The Stimulatory Effect of Leptin on the Neuroendocrine Reproductive Axis of the Monkey*

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ABSTRACT

Leptin acts as a metabolic activator of the neuroendocrine reproductive axis in several rodent species, but whether leptin plays a similar role in primates is unknown. To explore this question, we examined the effects of leptin on gonadotropin and testosterone secretion in male rhesus monkeys that were fasted for 2 days. Mean plasma levels of LH and FSH, LH pulse frequency, and LH pulse amplitude were significantly higher in leptin-treated animals compared with saline-treated controls during the second day of the fast. To identify targets for leptin's action, we used *in situ* hybridization and computerized imaging to map leptin receptor (Ob-R) messenger RNA (mRNA) distribution. Ob-R mRNA was observed in the anterior pituitary and several areas of the brain, including the arcuate and ventromedial nuclei of the hypothalamus. Ob-R mRNA was coexpressed in both POMC and neuropeptide Y neurons in the arcuate nucleus, whereas little or no coexpression of Ob-R mRNA was evident in GnRH neurons. These results suggest that leptin is a metabolic signal to the reproductive axis in primates and imply that both POMC and neuropeptide Y neurons are involved in mediating leptin's effects in the brain. (*Endocrinology* **139:** 4652–4662, 1998)

THE INITIATION and maintenance of reproductive function are governed by physiological factors associated with nutrition, adiposity, and metabolic rate (1, 2). Abnormalities in body weight and metabolism can influence the activity of the reproductive system. For example, in response to severe dietary restriction (*e.g.* anorexia nervosa) or heavy exercise (*e.g.* ballet dancing and long distance running), there may be delays in the onset of puberty and infertility in adulthood (3). Reproductive failure caused by metabolic stress is attributable to hypothalamic-pituitary dysfunction (1–3), and indeed, even subtle changes in diet produce significant alterations in LH secretion (4–7). The hypothalamic-pituitary-gonadal axis integrates information derived from the body's metabolic control centers and responds to maximize the organism's reproductive fitness (1, 2); however, the physiological mechanisms linking nutrition, metabolism, and reproduction remain poorly understood.

Leptin is a protein product of the *obese* (*ob*) gene. It is secreted as a hormone by adipocytes and acts on the brain to regulate body weight and metabolism (8). Leptin also appears to act as a metabolic signal to the reproductive axis. Animals that are either leptin deficient (*ob/ob* mice) or have defective leptin receptors (*db/db* mice and *fa/fa* rats) are hyperphagic, obese, and infertile (8, 9). We and others have shown that exogenous leptin stimulates the reproductive

system of the *ob/ob* mouse (10, 11) and counteracts the inhibitory effects of fasting on gonadotropin secretion in both the rat and the mouse (12, 13). Whether these effects of leptin can be generalized to other species is unknown. One of our experimental aims was to determine whether leptin acts as a metabolic signal to the reproductive axis of the primate. We hypothesized that the inhibition of gonadotropin secretion during dietary restriction in the monkey is at least in part attributable to low plasma levels of leptin. To test this hypothesis, we administered leptin to food-restricted monkeys. We provide evidence that leptin prevents the fasting-induced suppression of LH and FSH secretion.

The effects of leptin on gonadotropin secretion are thought to reflect its action on the neural circuitry governing GnRH secretion (13–15). However, neither the identity of leptin's target cells nor the cellular mechanisms by which this activational effect occurs are fully known. In the rodent brain, leptin receptor (Ob-R) messenger RNA (mRNA) and protein are expressed by cells in the arcuate nucleus (Arc) as well as other regions in the hypothalamus (16–21) that have been implicated in the regulation of both feeding and reproduction (22). Furthermore, cells that express neuropeptide Y (NPY) mRNA and POMC mRNA in the Arc coexpress the Ob-R mRNA (23, 24), but whether these same neuronal phenotypes also express Ob-R mRNA in a primate species is unexplored. Our second experimental objective was to map the distribution of Ob-R mRNA in the brain and pituitary of the monkey and to test the hypotheses that GnRH neurons in the forebrain and/or NPY and POMC neurons in the Arc are direct targets for the action of leptin. This was accomplished by conducting single and double label *in situ* hybridization (ISH) studies. We report that both POMC and

NPY neurons coexpress Ob-R mRNA, whereas few, if any, GnRH neurons appear to do so.

Materials and Methods

Animals

Exp 1. Peripubertal male rhesus macaques (*Macaca mulatta*; 3–5 yr old) were housed individually in a room with controlled temperature (23 ± 2 C) and photoperiod (12-h light, 12-h dark cycle) at the Oregon Regional Primate Research Center with the approval of the animal care committee and in accordance with the NIH Guide for the Care and Use of Laboratory Animals. The animals were fed monkey chow twice daily (at ~0800 h and 1530 h) and fresh fruit once daily (at ~1530 h). Fresh water was provided *ad libitum*. Each monkey was fitted with a vest that connects to a tether/swivel system as previously described (25). The monkeys were acclimated to the vest/tether/swivel system for 2 weeks before catherization of the subclavian and femoral veins during surgical anesthesia (25). Immediately after surgery, the animals were returned to the home cage. The lines from the catheters were protected by the vest/tether and connected to a two-channel swivel on the cage top. The two catheters were extended from the swivel through a wall into an adjacent room where blood sample collection and leptin infusion could be performed via the subclavian and femoral catheters, respectively. Patency of the catheters was maintained by continuously infusing heparinized saline (5 U/h) with a syringe pump (Harvard Apparatus, South Natick, MA).

Exp 2 and 3. All tissue used was obtained from gonadally intact female pigtailed macaques (*Macaca nemestrina*; 8–15 yr old) or from an intact male rhesus macaque (8 yr old) through the Tissue Distribution Program at the Regional Primate Research Center, University of Washington. All procedures were approved by the animal care committee of the School of Medicine at the University of Washington in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Experimental design

Exp 1. To determine whether exogenously administered leptin could prevent fasting-induced suppression of gonadotropin and testosterone secretion, four male rhesus macaques were fasted for 2 days (see Fig. 1 for a schematic representation of the experimental design). An experimental day was considered to begin and end at 0700 h. On the day before the fast began (day 0), the monkeys were fed at 0800 and 1530 h as usual, and blood (0.5 ml) was collected via the subclavian (or, if necessary, the femoral) vein every 20 min for 12 h from 1600–0400 h. Food was withheld from the animals for the next four meals (days 1 and 2 of the fast). On day 1 of the fast, infusion of leptin or saline began at 0700 h (15.5 h after the last meal) and continued for 45 h. Two of the monkeys received human recombinant leptin (134 µg/kg BW·h; provided by ZymoGenetics, Inc., Seattle, WA) (26), and two received saline through the
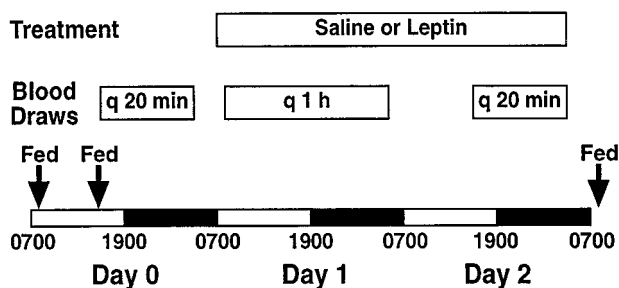

FIG. 1. Experimental design. The *bottom bar* represents time (*dark bar*, lights off). An experimental day was considered to begin and end at 0700 h. *Arrows* point to times when the animals were fed, and *middle bars* represent times when blood was drawn from the animals (*inset*, sampling frequency). Blood was drawn from 1600–0400 h on day 0, from 0800–0400 h on day 1, and from 1600–0400 h on day 2. The *top bar* represents the time of infusion of either vehicle (saline) or leptin (134 µg/kg BW·h).

femoral vein catheter. This dose of leptin was calculated to achieve a high physiological serum level of leptin (20–30 ng/ml) (27, 28) based on an assumed half-life of 30 min. Blood was sampled hourly from 0800–0400 h on day 1 of the fast. On day 2 of the fast, blood was collected every 20 min for 12 h from 1600–0400 h. Six weeks later, all of the monkeys were tested again in the same paradigm, and each monkey received the opposite treatment. During the first test session, the catheter of one of the monkeys treated with leptin became disconnected during the fast period, making it impossible to collect blood samples from 2400 h onward. Because these data were incomplete, they were excluded from the analysis. This monkey's catheter was replaced, and it received saline treatment during the second test session. To replace the data from this monkey's leptin treatment session, an additional monkey was added on the second test session and treated with leptin.

Exp 2. The distribution of Ob-R mRNA-expressing neurons in the forebrain, pons, and pituitary of the macaque was determined by single label ISH.

Exp 3. To determine whether GnRH, POMC, or NPY neurons are targets for leptin's action, we performed three separate double label ISH assays.

Tissue preparation

Animals used in Exp 2 and 3 were first sedated with ketamine and then overdosed with sodium pentobarbital. Approximately 20 min later, the brain was removed. The pituitary and two blocks of brain tissue were obtained. Forebrain tissue was blocked by making coronal cuts through the caudal diagonal band of Broca (DBB) and rostral midbrain, a horizontal cut through the corpus callosum, and saggital cuts lateral to the hypothalamus. Pontine tissue was blocked by making transverse cuts through the caudal midbrain and the rostral medulla and removing the cerebellum. The forebrain tissue was embedded in OCT (Tissue-Tek, Miles, Elkhart, IN) before freezing. All tissues were frozen on dry ice and stored at −80 C. Before cryostat sectioning, the tissue was allowed to equilibrate to −20 C. The forebrain and pons were sectioned in coronal and transverse planes, respectively. Sections were cut at 20 µm, thaw mounted onto SuperFrost Plus slides (Fisher Scientific International, Inc., Fairlawn, NJ), and stored in air-tight boxes at −80 C until needed. The forebrain and pons sections were collected onto 10 sets of slides, each representing a 1 in 10 series of sections. Thus, within each set, there were 200 µm between each section. The pituitary sections were collected onto 5 sets of slides, each representing a 1 in 5 series of sections. Thus, within each set, there were 100 µm between each section.

Riboprobe preparation

[33]P-Labeled Ob-R complementary RNA (cRNA) riboprobe. A 380-bp fragment of complementary DNA (cDNA) coding for human Ob-R in Bluescript S/K (Stratagene, La Jolla, CA) was provided by Joseph Kuijper (ZymoGenetics). This fragment is complementary to bases 1016–1396, which code for amino acids 275–402 in the extracellular domain of the receptor. The cDNA was linearized with *Eco*RI (New England Biolabs, Inc., Beverly, MA) and the ^{33}P-labeled antisense cRNA probe was synthesized *in vitro* by inclusion of the following ingredients in a volume of 20 µl: 50 pmol [^{33}P]UTP (New England Nuclear-DuPont, Wilmington, DE); linearized cDNA (2 µg); T7 RNA polymerase (80 U; Life Technologies, Grand Island, NY); transcription buffer (provided with polymerase); 500 µM ATP, CTP, and GTP; ribonuclease (RNase) inhibitor (80 U; Stratagene); and 10 mM dithiothreitol. Residual DNA was digested with deoxyribonuclease (DNase; 10 U; Stratagene), and the DNase reaction was stopped by adding 2 µl 0.5 M EDTA (pH 8.0). Finally, 1 µl yeast transfer RNA (tRNA; 10 mg/ml) was added as carrier. The cRNA was separated from unincorporated nucleotides on a Quick Spin Sephadex G-50 column (Boehringer Mannheim, Indianapolis, IN).

Digoxigenin-labeled GnRH cRNA riboprobe. The original pMSG plasmid containing a 470-bp fragment of cDNA coding for human prepro-GnRH was provided by John Adelman (29). The DNA fragment was isolated and subcloned into the *Sal*I site of Bluescript (Stratagene). This fragment contains 32 bp of the 5′-untranslated coding region, the entire coding region of prepro-GnRH, and 159 bp of the 3′-untranslated region. The cDNA was linearized with *Eco*RI, and the digoxigenin-labeled cRNA probe was transcribed *in vitro* in a 40-µl reaction containing the follow-

ing ingredients: linearized DNA (4 μg), DIG RNA labeling mixture (Boehringer Mannheim), T3 RNA polymerase (80 U; Boehringer Mannheim), transcription buffer, RNase inhibitor (80 U), and 10 mM dithiothreitol. Residual DNA was digested with DNase (20 U), and the reaction was stopped by adding 80 mM EDTA. The cRNA probe was separated from unincorporated nucleotides on a Quick Spin Sephadex G-50 column.

Digoxigenin-labeled NPY cRNA riboprobe. The human NPY cDNA-containing plasmid (pUC 8) was provided by Carolyn Worby (University of Michigan, Ann Arbor, MI). A 521-bp *Eco*RI fragment of the cDNA containing 86 bp of the 5'-untranslated sequence, the entire coding region for prepro-NPY, and 105 bp of the 3' untranslated sequence was subcloned into Bluescript II KS (Stratagene). The cDNA was linearized with *Hin*dIII (New England Biolabs), and the antisense digoxigenin-labeled cRNA probe was synthesized with T7 polymerase and purified as described above.

Digoxigenin-labeled POMC cRNA riboprobe. The original pGEM3 vector containing a 1040-bp fragment of cDNA coding for monkey (*Macaca nemestrina*) POMC was provided by Paresh Patel and Stanley J. Watson (30). A 957-bp *Hin*dIII-*Sal*I fragment containing 103 bp of the 5'-untranslated sequence, the entire 792-bp coding region, 46 bp of 3'-untranslated sequence, and 16 bp of the polylinker site was subcloned into pGEM4 (31). The cDNA was linearized with *Hin*dIII, and the antisense digoxigenin-labeled cRNA probe was synthesized with SP6 polymerase (Boehringer Mannheim) and purified as described above.

Preliminary test assays determined the optimal working dilutions for each of the digoxigenin-labeled riboprobes.

Single label ISH

To identify cells containing Ob-R mRNA, we performed single label ISH on forebrain (caudal DBB through the rostral mammillary bodies) and pontine (caudal midbrain through rostral medulla) tissue collected from three female pigtailed macaques and on pituitary tissue collected from three different female pigtailed macaques. In addition, we examined the distribution of Ob-R mRNA in forebrain tissue collected from one male rhesus macaque. Tissues were processed using a previously described protocol (32), with slight modification. The tissue was fixed, acetylated, and delipidated. Next, 45 μl/slide prehybridization solution containing hybridization buffer [52% deionized formamide, 10% dextran sulfate, 0.3 M NaCl, 8 mM Tris (pH 8.0), 0.08 mM EDTA, 0.02% BSA, 0.02% Ficoll, and 0.02% polyvinylpyrrolidone] and 15% tRNA (10 mg/ml; freshly denatured) were applied to the tissue, and the slides were covered with silane-coated glass coverslips and incubated in humid chambers for 2 h at 54 C. The sections were then rinsed in 2 × SSC (standard saline citrate), dehydrated with 70% and 95% ethanol, and air-dried. Next, 45 μl/slide hybridization buffer containing freshly denatured ³³P-labeled Ob-R probe (365,400 cpm/μl) and yeast tRNA (875 μg/ml) were applied to the tissue, and the slides were covered with silane-coated glass coverslips and incubated in humid chambers overnight at 54 C. The next day, the tissue was treated with RNase A and washed under conditions of increasing stringency, including a 30-min wash at 60 C in 0.1 × SSC. The tissue was then dehydrated and air-dried. The slides were dipped in Kodak NTB-3 emulsion (Eastman Kodak Co., Rochester, NY), exposed for 8 days at 4 C, and developed.

Double label ISH

³³P-Labeled Ob-R and digoxigenin-labeled GnRH mRNA. To identify cells that express Ob-R mRNA and GnRH mRNA, we performed double label ISH on forebrain tissue (caudal DBB through the rostral mammillary bodies) collected from three female pigtailed macaques, using a previously described procedure with slight modifications (32). In brief, sections were pretreated as described above for single label ISH (fixation through delipidation/rehydration). Sections were then prehybridized with 25% tRNA (10 mg/ml) in hybridization buffer at 54 C. Next, 45 μl hybridization solution, consisting of hybridization buffer containing freshly denatured ³³P-labeled Ob-R probe (661,000 cpm/μl), digoxigenin-labeled GnRH probe, and tRNA (1.8 mg/ml) were applied to the tissue. The slides were covered with silane-coated coverslips and incubated in humid chambers overnight at 54 C. The next day, the tissue was

treated with RNase A and washed under conditions of increasing stringency, including a 30-min wash at 60 C in 0.1 × SSC. The sections were then incubated for 60 min in blocking buffer, rinsed in buffer 1 (100 mM Tris-HCl and 150 mM NaCl, pH 7.4), and then incubated in buffer 1 containing antidigoxigenin fragments conjugated to alkaline phosphatase (Boehringer Mannheim) diluted 1:1000, 1% normal sheep serum, and 0.3% Triton X-100 for 3 h at 37 C and then overnight at 4 C. Next, the sections were rinsed in buffer 1, rinsed in buffer 2 (100 mM Tris-HCl, 50 mM MgCl₂, and 100 mM NaCl, pH 9.5), and then incubated in buffer 2 containing 4-nitro blue tetrazolium-chloride (340 μg/ml; Boehringer Mannheim), 5-bromo-4-chloro-3-indolyl phosphate (175 μg/ml; Boehringer Mannheim), and levamisole (240 μg/ml). When cells containing dark purple precipitate (corresponding to cells containing hybridized digoxigenin-labeled riboprobe) were clearly visible at the light microscopic level, the reaction was stopped by rinsing the sections in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Next, the slides were dipped in 70% ethanol, air-dried, and coated in 3% parlodion dissolved in isoamyl acetate. After air-drying, the slides were dipped in Kodak NTB-3 emulsion, exposed for 10 days at 4 C, and developed.

³³P-Labeled Ob-R and digoxigenin-labeled NPY mRNA. We performed double label ISH to identify hypothalamic cells (retrochiasmatic area through the caudal Arc) containing both Ob-R mRNA and NPY mRNA in tissue obtained from the same three pigtailed macaques used in the double label ISH assay described above. The protocol used was similar to the one described immediately above with the following modifications: 1) the hybridization solutions were applied to the tissue sequentially and consisted of hybridization buffer containing freshly denatured ³³P-labeled Ob-R probe (672,960 cpm/μl hybridization solution) and yeast tRNA (1.9 mg/ml) and of hybridization buffer containing freshly denatured digoxigenin-labeled NPY probe and yeast tRNA (2.0 mg/ml); 2) the tissue was incubated for 9.5 and 6.5 h in the two hybridization solutions, respectively; and 3) slides were exposed for 5 days at 4 C.

³³P-Labeled Ob-R and digoxigenin-labeled POMC mRNA. We performed a double label ISH to identify neurons in the retrochiasmatic area and Arc that contain both Ob-R mRNA and POMC mRNA in tissue obtained from the same three pigtailed macaques as those used in the double label ISH assays described above. The protocol used was similar to the one described above with the following modifications: 1) the slides were prehybridized with 15% tRNA in hybridization buffer; 2) the hybridization solution consisted of freshly denatured ³³P-labeled Ob-R probe (491,000 cpm/μl hybridization solution), digoxigenin-labeled POMC probe, and yeast tRNA (1.3 mg/ml) in hybridization buffer; and 3) the slides were exposed for 7 days at 4 C.

Control experiments

The identity and integrity of the ³³P-labeled cRNA probe for Ob-R mRNA were verified by PAGE against known standards. The labeling seen in brain regions treated with radiolabeled antisense riboprobe was absent in tissue hybridized with a ³³P-labeled sense riboprobe. In addition, specific labeling was abolished when the tissue was hybridized with a ³³P-labeled antisense riboprobe in the presence of a 100-fold excess of nonradiolabeled antisense riboprobe.

The identity and integrity of the digoxigenin-labeled cRNA probes were verified by agarose gel electrophoresis against known standards. Control experiments to validate the specificity of the POMC probe have been previously described (31). The anatomical distributions of digoxigenin-labeled cells that we observed in these studies agree with previously published data on the localization of GnRH, NPY, and POMC cell bodies (33–35) and suggest that our probes specifically hybridized to the correct mRNAs.

Hormone assays

LH and testosterone assays were performed by the Oregon Regional Primate Research Center Endocrine Assay Laboratory. Plasma LH was determined by the mouse Leydig cell bioassay (36) in duplicate samples, and plasma testosterone concentrations were measured by RIA in single samples (37). The intra- and interassay coefficients of variation for LH were 15.3% and 23.9%, respectively, and those for testosterone were 4.7% and 7.9%, respectively. FSH levels were measured in single samples by the Center for Research in Reproductive Physiology, Assay Core, at the

University of Pittsburgh (Pittsburgh, PA) using homologous RIA reagents supplied by the National Hormone and Pituitary Program. Recombinant cynomolgus FSH (NICHHD Rec-MoFSH-RP-1, AFP 6940A) was employed for the reference preparation and the radioiodinated trace, and a polyclonal rabbit antiserum (AFP782594) raised against recombinant cynomolgus FSH was used as the first antibody. The intraassay coefficient of variation was 6%. Leptin levels were measured in duplicate by RIA using the Primate Leptin RIA Kit (Linco Research, Inc., St. Louis, MO). The intraassay coefficient of variation was 18%.

Data analysis

Exp 1. The peripubertal animals used in this study exhibited LH and testosterone pulses primarily during the dark phase of the light-dark cycle. Therefore, analyses of hormone levels were restricted to the dark phase (9 h) on day 0 (prefast period), day 1 of the fast (fast day 1 period), and day 2 of the fast (fast day 2 period). LH pulse frequency and amplitude were determined by the DC3 pulse analysis program (38). Means and SEMs for LH and testosterone levels, LH pulse frequency, and LH pulse amplitude were calculated for the prefast and fast day 2 periods. For additional hormone measurements during the prefast, fast day 1, and fast day 2 periods, equal amounts of plasma were pooled from blood samples collected during each of these periods. Means and SEMs for plasma levels of FSH and leptin were calculated from these pooled samples. (During blood sampling, one animal's subclavian vein catheter became clogged when it received leptin treatment. This required further samples to be obtained through the femoral vein catheter, which was used for the infusion of recombinant leptin. The monkey's blood samples from this session were contaminated with the infused leptin and thus were omitted from the analysis.) Unpaired *t* tests were used to determine group (treatment) differences in mean levels of hormones for the prefast, fast day 1, and fast day 2 periods. The Mann-Whitney U test was used to determine group differences in LH pulse frequency and amplitude for the prefast and fast day 2 periods. The rejection level for all statistical tests was set at $\alpha = 0.05$.

Exp 2. For the single label ISH assay, tissue was viewed with a Zeiss Axioskop microscope (Zeiss, New York, NY), and Ob-R mRNA-expressing neurons were identified under brightfield illumination by the presence of a discrete grain cluster. The distribution of these clusters was transferred to standard atlas plates modified from those provided by the Regional Primate Research Center at the University of Washington, Seattle, WA (Template Atlas of the Primate Brain; http://rprcsgi.rprc.washington.edu/atlas/)

Exp 3. For the double label ISH assays, the tissue sections were analyzed according to a protocol previously published (39). Briefly, sections were viewed under a microscope, and images were captured with a Dage model 85 camera (Dage-MTI, Inc., Michigan City, IN) for computer analysis. GnRH, NPY, or POMC mRNA-containing neurons were outlined under brightfield illumination, and then silver grains were counted over the identified neurons under darkfield illumination. Tissue from each animal was sampled by reading grains over all clearly discernible GnRH, NPY, or POMC neurons on one side of the brain in sections taken every 400 μm. Signal to background ratios (SBRs) for individual cells were calculated as previously described (40). For each animal, the number of counted cells with a SBR greater than a particular value (*e.g.* 0, 1, 2, *etc.*) was computed and then converted to a percentage of the total counted cells (cumulative frequency curve). The percentages at each SBR interval were averaged among the three animals in each assay to produce the means and SEMs.

Because GnRH, NPY, and POMC double label assays were performed separately, we made two additional measurements to assess the similarity of Ob-R mRNA labeling across experiments. First, a small, random sample of clusters corresponding to Ob-R mRNA was counted for grains in the Arc of each animal in each assay without regard to whether the clusters were over cells expressing GnRH, NPY, or POMC mRNA. These data were used to compute mean SBRs for Ob-R mRNA-containing cells in the Arc. Second, we sampled background grain density from all sections analyzed in each of the three double label experiments. Results from both methods were analyzed by ANOVA followed by Fisher's protected least significant difference (PLSD) test.

Results

Exp 1

Peripheral infusion of leptin at the dosage of 134 μg/kg BW·h resulted in a significant difference in mean levels of leptin between leptin-treated and saline-treated animals during the fast day 1 period (11.9 ± 2.5 *vs.* 2.6 ± 0.9 ng/ml; $P < 0.05$) and the fast day 2 period (24.8 ± 3.3 *vs.* 5.3 ± 1.2 ng/ml; $P < 0.05$). This dose of leptin prevented fasting-induced suppression of LH, FSH, and, to a lesser extent, testosterone secretion in male monkeys. Figure 2 shows concomitant patterns of bioactive LH and testosterone in plasma before and during fasting with either saline or leptin in one animal. During the prefast period, FSH levels, LH levels, LH pulse frequency, and LH pulse amplitude were not significantly different between leptin-treated and saline-treated monkeys ($P > 0.05$; data not shown). In contrast, during the fast day 2 period, FSH levels, LH levels, LH pulse frequency, and LH pulse amplitude were significantly higher in leptin-treated than in saline-treated monkeys [FSH levels, 0.11 ± 0.01 *vs.* 0.06 ± 0.01 ng/ml ($P < 0.005$, Fig. 3A); LH levels, 8.5 ± 3.0 *vs.* 1.3 ± 0.2 ng/ml ($P < 0.05$; Fig. 3B); LH pulse frequency, 3.0 ± 1.3 *vs.* 0 ± 0 pulses/9 h ($P < 0.05$; Fig. 3C); LH pulse amplitude, 16.0 ± 7.0 *vs.* 0 ± 0 ng/ml ($P < 0.05$; Fig. 3D)]. The differences in LH secretion arose from the complete absence of LH pulses in all four saline-treated monkeys during the fast day 2 period. [The monkey whose catheter become disconnected during the leptin test session showed no pulsatile release of LH throughout the part of the fast period for which we were able to obtain samples (see *Materials and Methods*).]

Leptin's effects on testosterone secretion were not as robust. During the prefast period, testosterone levels were not significantly different between leptin-treated and saline-treated monkeys ($P > 0.05$). Although testosterone levels were higher in leptin-treated than in saline-treated monkeys during the fast day 2 period, this difference was not statistically significant (2.6 ± 0.6 *vs.* 1.3 ± 0.5; $P < 0.09$).

Exp 2

The overall distribution of Ob-R mRNA-expressing cells in ventral regions of the forebrain (DBB through the mammillary bodies), pons, and pituitary was similar in all female pigtailed macaques examined. We also found a similar distribution of Ob-R mRNA in the forebrain of the one male rhesus macaque we examined. Figure 4 shows the distribution of Ob-R mRNA-expressing cells in various regions of the forebrain and pons. We found that Ob-R mRNA is highly expressed in the choroid plexus. Within the forebrain, the highest density of cells that express Ob-R mRNA was seen in the retrochiasmatic area, Arc (Figs. 4, B and C, and 5A), and the medial portion of the ventromedial nucleus of the hypothalamus (VMN; Figs. 4, B and C, and 5A). A lower density of Ob-R mRNA-expressing cells was seen in the bed nucleus of the stria terminalis and in a distinct region of the medial preoptic area (MPO) just rostral to the level of the decussation of the anterior commissure. These cells were distributed in the shape of an inverted V and were found just lateral to the preoptic periventricular nucleus and in the ventral portion of the median preoptic nucleus (Fig. 4A). Ob-R mRNA-expressing cells were seen scattered throughout the ventral

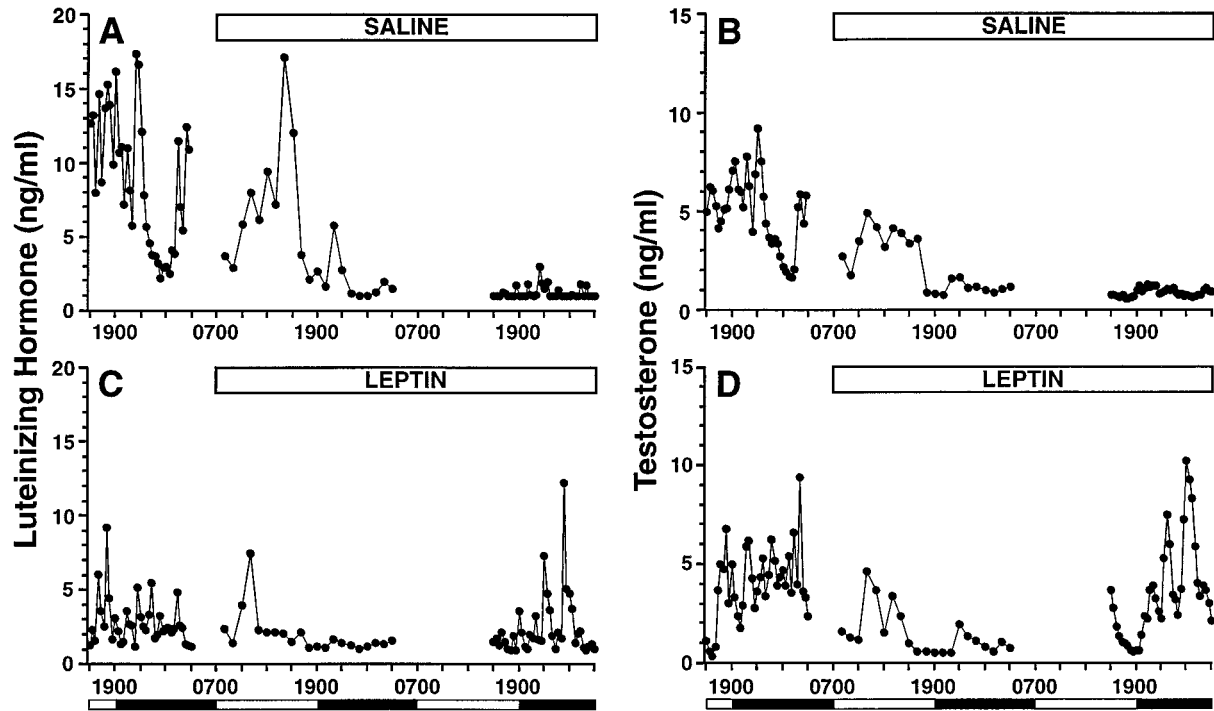LEPTIN STIMULATES THE MONKEY REPRODUCTIVE AXIS

FIG. 2. A and C, Effect of leptin on plasma LH in a single monkey. B and D, Effect of leptin on testosterone in the same monkey. *Inset box*, Time of treatment with either saline (A and B) or leptin (C and D). Values represent means of duplicates (A and C) or single measurements (B and D).

hypothalamus and were present in the lateral hypothalamic area (Fig. 4, B and C), the periventricular nucleus (Fig. 4, B and C), the lateral portion of the VMN (Figs. 4, B and C, and 5A), the median eminence (Fig. 4B), the lateral tuberal nuclei (Fig. 4C), the tuberomammillary nucleus (Fig. 4C), the perifornical area, and the ventral premammillary nucleus. A high density of Ob-R mRNA was seen in the medial mammillary nucleus; however, this labeling was diffuse and was not restricted to discrete clusters. Ob-R mRNA was not expressed at detectable levels in the DBB, nucleus basalis of Meynert, striatum, septum, thalamus, or dorsomedial or paraventricular nuclei of the hypothalamus.

In the pons, there was a high density of Ob-R mRNA-expressing cells restricted to the dorsal raphe nucleus (Fig. 4D). Ob-R mRNA-expressing cells were clearly visible in the anterior pituitary (Fig. 5B), but were not detectable in the intermediate or posterior lobes of the pituitary.

Exp 3

Ob-R mRNA expression in GnRH mRNA-expressing neurons. GnRH mRNA-expressing cells were widely scattered in the DBB, medial septum, MPO, and mediobasal hypothalamus. Although distinct clusters of silver grains, indicating the presence of Ob-R mRNA, were found near GnRH mRNA-expressing neurons (Fig. 6A), virtually no GnRH-expressing neurons were associated with an overlying cluster of grains.

Ob-R mRNA expression in NPY mRNA-expressing neurons. NPY mRNA-expressing neurons were found in the Arc as well as the caudate nucleus. In the Arc, some NPY mRNA-expressing neurons had overlying clusters of silver grains,

suggesting that they coexpressed Ob-R mRNA (Fig. 6B); however, Ob-R mRNA expression was not restricted to NPY neurons.

Ob-R mRNA expression in POMC mRNA-expressing neurons. POMC mRNA-expressing neurons were restricted to the retrochiasmatic area and the Arc. Many POMC mRNA-expressing neurons had overlying clusters of silver grains, indicating that they coexpressed Ob-R mRNA (Fig. 6C). Ob-R mRNA expression was not restricted to POMC mRNA-expressing neurons.

Semiquantitative assessment of Ob-R mRNA coexpression. Measurements of SBR in all three double label assays are displayed as a cumulative density function in Fig. 7. The *ordinate* value of each point represents the percentage of neurons having a SBR greater than a particular integer (*abscissa*). This graph reveals the percentage of neurons considered to be double labeled at any arbitrarily determined threshold or SBR level. For example (see Table 1), with a threshold criterion of signal at least 6 times the background (SBR = 6), 7% of GnRH mRNA-labeled, 19% of NPY mRNA-labeled, and 46% of POMC mRNA-labeled cells would be considered to also express Ob-R mRNA. Our tests of similarity in Ob-R mRNA labeling across assays revealed that, on the average, Ob-R mRNA clusters in the Arc in the GnRH double label experiment had a reduced SBR compared with those in both the NPY and POMC experiments (data not shown). Average SBRs for NPY and POMC experiments were not different from each other. This assessment was confirmed by grain density readings taken from the septum of each animal, which demonstrated that tissues from the GnRH experiment
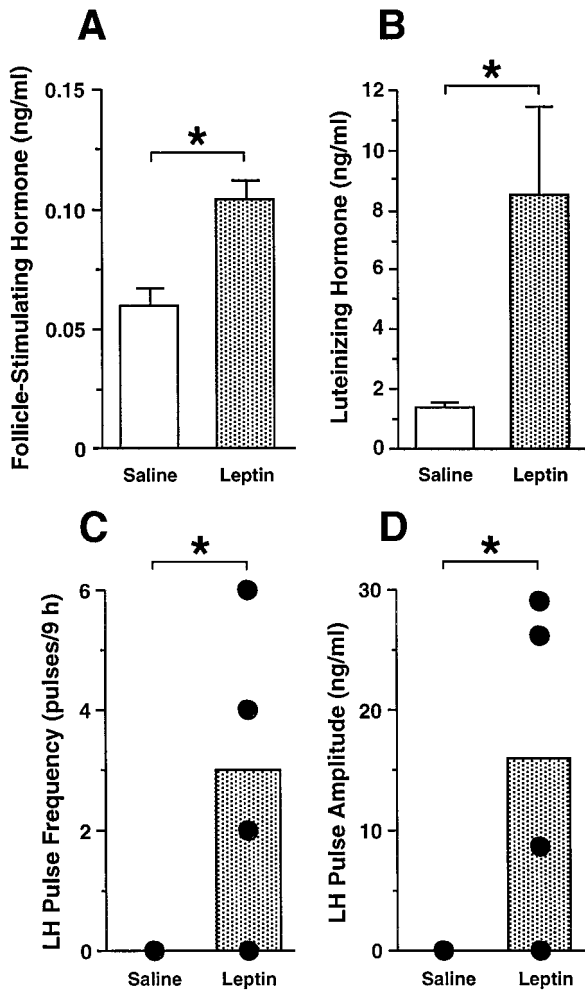
FIG. 3. A, FSH values (mean ± SEM) during the fast day 2 period (dark phase of the sampling period on day 2) for each treatment group. B, LH values (mean ± SEM) during the fast day 2 period for each treatment group. C, LH pulse frequency (mean ± SEM) during the fast day 2 period for each treatment group. D, LH pulse amplitude (mean ± SEM) during the fast day 2 period for each treatment group. In each panel, n = 4/group. *, $P < 0.05$ compared with saline-treated controls. In C and D, *circles* represent values from individual animals.

had elevated background grain levels compared with those in both the NPY and POMC experiments (which were not different from each other; data not shown).

Discussion

In the present study, we have provided evidence that leptin relays nutritional and metabolic information to the reproductive axis of the monkey. Our results confirm previous observations that gonadotropin and testosterone secretion are markedly suppressed in fasted male monkeys (4–7). Fasting is associated with reduced plasma levels of leptin in all species studied to date, including humans (12, 13, 41–43). Based on the results of this study, we infer that reduced plasma levels of leptin are at least in part responsible for the fasting-induced inhibition of the reproductive axis. Our results are reminiscent of the effects of leptin on LH secretion in fasted mice and rats (12, 13), and together these
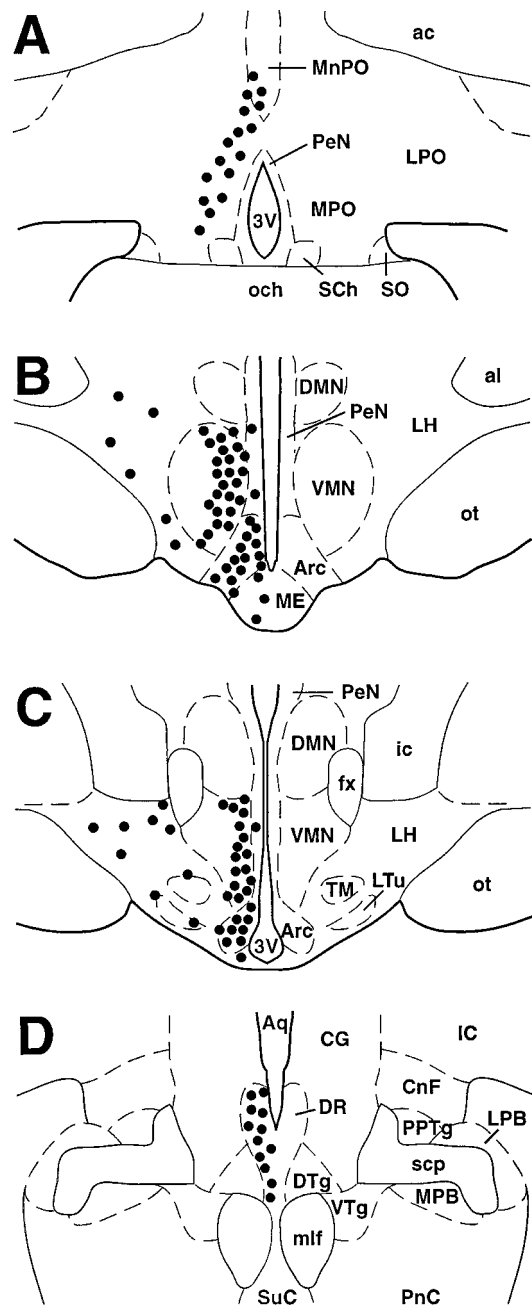
FIG. 4. Distribution of leptin receptor (Ob-R) mRNA-containing cells in the brain. *Circles* represent areas of silver grain clusters corresponding to Ob-R mRNA. A–C, Representative coronal sections through the forebrain. D, Representative coronal section through the pons. 3V, Third ventricle; ac, anterior commissure; al, ansa lenticularis; Aq, cerebral aqueduct; CG, central gray substance; CnF, cuneiform nucleus; DMN, dorsomedial nucleus of hypothalamus; DR, dorsal raphe nucleus; DTg, dorsal tegmental nucleus; fx, fornix; IC, inferior colliculus; ic, internal capsule; LPB, lateral parabrachial nucleus; LH, lateral hypothalamic area; LPO, lateral preoptic area; LTu, lateral tuberal nuclei; ME, median eminence; mlf, medial longitudinal fasciculus; MnPo, median preoptic nucleus; MPB, medial parabrachial nucleus; och, optic chiasm; ot, optic tract; PeN, periventricular nucleus of hypothalamus; PnC, caudal pontine reticular nucleus; PPTg, pedunculopontine nucleus; SCh, suprachiasmatic nucleus; scp, superior cerebellar peduncle; SO, supraoptic nucleus; SuC, superior central nucleus; TM, tuberomammillary nucleus; VTg, ventral tegmental nucleus.
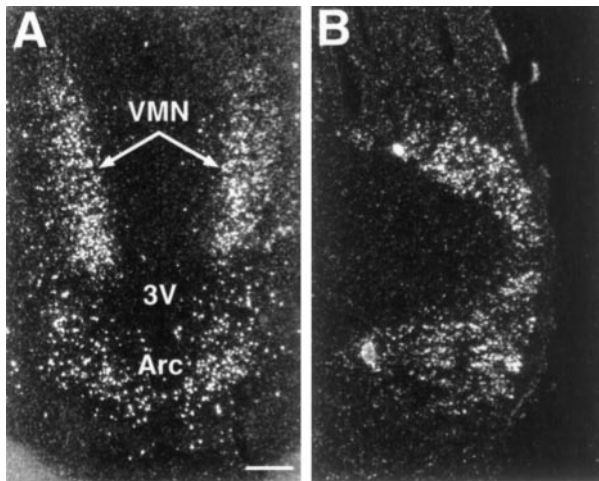
FIG. 5. Ob-R mRNA expression, seen as clusters of white dots, in the brain and pituitary. A, A darkfield photomicrograph showing intense signal in the VMN and Arc of a male monkey. B, A darkfield photomicrograph showing signal in a restricted region of the anterior lobe of the pituitary gland of a female monkey. *Bar* = 500 μm.

observations suggest that leptin acts via similar mechanisms on the reproductive axes of primate and rodent species.

In rodents, at least some of the effects of leptin on the reproductive system are thought to be mediated by the brain. LH secretion is increased by the central administration of leptin to female rats (15), whereas LH is suppressed and estrous cycles are disrupted by central infusion of leptin antiserum (14). Furthermore, Ob-R mRNA and protein have been found in the rat and mouse brain, particularly in the hypothalamus (16–21). Taken together, these data suggest that leptin acts on the brain to regulate GnRH secretion. Ob-R mRNA is also expressed in the pituitary (44–46), identifying this as another potential site for leptin's action on gonadotropin secretion. However, because Ob-R mRNA is also expressed in the gonads (46, 47) as well as in many other sites throughout the body, including the liver, kidneys, and adrenals (46, 48), leptin could influence hormones of the reproductive system through indirect mechanisms. For example, it is possible that leptin alters plasma levels of hormones (such as cholecystokinin and insulin) and metabolic fuels (such as glucose and fatty acids), which, in turn, influence gonadotropin secretion (1, 2). The relative contributions of the central and peripheral effects of leptin in the regulation of reproduction are important issues that remain to be resolved.

To evaluate the possible action of leptin on the brain and pituitary of the primate, we examined the expression of Ob-R mRNA in these tissues. Ob-R exists as several isoforms generated from differential splicing (49). The long form, designated Ob-Rb, has a complete cytoplasmic tail and is thought to be the signaling variant (49). Although the functions of the short forms are not well delineated, at least one of the short forms, Ob-Ra, is thought to act as a transporter for leptin (49). In the present study, we used a cRNA probe made to the extracellular domain of the Ob-R, which does not discriminate between alternatively spliced forms of Ob-R mRNA. We found Ob-R mRNA-expressing cells in restricted portions of the anterior pituitary. At present, the identity of the cells that
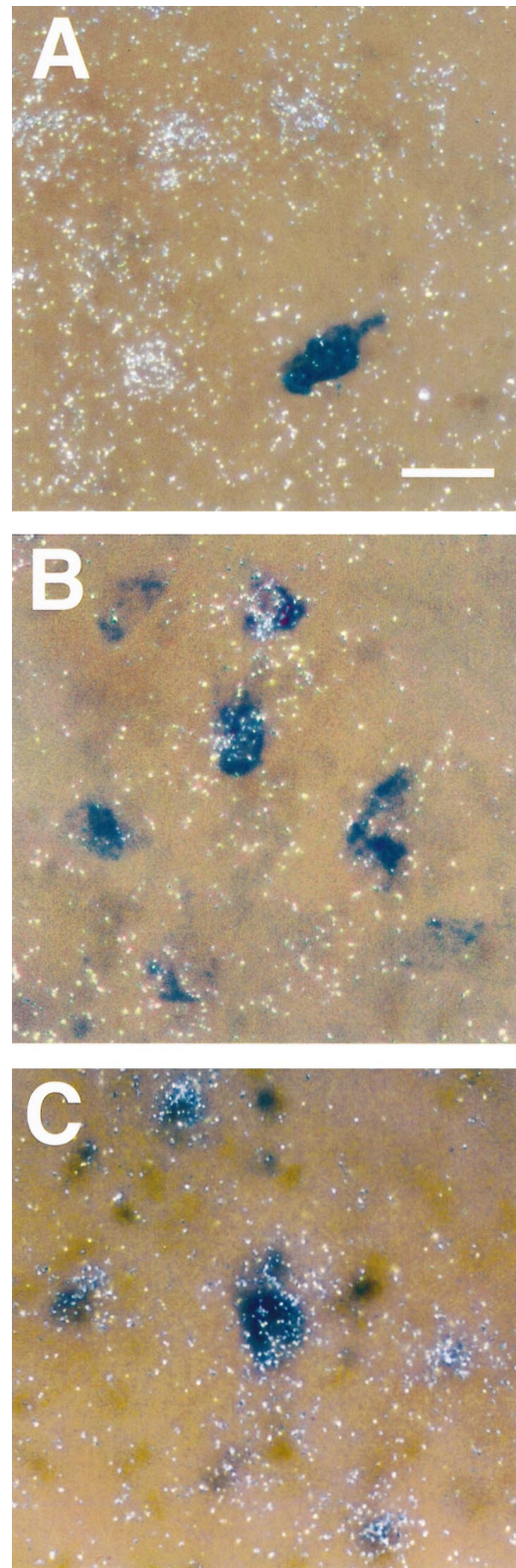

FIG. 6 A, Leptin receptor (Ob-R) mRNA expression (*clusters of white dots*) near, but not in, GnRH mRNA-expressing neurons (cells containing *purple-black* precipitate). B, Ob-R mRNA expression (*clusters of white dots*) in a subset of NPY mRNA-expressing neurons (cells containing *purple-black* precipitate). C, Ob-R mRNA expression (*clusters of white dots*) in a subset of POMC mRNA-expressing neurons (cells containing *purple-black* precipitate). *Bar* = 30 μm.
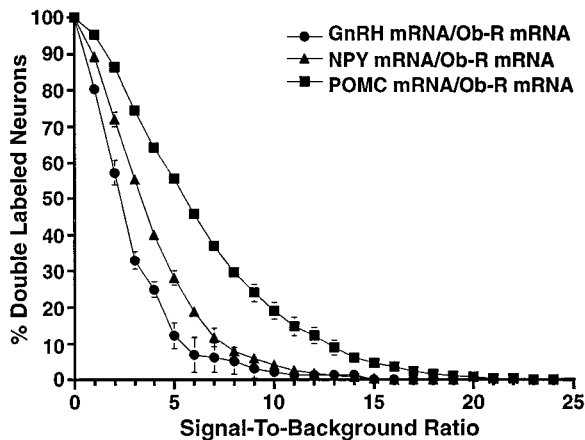
FIG. 7. Percentage of neurons accepted as being double labeled as a function of the SBR. Grain counts over each GnRH, NPY, and POMC mRNA-containing neuron were used to create SBR frequency curves for each double label *in situ* hybridization experiment (data presented are the mean ± SEM for three animals per experiment). The *ordinate* value represents the percentage of neurons considered double labeled at a particular SBR (*abscissa*). Representative amounts of double labeling for each experiment at several arbitrary SBR thresholds are shown in Table 1.

TABLE 1. Percentage of GnRH, NPY, and POMC mRNA-containing neurons accepted as expressing Ob-R mRNA at different signal SBR criterion

Double label assay: SBR criterion	GnRH/Ob-R (%)	NPY/Ob-R (%)	POMC/Ob-R (%)
3	33 ± 3	55 ± 1	74 ± 1
6	7 ± 5	19 ± 2	46 ± 1
9	3 ± 3	6 ± 1	24 ± 2

express Ob-R mRNA in the anterior pituitary are unknown; however, based on the observation that leptin can elicit LH secretion from isolated rat hemipituitaries in culture (15), gonadotropes themselves are good candidates. In the brain, we found Ob-R mRNA in the choroid plexus, the bed nucleus of the stria terminalis, the MPO, the hypothalamus, and the dorsal raphe nucleus. Ob-R mRNA expression was highest in the hypothalamus, particularly in the retrochiasmatic area, Arc, and VMN. These hypothalamic areas overlap with those implicated in the regulation of LH secretion and feeding (22), highlighting these regions as potential target sites for the central effects of leptin on nutrition and reproduction. Ob-R mRNA expression in the choroid plexus most likely reflects the expression of the short form that is thought to transport leptin into the cerebrospinal fluid (49), whereas the presence of Ob-R mRNA in other extrahypothalamic areas suggests that leptin may also influence physiological functions not directly (or obviously) related to either body weight regulation or reproduction.

The distribution of Ob-R protein has been previously examined in human forebrain with an antibody that, like the riboprobe we used, does not distinguish among isoforms (50). In humans, Ob-R protein is found in the choroid plexus, the Arc, and mammillary nucleus (50), areas in which we also found the expression of Ob-R mRNA in the monkey. Ob-R protein is also detectable in the nucleus basalis of Meynert, the posterior hypothalamus, and the suprachiasmatic, para-

ventricular, and dorsomedial hypothalamic nuclei in humans (50), whereas Ob-R mRNA was undetectable in all of these areas in the monkey brain. Conversely, Ob-R mRNA was expressed in several areas in monkey brain where the Ob-R protein was not reported in the human brain, *i.e.* the bed nucleus of the stria terminalis, MPO, lateral hypothalamic area, VMN, ventral hypothalamus, and dorsal raphe nucleus (50). On the one hand, the apparent discrepancies between the human and monkey in the patterns of expression of Ob-R mRNA and Ob-R protein could represent true species differences in the distribution of Ob-R. On the other hand, the apparent discrepancies could arise from the differential localization of Ob-R protein and mRNA within neurons, the lack of translation of Ob-R mRNA into protein, and/or the expression of Ob-R or its mRNA below the level of detectability in certain areas. Studies that combine immunocytochemistry and ISH in humans and monkeys will be required to clarify this issue.

There are similarities as well as differences in the distribution of Ob-R mRNA in brain and pituitary across species. In all species studied to date, Ob-R mRNA is found in the Arc, VMN, and lateral hypothalamus (16–20, 51). Ob-R mRNA has also been detected in the choroid plexus with probes that do not distinguish between the isoforms of Ob-R (17–20, 51), and Ob-R mRNA has been found in the pituitary of sheep, rats, and mice (44–46). Differences in Ob-R gene expression among species include the expression of Ob-R in the paraventricular and dorsomedial hypothalamic nuclei in rodents but not monkeys (17–20), its expression in the lateral parabrachial nucleus in mice but not monkeys or rats (52), and its expression in the thalamus in rats but not mice or monkeys (17, 19, 20). Whether the differences in the patterns of expression of Ob-R mRNA in the brain among species reflect differences in the mechanisms of leptin's action remains to be determined.

The results of our Ob-R distribution study indicate that leptin could be acting at numerous sites in the brain and pituitary to regulate gonadotropin and testosterone secretion. Although pituitary gonadotropes would seem to be attractive candidates for mediating the effects of fasting on LH secretion, the sensitivity of the pituitary to GnRH is apparently not altered in fasted monkeys (4), making this explanation seem less plausible. Another conceivable mechanism for leptin's regulation of gonadotropin secretion would be a direct action of leptin on GnRH neurons; however, we were unable to identify any distinct clusters of silver grains over GnRH neurons that would indicate clear expression of Ob-R mRNA by these cells. This subjective evaluation does not preclude the possibility that low levels of Ob-R mRNA are expressed in GnRH neurons. To obtain a more objective estimate of the frequency of Ob-R mRNA expression in GnRH neurons, we compared the grain counts over GnRH neurons to the background level by determining individual SBRs for a large sample of GnRH neurons. From this sample, a cumulative frequency histogram was constructed showing the percentage of double labeled cells based on the SBR. It is clear from this graph that the percentage of GnRH neurons accepted as coexpressing Ob-R mRNA depends on the threshold selected and that a precise determination is impossible, because the selection of the threshold is arbitrary.

Based on a conservative SBR of 6, fewer than 7% of the GnRH neurons would appear to express Ob-R mRNA. However, the elevated background in the GnRH/Ob-R double label ISH assay raises the possibility that the percentage of GnRH neurons accepted as coexpressing Ob-R mRNA has been underestimated compared with the percentage of double labeled NPY and POMC neurons. Our observation in the monkey is consistent with a recent report indicating that few, if any, GnRH neurons express Ob-R protein in the rat (21). Although it is possible that a small population of Ob-R-expressing GnRH neurons mediates leptin's stimulatory effect on gonadotropin secretion in fasted monkeys, it seems more likely that leptin influences GnRH secretion indirectly through other intermediate neurons.

A variety of neurons are thought to interact with GnRH neurons, but two of these, those that make NPY and POMC, are particularly good candidates for mediating the effects of leptin. NPY influences GnRH and LH secretion across species and has been implicated in the regulation of feeding (22). In contrast to our observations in GnRH neurons, silver grain clusters representing Ob-R mRNA were clearly visible over NPY mRNA-expressing neurons in the Arc. SBR measurements over NPY neurons confirmed our subjective impressions of coexpression, almost 20% of Arc NPY neurons had a SBR for Ob-R mRNA of 6 or greater. The possibility that NPY neurons are targets of leptin is also supported by studies in rodents showing that in the Arc, a subset of NPY neurons expresses Ob-R mRNA and protein (21,23) and that NPY gene expression is regulated by leptin (18, 53–56). POMC neurons located in the Arc also appear to be targets of leptin's action in the brain. We found many POMC mRNA-expressing neurons in the Arc with obvious Ob-R mRNA silver grain clusters and estimate that almost half of the Arc POMC neurons express Ob-R mRNA based on a SBR of 6. We have previously reported similar results in rats (24), and we and others have demonstrated that leptin regulates POMC mRNA levels in the Arc in *ob/ob* mice (57–59). POMC neurons make direct synaptic contact with GnRH neurons (60–62), suggesting that peptide products of the POMC gene can influence GnRH neurons directly. Two peptide products deriving from the POMC gene, β-endorphin and αMSH, are possible mediators of leptin's effects on LH secretion. In both monkeys and rats, β-endorphin exerts inhibitory effects on GnRH and LH secretion (22), and αMSH has been shown to modulate LH secretion in rats (63, 64). Furthermore, *agouti* mice, which congenitally overproduce an endogenous antagonist of the melanocortin receptors, have adult-onset infertility (65), suggesting that αMSH may act through melanocortin receptors to mediate the effects of leptin on the brain. Taken together, these data indicate that NPY and peptide products of the POMC gene mediate at least some of leptin's effects on the brain and may be involved in the regulation of gonadotropin secretion by leptin.

There are several other mechanisms by which leptin could affect reproductive function in addition to acting through NPY and POMC neurons. One possibility is that leptin regulates the expression of sex steroid hormone receptors. This suggestion is supported by the findings that estrogen receptors are up-regulated in the paraventricular nucleus of the hypothalamus in fasted rats (66), which are presumed to have decreased circulating levels of leptin (13), and earlier studies showing that *ob/ob* mice are more sensitive than wild-type controls to the negative feedback effects of testosterone (67). Leptin could also influence neuroendocrine reproductive function by modulating hormones of the hypothalamic-pituitary-adrenal (HPA) axis. Activation of the HPA axis inhibits reproduction (1). During fasting, leptin levels decrease (12, 13, 41–43), and HPA axis activity is increased with a concurrent decrease in hypothalamic-pituitary-gonadal axis activity (7, 12). These effects of fasting on the HPA and reproductive axes can be ameliorated in male mice by exogenous leptin treatment (12), suggesting that leptin's effects on LH secretion may be mediated by hormones of the HPA axis, namely CRH and/or glucocorticoids. Further studies are necessary to determine whether these or other mechanisms mediate leptin's effects on the reproductive axis.

In summary, we have confirmed that fasting suppresses LH and testosterone secretion in the male monkey. Although we cannot be certain that this inhibition is caused by reduced circulating levels of leptin, we have demonstrated that exogenous leptin prevents the effects of fasting on circulating levels of LH and FSH and, to a lesser extent, testosterone. These results suggest that at least one of leptin's actions is to sustain reproduction in animals with adequate metabolic reserves. We have also shown that Ob-R mRNA is detectable in the brain and the anterior pituitary of monkeys, making it likely that leptin acts at either or both of these sites to modulate gonadotropin secretion. If exogenous leptin stimulates LH and FSH secretion during fasting by modulating GnRH release, it is unlikely that these effects are direct, because few, if any, GnRH neurons appear to express Ob-R mRNA. However, leptin may indirectly modulate GnRH secretion by acting on NPY or POMC neurons in the Arc that clearly express Ob-R mRNA.

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