0013-7227/06/$15.00/0
Printed in U.S.A.

Endocrinology 147(2):647–658

doi: 10.1210/en.2005-0791

The Role of Leptin in the Development of the Cerebral Cortex in Mouse Embryos

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Leptin is detected in the sera, and leptin receptors are expressed in the cerebrum of mouse embryos, suggesting that leptin plays a role in cerebral development. Compared with the wild type, leptin-deficient (*ob/ob*) mice had fewer cells at embryonic day (E) 16 and E18 and had fewer 5-bromo-2′-deoxyuridine$^+$ cells at E14 and E16 in the neuroepithelium. Intracerebroventricular leptin injection in E14 *ob/ob* embryos increased the number of neuroepithelium cells at E16. In cultured neurosphere cells, leptin treatment increased *Hes1* mRNA expression and maintained neural progenitors. Astrocyte differentiation was induced by low-dose (0.1 μg/ml) but not high-dose (1 μg/ml) leptin. High-dose leptin decreased *Id* mRNA and increased *Ngn1* mRNA in neurosphere cells. The *neuropeptide Y* mRNA level in the cortical plate was lower in *ob/ob* than the wild type at E16 and E18. These results suggest that leptin maintains neural progenitors and is related to glial and neuronal development in embryos. (*Endocrinology* 147: 647–658, 2006)

LEPTIN, WHICH IS secreted from adipocytes, decreases appetite and increases energy expenditure in adults (1–4) by acting on the arcuate nucleus (ARH) in the hypothalamus, in which leptin receptors (Ob-Rs) are highly expressed (5–7). Leptin exhibits these functions by activating ARH neurons that express anorexigenic peptides, proopiomelanocortin and cocaine- and amphetamine-related transcript, and suppressing ARH neurons that express orexigenic peptides, neuropeptide Y (NPY) and agouti-related gene product (5–7). In humans, fetal plasma leptin concentration has correlation with birth weight, birth length, and head circumference, but maternal leptin concentration has negative correlation with fetal growth (8, 9). In transgenic skinny mice that overexpress leptin after birth, maternal leptin concentration was increased and fetal body weight was less than in nontransgenic mice (10, 11). These reports suggest that both fetal leptin and maternal leptin are related to fetal development.

First Published Online November 10, 2005

Abbreviations: ARH, Arcuate nucleus; bHLH, basic helix-loop-helix; BMP, bone morphogenetic protein; BrdU, 5-bromo-2′-deoxyuridine; CP, cortical plate; CRL, crown-rump length; E, embryonic day; EGF, epidermal growth factor; F, forward; FITC, fluorescein isothiocyanate; GFAP, glial fibrillary acidic protein; Hes, hairy and enhancer of split; Id, inhibitor of differentiation; IZ, intermediate zone; JAK, Janus kinase; Mash1, mammalian achaete-scute complex homolog-like 1; NE, neuroepithelium; NeuroD, neurogenic differentiation; Ngn, Neurogenin; NPC, neural progenitor cell; NPY, neuropeptide Y; NSC, neural stem cell; Ob-R, leptin receptor; P19EC, P19 embryonic carcinoma; QRT-PCR, quantitative real-time PCR; R, reverse; RA, retinoic acid; SSC, saline sodium citrate; ssDNA, single-stranded DNA; STAT, signal transducer and activator of transcription; STE, 4× SSC, 20 mM Tris, and 1 mM EDTA.

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

Brain weight and total brain protein content were reduced in juvenile and adult *ob/ob* (leptin-deficiency) and *db/db* (lack of long form of leptin receptor) mice, compared with the wild-type (C57BL/6) mice (12–14). Brain DNA content was also reduced in *ob/ob* mice, compared with the wild type (15). In the hypothalamus, the neural projection from ARH was disrupted in *ob/ob* mice, and the ip injection of leptin in juvenile *ob/ob* mice rescued the development of ARH projections (16). These reports suggest that leptin affects the proliferation and differentiation of neural cells, at least postnatally. In the cerebrum, cortical brain volume was reduced in adult *ob/ob* mice, compared with the wild type (12). The *ob/ob* and *db/db* mice exhibit reduced locomotor activity and impairment of cognitive function (13, 17), and the cerebral cortex is involved in the locomotor and cognitive functions in rodents (18–20).

Leptin receptor mRNA was expressed in the mouse embryonic cerebral cortex (21) and detected in the sera of mouse embryos (22), suggesting that leptin plays a role in embryonic cerebrocortical development. In this study, we examined the roles of leptin in the maintenance, proliferation, and differentiation of neuroepithelial cells as well as in the differentiation of neurons in the embryonic cerebral cortex, by both comparing brains of *ob/ob* embryos against those of a wild type (C57BL/6J) and intracerebroventricular injection of leptin to *ob/ob* embryos with an *exo utero* development system (23–25). We also examined the effect of leptin on neurosphere cells originating from the mouse embryonic cerebrum.

Materials and Methods

Animals

We purchased pregnant ICR mice from the Central Institute of Laboratory Animals (Kawasaki, Japan). In these mice, fertilized eggs of

leptin-deficient (*ob/ob*) or wild-type (C57BL/6J) mice had been transplanted, and the day of transplantation was defined as embryonic day (E) 0. C57BL/6J and BKS.Cg-m+/+ Lepr^{db} (*db/+*) mice were purchased from Clea Japan (Tokyo, Japan) and were mated from 1700 to 0800 h. We defined 0:00 of the day when a vaginal plug was observed as E0.

We maintained these mice at 22–24 C under a 12-h light, 12-h dark cycle at the Institute of Animal Experiment of Shimane University. Food and water were available *ad libitum*. All animal studies were approved by the Ethics Committee for Animal Experimentation of Shimane University, and the animals were handled according to the institutional guidelines.

The pregnant mice were injected with 5-bromo-2′-deoxyuridine (BrdU; 100 mg/kg) ip. Three hours later, *ob/ob* and wild-type (C57BL/6J) embryos at E14, E16, and E18 were obtained from ICR surrogate mothers, which were killed by ether anesthetization. Embryos were also killed by ether anesthetization. We measured crown-rump length (CRL) and body weight of nine or more embryos in total from two or more litters at each of E14 to E18. We also measured the total brain weight in *ob/ob* and wild-type embryos from two or more ICR transplantation litters at E16 and E18. E14 *db/db* (BKS.Cg-+ Lepr^{db}/+ Lepr^{db}) embryos from *db/+* mothers were distinguished from +/+ (BKS.Cg-m +/m +) or *db/+* embryos by the size of cDNA fragment of *Ob-Rb* mRNA in RT-PCR (26). The brains of embryos were embedded in OCT compounds and stored at −80 C until use as frozen sections. For the paraffin sections, the brains were fixed in 10% formalin solution containing 70% methanol at 4 C overnight, dehydrated with methanol, and embedded in paraffin.

Exo utero surgery and microinjection of leptin

Exo utero surgery was performed as described previously (23). Briefly, pregnant mice were anesthetized with pentobarbital (60 mg/kg), and the abdominal wall and uterus were incised. Fifty nanograms of leptin were injected into the lateral ventricle of E14 *ob/ob* embryos in the right uterine horn using a 40-μm-diameter micropipette, whereas the vehicle [1 mM sodium citrate in PBS (pH 7.4)] was injected in control *ob/ob* embryos in the left uterine horn of the same dam. The embryonic brain was obtained at E16 and embedded in paraffin as described above.

Intracerebroventricular injection of 1 μg leptin exhibits the function in food intake and metabolic rates in neonates and adults (27–29). Fifty nanograms of leptin for an E14 embryo is equivalent to 1 μg for an adult mouse because the embryonic brain at E14 weighed approximately one twentieth that of the adult.

Serum leptin concentration

We measured the pooled serum leptin concentration in ICR-transplanted *ob/ob* embryos and nontransplanted wild-type (C57BL/6J) embryos at each of E14, E16, and E18. The serum leptin concentration was measured by RIA with a leptin RIA kit (Linco Research, St. Charles, MO).

Immunohistochemistry in the brain

We performed immunohistochemistry with mouse monoclonal antinestin (1:200, Rat 401, Developmental Studies Hybridoma Bank), anti-Tuj1 (neuron-specific class III β-tubulin) (1:200, Covance, Berkeley, CA), and anti-BrdU (1:1000, BD PharMingen, San Diego, CA) antibodies, and rabbit polyclonal antileptin receptor long form (Ob-Rb) (1:100, Linco Research) and anti-single-stranded DNA (ssDNA) (1:400, DakoCytomation, Carpinteria, CA) antibodies. Nestin is an intermediate filament protein selectively expressed in the neural stem cells (NSCs) and neural progenitor cells (NPCs) in the brain (30, 31) and ssDNA is specific for apoptotic cells (32).

A coronal frozen section of the brain was cut at a thickness of 10 μm and fixed with acetone at −20 C for 10 min, incubated with 10% goat serum for 1 h at room temperature, and incubated with anti-Ob-Rb antibody and either antinestin or anti-Tuj1 antibody at 4 C overnight. The same section was then incubated with biotinylated goat antirabbit IgG (Vector Laboratories, Burlingame, CA) and Alexa Fluor 546-labeled goat antimouse IgG antibodies (Molecular Probes, Inc., Eugene, OR) and incubated with fluorescein isothiocyanate (FITC)-conjugated ExtrAvidin (Sigma, St. Louis, MO). The sections were observed by a confocal laser microscopy (FLUOVIEW FV300, Olympus, Tokyo, Japan). The brain sections of E14 *db/db* and +/+ (BKS.Cg-m +/m +) embryos were treated in the same way as described above and incubated with anti-Ob-Rb antibody at 4 C overnight and with Histofine simple stain mouse MAX PO (rabbit) (Nichirei, Tokyo, Japan) for 30 min at room temperature.

For BrdU immunostaining, coronal paraffin sections of the brain were cut at a thickness of 5 μm and treated with methanol containing 0.3% hydrogen peroxide for 30 min to inactivate intrinsic peroxidase. The section was treated with 0.05% trypsin and 0.1% $CaCl_2$ solution at 37 C for 3 min 30 sec and then with 2 N HCl at room temperature for 30 min. We used the M.O.M. immunostaining kit (Vector Laboratories) for BrdU immunostaining. Staining was performed according to the protocol provided with the kit.

For ssDNA immunostaining (33), a coronal paraffin section of the brain was treated with 20 μg/ml proteinase K at 37 C for 20 min and incubated with anti-ssDNA at 4 C overnight. The section was incubated with ENVISION+ System HRP rabbit (DakoCytomation). We used liquid diaminobenzidine chromogen (DakoCytomation) for chromogenic reaction. Nuclei in the sections were counterstained with hematoxylin.

Semiquantitative study on the cells in the cerebral cortex

The numbers of cells in the neuroepithelium (NE), intermediate zone (IZ), and cortical plate (CP) of a hemisphere in the cerebral cortex were counted blindly as described previously with random and systematic sampling (24, 34, 35). Coronal sections with a thickness of 5 μm were sampled from E14 to E18 transplanted wild-type and *ob/ob* embryos and stained by Nissl's staining. Four or more embryos from two or more litters were used for the cell counts. We counted the total cell number in the volume delimited as follows: 1) rostrocaudally, between the section at the rostral end of the corpus callosum and the section at the interventricular foramen in E16 and E18 brains, or between the section in which the corticostriatal sulcus is first observed in the rostral end and the section at the interventricular foramen in E14 brains and 2) mediolaterally, between the perpendicular lines that come in contact with the medial and lateral edges of the lateral ventricle. Every 18th coronal section for E14 and E16 brains and every 30th for E18 brains were chosen. In embryos microinjected during *exo utero* surgery, every 12th section was chosen. Six to eight sections were counted in each embryonic brain. A one-sided 20-μm grid (in the NE at E14, the NE, IZ, and CP at E16 and the NE at E18) or 50-μm grid (in the IZ and CP at E18) was superimposed on a photograph of the cerebral cortex, and the cells within the box were counted. In total, we used about 100 boxes for the NE at E14 embryos; 250 boxes for each of the NE, IZ, and CP at E16; and 200 boxes for the NE and 70 boxes for each of the IZ and CP at E18 per brain. The mean cell density in the boxes in each section was calculated and the total cell counts in each section were calculated by multiplying mean cell density by the area of the NE, IZ, or CP in each section. We multiplied the cell number in the section by the distance between the sections and totaled the cell numbers in the defined volume. This calculated value was corrected with the diameter of the nuclei and thickness of the section, and the total cell number in NE, IZ, and CP in the defined volume was estimated (24, 34, 35).

BrdU$^+$ cells were estimated in the NE at E14 and E16 as described above, and the BrdU index was calculated.

To count apoptotic cells in the NE at E16 and E18, every sixth paraffin section stained by ssDNA immunostaining and Hechst 33258 was chosen. The total apoptotic cell number was estimated as described above.

Culture and immunocytochemistry of neurosphere cells

We purchased neurosphere cells isolated from murine E14 cerebral cortex (Stem Cell Technologies, Vancouver, Canada). These cells were maintained in the NeuroCult NSC proliferation medium (Stem Cell Technologies) containing 20 ng/ml epidermal growth factor (EGF; Stem Cell Technologies) at 37 C under 5% CO_2.

Neurosphere cells were fixed with acetone and dried on silicon-coated slides. The cells were washed with PBS, treated with 0.3% Triton X-100 for 10 min, and incubated with 1% BSA for 30 min. They were incubated with mouse monoclonal antinestin (1:200) and rabbit polyclonal anti-Ob-Rb (1:100) antibodies, followed by Alexa Fluor 546-labeled antimouse IgG antibody and biotinylated antirabbit IgG antibody, and finally by FITC-conjugated ExtrAvidin.

TABLE 1. CRL, body weight, and total brain weight in *ob/ob* and wild-type embryos

		CRL (mm)	Body weight (g)	Brain weight (mg)
E14	*ob/ob*	11.9 ± 0.8 (10)	0.27 ± 0.05 (9)	Not examined
	Wild type	12.0 ± 0.7 (11)	0.27 ± 0.05 (11)	Not examined
E16	*ob/ob*	17.2 ± 1.8 (13)	0.66 ± 0.07 (13)	58 ± 5 (19)
	Wild type	16.3 ± 1.0 (20)	0.62 ± 0.06 (20)	57 ± 7 (14)
E18	*ob/ob*	22.6 ± 1.0 (16)	1.23 ± 0.11 (16)	80 ± 9 (21)
	Wild type	22.5 ± 1.1 (16)	1.20 ± 0.08 (16)	86 ± 5 (21)

Values are means ± SD (n).

Proliferation of neurosphere cells

We used the cell proliferation ELISA BioTrak system (Amersham Biosciences, Piscataway, NJ) to detect BrdU incorporation into the cells. Quadruplicated samples were prepared from each of the following groups.

Neurosphere cells (5×10^4 cells/ml) were cultured in a 96-well plastic plate in the NSC proliferation medium containing: 1) leptin (0.01–1 μg/ml) and/or NPY (0.01–1 μM) for 2 d; 2) leptin (0.01–1 μg/ml) and/or NPY (0.01–1 μM) for 1 or 2 d, followed by EGF (20 ng/ml) administration for an additional day; or 3) EGF with leptin (0.01–1 μg/ml) or EGF with NPY (0.01–1 μM) for 1 d.

We examined BrdU incorporation into these cells by ELISA after 3 h of exposure to BrdU (10 nM).

These doses (0.01–1 μg/ml) of leptin and NPY (0.01–1 μM) exhibit neuronal function *in vitro* (36–39).

Differentiation assay and clonal analysis of leptin-treated neurosphere cells

Neurosphere cells were plated on Lab-Tek II CC2 chamber slides (8 wells, Nalge Nunc, Rochester, NY) at 1000 cells/chamber with leptin (0.1 μg/ml)-added NSC proliferation medium for 2 d. The cells were cultured in EGF (20 ng/ml)-contained medium for 6 d and cultured in NeuroCult differentiation medium for additional 6 d. The cells were incubated with mouse monoclonal anti-Tuj1 (1:500), rabbit polyclonal antiglial fibrillary acidic protein (GFAP) (1:2000, DakoCytomation) and mouse IgM anti-O4 (1:100) antibodies and followed by the incubation with biotinylated antimouse IgG, antirabbit IgG, and antimouse IgM antibodies, respectively (Vector Laboratories). We used the VECSTATIN immunostaining kit (Vector Laboratories). Diaminobenzidine was used for chromogenic reaction and nuclei in the sections were counterstained with hematoxylin. More than 500 cells were counted in the leptin-treated and control groups (n = 3, respectively) and the proportion of $Tuj1^+$, $GFAP^+$, and $O4^+$ cells was calculated.

Clonal analysis was performed as described previously (40, 41). Single neurosphere cells were plated on Lab-Tek II CC2 chamber slides (8 wells, Nalge Nunc) at 50 cells/chamber. These cells were cultured for 2 d in leptin (0.1 or 1 μg/ml)-added NSC proliferation medium. The cells were cultured in EGF (20 ng/ml)-contained medium for 8 d and cultured in NeuroCult differentiation medium for additional 7 d. The cells were incubated with mouse monoclonal anti-Tuj1, rabbit polyclonal anti-GFAP, and mouse IgM anti-O4 antibodies followed by the incubation with Alexa 633-labeled antimouse IgG (Molecular Probes), Cy3-labeled antirabbit IgG (Chemicon, Temecula, CA), biotinylated antimouse IgM (Vector Laboratories) antibodies, and finally FITC-conjugated ExtrAvidin. $Tuj1^+$, $GFAP^+$, and/or $O4^+$ clones were counted under a confocal laser microscopy. The ratio of viable colony number to plated cell number was counted and the proportion of $Tuj1^+$, $GFAP^+$, and/or $O4^+$ clones was calculated in the leptin-treated and control groups (n = 3, respectively).

Probe synthesis

Total RNA was extracted from the mouse brain using TRI reagent (Molecular Research Center, Cincinnati, OH), and fragments of mouse *Ob-R* and *NPY* cDNAs were made by RT-PCR with Ready-To-Go RT-PCR beads (Amersham Biosciences). To generate 429- and 331-bp PCR products characteristic of the *Ob-R* and *NPY* mRNA sequences, the primers used were 5′-AGAGCCAAACTCAACTACGCTCTT-3′ (+661 to +684) and 5′-TCCAACACTAGTCAGAATTTTGGG-3′ (+1089 to +1066; GenBank U42467) and 5′-AGCAGAGGACATGGCCAGAT-3′ (+123 to +142) and 5′-TTAAACACACATATATACAACAAC-3′ (+453 to +430; GenBank AF273768), respectively. The PCR product was purified and cloned into pBluescript II SK(+) using a PCR-Script Amp SK(+) cloning kit (Toyobo, Osaka, Japan). The positive clone insert was verified by DNA sequencing with T7 and T3 primers using an ABI PRISM BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA). To generate antisense and sense ^{35}S-labeled riboprobes for *NPY* mRNA, the plasmid was linearized with *Sac*I and *Kpn*I and subjected to *in vitro* transcription with T7 and T3 RNA polymerases, respectively.

Quantitative real-time PCR (QRT-PCR)

Total RNA of the brains of three or more *ob/ob* and wild-type embryos from two or three litters at each of E16 and E18 was extracted using TRI Reagent (Molecular Research Center). For reverse transcription reaction, 200 ng RNA were applied using the Ready-To-Go RT-PCR beads. The primers for *NPY* mRNA were 5′-AGCAGAGGACATGGCCAGAT-3′ (+123 to +142) and 5′-AATCAGTGTCTCAGGGCTGGAT-3′ (+222 to +201; GenBank AF273768). Primers and the SYBR GREEN PCR master mix (Applied Biosystems, Warrington, UK) were added to the reverse transcription mixture, and the ratio of the *NPY* mRNA expression level to the amount of 18S rRNA was examined with ABI PRISM 7000 (Applied Biosystems, Foster City, CA).

Total RNA was extracted from neurosphere cells that were cultured on 75-cm^2 flasks at a density of 1×10^5 cells/ml in the NSC proliferation medium with 0, 0.1, and 1 μg/ml of leptin for 1 d (n = 3). The expression level of the hairy and enhancer of split (*Hes*) *1*, *Hes5*, inhibitor of differentiation (*Id*) *2*, *Id4*, Neurogenin (*Ngn*) *1*, *Ngn2*, neurogenic differentiation(*NeuroD*), mammalian achaete-scute complex homolog-like 1 (*Mash1*) and *4* bone morphogenetic protein (*BMP*) 4 mRNAs to that of *18S* rRNA was examined as described above. Primers were as follows (F, forward; R, reverse): *Hes1* F, AGAAAGATAGCTCCCGGCAT; R, TCGTTCATGCACTCGCTGAA; *Hes5* F, AACACAGCAAAGCCTTCGCCG; R, TGGAAGTGGTAAAGCAGCTTC; *Id2* F, CAAAGGTGGAGCGTGAATTCCAGG; R, CACAGCATTCAGTAGGCTCGTGTC; *Id4* F, GCGATATGAACGACTGCTAC; R, TCTCAGCAAAGCAGGGTGAG.; *Ngn1* F: ATGCCTGCCCCTTTGGAGACCT; R, TGTAGCCTGGCACAGTCCTCCT; *Ngn2* F, CCGGGTCAGACGTGGACTACT; R, GGCGGGAGAAGGATGGGAAGA; *NeuroD* F, ATCTGCCAACCGCCAGCGCTTCCTT; R, TTGACGTGGAAGACGTGGGAGCTGT; *Mash1* F, CTCGTCCTCTCCGGAACTGATG; R, ATGCTCCCGGAGGGTGGCAAAA; *BMP4* F, ATTCTCTGGGATGCTGCTGAGG; R, CCGAGCCAACACTGTGAGGAGT.

The cDNAs were verified by DNA sequencing with respective primers using an ABI PRISM BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems USA).

TABLE 2. Serum leptin concentration in mouse embryos

		Leptin concentration (ng/ml)
E14	*ob/ob*	nd
	Wild type	1.222
E16	*ob/ob*	nd
	Wild type	0.747
E18	*ob/ob*	nd
	Wild type	0.958

nd, Not detected.

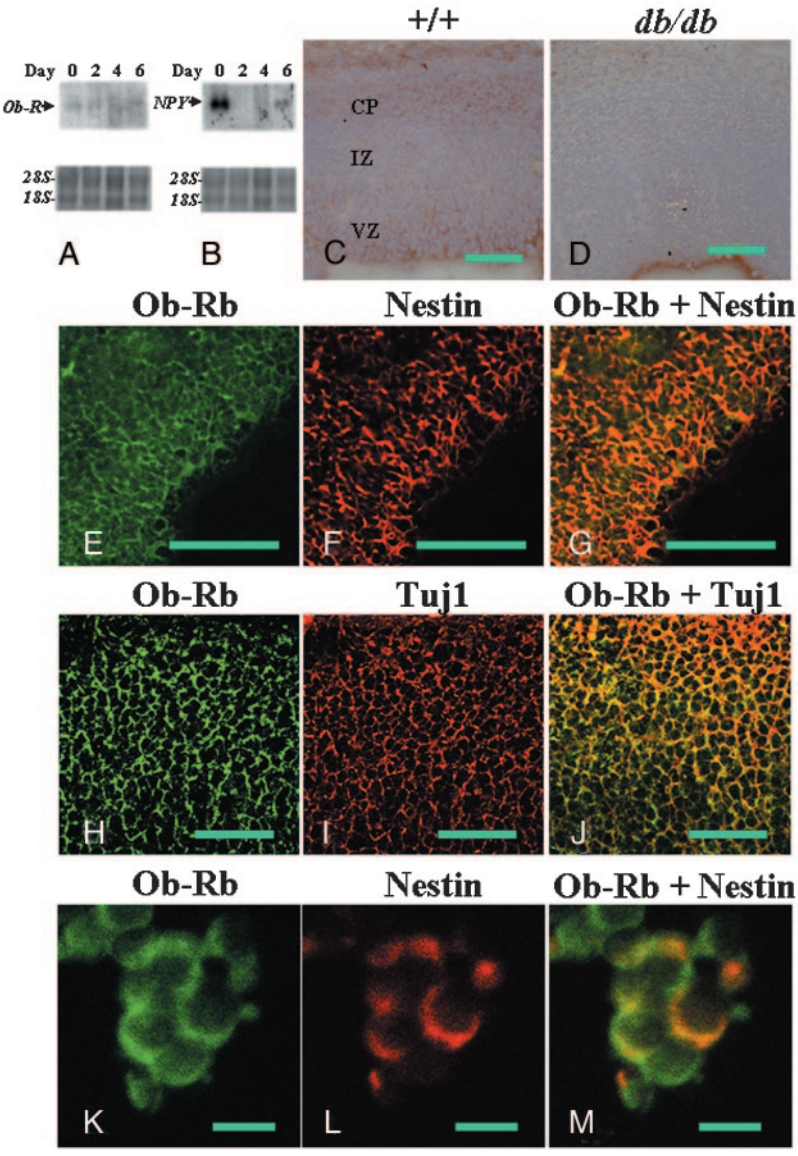

FIG. 1. *Ob-R* (A) and *NPY* (B) mRNA expression during RA-induced neuronal differentiation of P19EC cells and Ob-Rb expression in the cerebral cortex (C–J) and neurosphere cells (K and L). Total RNA was prepared from P19EC cells treated with RA for 0, 2, 4, and 6 d, separated by 1% agarose formaldehyde gels, transferred onto nylon membranes, and hybridized with radiolabeled probes for *Ob-R* mRNA (A) and *NPY* mRNA (B). *Ob-R* mRNA expression was observed in P19EC cells from d 0, when P19EC cells were undifferentiated, to d 6, when P19EC cells were differentiated into neurons (A). *NPY* mRNA was expressed in P19EC cells before RA treatment (0 d) and had disappeared by 2 and 4 d of RA treatment. However, it had become reinduced by 6 d of RA treatment, when P19EC cells were differentiated into neurons (B). The filters were stained with methylene blue to show 18S and 28S ribosomal RNAs. Ob-Rb was expressed in the NE and CP of +/+ (BKS.Cg-m +/m +) embryos (C), but not in those of *db/db* embryos (D), at E14 *in vivo*. Fluorescent double stainings of coronal sections of the neocortex were made for Ob-Rb (*green*) and nestin (*red*) at E14 (E–G), and Ob-Rb (*green*) and Tuj1 (*red*) at E18 (H–J) in wild-type (C57BL/6J) embryos. Ob-Rb and nestin were coexpressed in the NE at E14, and Ob-Rb and Tuj1 were coexpressed in the CP at E18. Ob-Rb (*green*) and nestin (*red*) were coexpressed in neurosphere cells (K–M). These results indicate that NSCs and/or NPCs as well as neurons express Ob-Rb. *Scale bars*, 100 μm (C–J), and 10 μm (K–M).

In situ hybridization

The section was fixed with 4% paraformaldehyde for 20 min at room temperature, treated with 20 μg/ml of proteinase K for 7 min at 30 C, and fixed with 4% paraformaldehyde again. It was acetylated in 0.1 M triethanol amine and 0.25% acetic anhydride for 10 min at room temperature and dehydrated with ethanol. The riboprobes for *NPY* mRNA were diluted with hybridization solution [50 mM dithiothreitol, 0.5 mg/ml poly ribo A, 10% dextran sulfate, 50 μg/ml of yeast tRNA, 0.3 M NaCl, 10 mM Trisma base, 10 mM NaH_2PO_4, 5 mM NaEDTA, 0.2% Ficoll 400, 0.2% polyvinyl pyrrolidone, 50% formamide, and 0.25 mM adenosine-5′-O-(1-thio triphosphate)] and applied to each slide, and the sections were incubated at 60 C overnight. The slide was washed twice with 50% formamide, 2× saline sodium citrate (SSC), and 20 mM 2-mercaptoethanol at 60 C for 30 min and twice with 4× SSC, 20 mM Tris, and 1 mM EDTA (STE) at 37 C for 10 min. The section was treated with RNase A (10 μg/ml) in STE at 37 C for 30 min. It was then washed once with STE containing 20 mM 2-mercaptoethanol at 37 C for 30 min; twice with 50% formamide, 2× SSC, and 20 mM 2-mercaptoethanol at 60 C for 45 min; and once with distilled water. It was then dehydrated with ethanol and dried. The section was developed and observed under a dark field.

Differentiation of P19 embryonic carcinoma (P19EC) cells and Northern blot analysis

P19EC cells were grown in MEM, αMEM (Sigma) with 10% (vol/vol) fetal calf serum (42). P19EC cells were induced to be differentiated with retinoic acid (RA) essentially as described previously (43).

Total RNAs from differentiated P19EC cells were prepared by using Isogen (Wako, Osaka, Japan). For RNA blot analysis, 10 μg of total RNA were electrophoresed on 1% agarose formaldehyde gels and transferred onto nylon membranes. Probe DNAs (~0.4 kb) were prepared from pBS II SK(+)-*Ob-R* and *NPY* by digestion with *Sma*I and *Sac*II, labeled with α-^{32}PdCTP using the MultiPrime labeling kit (Amersham), and hybridized as described previously (44, 45). Specific activity was approximately 1×10^6 cpm/ng for all of the probe DNAs.

Statistical analysis

We used an ANOVA and Fisher's *post hoc* test to analyze the cell counts in the cerebral cortex, BrdU labeling index in the NE, proliferation activity of the neurosphere cells, and expression of *NPY* mRNAs with QRT-PCR. Scheffé's *post hoc* test was used for the clonal analysis and analysis of *Hes*, *Id*s, *Ngn*s, *NeuroD*, *Mash1*, and *BMP4* mRNA expression in neurosphere cells because sample sizes were different among exper-

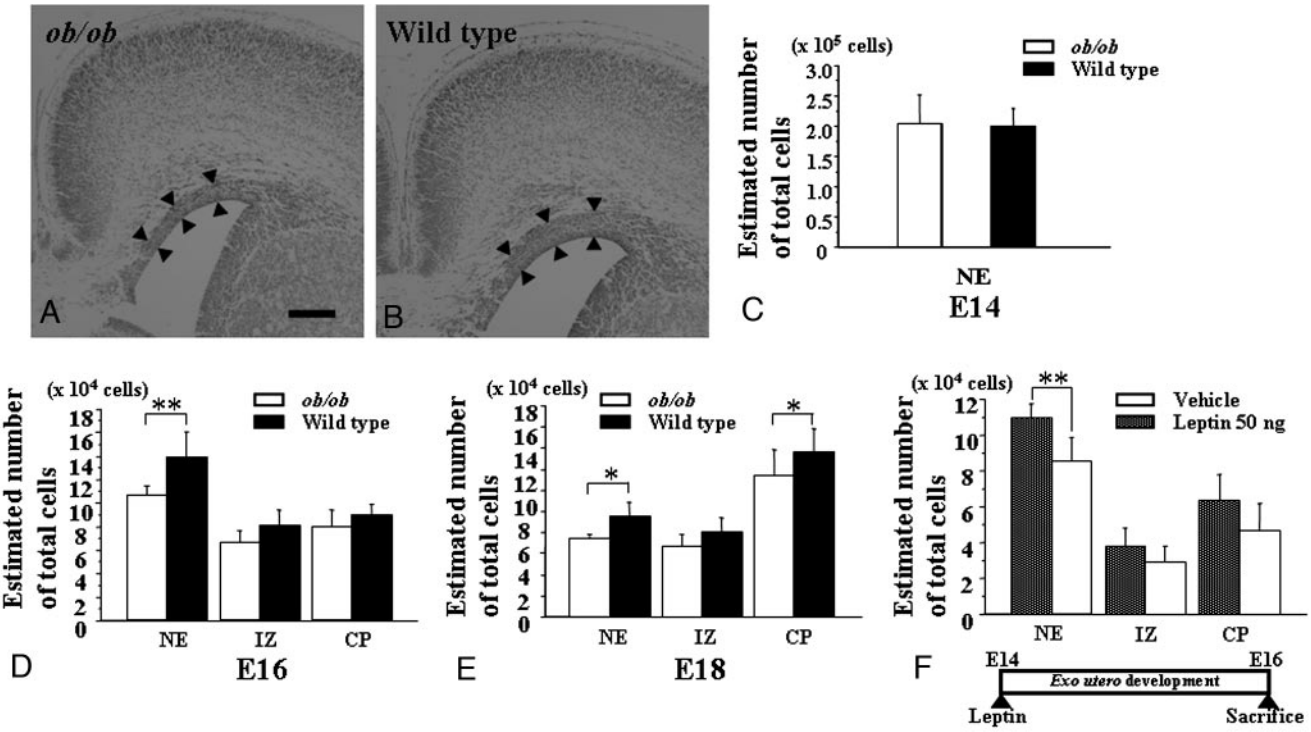

FIG. 2. The cell numbers in the cerebral cortex. The NE in the cerebral cortex was thinner in *ob/ob* (A) than wild type (B). Cell numbers in the NE did not differ between *ob/ob* and wild-type embryos at E14 (C), but there were fewer cells in *ob/ob* than the wild type at E16 (D) (n = 4). There were fewer cells in the NE and CP in *ob/ob* than the wild type at E18 (E) (n = 4). The number of cells in the NE at E16 increased by leptin injection at E14 in *ob/ob* embryos (F) (n = 4). *Scale bar*, 200 μm. *, $P < 0.05$; **, $P < 0.01$.

imental groups. Student's *t* test was used for analysis of the differentiation assay.

Results

CRL, body weight, and total brain weight

There were no significant differences in CRL, body weight, or total brain weight between *ob/ob* and wild-type (C57BL/6J) embryos at E14, E16, or E18 (Table 1) or between litters, according to ANOVA (data not shown).

Serum leptin concentration

In normal development, leptin was detected in the mouse embryonic serum at a concentration of approximately 1 ng/ml during E14 to E18 (Table 2). Leptin was not detected in the serum of the *ob/ob* embryos from ICR surrogate mothers (see *Materials and Methods*) at E14, E16, or E18 (Table 2). This result indicates that maternal leptin did not pass through the placenta and that the *ob/ob* embryos were deficient in leptin in the embryonic stages.

Expression of leptin receptors in the cerebral cortex and cultured cells

Ob-Rb was detected by the present anti-Ob-Rb antisera in the NE and CP of +/+ (BKS.Cg-m +/m +) embryos at E14 (Fig. 1C) but not in those of *db/db* embryos (Fig. 1D); therefore, this antibody is specific to Ob-Rb. Nestin was coexpressed with Ob-Rb in the NE at E14 (Fig. 1, E–G), and Tuj1 was coexpressed with Ob-Rb in the CP at E18 wild-type (C57BL/6J) embryos (Fig. 1, H–J). Neurosphere cells coexpressed nestin and Ob-Rb (Fig. 1, K–M). Treatment of aggregated P19EC cells with RA results in their differentiation into neuronal cells (43). After RA treatment for 6 d, most cells are neurons with elaborate axons and dendrites (43, 45). *Ob-R* mRNA expression was observed as a single band (~3 kb) by Northern blot analysis in P19EC cells before RA treatment (0 d) and continued to be observed at 6 d after RA treatment (Fig. 1A).

Cell proliferation and cell death in the cerebral cortex

At E18, the NE of the cerebral cortex was thinner in the *ob/ob* embryos than wild type (Fig. 2, A and B). At E16 and E18 but not E14, the *ob/ob* had significantly fewer cells in the NE than the wild type did ($P < 0.01$ and 0.05) (Fig. 2, C–E). At E18, *ob/ob* had significantly fewer cells in the CP than the wild type did ($P < 0.05$). The BrdU index in the NE was significantly lower in the *ob/ob* than wild type at E14 (Fig. 3, A, B, and E) and E16 (Fig. 3, C, D, and F) ($P < 0.05$). There was no significant difference between *ob/ob* and the wild type in the number of apoptotic cells in the NE at E16 or E18 (data not shown).

Intracerebroventricular injection of 50 ng of leptin to E14 *ob/ob* embryos increased the number of cells in the NE at E16 ($P < 0.01$), whereas leptin did not change the cell count in the IZ or CP (Fig. 2F). Although the cell number tended to be lower in leptin-treated *ob/ob* (Fig. 2D) than wild-type embryos (Fig. 2F), this is probably due to the stress of *exo utero* surgery.

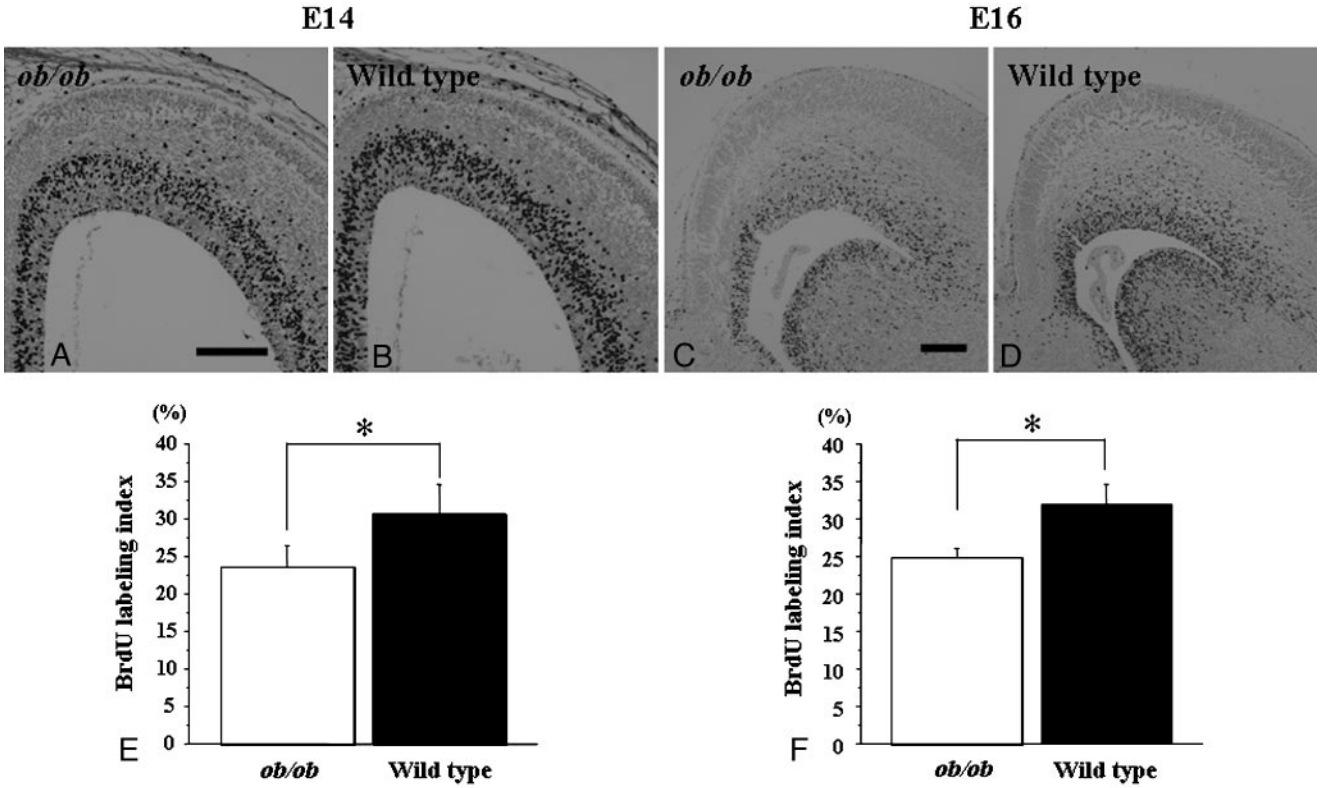

FIG. 3. The number of BrdU$^+$ cells in the NE of the cerebral cortex. Coronal sections of the cerebral cortex in *ob/ob* (A) and wild-type (B) embryos at E14 and in *ob/ob* (C) and wild type (D) at E16. There were fewer BrdU$^+$ cells in *ob/ob* embryos than in the wild type at both E14 (E) and E16 (F). *Scale bar*, 100 μm. *, $P < 0.05$. n = 4 (E14 *ob/ob*); n = 3 (E14 wild type; and E16 *ob/ob* and wild type).

Effects of leptin and NPY on proliferation activity of neurosphere cells

Leptin treatment for 2 d increased BrdU incorporation into neurosphere cells from E14 cerebral cortex cultured in medium containing no EGF ($P < 0.05$) (Fig. 4A). The EGF-responsive proliferation of neurosphere cells was enhanced by leptin pretreatment (0.1 and 1 μg/ml) for 1 (Fig. 4B) and 2 d (Fig. 4C) ($P < 0.01$ and 0.05). There was no synergistic effect on the proliferation of neurosphere cells when leptin and EGF were added simultaneously (data not shown). Without EGF, NPY did not increase BrdU incorporation into neurosphere cells (data not shown), but a synergistic effect was observed on the proliferation of neurosphere cells by the simultaneous administration of NPY and EGF ($P < 0.01$) (Fig. 4D).

Clonal analysis and differentiation assay of leptin-treated neurosphere cells

In clonal analysis, leptin increased the ratio of viable colony number to plated cell number in a dose-dependent manner ($P < 0.01$) (Fig. 5A). The proportion of GFAP$^+$/O4$^+$ progenitor colonies was higher and that of O4$^+$ colonies was lower in cells treated with a low dose of leptin (0.1 μg/ml) than in control cells ($P < 0.05$) (Fig. 5, B, C, and E). The proportions of multipotent progenitor colonies tended to be increased, and that of Tuj1$^+$/O4$^+$ progenitor colonies were not altered by the low-dose treatment (Fig. 5, D and E). In the differentiation assay, low-dose leptin significantly increased the proportion of astrocytes ($P < 0.01$) (Fig. 5, F–H) but did not affect the proportions of neurons and oligodendrocytes (data not shown). In clonal analysis, high-dose leptin (1 μg/ml) did not alter the proportion of any progenitor colony (Fig. 5E).

The mRNA expression of basic helix-loop-helix (bHLH) factors in leptin-treated neurosphere cells

Hes and Id are bHLH factors (46). Hes1 and Hes5 each play an important role in the maintenance of NSCs (46–48). Id2 and Id4 blocked both neuronal differentiation and oligodendrocyte formation and promoted the proliferation of cortical progenitors (46). Ngn1, Ngn2, and NeuroD are known as proneural bHLH factors (46). By QRT-PCR analysis, the *Hes1* mRNA expression level was higher in the leptin-treated neurosphere cells than control cells ($P < 0.05$), and this expression increased in a dose-dependent manner (Fig. 6A). *Id2* and *Id4* mRNA expression levels were lower in neurosphere cells treated by high-dose leptin (1 μg/ml) than those treated by low-dose leptin (0.1 μg/ml) ($P < 0.05$) (Fig. 6, B and C). The *Ngn1* mRNA expression level was higher in neurosphere cells dosed with 1 μg/ml of leptin than in the control and low-dose-treated neurosphere cells ($P < 0.05$) (Fig. 6D). There was no significant difference in *Ngn2* or *NeuroD* mRNA expression levels between the leptin-treated and control neurosphere cells, although both tended to be increased by 1 μg/ml leptin treatment ($P < 0.1$) (Fig. 6, E and F). Leptin

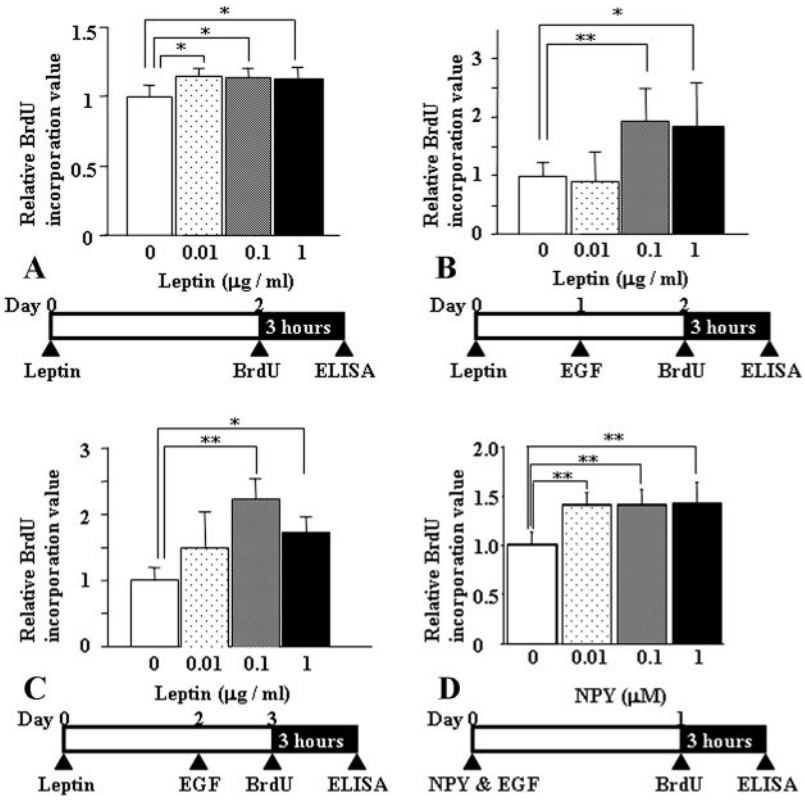

FIG. 4. BrdU incorporation into neurosphere cells. Incorporation increased slightly by leptin treatment for 2 d in medium containing no EGF (A). Leptin pretreatment for 1 (B) and 2 (C) d increased the responsiveness of EGF-dependent proliferation. NPY had an additive effect with EGF on the proliferation of neurosphere cells (D). EGF concentration was 20 ng/ml. *, $P < 0.05$; **, $P < 0.01$. n = 4.

treatment did not affect the mRNA expression level of *Mash1*, a proneural bHLH factor (46), or *BMP4* (data not shown).

The expression of NPY *mRNA in the brain and P19EC cells*

By QRT-PCR analysis, the *NPY* mRNA expression level in the total brain was higher at E18 than E16 in wild-type embryos ($P < 0.01$) (Fig. 7A). It was lower in the *ob/ob* embryos than the wild type at both E16 and E18 ($P < 0.01$) (Fig. 7A). By *in situ* hybridization, *NPY* mRNA expression was observed mainly in the CP of the cerebrum at E16 and E18, and this expression was weaker in *ob/ob* embryos than the wild type (Fig. 7, B–E).

The *NPY* probe detected a single band (~0.5 kb) by Northern blot analysis during the differentiation of P19EC cells (Fig. 1B). *NPY* mRNA expression was observed in P19EC cells before RA treatment (0 d), disappeared at 2 d, and was induced again 6 d after RA treatment upon neuronal differentiation (Fig. 1B).

Discussion

Maintenance, proliferation, and differentiation of NSCs and NPCs

Brain DNA content, as well as brain weight and total brain protein content, was reduced in *ob/ob* mice, compared with their lean counterparts, and leptin injection into juvenile *ob/ob* mice increased brain DNA (13, 15), indicating the increase in cell number by leptin. Multipotent NSCs may be a target of leptin because these cells exist in even the adult cerebral cortex (49). Leptin was detected in the sera of wild-type embryos (Table 2), and both NE at E14 (Fig. 1G) and neurosphere cells from E14 mouse cerebral cortex (Fig. 1M), both of which include NSCs and NPCs, coexpressed Ob-Rb and nestin. Compared with wild-type (C57BL/6J) mice, in *ob/ob* the BrdU index in the NE was reduced at E14 and E16 (Fig. 3), there were fewer cells in the NE at E16 and E18 (Fig. 2, D and E), and the NE layer was thinner at E18 (Fig. 2, A and B). Leptin supplementation at E14 rescued the reduced number of cells in the NE at E16 in *ob/ob* embryos (Fig. 2F). These findings support the contention that leptin increases proliferation activity in the NE.

The present cell proliferation analyses by ELISA in neurosphere cells from E14 mouse cerebral cortex showed that: 1) leptin enhanced EGF-responsive proliferation of neurosphere cells (Fig. 4, B and C); 2) the increase in proliferative activity by leptin was slight without EGF (Fig. 4A); and 3) the simultaneous administration of leptin and EGF had no synergistic effect on proliferation activity (data not shown). These findings in cultured cells suggest that leptin maintains either the cells' EGF responsiveness or the number of EGF-responsive cells rather than promoting proliferation.

The present clonal analysis supported the contention that leptin maintained neurosphere cells because leptin increased the ratio of viable colony number to plated cell number in a dose-dependent manner (Fig. 5A). Low-dose leptin (0.1 μg/ml) significantly increased the proportion of $GFAP^+/O4^+$ progenitor colonies, significantly decreased that of $O4^+$ progenitor colonies, and tended to increase the proportion of multipotent progenitor colonies ($P = 0.06$) (Fig. 5, B–E). These results suggest that low-dose leptin preferentially maintains astrocytes/oligodendrocytes and multipotent

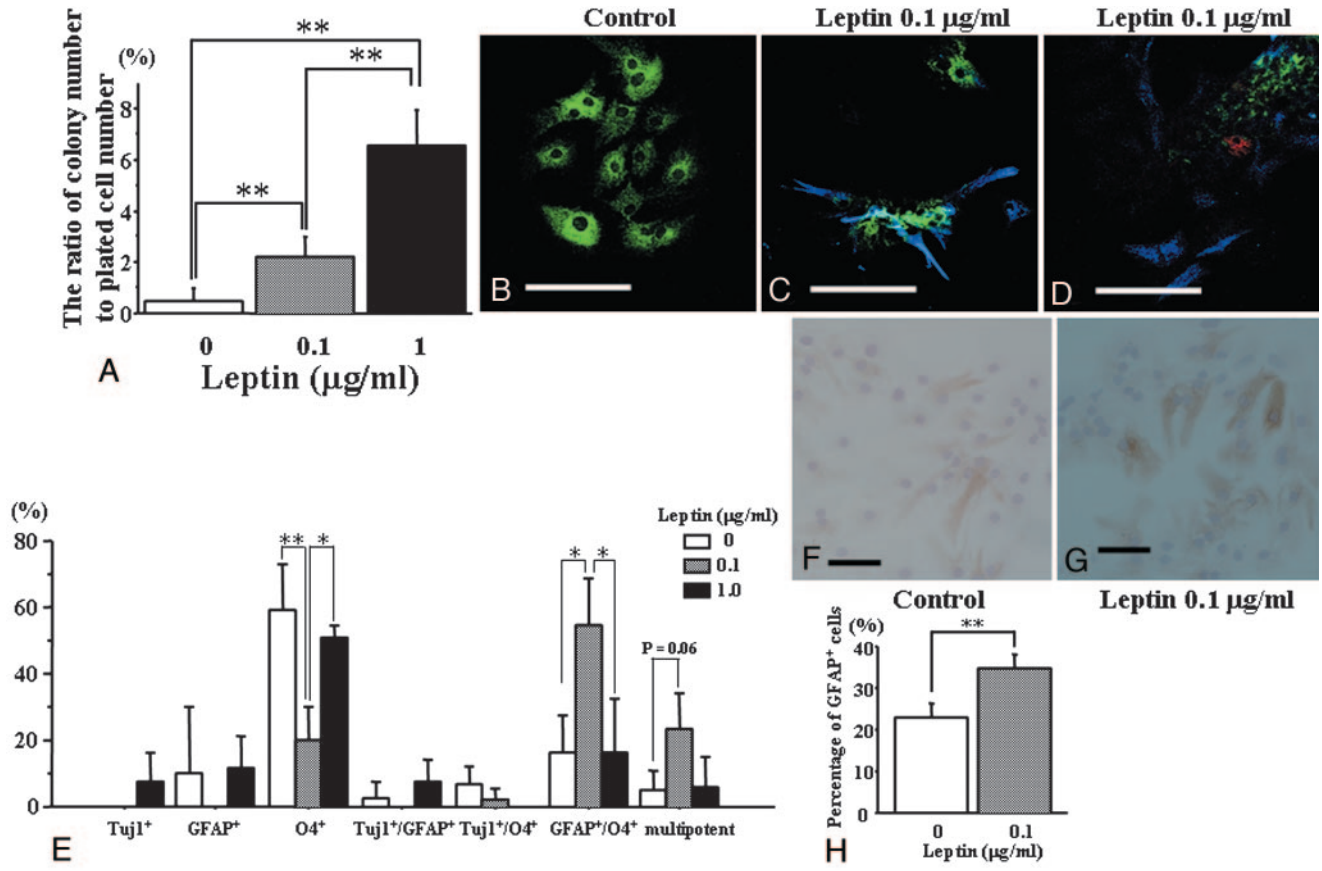

FIG. 5. Clonal analysis and differentiation assay of neurosphere cells. In clonal analysis, the ratio of colony number to plated cell number was increased by leptin pretreatment in a dose-dependent manner (A). Fluorescent staining of the differentiated cells revealed the proportion of the $O4^+$ (*green*) colonies (B) was smaller (E) and that of $GFAP^+$ (*blue*)/$O4^+$ colonies (C) was larger (E) by low-dose leptin. The proportion of $Tuj1^+$ (*red*)/$GFAP^+$/$O4^+$ multipotent progenitor colonies (D) tended to be increased by low-dose leptin administration ($P = 0.06$) (E). The proportion of $GFAP^+$ astrocytes was increased by low-dose leptin treatment in the differentiation assay (F–H). *Scale bar*, 50 μm. *, $P < 0.05$; **, $P < 0.01$. n = 3 or 4.

progenitor cells. This is consistent with the result in the differentiation assay that low-dose leptin treatment significantly increased the proportion of astrocytes (Fig. 5, F–H) without significant decreases in the proportions of neurons and oligodendrocytes (data not shown).

High-dose leptin, while maintaining a larger number of viable colonies (Fig. 5A), did not alter the proportion of colonies of each progenitor (Fig. 5E), suggesting that high-dose leptin maintained all types of NPCs. Hes1 and Hes5 are Notch effectors in mammalian neuronal differentiation (50), block neurogenesis, and increase the proportion of mitotically active cortical progenitors (46). Hes1-expressing cells are maintained as NSCs in the embryonic telencephalon (51). The increased expression of *Hes1* mRNA in leptin-treated neurosphere cells (Fig. 6A) suggests that leptin maintains the NSCs and NPCs by inducing Hes1. The reduced number of cells in the CP of *ob/ob* embryos at E18 (Fig. 2E) may be attributable to the reduction of NSCs and NPCs at earlier stages than E17 because neocortical cytogenesis continued from E11 to E17 and because postmitotic cells, which exist in NE from E14 to E16, reached the CP at E18 (52–54). Ob-Rb belongs to a family of IL-6-related cytokine receptors, and leptin signal is thought to be transmitted mainly by Janus kinase (JAK)/signal transducer and activator of transcription (STAT)3 pathway (7). Leukemia inhibitory factor is a member of IL-6-related cytokines, led to activation of STAT3 (55), and increased the number of NSCs (24, 56). Leptin may exhibit the maintenance effect on NSCs and NPCs through the activation of JAK/STAT3 pathway.

The subventricular zone enlarges during the peak of gliogenesis (postnatal 5 to 20 d) in the mouse forebrain, and astrocytes and oligodendrocytes are generated from subventricular zone postnatally (57). The serum leptin concentration peaked in 10-d-old mice (58). The present *in vitro* study showed that low-dose leptin preferentially maintained astrocyte/oligodendrocyte progenitor cells in clonal analysis and increased the proportion of astrocytes in the differentiation assay (Fig. 5, B and C). These results suggest that leptin has a maintenance effect on the glial progenitor cells in the embryonic and neonatal brains. Hes1 can drive glial-restricted progenitor cells to an astrocyte cell fate at the expense of oligodendrocyte differentiation (59). The increased expression of Hes1 in the present study (Fig. 6A) may have caused the astrocyte differentiation in leptin-treated neurosphere cells (Fig. 5, E–H). However, high-dose leptin did not promote astrocyte differentiation (Fig. 5E). The inhibition of Id2 and Id4 expression by high-dose leptin treatment (Fig. 6, B and C) may offset the driving force to astrocyte differen-

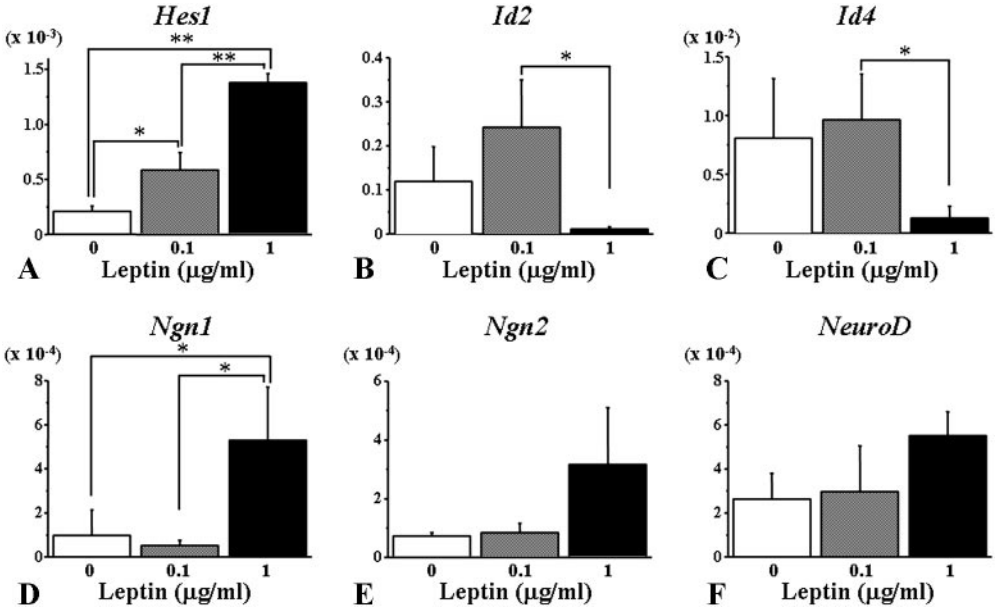

FIG. 6. The mRNA expression of bHLH proteins in neurosphere cells. QRT-PCR revealed that high-dose (1 μg/ml) and low-dose (0.1 μg/ml) leptin significantly increased *Hes1* mRNA expression (A). *Id2* (B) and *Id4* (C) mRNA expression was significantly lower in high-dose than low-dose leptin-treated cells. *Ngn1* mRNA expression (D) was significantly higher in high-dose leptin-treated cells than control cells. *Ngn2* (E) and *NeuroD* (F) mRNA expression tended to increase in high-dose leptin-treated cells ($P < 0.10$). *, $P < 0.05$; **, $P < 0.01$. n = 3–5.

tiation in leptin-treated neurosphere cells. Id2 and Id4 each sequester both OLIG and E2A proteins, which bind to promoter regions in the oligodendroglial gene, inhibit oligodendrocyte development, and enhance commitment to the astrocytic fate (60).

The expression of *NPY* mRNA was lower in *ob/ob* than wild-type embryos at E16 and E18 (Fig. 7A). The reduced proliferative activity in *ob/ob* embryos might also be caused by a low amount of NPY because NPY had an additive effect with EGF on the proliferative activity of neurosphere cells (Fig. 4D). This is consistent with a report that NPY acted on multipotent neuronal precursor in the olfactory epithelium and promoted proliferation of neuronal precursors (61). Because leptin and NPY had no synergistic effect on the proliferation of neurosphere cells (data not shown), these two factors may not have a cross-talk in the maintenance of NSCs and/or NPCs. This contention is not contradictory to the previous reports that NPY activates the ERK 1/2 subgroup of MAPKs (61), whereas leptin activates the JAK-STAT pathway (7).

Differentiation of neurons

Yura *et al.* (62) reported that the nutritional status in the fetus was related to neonatal leptin surge, neuronal development, and obesity. Offspring with fetal undernutrition exhibited a premature onset of neonatal leptin surge and develops pronounced weight gain and adiposity (62). The premature leptin surge led to accelerated weight gain with a high-fat diet and offspring with fetal undernutrition (exhibited an impaired response to acute peripheral leptin administration with impaired leptin transport to the brain as well as an increased density of hypothalamic nerve terminals (62). Bouret *et al.* (16) reported that leptin promoted the development of the neural projection in the hypothalamus in postnatal mice. These reports suggest that the intrauterine circumstances alter the onset of postnatal leptin surge and postnatal leptin surge affects hypothalamic development; however, the role of leptin in embryonic neuronal development is still unknown.

Terminally postmitotic neurons migrate into the CP, and migratory cohorts are added in succession to the CP's most superficial level (53). Ob-Rb was already expressed in the CP at E14 (Fig. 1, C–E). At E18, this expression was strong (Fig. 1 H) and Tuj1$^+$ neurons in the CP expressed Ob-Rb (Fig. 1J). In P19EC cells, *NPY* mRNA expression disappeared once after RA treatment, but it was induced again when the cells were differentiated into neurons (Fig. 1B). *NPY* mRNA was expressed in the inner layer of the CP, which contained neurons that had migrated earlier, and the expression area was tangentially wider at E18 than E16 (Fig. 7, B–E). Taken together, these results suggest that well-differentiated neurons expressed *NPY* mRNA in the embryonic cerebral cortex. *NPY* mRNA expression was lower in the brain (Fig. 7A) and weaker in the CP in *ob/ob* than wild-type embryos at E16 and E18 (Fig. 7, B–E).

Leptin may promote the differentiation of neurons into *NPY*-expressing ones in the embryonic stage. According to the present QRT-PCR, *NPY* mRNA expression in the brain of *ob/ob* embryos at E16 was restored to the level of wild type [the average value of *NPY* mRNA/18S was 0.140 (n = 6)] (Fig. 7A) by intracerebroventricular injection of 200 ng leptin at E14 [the values were 0.127 and 0.159 in leptin-injected *ob/ob* embryos (n = 2), and 0.079, 0.085, and 0.051 in vehicle-injected *ob/ob* embryos (n = 3)]. In the adult mature hypothalamus, the functions of leptin and NPY in appetite and energy expenditure are opposite to each other, and leptin inhibits the expression of NPY (7). This mature leptin-NPY axis may not be established in the embryonic cerebral cortex.

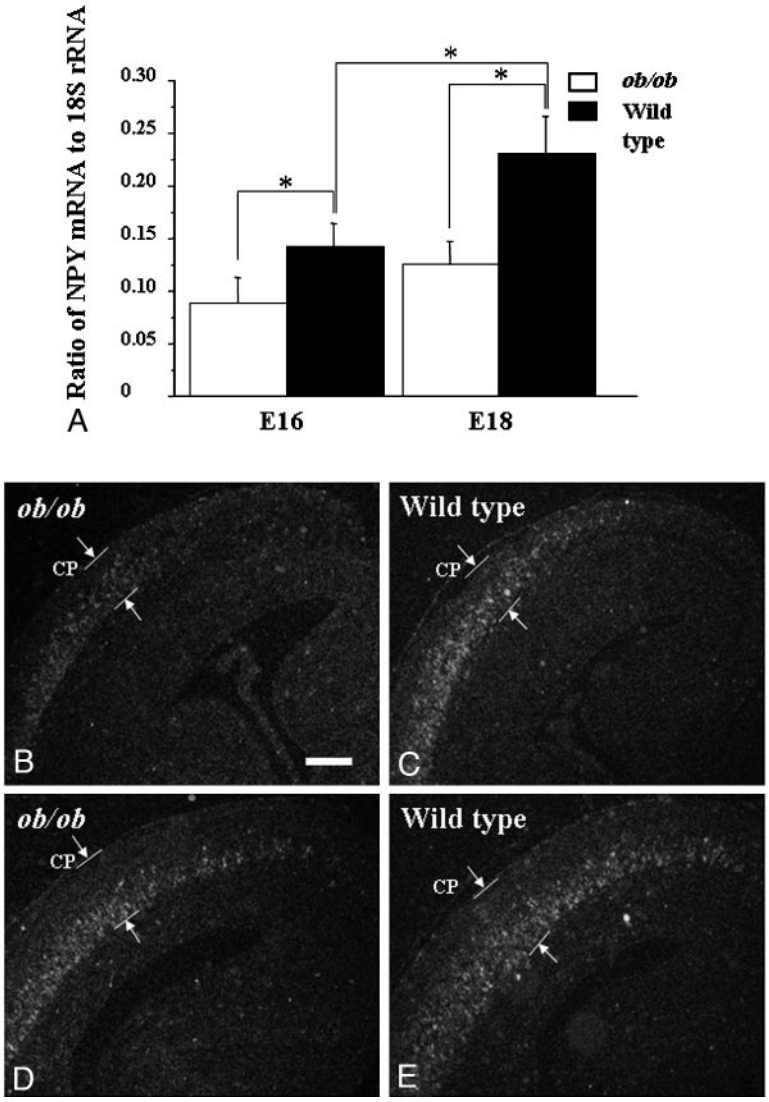

FIG. 7. The expression of *NPY* mRNA in the brain. QRT-PCR revealed that *NPY* mRNA expression in the total brain was lower at E16 than E18 and lower in *ob/ob* than the wild type at both E16 and E18 (A). *NPY* mRNA expression in the CP was weaker in *ob/ob* (B) than the wild type (C) at E16 and was limited to the inner layer in *ob/ob* mice (D), compared with the wild type at E18 (E). *Scale bar*, 100 μm. *, $P < 0.01$. n = 3 (E16 *ob/ob*); n = 6 (E16 wild type); n = 7 (E18 *ob/ob* and wild type).

This would be consistent with a previous report that leptin injection did not decrease NPY expression in the neonatal mouse hypothalamus (63). In the immature brain, leptin may play a role in the functional differentiation of neurons. Such a role is supported by the present QRT-PCR data that 1 μg/ml leptin treatment significantly increased *Ngn1* mRNA expression (Fig. 6D) because Ngn1 is expressed in newly committed neuronal progenitors and immature neurons and plays a role in neurogenesis together with Ngn2 (64). Leptin treatment tended to increase *Ngn2* and *NeuroD* mRNA expression in neurosphere cells (Fig. 6, D–F). Leptin did not increase the proportion of Tuj1$^+$ progenitor cells (Fig. 5E) but may promote the differentiation of neuronal restricted progenitor cells into neurons. Leptin treatment of juvenile *ob/ob* mice restored brain weight and protein content but did not restore neurodegeneration or the expression of synaptobrevin in the neocortex (13). Brain DNA content was increased by leptin injection to juvenile *ob/ob* mice but was not normalized to the level of lean mice (15). The present study suggests that exposure to leptin in the embryonic or early postnatal stage may be required to rescue these abnormalities.

Leptin has effects on other brain regions than the neocortex and hypothalamus. In the adults, leptin receptor is expressed in the hippocampus, and leptin facilitates hippocampal synaptic plasticity and inhibits hippocampal epileptiform-like activity (21, 65, 66). Neuronal soma size was smaller in the cingulate cortex of *ob/ob* mice than that of wild type (12). The cingulate girus is involved in emotion and sensory, motor, and cognitive processes (67–70). Impairment of the anterior cingulate causes the motor deficit, akinetic mutism, and apathy (69, 71), and the *ob/ob* mice has low locomotor activity (13). In E18 *ob/ob* embryos, pyknosis was caused in the cingulate cortex (72). Leptin may be related to the development of the hippocampus and the cingulate cortex and may affect the memory and cognitive function.

In summary, this study has suggested that leptin maintains neural stem and progenitor cells and is related to neuronal and glial development in the mouse embryonic brain.

Acknowledgments

We thank Ms. Y. Takeda for the histological preparation.

Received June 28, 2005. Accepted November 1, 2005.

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This work was supported by a grant from the Japan Society for the Promotion of Science.

The authors have no conflict of interest.

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