20 YEARS OF LEPTIN

Leptin at 20: an overview

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Abstract

Historically, adipose tissue was considered to be a passive storage vessel discharging nutrients in times of famine and accumulating fat in times of surfeit. This view changed with the identification of leptin as an adipocyte hormone. Leptin functions as an afferent signal in a negative feedback loop that regulates food intake and metabolism to maintain homeostatic control of adipose tissue mass. Before this, the existence of a system maintaining homeostatic control of energy balance was unclear. The identification of leptin has thus uncovered a new endocrine system that also links changes in nutrition to adaptive responses in most if not all other physiologic systems. Further studies have revealed a set of clinical syndromes caused by leptin deficiency, including lipodystrophy and hypothalamic amenorrhea. This work has led to new therapeutic approaches for a number of human conditions and has also established a conceptual framework for studying the pathogenesis of obesity.

Key Words
- leptin
- food intake
- energy homeostasis
- obesity

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Introduction

Early one morning, slightly longer than 20 years ago, I developed a northern blotting showing changes in the levels of adipose tissue RNA detected by a probe named 2G7 (Ingalls *et al.* 1950, Zhang *et al.* 1994). The RNA in this experiment was derived from two mutant mouse strains each of which was homozygous for mutations in the mouse obese (*ob*) gene. *ob* is a fully penetrant autosomal recessive gene, which causes affected animals to eat for five times compared with a normal mouse, resulting in a profound increase in weight and adipose tissue mass. 2G7 was a clone that mapped to the region of chromosome 6 where the *ob* gene had been found to reside (Friedman *et al.* 1991, Bahary *et al.* 1993). The development of this film was the culmination of a 12-year odyssey that began with the demonstration that cholecystokinin, then considered to be a candidate for the *ob* gene, mapped to chromosome 9 and could not be *ob* (Friedman *et al.* 1985, 1989), to the identification of 2G7 from an exon-trapping experiment in which 182 different clones from an ~100-kB P1 clone in the region of the *ob* gene on mouse chromosome 6 were arrayed on two microtiter plates (the *ob* gene was on plate 2, row G, column 7) (Zhang *et al.* 1994).

The 2G7 probe identified a 4.5-kB RNA in adipose tissue that was absent in RNA from CMC dac *ob/ob* mice but was increased 20-fold in adipose RNA from C57Bl/6J *ob/ob* mice, first characterized by Ingalls *et al.* (1950). CMC dac *ob/ob* mice were unpublished at the time but had been kindly provided by Skippy Lane at the Jackson Laboratory, as were many other mice that were critical for this study.

The demonstration that the same RNA was absent in one mutant and increased in the second mutant provided definitive proof the 2G7 was an exon from the *ob* gene. These data by themselves confirm that the *ob* gene had been isolated because, had the observed RNA changes been secondary to some other genetic defects, the expression levels would have to be similar in both *ob* mutant strains. Further studies revealed that a viral insertion in CMC dac *ob/ob* mice interfered with *ob*

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expression, while in C57Bl/6J *ob/ob* mice a point mutation in the second exon of the *ob* gene introduced a nonsense mutation at amino acid 105 of this 167 amino acid protein (Zhang *et al*. 1994, Moon & Friedman 1997). The marked overexpression of the *ob* gene in C57Bl/6J *ob/ob* adipose tissue further suggested that the gene was under feedback control with an increased level of gene expression in the obese state.

This finding that the *ob* gene was induced in *ob/ob* fat tissue was consistent with data from classic parabiosis experiments of Doug Coleman in a now-iconic set of experiments that involved stitching together the skins of living mice so that animals with different mutations shared a circulatory system (Coleman 1978). *ob* mice surgically joined to normal or *db* mice (on the same inbred strain background) ate less and lost weight. In contrast, normal mice paired to *db* mice starved to death. From this, Coleman concluded that *ob* mice normally lacked a circulating factor that was provided by the conjoined partner, which suppressed food intake and body weight. He further suggested that *db* mice lacked a receptor to detect the weight-suppressing factor in their blood and so overexpressed it, producing levels so high that conjoined mice sensitive to the factor stopped eating. Implicit in this hypothesis was the prediction that the *ob* gene was under feedback control and that obesity would be associated with increased levels of *ob* RNA. However, Coleman's experiments did not predict where the hormone that was missing in *ob* mice was expressed, though prior experiments from Hervey predicted that the receptor would be expressed in the hypothalamus (Hervey 1969).

Putting this all together in that moment in a darkroom, it became evident in an instant that not only had the *ob* gene been cloned but also that the data were consistent with Coleman's predictions. The data thus suggested the hypothesis that the *ob* gene encoded a novel adipocyte hormone which functioned as the afferent signal in a negative feedback loop that maintains homeostatic control of adipose tissue mass. Subsequent studies have confirmed this possibility in animals and humans, and some of the fruits of this set of observations are summarized in this timely volume and elsewhere (Halaas *et al*. 1995, Tartaglia *et al*. 1995, Lee *et al*. 1996, Friedman & Halaas 1998). While some of the features of this new hormonal system were predicted at the time, others were not. Science seldom proceeds in a straight line, and the field spawned by the identification of leptin and other genes that cause obesity is no exception.

This year is likely to mark the beginning of a new chapter in the biology of leptin as last February, 20 years after its discovery, leptin is now an FDA-approved human therapeutic for the treatment of severe lipodystrophy with potential for the treatment of other disorders http://www.fda.gov/newsevents/newsroom/pressannouncements/ucm387060.htm. In addition, though leptin's utility as a monotherapy for obesity appears limited, other evidence suggests that it still has potential as part of a combination therapy for this disorder.

Thus, there have been surprises and disappointments and the passage of time now provides an opportunity to chronicle, as this timely volume does, some of what has been learned, what was surprising and where the field is headed. Some of the key lessons from the last 20 years of research and their context are summarized below.

Wiring diagram of a complex behavior

One can describe the phenotype of *ob* and *db* mice in several different ways. Historically, these animals have been described as obesity mutations, but one could also think of these animals as showing a behavioral phenotype. *ob* and *db* mice show abnormalities in numerous behaviors (Bray 1991). They are massively hyperphagic, show a dramatic decrease in locomotor activity, are quite gentle and non-aggressive, and are not sexually active. Thus, the identification of leptin and later the localization of the leptin receptor (Tartaglia *et al*. 1995, Lee *et al*. 1996), encoded by the *db* gene, have provided an entry point for delving into the neural mechanisms that control complex behaviors. Moreover, the elucidation of the pathogenesis of the obesity resulting from the yellow agouti (Ay) mutation has identified hypothalamic neurons expressing POMC, the precursor of αMSH, as a key neural target of leptin, and more generally, integrators of numerous metabolic signals (Lu *et al*. 1994). αMSH acts on the MC4 receptor, a GPCR, and MC4R mutations replicate the obese phenotype of Ay mice (Huszar *et al*. 1997). The subsequent identification of AGRP as an endogenous inhibitor in a second group of hypothalamic neurons, also expressing NPY, added another population of leptin-responsive neurons (Stephens *et al*. 1995, Ollmann *et al*. 1997). We now know that leptin acts in part by activating POMC neurons and inhibiting NPY/AGRP neurons though clearly many other neural populations also play a role either as direct neural targets or downstream of these neurons (Friedman 1997). Indeed, enormous progress has been made in defining a set of overlapping neural circuits that control food intake and body weight. With time, these findings are likely to lead to a deeper understanding

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of how feeding behavior is controlled as well as our understanding of the control of other behaviors.

Thus, leptin's neurobiologic effects are not limited to feeding circuits. Extreme weight loss in human has been shown to induce a set of emotional sequelae including depression. A possible role for a reduction in leptin in mediating some portion of this was suggested by the finding that leptin injection into the hippocampus can improve the performance of animals in a forced swim test (Lu *et al*. 2006). This assay provides a quantitative indication of the level of depression in animals and robustly predicts the efficacy of anti-depressant drugs in human. Other studies have shown that leptin has significant effects on reward processing by dopaminergic centers in the midbrain and that it can reduce the value of a sucrose reward (Domingos *et al*. 2011). This is important because it shows that the pleasure we derive from eating is not fixed but rather reflects the status of metabolic signals such as leptin. Leptin also has potent effects on many other neural circuits including those controlling hormones that regulate reproduction and reproductive behaviors, activity, thermoregulation, and stress (Lu *et al*. 2006, Wu *et al*. 2009, Atasoy *et al*. 2012).

Obesity has a substantial genetic component

The identification of mutant genes that cause obesity in mice provided a molecular framework for identifying mutant genes that cause obesity in human. Thus, mutations in leptin, the leptin receptor (LepR), the MC4R as well as PCSK1, and enzymes required for the processing of POMC cause human obesity as do other components of the neural circuit that regulates food intake including brain-derived neurotrophic factor (BDNF) and Sim1. Indeed, it now appears that >10% of morbid human obesity is a result of Mendelian defects in these (and other) genes, which in the majority of cases are in MC4R and LepR (Barsh *et al*. 2000). This is a level of Mendelian inheritance that exceeds that for nearly every other complex trait that has been studied. The realization that obesity is often the result of genetic mutations in human provides strong evidence that this condition is a result of alterations in a neural circuit that controls the basic drive to eat as well as metabolism (and perhaps other behaviors) and provides an alternative to the widely held view that obesity develops from a failure of willpower or consequent to the modern environment.

Furthermore, it is interesting that all of the obesity genes identified thus far are expressed in the brain. This is despite the fact that there is a large body of evidence indicating that differences in metabolic rate can predict changes in weight (Ravussin *et al*. 1988) and that an increase in peripheral metabolism such as after treatment with thyroid hormone or uncouplers of respiration such as dinitrophenol leads to weight loss (Grundlingh *et al*. 2011, Pearce 2012). Moreover, while a defect in leptin signaling is associated with hyperphagia and a marked decrease in energy expenditure in mice, the principal effect in human is on appetite with little or no discernible effect on metabolism (Farooqi *et al*. 1999). However, while leptin does not appear to cause a net increase in energy expenditure, it does blunt the reduction of energy expenditure that is normally associated with weight loss (Farooqi *et al*. 2002, Galgani *et al*. 2010).

The hereditability of obesity has been reported to be between 0.7 and 0.8, which is higher than that for most other traits (Stunkard *et al*. 1990). That there is a substantial genetic contribution to obesity is also supported by adoption and familial aggregation studies (Stunkard *et al*. 1986, Adams *et al*. 1993). However, while some fraction of obesity can be attributed to the aforementioned Mendelian defects as well as variation in genes identified in GWAS studies such as FTO, there is a reason to expect that many new genes remain to be discovered (Fawcett & Barroso 2010). The use of high-throughput genomic sequencing to look for variation in patients with extreme phenotypes, as pioneered by several authors in this volume, is likely to lead to the identification of new genes (Ku *et al*. 2011). It will be of particular interest to learn whether these new genes also function in the neural circuit that is modulated by leptin and other metabolic signals. It is thus quite likely that future volumes on leptin will need to take into account additional as yet unidentified components of the neural circuit that regulates weight that are identified genetic analyses of human patients.

Leptin deficiency syndromes

Leptin-deficient *ob* mice show abnormalities in most, perhaps all, physiologic systems (Bray 1991). Thus, these animals show defects in the entire neuroendocrine axis and are infertile or subfertile, euthyroid sick with markedly increased corticosterone levels. In addition to these global effects on the neuroendocrine axis, *ob* mice are hypothermic, diabetic and have profoundly abnormal immune and hematologic function. Indeed, after they were first identified, the complex phenotype of these animals led some to question whether the identification of the *ob* gene would advance our understanding of how food

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intake and body weight are regulated. In retrospect, the complex phenotype of these mice can be most easily understood by noting that the abnormalities they manifest are generally associated not with obesity, but rather starvation (Lord 1998, Frisch 2002). This prediction was supported by the observation that leptin administration suppresses the neuroendocrine response to fasting in mice (Ahima *et al*. 1996). These findings and others suggest that a key function of leptin is to communicate information to the brain and other organs that there are adequate fat stores and that the organism is not starving. In the absence of leptin, or with the reduced levels seen after fasting, a set of physiologic responses are elicited the aggregate effect of which is to reduce energy expenditure, at the same time as appetite is stimulated.

In addition to its intrinsic importance, this aspect of leptin function provides a framework for understanding the efficacy for treating a series of leptin-deficient states in human. In each case, leptin treatment improves one or more abnormalities generally associated with starvation. As outlined in this volume, lipodystrophy, the complete or partial absence of fat, is a heterogeneous disorder associated with leptin deficiency and a severe sometimes intractable insulin resistance and diabetes as well as hyperlipidemia and nonalcoholic steatohepatitis (NASH) (Oral *et al*. 2002). Similarly, as also discussed in this volume, the leanness of young women who often exercise with a great avidity is also associated with leptin deficiency and hypothalamic amenorrhea (Welt *et al*. 2004). This condition is characterized by a failure to menstruate, infertility and also osteoporosis (Sienkiewicz *et al*. 2011). Leptin-replacement therapy improves the abnormalities associated with lipodystrophy and hypothalamic amenorrhea (HA) and even causes a significant improvement of bone mineral density in HA patients. Leptin confers these beneficial effects despite causing weight loss in treated patients. Similarly, patients with mutations also show extreme weight loss after leptin therapy but also show improvements in this same set of abnormalities, more typically associated with starvation. Thus, these patients show marked improvements in their metabolic profile, a restoration of fertility and improvements in immune function with leptin treatment. In aggregate, these data strongly suggest that organismal sensing of overall nutritional state (i.e. adipose tissue mass) is conveyed by leptin and not by the actual amount of fat stored in adipose tissue.

In general, the more extreme the abnormalities of patients with low leptin levels, the more significant the clinical response to leptin therapy. This raises the possibility that leptin might have potential as a treatment for other pathologies that develop in settings of leptin deficiency. For example, one female leptin-deficient patient failed to enter puberty in adolescence even though her bone age indicated they should have and leptin treatment led to the onset of menses. This suggests that leptin might be used to induce puberty in very lean young women with a delayed onset of puberty (Farooqi *et al*. 1999). Both leptin-deficient and starved individuals show immune abnormalities with a shift from TH1 to TH2 immunity and an increased susceptibility to infectious disease. Here again, leptin treatment of leptin-deficient humans and starved animals reverses these changes (Ahima *et al*. 1996, Lord 1998, Ozata *et al*. 1999). Thus, it is possible that leptin could be used as an immune adjuvant in settings of extreme cachexia such as starvation, cancer, or chronic inflammatory disease. It has even been proposed that leptin might be useful in patients with end-stage anorexia nervosa with the hope that low-dose leptin treatment could ameliorate some of the pathologies associated with leptin deficiency without significantly reducing food intake (further) and/or as an adjunct to parenteral nutrition (C Montzoros, personal communication).

Leptin might also be of benefit in patients who do not manifest signs or symptoms of pathologic deficiency of leptin (i.e., starvation) but who nonetheless are leptin sensitive (this would be in contrast to most obese patients who are leptin resistant, see below). Prior studies in animals have shown that leptin stimulates glucose metabolism in WT mice independent of weight loss and that it can improve the diabetes of lipodystrophic mice independent of insulin (Kamohara *et al* 1997, Asilmaz *et al*. 2004). This raised the possibility that leptin might show efficacy for the treatment of type 1 diabetes. This possibility has now been tested in streptozotocin-treated mice who are either partial or completely insulin deficient. In both cases, leptin markedly lowered blood glucose. Indeed in one study, untreated animals all died within one month, while treated animals survived as long as leptin continued to be expressed from an adenoviral vector (Wang *et al*. 2010). Further evidence has suggested that leptin elicits its antidiabetic effects by inhibiting glucagon. This has raised the possibility that leptin might also be of benefit for patients with type 1 diabetes who often present with weight loss and hyperphagia as a consequence of complete or partial insulin deficiency. In this setting, leptin could either alleviate the demands on β cells at the onset of the disease to extend the 'honeymoon' period and/or be used to supplement insulin at later stages of the disease as a means for smoothing glucose control with less hyperglycemia. Leptin therapy might also minimize the weight gain that is

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associated with increased doses of insulin. Further studies will be necessary to evaluate these possibilities.

Leptin resistance

Physiologic increases in plasma leptin level in WT mice lead to a dose-dependent reduction of food intake and loss of weight (Halaas *et al*. 1997). While leptin has potent effects to reduce food intake and body weight in *ob* and WT animals, its efficacy in obese animals is variable and generally reduced (Halaas *et al*. 1997). Animals with mutations in the leptin receptor fail to respond to leptin treatment at all as do Ay mice that have a defect in melanocortin signaling. Diet-induced animals show only a small response, while New Zealand obese mice (NZO), a strain that develops a polygenic form of obesity, fail to respond to leptin delivered peripherally but lose significant amounts of weight when leptin is delivered through intracerebroventricular (i.c.v.) administration. Each of these strains has high plasma levels of leptin suggesting that they are leptin resistant. The most extreme case of leptin resistance is the *db* mouse which has a mutation in the leptin receptor (Tartaglia *et al*. 1995, Lee *et al*. 1996). In the absence of leptin action, these animals become obese and secondarily overproduce the hormone. Thus, obesity satisfies the hallmarks of a hormone resistance syndrome, with an attenuated response to exogenously administered hormone and elevated endogenous levels (Maffei *et al*. 1995). In addition, mutations in genes in the leptin signal transduction pathway, PTP1B and SOCS3, increase leptin signaling and lead to a resistance to obesity identifying potential biochemical mechanisms (Bjorbak *et al*. 2000, Bence *et al*. 2006). However, leptin resistance is complex and can develop at many points in the neural circuit that regulates feeding. Thus, leptin resistance can also develop downstream of leptin target neurons as in Ay and MC4R knockout mice in which melanocortin signaling from POMC neurons is abrogated (Lu *et al*. 1994). Similar to other hormones, leptin resistance can also develop in response to chronically elevated hormone levels via tachyphylaxis (Knight *et al*. 2010). Finally, as mentioned, in NZO mice, leptin resistance can develop because of impaired leptin transport although little is known about the transcytotic mechanism (Schwartz *et al*. 1996).

In human, leptin is highly potent in patients with low endogenous levels though its effects in otherwise normal lean patients have never been comprehensively studied (Farooqi *et al*. 1999, Oral *et al*. 2002, Welt *et al*. 2004, Sienkiewicz *et al*. 2011). In contrast, leptin has variable effects as monotherapy for obesity in the general population. Initial studies showed encouraging effects at very high doses (0.3 mg/kg bid), but this dose was too high for general usage and a lower dose (0.1 mg/kg bid) did not show efficacy (Heymsfield *et al*. 1999). However, a more recent study treating obese patients with an even lower dose (0.05 mg/kg) led to ~5% weight loss equivalent with efficacy equivalent to other pharmacotherapies for obesity (Roth *et al*. 2008). It is thus possible that higher doses of leptin led to tachyphylaxis and that a larger study of patients treated with leptin at 0.05 mg/kg or lower could replicate the weight loss observed in the earlier study. There is also evidence that some obese patients show a greater response to leptin than others. In light of the potency of leptin in patients with low endogenous leptin levels, it is possible that the one could enrich for a responder subset by selecting obese patients with low leptin levels. Indeed, while leptin level is highly correlated with adipose tissue mass ($r=0.9$), plasma leptin can still vary by tenfold or more among patients of the same BMI (Maffei *et al*. 1995). Furthermore, mice with low levels of leptin are obese and remain leptin sensitive, while patients with heterozygous leptin mutations are obese with low leptin levels (Ioffe *et al*. 1998, Farooqi *et al*. 2001).

The efficacy of leptin for the treatment of obesity has been augmented by combining it with other agents that cause weight loss, in particular short-term signals including intestinal peptides that modulate meal pattern. For example, both leptin (0.05 mg/kg) and amylin (pramlintide), a pancreatic peptide that is approved for the treatment of diabetes, each caused ~5% weight loss in a selected group of patients. However, when the two agents were combined, a synergistic effect was observed with an average weight loss of 13% which is significant efficacy for an anti-obesity agent (Roth *et al*. 2008). Studies in animals also showed that pre-treatment of diet-induced obese animals with amylin restored leptin's ability to phosphorylate Stat3 in the hypothalamus, suggesting that this gut peptide might reduce the activity of neural circuits that cause leptin resistance (Turek *et al*. 2010). In animals, leptin's efficacy has also been augmented in combinations with other peptides or hormones raising the possibility that in time it could emerge as part of a combination therapy for obesity (Muller *et al*. 2012). Bariatric surgery is an alternative means for inducing weight loss and, while invasive, can be extremely effective. Leptin falls after this procedure, in proportion to the amount of the weight loss, and it is also possible that leptin treatment in this setting could reduce recidivism and/or mitigate some of the sequelae of the procedure that might be secondary to the relative leptin deficiency that develops (Rubino *et al*. 2004).

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Questions for the future

While much has been learned, the road ahead is likely to lead to new advances and some surprises. Still some key questions remain unanswered including the aforementioned.

How is leptin transported into the CNS? Previous studies suggested that leptin entered the brain by transport across the vascular endothelium (Banks & Farrell 1999, Banks & Farrell 2003). However, recent evidence indicates that leptin enters the circumventricular organ in the median eminence where it is then taken up by processes from tanycytes and transcytosed into the III ventricle and then circulates through the ventricular system and accesses deeper brain structures (Langlet *et al.* 2013, Balland *et al.* 2014).

What regulates leptin gene expression? Leptin gene expression is correlated with the intracellular lipid content of adipocytes, suggesting that its regulation might be coupled to a lipid-sensing mechanism (Maffei *et al.* 1995, Fei *et al.* 1997). The nature of this putative lipid-sensing mechanism is unknown.

How does leptin control metabolism? What is the role of leptin signaling at peripheral tissues or are most or all of its effects mediated by the CNS? What are the physiologic and cellular mechanisms by which leptin reduces adipose deposits in fat and other tissues? What are the physiologic and cellular mechanisms by which leptin improves glucose metabolism?

Finally, how does leptin modulate a complex motivational behavior? Leptin acts directly on a number of CNS sites to reduce food intake and body weight in animals and humans and provides an entry point to study the control of feeding (Halaas *et al.* 1997). Feeding is a complex motivational behavior controlled by many inputs including smell, taste, hormonal state, cognitive inputs, etc. Recently, it has been shown that leptin acts in part by regulating hedonic circuitry but the anatomic site(s) responsible for initiating feeding behavior (Domingos *et al.* 2011). Thus, it is not known how or even when the multiple inputs are processed to formulate a 'binary' decision. So perhaps the biggest question is, how do we decide to eat or do not eat? Perhaps, the answer to this timeless question will be part of future volumes on leptin biology.

Declaration of interest

Leptin is now an approved drug. While Rockefeller owns the patent for leptin, I am named as an inventor on the patent for leptin and as per university policy receive a portion of the milestone payments and royalty payments from Astra Zeneca, the company that owns the license to the patents, to Rockefeller University.

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