

# A Reassessment of Leptin's Role in Triggering the Onset of Puberty in the Rat and Mouse

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## Key Words

Gonadotropins · Leptin · Leptin receptor · Puberty · Food restriction · Sex dimorphism · In situ hybridization · Mouse

## Abstract

Leptin is an adipocyte-derived hormone that has been implicated to serve as a metabolic signal to the reproductive axis. The role of leptin in pubertal maturation, however, has been a much-debated topic. We have previously reported that leptin serves as a permissive signal to the onset of puberty in the female rat. In an attempt to further understand the mechanics of leptin during pubertal maturation in rodent species, we had three experimental objectives: first, to describe the temporal relationship of leptin with development in the male and female rat; second, to seek evidence for an increase in responsiveness of the neuroendocrine axis to leptin by assessing for possible changes in leptin receptor expression during pubertal developmental in the female rat; and, third, to reevaluate the possible role of leptin as a permissive signal to the onset of puberty in the mouse. We found that serum leptin levels remain relatively constant during the prepubertal and postpubertal stages of both sexes. In addition, we could not detect any significant developmental changes in leptin receptor gene ex-

pression in the hypothalamus of the female rat. Lastly, we corroborated our findings in the female rat that leptin reversed the delay in pubertal maturation secondary to food restriction but did not advance the onset of puberty in female mice. Together, these results suggest that leptin is not a metabolic trigger for the onset of puberty in the rodent; instead, leptin is one of several permissive factors, whose presence may be necessary but alone is not sufficient to initiate sexual maturation in these species.

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## Introduction

The timing of the onset of puberty in the rat is correlated with body weight and adiposity [1]. Many factors linked to metabolism, growth, and fat reserves have been implicated in the timing of sexual maturation in the rodent, including growth factors such as insulin-like growth factor-1 (IGF-1) and insulin; however, these factors are not thought to be direct triggers for the onset of puberty [2–5]. The adipocyte-derived hormone leptin has emerged as an attractive hormonal candidate for linking the attainment of critical fuel reserves to the activation of reproductive function at puberty based on several observations. First, leptin-deficient *ob/ob* mice fail to undergo normal sexual maturation and remain infertile

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throughout life [6], and, second, administering leptin to *ob/ob* mice stimulates all aspects of reproduction in both sexes [7–9]. Because plasma levels of leptin are positively correlated with adiposity and leptin is apparently necessary for reproductive function, at least in rodents, it seems plausible that the onset of puberty may reflect the activation effects of leptin on the neuroendocrine reproductive axis; however, results from several laboratories argue that although leptin might be permissive to pubertal maturation in the rat, its presence alone in a prepubertal animal, even at high levels, is not sufficient to initiate puberty before the time it would normally occur in a well-fed animal [10–13]. Nevertheless, there are reports in the mouse to suggest that in this species, the administration of leptin to normal prepubertal animals can advance the onset of puberty [14–16]. Whether these apparently contradictory findings reflect real species differences or simple chance differences in experimental results is unknown.

We had three primary experimental objectives for our work. First, we argued that if leptin were the primary signal for initiating puberty onset in the rat, one would predict that there would be a change in plasma levels of leptin during the prepubertal period that might trigger the onset of puberty. To test this hypothesis, we measured serum levels of leptin across development in both male and female rats.

Our second objective was to seek evidence for possible changes in leptin receptor expression as a function of pubertal development in the rat. We argued that across the pubertal transition, animals might become increasingly sensitive to leptin through an increase in leptin receptor expression in the hypothalamus. If this were the case, the stimulatory effect of leptin on the central mechanisms controlling gonadotropin-releasing hormone (GnRH) and gonadotropins secretion might be enhanced and thus help to initiate the onset of puberty. To test this hypothesis, we compared levels of leptin receptor mRNA in various hypothalamic nuclei across pubertal development in the female rat, using single-label *in situ* hybridization.

Our third objective was to revisit the question of leptin's role in initiating the onset of puberty in the mouse, in an effort to reevaluate the contradictory findings in this species and perhaps reconcile conclusions drawn from published studies of the rat and mouse [11–16]. To accomplish this, we used an experimental paradigm similar to that used in the rat to assess the possible role of leptin as either a trigger or permissive signal to the onset of puberty in the mouse. We argued that if leptin played a similar role in the mouse as that in the rat [11–13], we would expect that leptin administered to prepubertal

mice would not advance the normal timing of puberty onset, but instead only reverse the delay of puberty that occurs as a consequence of reduced food intake.

## Materials and Methods

### *Animals*

Female and male Sprague-Dawley rats at various days of age were purchased from B&K Universal (Kent, Wash., USA) and Simonsen (Gilroy, Calif., USA). Prepubertal C57BL/6J female mice at 21 days of age were purchased from Jackson Laboratory (Bar Harbor, Me., USA). All animals were housed in the animal care facilities of the University of Washington Department of Comparative Medicine. Rats were maintained on a 14:10 h light/dark cycle. Mice were maintained on a 12:12 h light/dark cycle. Standard rodent chow and water were available *ad libitum* to the animals unless otherwise stated. The University of Washington's Animal Care Committee approved all animal procedures in accordance with the NIH Guide for Care and Use of Laboratory Animals.

### *Experimental Design*

*Experiment 1a.* Leptin levels were measured in male and female rats at 5, 10, 15, 20, 25, and 90 days of age. To obtain animals for the younger age groups (5 to 25 days old), we purchased 8 lactating rats, each with 6 male and 6 female newborns. All the pups were cross-fostered on the day of birth. A set of male and female pups was sacrificed every 5 days from day 5 to day 25. Day 90 animals were also obtained and sacrificed. All animals were asphyxiated in carbon dioxide, decapitated, and trunk blood collected at 1 h before lights off.

*Experiment 1b.* Leptin levels were measured in male and female rats at 5, 15, 25, 30, 35, 40, 50, 70, and 90 days of age. To obtain animals for the age groups of 5–40, we purchased 12 lactating rats that were timed pregnant and arrived each with 4 male and 4 female newborns. All pups were cross-fostered on the day of birth. At each time point from day 5 to day 40, 8 pups of each sex were sacrificed. Animals for the older age groups were obtained at 1 week younger than the desired age; each age group consisted of 8 animals per sex. All animals were asphyxiated in carbon dioxide, decapitated, and trunk blood collected at 1 h before lights off. Serum leptin levels were measured in blood samples collected at the time of sacrifice.

*Experiment 2.* Female Sprague-Dawley rats at 20 (juvenile,  $n = 8$ ), 30 (prepubertal,  $n = 6$ ), 45 (postpubertal,  $n = 6$ ), and 90 days of age (adult,  $n = 6$ ) were purchased from Simonsen Laboratory. They were sacrificed by CO<sub>2</sub> asphyxiation; brains were removed and sectioned at 20  $\mu\text{m}$ . Trunk blood was collected to measure LH, FSH, and estradiol.

*Experiment 3.* Twenty-one-day old female C57BL/6J mouse pups were randomly assigned to three groups ( $n = 15/\text{group}$ ). The first group received daily injections (i.p.) of 2  $\mu\text{g/g}$  leptin (a gift of Amgen, Inc., Thousand Oaks, Calif., USA) and were fed *ad libitum*; the second group received placebo injections and was pair-fed as in the leptin-treated group; the third group was the control group that received placebo injections alone and was fed *ad libitum*. Animals were weighed and food intake and vaginal opening (VO) were checked daily. Vaginal smears were collected daily from those mice that had shown VO to determine first estrus (FE). Starting on day 31, animals that had begun to cycle were sacrificed on the day of diestrus I. All remaining animals were sacrificed on day 36.

*<sup>33</sup>P-Labeled Rat Long-Form Leptin Receptor (Ob-Rb) cRNA Probe*

The plasmid vector containing a full-coding cDNA sequence of 3489 bp with a 95 bp 3' noncoding flanking sequence and 587 bp 5' noncoding flanking sequence of the rat Ob-Rb cDNA was kindly provided by Dr. Charles Rosenblum at Merck & Co., Inc. The cDNA was subcloned in the *Hind*III and *Xba*I sites of pcDNA3 expression vector (Invitrogen, Carlsbad, Calif., USA). A 138 bp *Hinc*II/*Kpn*I fragment (bases 2895–3033) that maps to the cytoplasmic tail region of the rat leptin receptor was subcloned into PDP18-T7/T3 vector (Ambion, Austin, Tex., USA). This fragment is selective for the long-form leptin receptor. To generate an antisense cRNA, the plasmid was linearized with *Eco*RI and transcribed with T7; to generate a sense cRNA, the plasmid was linearized with *Hind*III and transcribed with T3.

In vitro transcription was performed with the following reagents: 0.25 mCi lyophilized <sup>33</sup>P-labeled UTP, 1.8 µg linearized DNA; 2 µl of nucleotide mix that consisted each of 0.5 mM ATP, CTP, and GTP; 2 µl DTT, 2 µl 10 × transcription buffer (Boehringer Mannheim, Indianapolis, Ind., USA); 80 U RNase blocker (Gibco, Gaithersburg, Md., USA); 40 U T3 (Boehringer Mannheim) and 8.5 µl DEPC-treated water to bring the total volume of the reaction mixture to 20 µl. Four transcription reactions were performed for this experiment to yield sufficient amount of probes. After 90 min of incubation at 37 °C, 20 U DNase was added to digest the template DNA for 15 min. The reaction was terminated with 1 µl tRNA and 4 µl 0.5 mM EDTA. After the addition of 20 µl DEPC-treated water, the entire reaction mixture was purified with Sephadex spin column (Boehringer Mannheim).

*In situ Hybridization*

*Tissue Fixation and Delipidation.* Slides with brain sections were first removed from the –80 °C freezer, racked into baked metal holders, and dried. Next, racked slides were placed in 4% paraformaldehyde fixative (pH 7.4) for 5 min. They were washed twice in stirring 0.1 M phosphate buffer (0.02 M NaH<sub>2</sub>PO<sub>4</sub>, 0.08 M Na<sub>2</sub>HPO<sub>4</sub>, pH 7.4) for 5 min each, dipped in DEPC-treated water followed by 0.1 M TEA (pH = 8.0), and were placed in 0.1 M TEA, which was rapidly stirring with 0.25% acetic anhydride for 10 min. After a 3-min equilibration in 2 × SSC, tissues were put through a series of dehydrating alcohol (70, 95, 100%), followed by a 5-min delipidation step in chloroform, a rehydration step of 3 min each of 100 and 95% ethanol, and then air-dried.

*Prehybridization and Hybridization.* Tissues were prehybridized at 50 °C with a solution containing 75% hybridization buffer and 25% 10 mg/ml tRNA [17]. Silane-coated coverslips were used to allow close contact between the prehybridization buffer and tissue sections. After 2 h and 25 min, coverslips were removed and tissues were rinsed in 2 × SSC for 3 min, dehydrated for 3 min each in 70% and 95% ethanol, and air-dried. Slides were arranged in a plastic container that was lined with moist paper towels, which had been saturated with DEPC-treated water. Prewarmed hybridization buffer was mixed well with an appropriate amount of riboprobes and tRNA and was applied at a volume of 45–60 µl onto each slide, at a final concentration of 4 pmol/ml. All slides were covered with silane-coated glass coverslips and incubated overnight at 55 °C. Next, they were rinsed twice in stirring 4 × SSC for 15 min each. Tissue sections were first treated with RNase (30 µg/ml in 10 mM Tris, pH 8.0; 1 mM EDTA; and 50 mM NaCl) at 37 °C for 30 min and were then rinsed in RNase buffer alone for an additional 30 min. Following a 30-min 2 × SSC

wash at room temperature, all slides underwent another stringent treatment in 0.1 × SSC at 60 °C. After the hot wash, all tissues sections were rinsed through 0.1 × SSC at room temperature for 3 min, dehydrated through an increasing concentration of alcohol [50, 85, (each in 300 mM ammonium acetate), and 100%], and air-dried.

*Photosensitive Emulsion Dippings and Development.* In the absence of light, slides were dipped in undiluted NTB3 (Eastman Kodak Co., Rochester, N.Y., USA) that had been prewarmed to 45 °C. The slides were air-dried, then placed in light-proof boxes, and stored at 4 °C. After an exposure of 23 days, slides were developed with Kodak Developer and Kodak Fixer, and counterstained with cresyl violet. All slides were mounted in Permount to preserve tissue for visualization.

*Image Analysis.* A rat brain atlas was used as a guide to match anatomical sections across animals [18]. The density of the <sup>33</sup>P-labeled rat Ob-Rb cRNA probe was analyzed with a computerized image analysis system. Briefly, anatomical areas of interest were visualized with a Nikon Optiphot II microscope equipped with a 4 × objective and dark-field condenser. Video images were obtained by a Dage model CCD-72 camera (Dage-MTI, Inc., Michigan City, Ind., USA) and were projected onto a monitor. The area of interest was outlined and its gray level and that of the background were then determined. Subsequently, average gray level/area was compared to average background gray level and were expressed as the signal-to-background ratio (SBR). The following hypothalamic nuclei were examined: retrochiasmatic area (RCh), arcuate nucleus (ARC), ventromedial nucleus (VMN), anterior and ventral portion of the dorsomedial nucleus (DMNa, DMNv), ventral premamillary nucleus (PMv), and posterior periventricular nucleus (PVp).

*Control Experiment.* The identity and integrity of the radioactively-labeled Ob-Rb cRNA probes were verified by polyacrylamide gel electrophoresis against known standards. Control experiment conducted with sense riboprobes revealed no specific labeling.

*Hormone Measurements*

*Leptin.* Serum leptin concentrations were measured in the laboratory of Dr. Margery Nicolson at Amgen, Inc. by a solid-phase sandwich enzyme immunoassay (EIA) with an affinity purified polyvalent antibody immobilized in microtiter wells. Bound leptin was detected with affinity purified antibody conjugated to horseradish peroxidase and quantified with a chromogenic substrate. Leptin concentration was calculated from a standard curve generated for each assay with recombinant murine leptin as a standard. The inter- and intra-assay coefficients of variation were 6.5 and 4.7%, respectively.

*Luteinizing Hormone.* Serum LH concentrations were assayed with reagents from NIH. The standards used for LH were rLH-PR3 (anti-rLH-S11). Tracers for the assay were obtained from Corning-Hazelton, Inc. (Vienna, Va., USA). The intra-assay coefficient of variation was 16%. Serum LH was measured in the laboratory of Dr. William Bremner of the Puget Sound Veterans Administration Medical Center under the auspices of the NICHD Population Research Center.

*Follicle-Stimulating Hormone.* Serum FSH concentrations were assayed with reagents from NIH. The standards used for FSH were rFSH-PR2 (anti-rFSH-S11). Tracers for the assay were obtained from Corning-Hazelton, Inc. (Vienna, Va., USA). The intra-assay coefficient of variation was 11%. Serum FSH was measured in the laboratory of Dr. William Bremner of the Puget Sound Veterans Administration under the auspices of the NICHD Population Research Center.

*Estradiol.* Serum estradiol concentrations were measured by radioimmunoassay after chromatography of the samples. The sensitivity of this assay was 7.5 pg/ml and the intra-assay coefficient of variation was 6.9%. The assay was performed in the laboratory of Dr. David Hess in the Division of Reproductive Biology and Behavior at the Oregon Regional Primate Research Center in Beaverton, Oreg., USA.

#### Statistical Analysis

Data are presented as mean  $\pm$  SEM. One-way ANOVA with repeated measures followed by Fisher's PLSD tests was used to analyze data on food intake and body weight. Hormone levels and SBR were measured with one-way ANOVA followed by Fisher's PLSD tests. The nonparametric Kruskal-Wallis test followed by the Mann-Whitney U test for two group comparisons was used to analyze the data on vaginal opening and first estrus. A probability of less than 0.05 was considered statistically significant.

## Results

### Experiments 1a and 1b

*Body Weight and Leptin Levels.* Females gained weight steadily and their mean body weight at each time point was significantly different from each other ( $p < 0.0001$ ; fig. 1a). Leptin levels at all the prepubertal stages examined were not different from each other but were significantly lower than the level measured at day 90 ( $p < 0.0001$ ; fig. 1a). Males gained weight steadily in a similar fashion to the females ( $p < 0.0001$ ; fig. 1b). Male serum leptin levels fluctuated slightly more than the female; however, there were no significant differences among levels between day 5 and day 50 in the males and between day 5 and day 70 in the females (fig. 1b).

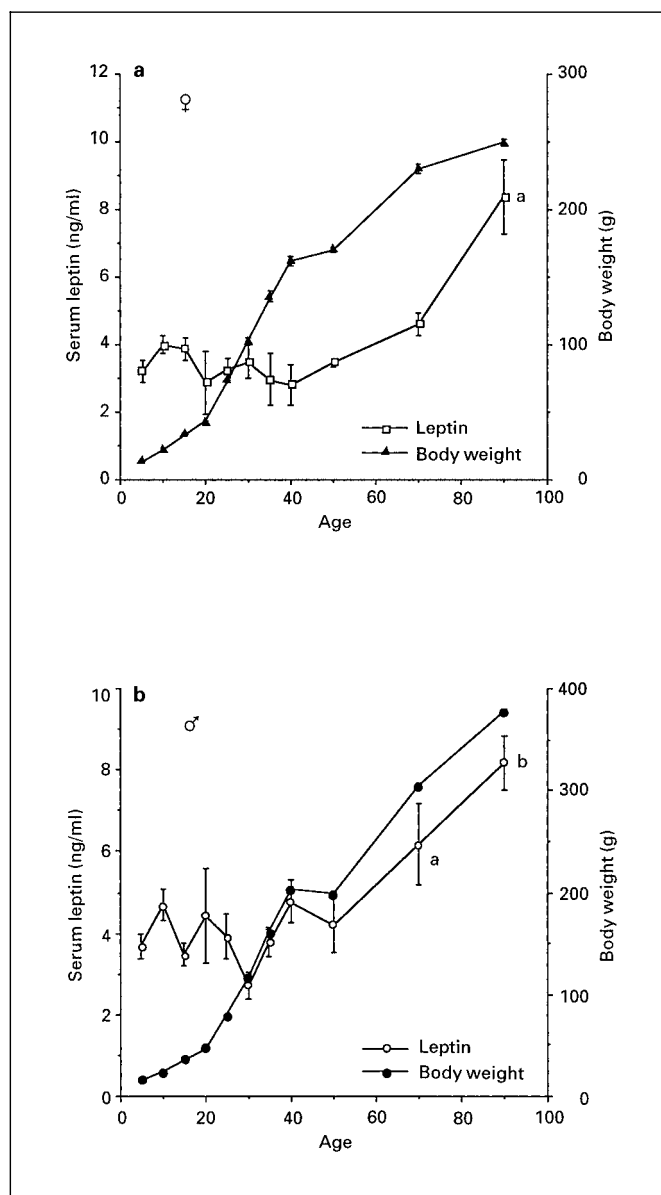
### Experiment 2

*Leptin Receptor Expression.* Levels of Ob-Rb mRNA, expressed in a ratio of signal-to-background gray level, were uniform across ages in all of the hypothalamic nuclei measured-RCh, ARC, VMN, DMNa, DMNv, PMv, and PVp (fig. 2).

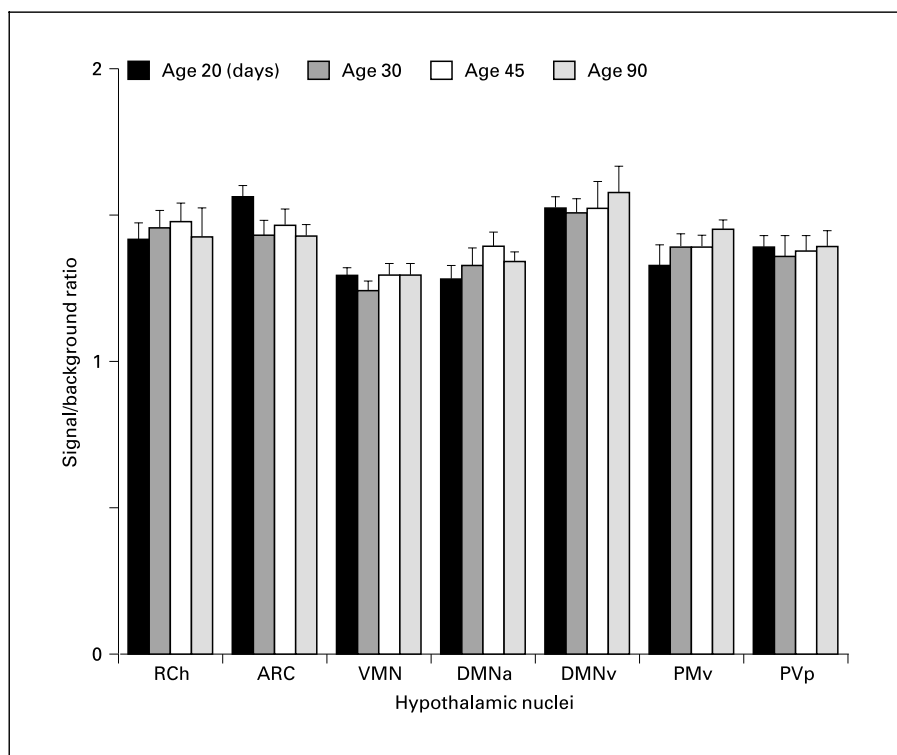
*Reproductive Hormone Levels.* As expected, FSH and LH levels were elevated at 20 days of age during the juvenile stage and remained low for the other three stages ( $p < 0.0001$ ; fig. 3a, b). Serum estradiol levels were elevated on day 20, and again on day 45 (n.s.; fig. 3c).

### Experiment 3

*Body Weight and Food Intake.* Leptin treatment induced a decrease in food intake in the developing female mouse; accordingly, food intake of the pair-fed animals was significantly decreased from that of the placebo-treated, ad libitum-fed animals but did not differ from the



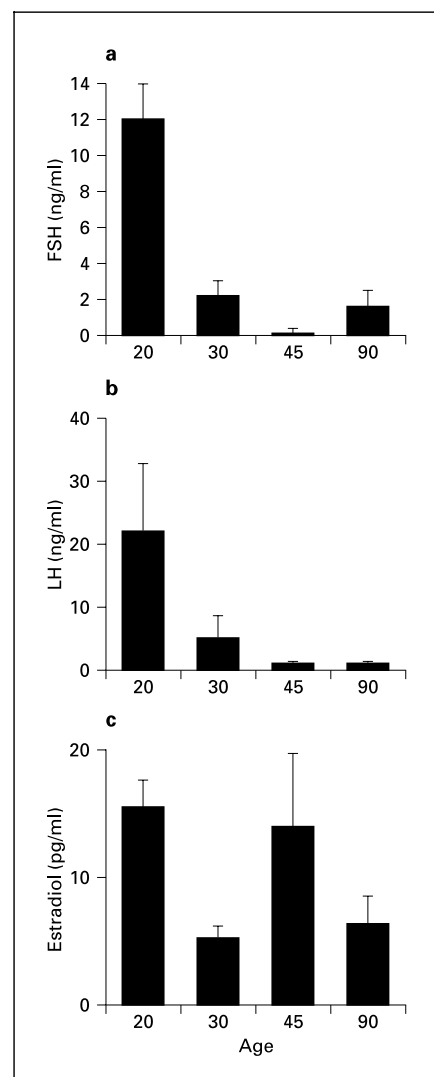
**Fig. 1.** Double-plot of serum leptin levels and body weight across development in (a) the female rat and (b) the male rat at various developmental ages. Body weight at each time point is significantly different from any other time point within a sex. The standard error bar for body weight is too small to be seen. The age and the number of animals used in the two combined studies are as follows. For females: d5 (n = 19), d10 (n = 12), d15 (n = 14), d20 (n = 6), d25 (n = 14), d30 (n = 8), d35 (n = 6), d40 (n = 6), d50 (n = 8), d70 (n = 9), d90 (n = 16). For males: d5 (n = 20), d10 (n = 12), d15 (n = 14), d20 (n = 6), d25 (n = 13), d30 (n = 8), d35 (n = 9), d40 (n = 9), d50 (n = 8), d70 (n = 7), d90 (n = 14). Different superscripts are significantly different from each other and from those without superscripts.



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**Fig. 2.** Comparison of long form leptin receptor (Ob-Rb) mRNA levels expressed as a ratio of signal-to-background gray level at 20 (n = 8), 30 (n = 6), 45 (n = 6) and 90 days of age (n = 6) in various hypothalamic nuclei of the female rat. There were no significant differences among groups in any of the nuclei examined. ARC = Arcuate nucleus; DMNa = anterior portion of the dorsomedial nucleus; DMNv = ventral portion of the dorsomedial nucleus; PMv = ventral premammillary nucleus; PVp = posterior periventricular nucleus; RCh = retrochiasmatic area; VMN = ventromedial nucleus.

**Fig. 3.** Comparisons of (a) serum FSH levels; (b) serum LH levels, and (c) serum estradiol levels measured at 20, 30, 45, and 90 days of age in the female rat.



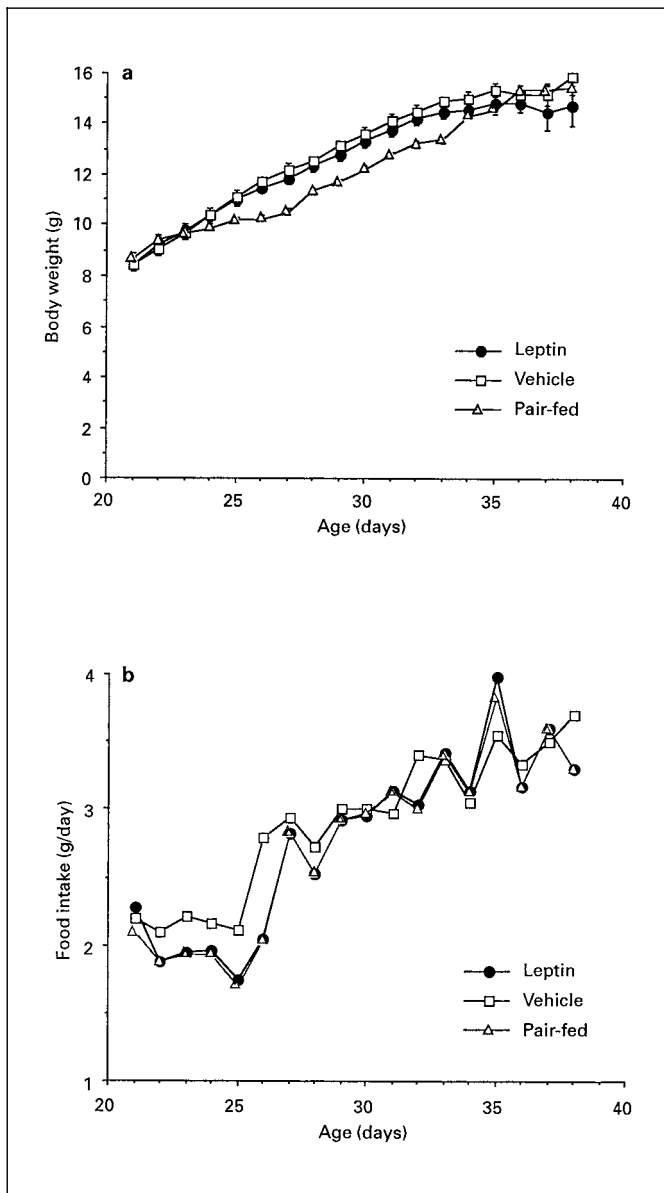
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leptin-treated animals ( $p < 0.0001$ ; fig. 4b). Body weight of the pair-fed animals was significantly decreased whereas there was no difference between the two ad libitum-fed groups (fig. 4a).

**Vaginal Opening and First Estrus.** The mean age of vaginal opening was not different among the three groups (placebo + ad libitum:  $28.8 \pm 0.9$ ; leptin + ad libitum:  $30.1 \pm 0.9$ ; placebo + pair-fed:  $30.2 \pm 0.9$ ;  $p = 0.48$ ; fig. 5a, 6a). The mean age of first estrus of the pair-fed group was significantly delayed compared to the other two groups (placebo + ad libitum:  $33.2 \pm 0.4$ ; leptin + ad libitum:  $32.4 \pm 0.6$ ; placebo + pair-fed:  $35.0 \pm 0.7$ ;  $p < 0.01$ ; fig. 5b, 6b).

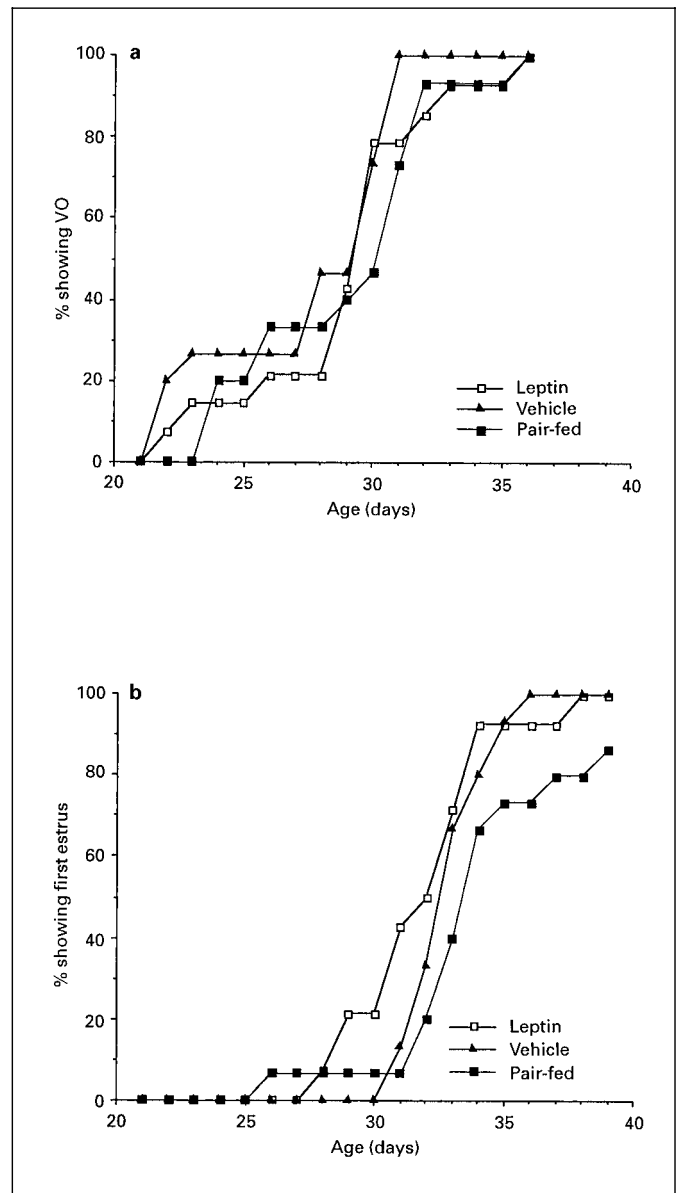
## Discussion

Previously published work in the rat has shown that although leptin can reverse the effects of caloric restriction on sexual maturation, it cannot advance the age of puberty onset beyond that time when it would normally occur in a well-nourished animal [11–13]. This would suggest that leptin, by itself, is not the primary trigger initiating puberty in this species. Indeed, we have previously shown that in severely food-restricted, prepubertal rats (fed ~ 70% of ad libitum-fed animals) treatment with leptin by itself, even at high doses, cannot overcome the hormonal and metabolic consequences of this caloric deprivation [11]. This would suggest that other factors, such as



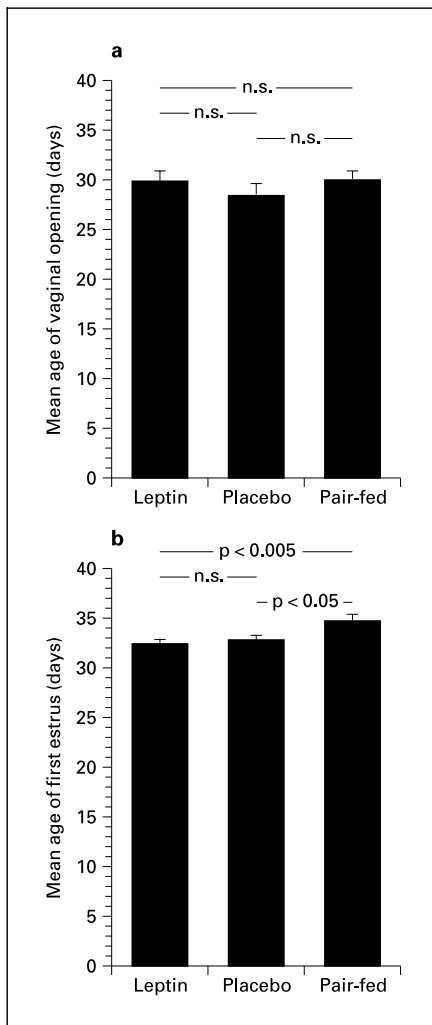
**Fig. 4.** Longitudinal measurements of (a) mean body weight and (b) mean food intake across groups of prepubertal female mice under different treatment regimens. The groups comprised vehicle-treated and ad libitum-fed animals (n = 15), leptin-treated and ad libitum-fed animals (n = 15), and vehicle-treated and pair-fed animals (n = 15). Leptin significantly decreased food intake, without affecting the animals' body weight.

fuel availability and insulin signaling, may be limiting for the effects of leptin to become manifest [4, 5, 19, 20]. From another perspective, if leptin were the metabolic trigger for the onset of puberty in the rat, one would predict that plasma levels of leptin would increase sometime



**Fig. 5.** Cumulative plots of the percent of animals showing (a) vaginal opening and (b) first estrus at each developmental stage, from 21 to 36 days of age, of groups of prepubertal female mice under different treatment regimens. The groups comprised vehicle-treated and ad libitum-fed animals (n = 15), leptin-treated and ad libitum-fed animals (n = 15), and vehicle-treated and pair-fed animals (n = 15). Leptin significantly advanced the percent of animals showing FE relative to the pair-fed animals.

shortly preceding puberty onset; however, the results of experiments 1a and 1b, demonstrating that serum levels of leptin stay relatively constant from day 10 to day 50 – spanning the full transition of pubertal development by several weeks on either side – do not support this hypothe-



**Fig. 6.** **a** Comparison of the age of VO in groups of prepubertal female mice under different treatment regimens. The groups comprised vehicle-treated and ad libitum-fed animals ( $n = 15$ ), leptin-treated and ad libitum-fed animals ( $n = 15$ ), and vehicle-treated and pair-fed animals ( $n = 15$ ). **b** A comparison of the age of FE among the same groups of prepubertal female mice. n.s. = Nonsignificant.

sis. Leptin levels begin to rise only after animals have entered the young adult stage, at approximately day 50 of postnatal life. Although leptin signaling may be permissive to puberty onset in the rat [6], the relatively constant plasma levels of leptin maintained across the boundary between prepubertal life and full sexual maturation, coupled with the fact that the administration of leptin to prepubertal rats does not advance the onset of puberty [11–13] is compelling evidence that leptin is not a metabolic trigger for puberty in this species.

It is noteworthy that other groups have studied developmental changes in plasma leptin levels in the rat and reported results that are similar, but not entirely congruent with those reported here. For instance, a report by Rayner et al. [21], demonstrated that leptin levels are low from day 21 to day 28, a trend that is similar to what we report; however, these same authors also observed a peak in plasma levels of leptin of 10 ng/ml that occurs at postnatal day 10, (apparently in parallel to levels of *ob* mRNA in the inguinal fat pad), which was not replicated in the present study. A similar peak in plasma leptin levels has been reported during the neonatal and infantile stage of development in the mouse; however, none of these studies report a significant change in plasma leptin levels across puberty [22, 23]. A more puzzling observation was found by Gruaz et al. [12] who reported a gradual rise in leptin levels from day 24 through day 59. In this longitudinal study, leptin levels were reported to almost triple from 290 pg/ml on day 24 to almost 1 ng/ml on day 59 of postnatal life. Levels of leptin reported in this study are much lower than those reported elsewhere (e.g.  $590 \pm 26$  pg/ml on day 30 in Gruaz et al. [12] vs.  $3.5 \pm 0.5$  ng/ml on day 30 in this study vs.  $\sim 3$  ng/ml on day 28 by Rayner et al. [21]). It could be that technical differences in methods of leptin preparation and measurement, animal housing and lighting schedules, blood sampling techniques, and feeding regimens account for the discrepancies in the results of these studies. In any case, there is no clear consensus about plasma leptin levels over development in any species (to date), and conclusions drawn from correlating these leptin data with indices of sexual maturation need to be treated with caution. Since the concentration of leptin in the circulation undergoes diurnal and pulsatile fluctuations, it would seem possible that changes in leptin during development occur, but do so in a more subtle fashion. If this were true, one might not be able to reveal the intricacies in the developmental changes of leptin by simply using a cross-sectional approach, with one time point/age measurement. This criticism is bolstered by a report from Nagatani et al. [24], who used frequent blood sampling in a longitudinal fashion to reveal the appearance of a nocturnal peak in plasma leptin levels that appears just prior to the onset of puberty in the female rat. Whether this nocturnal incident of leptin secretion plays a causal role in activating the reproductive axis at puberty remains to be determined. In any case, a consensus of experimental studies suggests that increasing plasma levels of leptin per se are unlikely to be the triggering event for initiating puberty in the rat. Nevertheless, it does seem likely that plasma levels of leptin must exceed some threshold to

support the process of sexual maturation, since leptin levels falling below this threshold (e.g. in the *fa/fa* rat, the *ob/ob* mouse or with food restriction in any species) impose a severe brake on the normal timing of pubertal onset. Although leptin levels were not measured in our earlier studies, we may infer from the work of others, in which fasting was reported to reduce leptin levels, that serum leptin concentrations in the food-restricted female rats that we studied were in fact lower than those in normal ad libitum-fed animals [25–27].

We also explored the possibility that leptin receptor expression in the hypothalamus increases across development, which could provide the means by which developing animals alter their sensitivity to an otherwise relatively stable plasma concentration of leptin. Evidence for such alteration in sensitivity involving pubertal changes in receptor expression can be found in other neuropeptide and neurotransmitter systems, such as galanin, insulin-like growth factor 1, and dopamine – all of which have been implicated to play a role in the onset of puberty in the female rat [28–30]. We reasoned that whether or not changes in circulating levels of leptin influence the timing of puberty onset in the normal female rat, it was conceivable that the responsiveness of the neuroendocrine axis to leptin might be increased at puberty, which would theoretically amplify leptin's biological effect on the neuroendocrine reproductive system across the transition from prepubertal to adult life – even in the presence of steady-state plasma levels of leptin. However, we did not detect any difference in expression of the leptin receptor message level across development in any brain area examined. It remains conceivable that an induction of leptin receptor expression does occur sometime preceding the onset of puberty – but involves some aspect of expression that does not require an increase in gene transcription (e.g. translational regulation). Nevertheless, leptin receptor mRNA can be transcriptionally regulated in the adult [31], and our results suggest that such regulation does not occur as a function of sexual development, at least in the female rat. Alternatively, it's possible that there is a subtle, but biologically significant, change in the expression of *Ob-Rb* mRNA over development, and that our methods simply lacked the sensitivity to resolve it; nonetheless, we simply could adduce no evidence to support the hypothesis that an age-dependent change in leptin receptor expression plays a role in governing the onset of puberty in the rat.

Although studies performed in the female rat indicate that leptin acts only as a permissive signal to the onset of puberty [11–13], similar studies performed in the mouse

paint a somewhat different and more controversial picture. Chehab et al. [14] reported an advancement in the date of VO in female C57BL/6J mice that were injected with 2 µg/g leptin daily, starting on 21 days of age. However, another study carried out by the same group of investigators under an identical experimental paradigm, which was reported in the footnote, showed no apparent difference in the date of VO between leptin-treated and PBS-treated mice [14]. These latter results are similar to the results we report here showing that injections of leptin in prepubertal female C57BL/6J mice starting at 21 days of age did not alter the average date of either VO or FE. However, the date of FE was delayed in pair-fed mice compared to leptin-treated mice despite their equivalent food intake. These results in the female mouse corroborate the data from our earlier studies in the female rat, suggesting that leptin may serve as a permissive signal to the onset of puberty in rodent species [11].

In a similar study by Ahima et al. [15], female C57BL mice that were injected with either 2 µg/g leptin or vehicle starting at 21 days of age showed similar weight gain, yet the onset of sexual maturation, as measured by the date of VO, FE, and vaginal cycling, occurred significantly earlier in the leptin-treated animals: the difference in the mean age of VO, FE, and cycling between the two groups amounted to 1–3 days. The physiological significance of such apparent advancement in pubertal indices by exogenous leptin administration is challenged by the concurrent finding that levels of endogenous leptin measured in animals on the date of VO did not differ significantly from those of the same age whose vaginas remained closed [15]. The absence of a rise in leptin levels prior to the occurrence of pubertal indices argues against the assertion that a threshold of leptin levels is sufficient for the onset of puberty. Again, any role of subtle changes in leptin levels in the peripubertal period, such as a selective nocturnal rise as reported by Nagatani et al. [24], remains unknown at this point. Nevertheless, our findings reported here as well as findings of other investigators generally show that circulating levels of leptin across puberty remain relatively constant [15, 21, 22], and that attaining a threshold leptin level is an essential but insufficient condition for the activation of the reproductive axis during puberty. The corollary is that when leptin levels deviate negatively from the threshold level, as is the case with food restriction or excessive demand on metabolic reserves, the normal progression of pubertal development becomes delayed or blocked [11–13].

In a recent study by Yura et al. [16], evidence is presented that transgenic skinny mice that overexpress the

leptin gene have accelerated puberty; however, this assertion is based on studies of relatively few animals and is based solely on the apparent differential rate of appearance of vaginal opening, for which its early occurrence in 3 of 9 animals provide the entire empirical basis of the argument. Furthermore, the conclusions about leptin's role in the onset of puberty in this study are muddled by the observation that there is no apparent effect of leptin's overexpression on puberty onset in the male mouse, unlike the female, making a generalized hypothesis about leptin's role in puberty difficult to defend. Given the inconsistency in the various reports of leptin's effects on the timing of the onset of puberty in rodents and the apparent lack of a rising plasma titer of leptin just prior to puberty, it would seem that conclusions about leptin's possible role in controlling the timing of puberty onset should be treated with considerable caution, if not skepticism. Species differences may account for some of these apparent discrepancies. In addition, the apparent differences in leptin's effect on body weight reported among the various mouse studies suggest that the different sources of recombinant leptin used in these studies is another uncontrolled variable that might help explain the disparate results.

In conclusion, we have demonstrated that serum leptin levels remain relatively constant during the prepubertal and postpubertal stages of both the male and the female rats. In addition, we could not detect any significant changes in leptin receptor gene expression in the hypothalamus across development of the female rat. Furthermore, exogenous administration of leptin to developing female mice did not advance the onset of puberty, thus corroborating our previous conclusions about leptin's role in the onset of puberty in the rat. Together, these results suggest that leptin does not serve as a triggering signal for the onset of puberty in either the rat or mouse, but rather may function as a permissive factor, whose presence is important but not sufficient.

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