

# Trophic Action of Leptin on Hypothalamic Neurons That Regulate Feeding

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In adult mammals, the adipocyte-derived hormone leptin acts on the brain to reduce food intake by regulating the activity of neurons in the arcuate nucleus of the hypothalamus (ARH). Here, we report that neural projection pathways from the ARH are permanently disrupted in leptin-deficient ( $Lep^{ob}/Lep^{ob}$ ) mice and leptin treatment in adulthood does not reverse these neuroanatomical defects. However, treatment of  $Lep^{ob}/Lep^{ob}$  neonates with exogenous leptin rescues the development of ARH projections, and leptin promotes neurite outgrowth from ARH neurons in vitro. These results suggest that leptin plays a neurotrophic role during the development of the hypothalamus and that this activity is restricted to a neonatal critical period that precedes leptin's acute regulation of food intake in adults.

It has been known for decades that circulating hormones such as adrenal steroids, sex steroids, and thyroid hormone exert widespread actions on brain development. Neuronal pathways in the limbic regions of the forebrain and hypothalamus are particularly sensitive to the developmental actions of hormones during perinatal life, as shown by hormone-dependent changes in cell number, innervation, and neurochemistry that have been documented for a variety of regions (1–5). The discovery of the adipocyte-derived hormone leptin led to a revolution in the understanding of neurobiological mechanisms regulating obesity (6–9). In adult animals, leptin stimulates ARH neurons that contain the anorexigenic peptide alpha melanocyte-stimulating hormone ( $\alpha$ -MSH) and inhibits neurons that coexpress the orexigenic peptides neuropeptide Y (NPY) and agouti-related protein (AgRP) (10), which relay this information to other parts of the hypothalamus involved in regulating food intake and energy expenditure (11–13). However, despite convincing evidence that leptin deficiency contributes to early-onset obesity (14), we know remarkably little about the development of the central circuits that regulate feeding and energy balance.

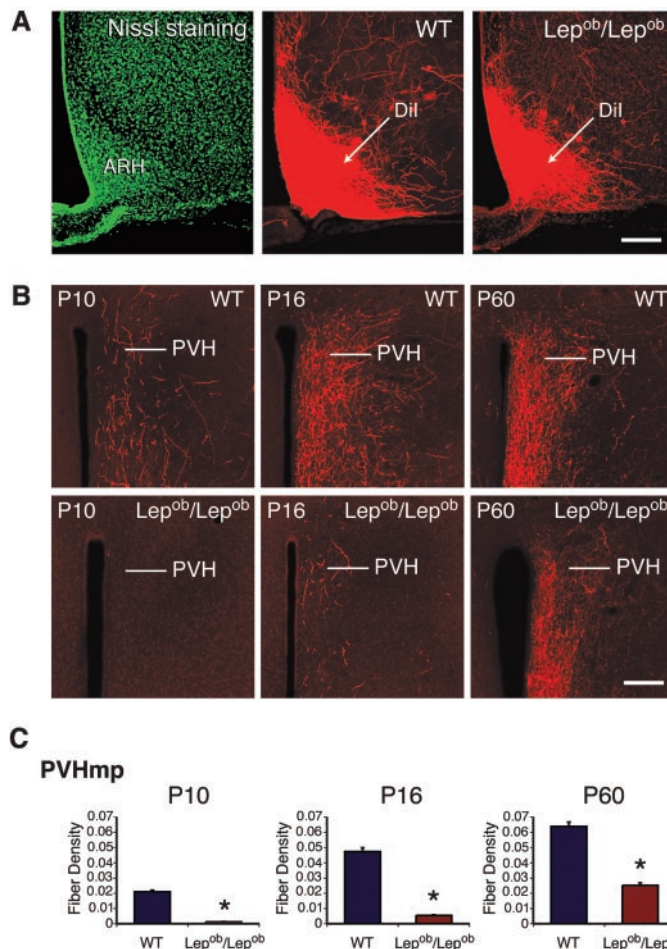
In adult mice, the ARH provides dense projections to three other hypothalamic nuclei that are involved in food intake: the paraventricular nucleus (PVH), the dorsomedial hypothalamic nucleus (DMH), and the lateral hypothalamic area (LHA) (15). In wild-type C57BL/6 mice, these projections form primarily during the second postnatal

week and innervate each hypothalamic target at different times (16). The projection from the ARH to the PVH develops relatively late in postnatal life [on postnatal day 8 (P8) to P10] with a mature pattern of innervation achieved by P16 (Fig. 1). Projections from the ARH to the DMH are established between

P5 and P6 and achieve the adult pattern by P12 (fig. S1, A and B). Projections to the LHA appear to be established by P12 but continue to increase in density through P16 (fig. S1, C and D).

Because food intake must be maximized to support growth during early development and leptin functions in adults to suppress food intake, it is surprising that there is a pronounced surge in leptin levels during the first week of life (17). This massive surge in leptin secretion is not matched by a corresponding reduction in food intake in neonatal mice, which was originally interpreted as an indication that the neonatal brain is relatively insensitive to leptin (18). An alternative view is that the neonatal leptin surge functions as a developmental signal (19–21).

To investigate the role of leptin in the development of ARH projections, we implanted crystals of 1,1',1'-diiodo-3,3',3'-tetramethylindocarbocyanine perchlorate (DiI), a fluorescent lipophilic tracer that labels axonal projections in fixed tissues (22), into the ARH of  $Lep^{ob}/Lep^{ob}$  and wild-type littermate mice at various ages (23) (Fig. 1A). The results showed that leptin deficiency caused profound disruptions in the development of ARH projections. In the PVH of



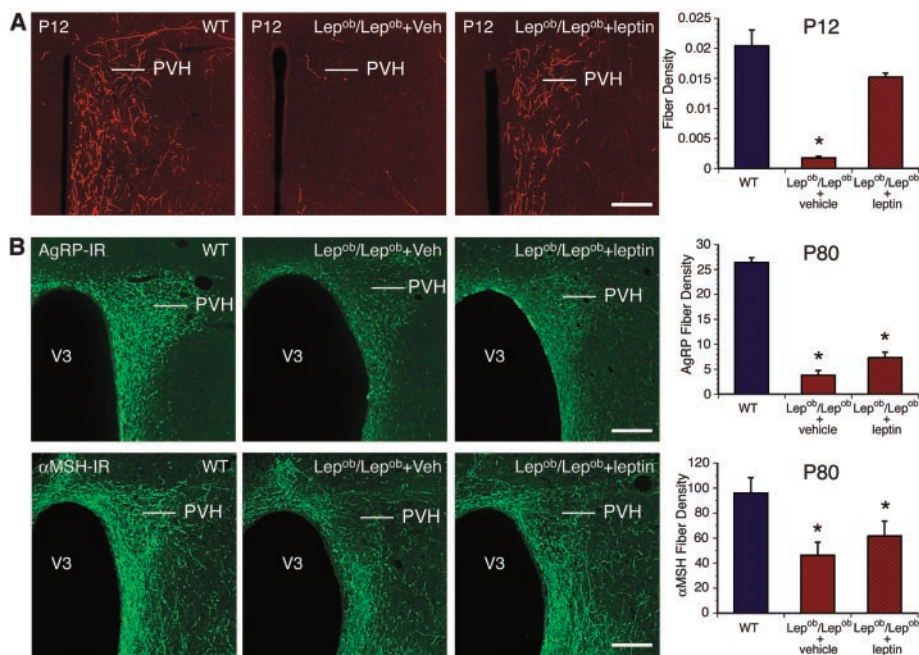
**Fig. 1.** Leptin deficiency disrupts the normal developmental pattern of projections from the ARH to the PVH. (A) Crystals of the anterograde tracer DiI were placed in the ARH of  $Lep^{ob}/Lep^{ob}$  and wild-type (WT) littermates. (B) DiI-labeled fibers in the PVH of P10, P16, and P60 wild-type and  $Lep^{ob}/Lep^{ob}$  mice. (C) Quantification of the density of arcuate DiI-labeled fibers innervating the medial parvocellular component of the PVH (PVHmp) in wild-type and  $Lep^{ob}/Lep^{ob}$  mice on P10 ( $n = 3$  per group), P16 ( $n = 3$  per group), and P60 ( $n = 3$  per group). Leptin deficiency severely diminished the density of arcuate fibers innervating each part of the PVH. The values shown are means + SEM. \*,  $P < 0.001$  between  $Lep^{ob}/Lep^{ob}$  and wild-type mice. Scale bars, 60  $\mu$ m (A); 190  $\mu$ m (B).

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10-day-old  $Lep^{ob}/Lep^{ob}$  mice, there were 10 times fewer labeled fibers compared with the number in wild-type littermates (Fig. 1, B and C). The average fiber densities in the PVH increased in both  $Lep^{ob}/Lep^{ob}$  mice and wild-type mice on P16, but those of  $Lep^{ob}/Lep^{ob}$  mice remained much lower. The disruption of ARH projections to the PVH appears to be permanent, because the density of labeled

fibers in the medial parvicellular component of the nucleus remained three to four times as high in wild-type adult (P60) mice relative to that of  $Lep^{ob}/Lep^{ob}$  mice (Fig. 1, B and C). The distribution pattern of labeled fibers in the PVH was similar in  $Lep^{ob}/Lep^{ob}$  mice and wild-type littermates, suggesting that leptin deficiency alters the density but not the pattern of innervation.



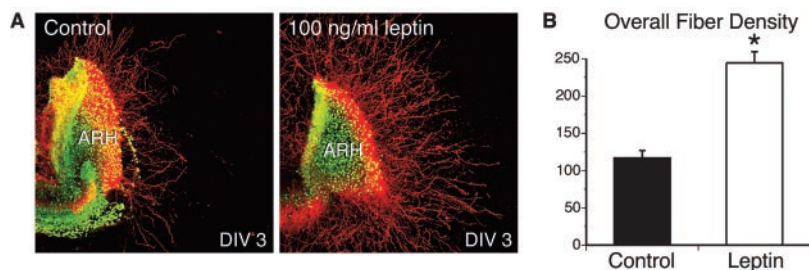
**Fig. 2.** Projections from the ARH to the PVH are rescued by leptin treatment of neonatal, but not adult,  $Lep^{ob}/Lep^{ob}$  mice. **(A)** Treatment of neonatal  $Lep^{ob}/Lep^{ob}$  mice from P4 to P12 with daily intraperitoneal injections of recombinant leptin (10 mg/kg) leads to increased innervation of the PVH by arcuate fibers. The magnitude of the leptin-induced changes is apparent in the graphical comparison between the density of arcuate DiI-labeled fibers innervating the medial parvicellular component of the PVH in P12 wild-type (WT) mice ( $n = 3$ ),  $Lep^{ob}/Lep^{ob}$  vehicle-treated mice ( $n = 3$ ), and  $Lep^{ob}/Lep^{ob}$  leptin-treated mice ( $n = 3$ ). \*,  $P < 0.001$  between  $Lep^{ob}/Lep^{ob}$  and wild-type littermates or leptin-treated animals. **(B)** Leptin treatment does not reverse the disruption of  $\alpha$ -MSH and AgRP pathways in adult  $Lep^{ob}/Lep^{ob}$  mice. Leptin deficiency reduces the density of AgRP-immunoreactive (AgRP-IR) and  $\alpha$ -MSH-immunoreactive ( $\alpha$ -MSH-IR) fibers in the PVH of  $Lep^{ob}/Lep^{ob}$  mice on P80 compared with that of wild-type littermates. Quantitative estimates of  $\alpha$ -MSH and AgRP fiber density reveal that treatment of adult  $Lep^{ob}/Lep^{ob}$  mice with leptin for 20 days (10 mg/kg; P60 to P80) did not restore the density of  $\alpha$ -MSH- and AgRP-immunoreactive fibers in  $Lep^{ob}/Lep^{ob}$  mice to that characteristic of wild-type littermates ( $n = 3$  per group). V3, third ventricle; \*,  $P < 0.001$  between  $Lep^{ob}/Lep^{ob}$  or leptin-treated animals and wild-type littermates. The values shown are means + SEM. Scale bars, 200  $\mu$ m (A); 160  $\mu$ m (B).

Similar reductions in DiI fiber density were observed in the DMH, LHA, and other terminal fields of  $Lep^{ob}/Lep^{ob}$  mice that received DiI into the ARH, indicating that leptin deficiency causes extensive disruption of ARH projections. For example, an analysis of DiI-labeled material revealed that rarely did axons from the ARH reach the DMH by P6 in  $Lep^{ob}/Lep^{ob}$  mice, and by P12, the density of labeled fibers remained five times lower than that in wild-type littermates (fig. S1, A and B). Thus, leptin deficiency appears to disrupt the formation of projections from the ARH to each of its major terminal fields.

To determine whether leptin promotes the development of other leptin-sensitive pathways, we examined the development of projections from the DMH to the PVH (23) (fig. S2A), but these projections appeared to be normal (fig. S2, B and C). We also examined the integrity of a limbic-hypothalamic pathway (fig. S2D) that is thought to be leptin insensitive; this pathway also appeared to be unaffected by leptin deficiency (fig. S2, E and F). Taken together, these observations suggest that leptin deficiency does not lead to widespread disruption of hypothalamic circuitry but specifically affects the development of ARH projections.

To determine whether leptin can reverse the disruption in ARH projections observed in leptin-deficient mice, we treated neonatal  $Lep^{ob}/Lep^{ob}$  mice with recombinant leptin (10 mg/kg by intraperitoneal injection) daily from P4 through P12 (23). Leptin treatment increased the density of DiI-labeled fibers in the medial parvicellular component of the PVH to levels that were comparable to those of wild-type littermates (Fig. 2A). In addition, neonatal leptin treatment reduced the food intake (4.5 g/day) of  $Lep^{ob}/Lep^{ob}$  mice on P32 to levels that were intermediate between wild-type mice (3.9 g/day) and vehicle-treated  $Lep^{ob}/Lep^{ob}$  mice (5.4 g/day; fig. S3). Because we observed substantial reductions in ARH projections to each target nucleus, we tested the hypothesis that leptin acts directly on the ARH by exposing isolated slice explant cultures derived from neonatal mice to leptin (23). By 72 hours after the addition of leptin (100 ng/ml) to the culture medium, there was a significant induction of neurites extending from the ARH explants relative to control cultures, suggesting that leptin acts on ARH neurons to promote axon elongation and proliferation (Fig. 3).

Hormonal signals that regulate development tend to act during restricted perinatal critical periods. To test the activity of leptin on ARH projections in adult  $Lep^{ob}/Lep^{ob}$  mice, we used immunohistochemical labeling of AgRP (23). Because it is known that in adult rodents AgRP expression is restricted to NPY-containing neurons in the ARH, AgRP immunoreactive fibers repre-



**Fig. 3.** Leptin promotes neurite outgrowth directly from the ARH in vitro. **(A)** Isolated organotypic cultures of ARH from P6 mice were incubated for 72 hours with or without leptin (100 ng/ml) and then immunostained with beta III tubulin (TuJ antibody), which stains neurites. DIV 3, 3 days in vitro. **(B)** The quantitative analysis shows that leptin induces an increase by a factor of 2 in fiber density from ARH explants. The values shown are means + SEM. \*,  $P < 0.001$  between control and leptin-treated animals.

sent projections of these neurons. As observed in the axon-labeling studies, the density of AgRP immunoreactive fibers was severely reduced in the PVH of Lep<sup>ob</sup>/Lep<sup>ob</sup> mice on P80 compared with that of wild-type littermates (Fig. 2B), and reductions in the density of  $\alpha$ -MSH fibers were also observed in Lep<sup>ob</sup>/Lep<sup>ob</sup> mice (Fig. 2B). In sharp contrast to the results seen in neonatal mice, treatment of adult Lep<sup>ob</sup>/Lep<sup>ob</sup> mice with leptin for 20 days did not restore the density of AgRP and  $\alpha$ -MSH immunoreactive fibers in the PVH to wild-type levels (Fig. 2B). The inability of leptin to alter AgRP and  $\alpha$ -MSH-immunoreactive fiber density in the PVH of adult Lep<sup>ob</sup>/Lep<sup>ob</sup> mice suggests that the developmental action of leptin is restricted largely to the perinatal period.

These data provide direct evidence that leptin functions as an essential factor for brain development, promoting formation of hypothalamic pathways that later convey leptin signals to brain regions regulating food intake and energy consumption. This developmental activity appears to be specific for ARH projections and is restricted to a neonatal window of maximum sensitivity that corresponds to a period of elevated leptin secretion. This neonatal "critical period" is also when ARH axons are guided to their targets. Although it is unknown whether leptin alters expression of local guidance cues in the periventricular zone of the hypothalamus, our *in vitro* findings suggest that leptin induces neurite outgrowth by acting on ARH neurons directly. Because of its direct access to these key components of homeostatic circuits, the neonatal leptin surge represents a likely peripheral signal capable of directing the development of hormone-sensitive central circuits during postnatal life. In this respect, the neonatal surge in leptin may be analogous to the surge in sex steroid secretion that occurs during neonatal life and is known to specify sexually dimorphic patterns of development in the mammalian forebrain (4, 5, 17). Our findings show that leptin has a similar programming action on the architecture of homeostatic neural circuitry, which raises the possibility that this developmental activity may contribute to the onset of leptin-dependent childhood obesity through a direct action on the brain.

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23. Materials and methods are available as supporting material on Science Online.
24. We thank M. S. Smith and S. R. Ojeda for comments on the manuscript. Supported by NIH grants NS37952, DK55819, DK65900, and RR00163.

Supporting Online Material

www.sciencemag.org/cgi/content/full/304/5667/108/DC1  
 Materials and Methods  
 Figs. S1 to S3  
 References

22 December 2003; accepted 1 March 2004

## Rapid Rewiring of Arcuate Nucleus Feeding Circuits by Leptin

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The fat-derived hormone leptin regulates energy balance in part by modulating the activity of neuropeptide Y and proopiomelanocortin neurons in the hypothalamic arcuate nucleus. To study the intrinsic activity of these neurons and their responses to leptin, we generated mice that express distinct green fluorescent proteins in these two neuronal types. Leptin-deficient (*ob/ob*) mice differed from wild-type mice in the numbers of excitatory and inhibitory synapses and postsynaptic currents onto neuropeptide Y and proopiomelanocortin neurons. When leptin was delivered systemically to *ob/ob* mice, the synaptic density rapidly normalized, an effect detectable within 6 hours, several hours before leptin's effect on food intake. These data suggest that leptin-mediated plasticity in the *ob/ob* hypothalamus may underlie some of the hormone's behavioral effects.

Administration of exogenous leptin to leptin-deficient mice and humans decreases food intake and body weight (1–5). These effects are mediated in part by leptin's ability to modulate hypothalamic function. In the arcuate nucleus (Arc) of the hypothalamus, the signaling form of the leptin receptor is co-expressed with neuropeptide Y (NPY) and agouti-related peptide (AgRP) in a group of orexigenic neurons and with proopiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) in a group of anorexigenic neurons (6–11). Increased NPY activity and reduced POMC

activity appear to increase feeding and fat deposition, whereas reduced NPY activity and increased POMC activity decrease feeding and body mass (12–16). Leptin increases the firing rate of POMC neurons in acute slice preparations from the Arc (17). In the *ob/ob* hypothalamus, the amounts of NPY RNAs are increased, whereas the RNAs for POMC are decreased and leptin treatment of these animals normalizes the amounts of these RNAs (8, 18).

To date, there is no direct evidence showing that leptin has differential effects on the activity or inputs of NPY and POMC neurons. One possibility is that there are differences in the synaptic input to these neurons in *ob/ob* mice, a possibility consistent with Cajal's neuronal doctrine. Previous studies of the inputs to NPY and POMC neurons have been difficult mainly because NPY and, to a lesser extent, POMC-derived peptides could only be identified by histochemistry after colchicine treatment in protocols that alter neuronal function. To overcome this obstacle and examine the electro-

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