRNA

Mice were euthanized by cervical dislocation. Brains were removed, arcuate-enriched tissue isolated, and cDNA were generated as described earlier (Huo et al., 2006). Real-time PCR was performed in a 96-well plate according to the manufacturer's instructions (Stratagene). The primers (Invitrogen) and probes (Biosearch Technologies; Novato, CA) were designed with the assistance of Primer Express software from Applied Biosystems (Life Technologies; Carlsbad, CA) as follows: mPOMCF (5′-ACCTCACCACGGAGAGCA-3′), mPOMCR (5′-GCGAGAGGTCGAGTTTGC-3′), and mPOMCP (5′-6-carboxy-fluorescein [Fam]-TGCTGGCTTGCATCCGGG-BHQ-1-3′); mNPYF (5′-CTCC GCTCTGCGACACTAC-3′), mNPYR (5′-AATCAGTGTCTCAGGGCT-3′), and mNPYP (5′-6-carboxy-fluorescein [Fam]-CAATCTCATCACCAGACAG-BHQ-1-3′); mAgRPF (5′-GCGGAGGTGCTAGATCCA-3′), mAgRPR (5′-AGGACTC GTGCAGCCTTA-3′), and mAgRPP (5′-6-carboxy-fluorescein [Fam]-CGAGTC TCGTTCTCCGCG -BHQ-1-3′). PCR reactions were run in a volume of 25.0 μl using 1.0 μl cDNA. A standard curve was generated from duplicate measurements of serial dilutions of arcuate cDNA. The housekeeping gene cyclophilin was used for normalization.

Hypothalamic Neuropeptides

The extraction and purification of the peptides from whole hypothalamic tissues were performed as previously described (Gamber et al., 2005; Guo et al., 2004). The samples were then assayed by enzyme immunoassays (EIAs) for α-MSH, NPY (both in-house assays), and AgRP (Phoenix Pharmaceuticals; Burlingame, CA). The sensitivities of the assays were 2, 8, and 9 pg per sample, respectively.

Statistical Analyses

Data are presented as means ± SEM, and significance level was set at $p \leqslant 0.05$. Analyses were done by Student's t tests. For insulin tolerance tests, treatment groups were analyzed using general linear models, and individual differences between the treatment groups were identified by one-way ANOVA followed by the protected least significant-differences technique (SAS version 8.2, SAS Institute; Cary, NC).

SUPPLEMENTAL DATA

Supplemental Data include six figures and can be found online at http://www.cell.com/cell-metabolism/supplemental/S1550-4131(09)00125-9.

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