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Expression of Leptin Receptor Isoforms in Rat Brain Microvessels

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ABSTRACT

Leptin acts on specific brain regions to affect body weight regulation. As leptin is made by white adipose tissue, it is thought that leptin must cross the blood-brain barrier or the blood-cerebrospinal fluid barrier to reach key sites of action within the brain. High expression of a short form leptin receptor has been reported in the choroid plexus. However, whether one or more of the known leptin receptor isoforms is expressed in brain capillaries is unknown. To identify and quantitate leptin receptor isoforms in rat brain microvessels, we applied quantitative RT-PCR to RNA from purified rat brain microvessels in parallel with *in situ* hybridization. The results show that the amount of short form leptin receptor messenger RNA (mRNA) in brain microvessels is extremely high, exceeding that in choroid plexus. In contrast, low levels of this mRNA were detected in the cerebellum, hypothalamus, and meninges. The long form leptin receptor mRNA is only present at low levels in the microvessels, but surprisingly, its level in cerebellum is 5 times higher than that in the hypothalamus. *In situ* hybridization experiments confirmed strong expression of short leptin receptors in microvessels, choroid plexus, and leptomeninges. The distribution and type of leptin receptor mRNA isoforms in brain microvessels are consistent with the possibility that receptor-mediated transport of leptin across the blood-brain barrier is mediated by the short leptin receptor isoform. (*Endocrinology* **139:** 3485–3491, 1998)

LEPTIN, the adipose tissue-derived hormone (1), plays an important role in the regulation of food intake, energy expenditure, and adiposity (2–4). Through alternative messenger RNA (mRNA) splicing, leptin receptors exists as several different isoforms with varying intracellular domains (5, 6), of which only the longest isoform has the capacity to activate STAT (signal transducer and activator of transcription) signaling (7–9). The mRNA of this isoform has been found at the highest levels within the hypothalamus and at much lower levels in other tissues (7). Several studies strongly suggest that the effects of leptin on body weight involve initial actions within the hypothalamus (10–14), although possible direct actions on peripheral tissues have been reported (15–18). The essential role of the long isoform of the leptin receptor is demonstrated by the fact that its absence results in extreme obesity in the *db/db* mice (6, 19). Among the other leptin receptor isoforms, a predominant form with a predicted short intracellular domain is expressed at very high levels in choroid plexus, from which it was cloned, as well as in lung and kidney, and is present at detectable levels in most other tissues (5, 7). This receptor is thought to play a role in the clearance of leptin from the circulation (20) and in the transport of leptin into the brain (5, 21, 22).

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Lack of functional leptin or of long form leptin receptors in *ob/ob* and *db/db* mice, respectively, causes severe obesity. Obesity in humans, therefore, may be related to low levels of functional circulating leptin or to decreased action at the target cells expressing long form leptin receptors. Supporting the latter possibility are data demonstrating that serum leptin levels are increased in obesity and correlate positively with body weight (23, 24). This raises the possibility that circulating leptin is not accessible to leptin receptor-expressing neurons in the hypothalamus, or that these cells are themselves deficient in leptin binding or in leptin action. A decreased cerebrospinal-fluid/serum ratio in human obesity has been reported, thus suggesting that a defect in leptin transport into the brain is a possible mechanism for leptin resistance (25, 26).

Very little is known at present about how leptin enters the brain. For leptin to reach most sites within the brain, the hormone must cross the blood-brain barrier (BBB) and/or the blood-cerebrospinal fluid (CSF) barrier. High expression of the short leptin receptor isoform in the choroid plexus (5), the major site for production of CSF, has resulted in speculation that this site is an important site for leptin entry into the brain. On the other hand, consistent with the BBB being a site for transport of leptin into the brain, a recent report demonstrated specific binding of [^{125}I]leptin to isolated human brain microvessels (22). However, whether brain microvessels have the capability to transport leptin has not been demonstrated directly, and the identity of the leptin receptor species on brain microvessels is at present unknown.

Here we applied quantitative RT-PCR in combination with *in situ* hybridization to identify and quantitate leptin receptor mRNA isoforms in rat brain microvessels. Our results show that the abundance of short form leptin receptor mRNA in brain microvessels is very high, exceeding that of choroid plexus. Other brain regions, including cerebellum, hypothalamus, and meninges express low amounts of this mRNA species. The long form leptin receptor mRNA is present at low levels in the microvessels. In cerebellum, however, the level of long form leptin receptor mRNA is 5 times greater than that in the hypothalamus, 7 times higher than that in microvessels, and 14 times higher than that in the choroid plexus. *In situ* hybridization revealed strong expression of short leptin receptors in brain microvessels, leptomeninges, and choroid plexus.

Materials and Methods

Animals and histology

Adult male pathogen-free Sprague-Dawley rats (250–350 g) were housed individually with food and water available *ad libitum* in a light- (12 h on/12 h off) and temperature-controlled environment (21.5–22.5 C). The animals and procedures used were in accordance with the guidelines and approval of the Harvard Medical School and Beth Israel Deaconess institutional animal care and use committees. For RT-PCR experiments, rats were deeply anesthetized by inhalation of Metofane (Mallinckrodt Veterinary, Mundelein, IL) and then decapitated. The skull was reflected from the brain, and the meninges were dissected from the scull. Meninges consisted mainly of dura together with some vascular elements of the leptomeninges pooled from 10 rats. Choroid plexi were carefully dissected from all ventricles and finally pooled from the same 10 animals. Samples of cerebellum and hypothalamus were also taken from these animals. Microvessels were isolated from brains after removal of cerebellum and brain stem as described previously using the method of Brendel *et al.* (27), as modified by Brecher *et al.* (28) and Sussman *et al*. (29). Sixteen rats were killed by decapitation, and their brains were placed in an aerated solution containing 118 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgSO_4$, 1.0 mM NaH_2PO_4, 5.5 mM D-glucose, 0.2% (wt/vol) fraction V albumin, and 28 mM HEPES (pH 7.4). The forebrains were dissected, stripped of pia-arachnoid mater using a cotton-tipped applicator, and then homogenized by hand in a Dounce glass homogenizer (Kontes Co., Vineland, NJ). Subsequently, nylon mesh filtration through (147 μm pore size) was performed, followed by trapping microvessels by filtering them through a 74-μm and a 37-μm nylon mesh. Microvessels were gently removed from the meshes with a rubber policeman and washed in PBS that was subsequently centrifuged at 1800 rpm. Aliquots were taken for assays of purity. Alkaline phosphatase and γ-glutamyl transpeptidase were measured in all microvessel preparations and compared to original brain homogenates according to the method of Hausamen *et al*. (30) and as described previously (29). This method results in a specific activity of this enzyme in microvessel samples that is increased approximately 20- to 30-fold compared with that in the respective original brain homogenates. Total rat brain RNA was purchased from Ambion (Austin, TX). For *in situ* experiments, rats were deeply anesthetized with ip chloral hydrate (7%; 350 mg/kg) and perfused transcardially with diethylpyrocarbonate-treated 0.9% saline followed by 500 ml phosphate-buffered 4% paraformaldehyde (pH 7.0). The brains were removed, stored in the same fixative for 4 h, and submerged in 20% sucrose in DEPC-PBS, and five series of coronal sections were cut at 30 μm. The sections were stored at −20 C in an antifreeze solution until being mounted for *in situ* hybridization histochemistry (31).

Quantification of leptin receptor (ObR) mRNA isoforms in rat brain by RT-PCR

Total RNA from the various tissues was isolated using the RNA-STAT-60 reagent as described by the manufacturer (Tel-Test, Friendswood, TX). The complementary DNA (cDNA) was synthesized from 1.0 μg total RNA using deoxythymidine-oligonucleotides and the Advantage RT-PCR kit from Stratagene (La Jolla, CA). The final volume of the cDNA samples was 100 μl. For amplification of 764 bp rat β-actin cDNA, the following primers were used; upstream primer, 5′-TTGTAACCAACTGGGACGATATGG-3′; and downstream primer, 5′-GATCTTGATCTTCATGGTGCTAGG-3′ (Clontech, Palo Alto, CA). The following primers were used for specific PCR amplification of 400 bp (C-terminal 129 amino acids) of rat leptin receptors (ObR) cDNA (short form): ObR-1, 5′-GTTATATCTGGTTATTGAATGG-3′; and ObR-2, 5′-GAGATACTTCAAAGAGTGTCC-3′ (GenBank accession no. D85558). The ObR-2 primer is complementary to the rat ObR short form (ObRs) cDNA in the region encoding the C-terminus of the receptor and to part of the 3′-untranslated region of the corresponding mRNA (GenBank accession no. D85557). The following primers were used for specific PCR amplification of 400 bp (amino acids 930-1063) of rat ObR long form (ObRl) cDNA: ObR-A, 5-AAAGAGCTCGAGATGGT-ACCAGCAGCTATGG-3′; and ObR-B, 5′-AAAAAGCTTCCCTCCAGTT-CCAAAAGCTCATCC-3′. The *single underlined* sequences are complementary to leptin receptor cDNA, and the *double underlined* sequences represent recognition sites for specific restriction enzymes. These primers were also used for generation of a probe for *in situ* hybridization (see below). Each 50-μl PCR reaction was performed with 5.0 μl cDNA as template. The assay conditions were 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.2 mM deoxy (d)-NTPs, 20 pmol of each primer, 2.5 U *Taq* polymerase (Stratagene, La Jolla, CA), and 0.50 μl [α-^{32}P]dCTP (29.6 tetrabecquerels/mmol; 370 megabecquerels/ml; New England Nuclear, Boston, MA). The mixture was overlaid with 25 μl mineral oil, and after initial denaturation at 96 C for 3 min, the samples were subjected to 24–32 cycles of amplification: denaturation at 95 C for 1 min, annealing at 55 C for 1 min, and extension at 72 C for 30 sec. Five microliters of the reaction were then combined with 5 μl sequencing stop solution (Amersham International, Aylesbury, UK) and heated to 85 C for 5 min before loading 4 μl onto a 4% urea-acrylamide gel (38 × 31 × 0.03 cm). Electrophoresis was performed at 65 watts of constant power for 3 h before the gels were transferred to filter paper, dried, and finally subjected to ^{32}P quantification by PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA).

Generation of rat leptin receptor complementary RNA (cRNA) probes

Fragments of rat leptin receptor cDNAs were generated by RT-PCR using total rat brain RNA (Ambion, Austin, TX) and the Advantage RT-PCR kit. The following primers were used for amplifying a 500-bp fragment corresponding to part of the extracellular domain of the leptin receptor (amino acids 293–460); ObR-C: 5′-AAAGAGCTCACAGCGTGCTTCCTGGGTCTTC-3′ and ObR-D: 5′-AAAAAGCTTCACAGTGCTTCCCACTAGTGATTGG-3′. The *single underlined* sequences are complementary to rat leptin receptor cDNA, and the *double underlined* sequences represent recognition sites for *Sac*I and *Hin*dIII in primers A and B, respectively. The PCR products obtained were digested with *Sac*I and *Hin*dIII restriction enzymes and cloned into pGEM-11Zf$^+$ (Promega, Madison, WI) using standard techniques. The ObR-A and ObR-B primers from above were used for the cloning of 400 bp from the ObR cDNA, corresponding to part of the intracellular domain of the long rat leptin receptor. The inserts of positive clones were verified by sequencing using standard, double stranded plasmid techniques. For generation of sense and antisense ^{35}S-labeled cRNA, the plasmids were linearized by digestion with *Hin*dIII or *Sac*I, respectively, and subjected to *in vitro* transcription with T7 or SP6 polymerase, according to the manufacturer's protocols (Promega).

In situ hybridization histochemistry

The protocol for *in situ* hybridization histochemistry was a modification of that previously reported (31–33). Tissue sections were mounted onto slides, air-dried, and stored in desiccated boxes at −20 C. Before hybridization, the slides were immersed in 10% neutral buffered formalin, incubated in 0.001% proteinase K (Boehringer Mannheim, Indianapolis, IN) for 30 min and then in 0.025% acetic anhydride for 10 min, and dehydrated in ascending concentrations of ethanol. The cRNA probes were then diluted to 10^6 cpm/ml in a hybridization solution of 50% formamide, 10 mM Tris-HCl (pH 8.0), 5 mg transfer RNA, 10 mM

dithiothreitol, 10% dextran sulfate, 0.3 M NaCl, 1 mM EDTA (pH 8), and 1 × Denhardt's solution (Sigma). Hybridization solution and a glass coverslip were applied to each slide, and sections were incubated for 12–16 h at 56 C. The coverslips were removed, and the slides were washed four times with 4 × SSC (standard saline citrate). Sections were then incubated in 0.002% ribonuclease A (Boehringer Mannheim) with 0.5 M NaCl, 10 mM Tris-HCl (pH 8), and 1 mM EDTA for 30 min at 37 C. Sections were rinsed in decreasing concentrations of SSC containing 0.25% dithiothreitol: 2 × at 50 C for 1 h, 0.2 × at 55 C for 1 h, and 0.2 × for 1 h at 60 C. Sections were next dehydrated in graded ethanol (50%, 70%, 80%, and 90%) containing 0.3 M NH_4OAc, followed by 100% ethanol. Slides were air-dried and placed in x-ray film cassettes with BMR-2 film (Eastman Kodak, Rochester, NY) for 3–5 days. Slides were then dipped in NTB2 photographic emulsion (Kodak), dried, and stored with desiccant in foil-wrapped slide boxes at 4 C for 2–3 weeks. Slides were developed with D-19 developer (Kodak), counterstained with thionin, dehydrated in graded ethanols, cleared in xylene, and coverslipped with Permaslip. Sections were analyzed with a Zeiss Axioplan light microscope using bright- and darkfield optics. Photomicrographs were produced by capturing images with a digital camera (Kodak, DCS) mounted directly on the microscope and an Apple Macintosh Power PC computer. Image-editing software (Adobe Photoshop) was used to combine photomicrographs into plates, and figures were printed on a dye sublimation printer (Kodak 8600). Only the sharpness, contrast, and brightness were adjusted.

Results

Isolation of tissue

In this study our primary aim was to identify and quantify mRNA levels of leptin receptor isoforms in brain microvessels. We, therefore, first isolated microvessels from rat brains according to methods described previously (27, 29). The total yield of microvessels from 16 rats was approximately 200 mg. We found approximately 20- and 30-fold enrichments of γ-glutamyl transferase and alkaline phosphatase, respectively, in the microvessel sample *vs.* the total brain homogenate, consistent with strong enrichment of cerebral endothelial cells in the purified microvessel sample (data not shown) (29). In addition, the preparation was highly enriched in fine threadlike strands, as determined by microscopy (Fig. 1). From another set of rats, we isolated choroid plexi and hypothalami as positive controls for receptor expression, because these tissues have been shown to express high amounts of short and long isoforms of leptin receptor, respectively (5, 7). Total RNA was purified in parallel from all tissue samples, including cerebellum and meninges (mainly dura mater). The total yields of RNA from microvessels (16 rats), choroid plexi (10 rats), and meninges (10 rats) were 40, 100, and 75 μg, respectively. The integrity of the RNA was tested by agarose-gel electrophoresis in combination with ethidium bromide staining, and no degradation of the ribosomal RNA bands were detected (data not shown). In addition, the quality of the RNA from each tissue sample was tested by amplifying β-actin cDNA by RT-PCR using limiting cycle numbers, under which conditions the amounts of β-actin PCR products were similar (data not shown).

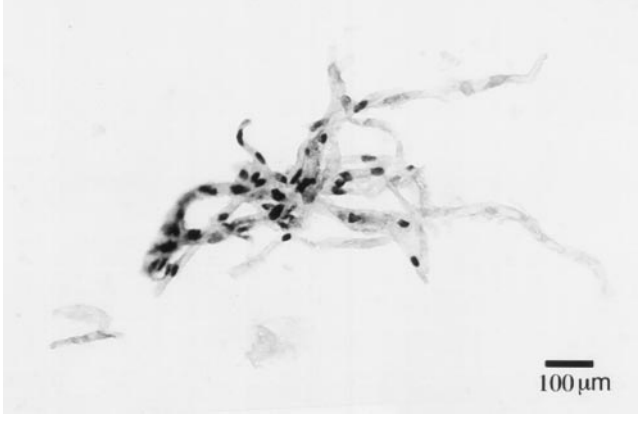

FIG. 1. Light photomicrograph of isolated rat brain capillaries. The microvessels were prepared from rat forebrains as described in *Materials and Methods*.

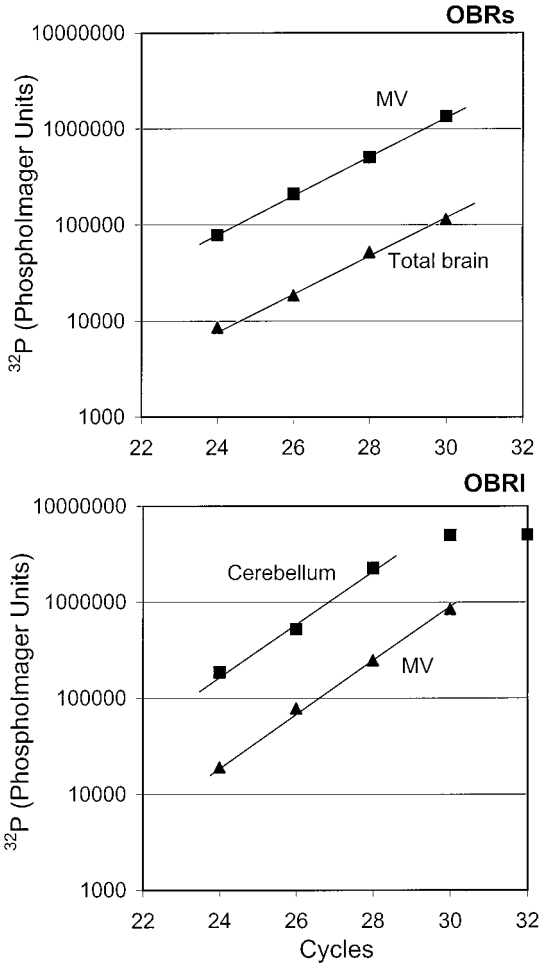

FIG. 2. Determination of exponential phase of RT-PCR amplification of the short and long forms of leptin receptor mRNA. *Top*, Equal amounts of cDNA derived from 1.0 μg total RNA from microvessels (■) and from total brain (▲) were subjected to PCR amplification reactions spiked with [^{32}P]dCTP and primers specific for the short leptin receptor cDNA. Reactions were terminated after 24–32 cycles of amplification. Equal amounts from each reaction were subjected to denaturing urea-PAGE and subsequent PhosphorImager quantification. Plotted are the average arbitrary units of measured ^{32}P from duplicate samples. *Bottom*, Equal amounts of cDNA derived from 1.0 μg total RNA from cerebellum (■]) and microvessels (▲) were subjected to PCR amplification reactions spiked with [^{32}P]dCTP and primers specific for the long leptin receptor cDNA.

Quantification of short and long isoforms of leptin receptor mRNA by RT-PCR in rat brain microvessels

First we determined the number of cycles necessary for detection and exponential amplification of ObRs and ObRl

cDNAs. Preliminary RT-PCR experiments showed that the levels of ObRs mRNA were relatively high in the microvessels and low in the total brain sample. In addition, ObRl mRNA levels were relatively high in cerebellum and low in microvessels (data not shown). These samples were then used for cycle number determination (Fig. 2). As shown at the *top* of Fig. 2, amplification of ObRs from microvessel and total brain cDNA proceeded at the same rate during the measured cycles. The two ObRl reactions were also amplified at the same rate during cycles 24–28, but at a faster rate than the ObRs PCR reactions (Fig. 2, *bottom*). However, the cerebellum reaction entered nonlinear amplification after 28 cycles. We, therefore, chose 26 cycles of amplification for both ObRs and ObRl in the subsequent quantitative PCR experiments. PCR reactions were then performed in triplicate for cerebellum, choroid plexus, meninges (dura mater), total brain, microvessels, and hypothalamus. An autoradiogram of the final results is shown in Fig. 3. The quantification of these results by PhosphorImager analysis is shown in Fig. 4 after normalization to the levels in hypothalamus. Clearly, the mRNA of the short form of the leptin receptor is most highly expressed in brain microvessels (Fig. 4, *top*). This level is even higher than that in the choroid plexus, the tissue in which ObRs mRNA levels are the highest tested to date. The mRNA level in the microvessels was more than 40-fold higher than that in the hypothalamus. The results obtained when amplifying the long form of the leptin receptor from the same samples are entirely different (Fig. 4, *bottom*). Surprisingly, the mRNA levels of ObRl in cerebellum exceeds those in all other tissues tested, including the hypothalamus, in which ObRl expression levels were previously thought to be the highest (7). Low levels of ObRl mRNA were detected in all other tissues. When no reverse transcriptase enzyme was added to the samples, no specific RT-PCR product was detected in any sample (data not shown). Expression of ObRl mRNA was not detected in samples of peripheral arteries and veins, whereas ObRs mRNA levels were barely detectable in these tissues (data not shown).

Detection of short leptin receptor mRNA by in situ hybridization in rat brain microvessels

To substantiate our finding of high levels of mRNA for the short form of the leptin receptor in rat brain microvessels, we generated ^{35}S-labeled RNA antisense probes for *in situ* hybridization to rat brain sections as described in *Materials and Methods*. The results with a probe specific to all forms of leptin receptors are shown in Fig. 5. Specific hybridization to perivascular cells from brain capillaries was found in most regions of the brain, with no particular enrichment in the hypothalamus compared with the rest of the brain. An example of a vessel from thalamus is shown in Fig. 5A. No expression of the long form leptin receptor was detected in microvessels by applying an antisense probe specific to this isoform (data not shown), thus supporting our RT-PCR data. Strong specific signals with the all form probe were also detected in the leptomeninges surrounding the brain (Fig. 5B), whereas no signal was found in these brain regions when sections were hybridized with the long form probe (data not shown). Furthermore, widespread hybridization with the all form probe was detected in several cell types of the choroid plexus (Fig. 5, C and D). Very little specific hybridization of the long form probe was seen in the choroid plexus (data not shown), which agrees with our RT-PCR results. Both the all form probe and the long form probe showed extensive hybridization to several hypothalamic nuclei, including the arcuate nucleus, and to several cellular regions of the cerebellum (data not shown). In addition, lower intensity specific signals were found in most parts of the brain with the long form probe (data not shown). Sense probes for the all form and long form probes did not exhibit any specific hybridization to any of the above regions of the rat brain.

Discussion

By applying RT-PCR and *in situ* hybridization we have demonstrated relatively high levels of mRNA encoding the short form of the leptin receptor in rat brain microvessels,

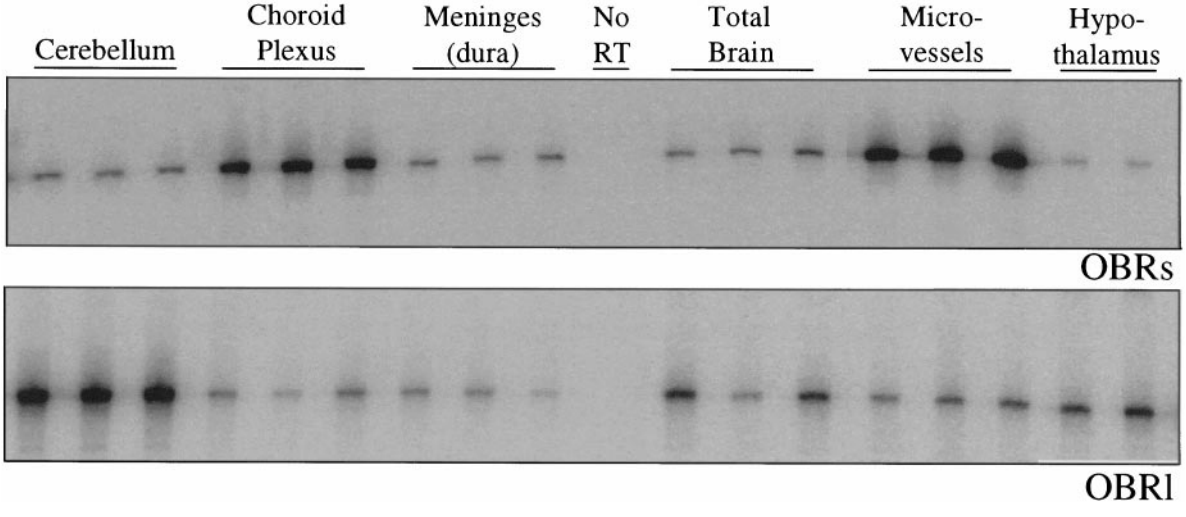

FIG. 3. RT-PCR of ObRs and ObRl mRNA from rat brain microvessels. Shown are autoradiograms of ^{32}P-labeled RT-PCR samples from cerebellum, choroid plexus, meninges, total brain, microvessels, and hypothalamus. The *upper* and *lower panels* show the results obtained applying primers specific for ObRs and ObRl, respectively. The PCR reactions were terminated after 26 cycles. Each lane represents independent PCR reactions. One RT-PCR reaction, in which no reverse transcriptase was added, was also loaded onto the gel (no RT).

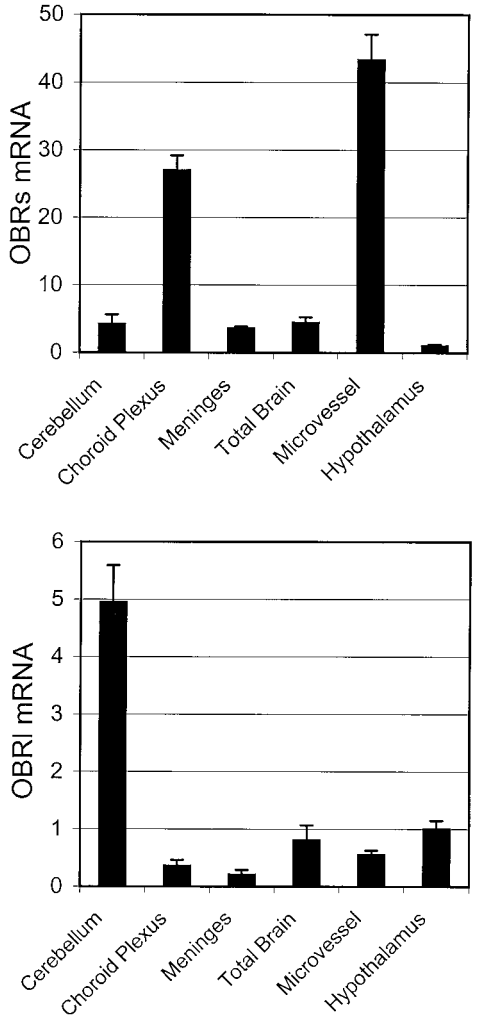

FIG. 4. Quantification of ObRs and ObRl cDNA in rat brain microvessels. The two gels from Fig. 3, were subjected to ^{32}P quantification by PhosphorImager analysis and normalization to hypothalamic levels (=1.0). The *upper* and *lower graphs* show the results obtained for ObRs and ObRl, respectively. *Error bars* are the mean ± SE.

which constitute the BBB (34). The levels in microvessels exceed those in choroid plexus, the site of the blood-CSF barrier. As the surface area of the BBB is estimated to be several thousand times greater than that of the blood-CSF barrier, our results are consistent with the possibility that the capillary endothelial cells comprising the BBB are the major site of leptin transfer from the circulation into the brain. Transport of leptin from the blood to brain interstitial fluid is likely to occur via a saturable transport mechanism. This probably involves binding of leptin to leptin receptors at the luminal surface of the capillaries, followed by transport across the endothelial cells and release of intact ligand at the basement membrane side. Leptin would then pass by diffusion through the interstitial fluid to activate leptin receptors expressed on neuronal cells. However, additional careful studies will clearly be needed to substantiate the above scenario.

The RT-PCR data also show high levels of the mRNA encoding the short isoform of the leptin receptor in the choroid plexus, thus confirming results published previously. The *in situ* hybridization results indicate that several cell types in the choroid plexus, including epithelial cells and microvessels, express high amounts of ObRs mRNA. These results are consistent with the possibility that the choroid plexus is a site at which leptin is transported into the CSF. Indeed, several studies show that when leptin is administered into the ventricles, it produces similar effects on body weight regulation as leptin injected peripherally (3, 10). When rats received much lower doses of leptin intracerebroventricularly, no effects on food intake were detected (11). The same dose delivered into the hypothalamus did evoke a marked reduction in food intake (11). These results support the possibility that transport of leptin into the CSF via the choroid plexus is not the major route by which leptin reaches the hypothalamus. Furthermore, the concentration of leptin in the CSF is about 0.26 ng/ml in lean humans (25, 26), which is about 40-fold lower than the equilibrium dissociation constant of the leptin receptor (5). Although the latter is based on *in vitro* results, these data also suggest that leptin is not transported to specific hypothalamic nuclei via the CSF, as the leptin concentration in the CSF is likely to be insufficient to activate significant Janus kinase-STAT signaling by leptin receptors. However, it is at present unknown what role ObRs plays in the choroid plexus and whether the choroid plexus is an important site for transport of leptin into or possibly out of the CSF.

Our *in situ* data show significant amounts of short form leptin receptor mRNA expression in the leptomeninges of the rat brain. However, the RT-PCR results demonstrated only low receptor mRNA levels in the dura mater of the meninges. Together, these results are consistent with the pia mater and/or the arachnoid being a site of significant expression of ObRs. The function of receptors in the leptomeninges is entirely unknown, and further studies are needed to establish a possible role for ObRs at these sites in the biology of leptin. The possibility that the leptomeninges may degrade CSF leptin should be considered.

The arcuate nucleus of the hypothalamus is a major site of leptin action in the brain, as leptin modulates the expression of neuropeptide Y (10, 35), POMC (13), and AGRP (12) at this site. A recent report demonstrated significant uptake of [^{125}I]leptin into the region of the arcuate nucleus 20 min after iv injection of radioiodinated leptin (21). However, the mechanism by which leptin gained access to this location is not known. As the arcuate nucleus is located near the median eminence, leptin may enter the interstitial fluid by diffusion through the fenestrated capillaries of the median eminence and/or through receptor-mediated transport across the BBB to the arcuate nucleus (21, 34). The Koletsky rat, which lacks all leptin receptor isoforms, has the same level of leptin in the CSF as control rats (36). This suggests that leptin can enter the brain by a mechanism independent of leptin receptors, and one such mechanism might be by diffusion through the circumventricular organs into the CSF. On the other hand, the level of plasma leptin in the Koletsky rat is 10 times higher than in control animals, suggesting saturation of leptin transport into the brain or lack of functional transporters. Careful dose-response and time-course studies of iv injections of

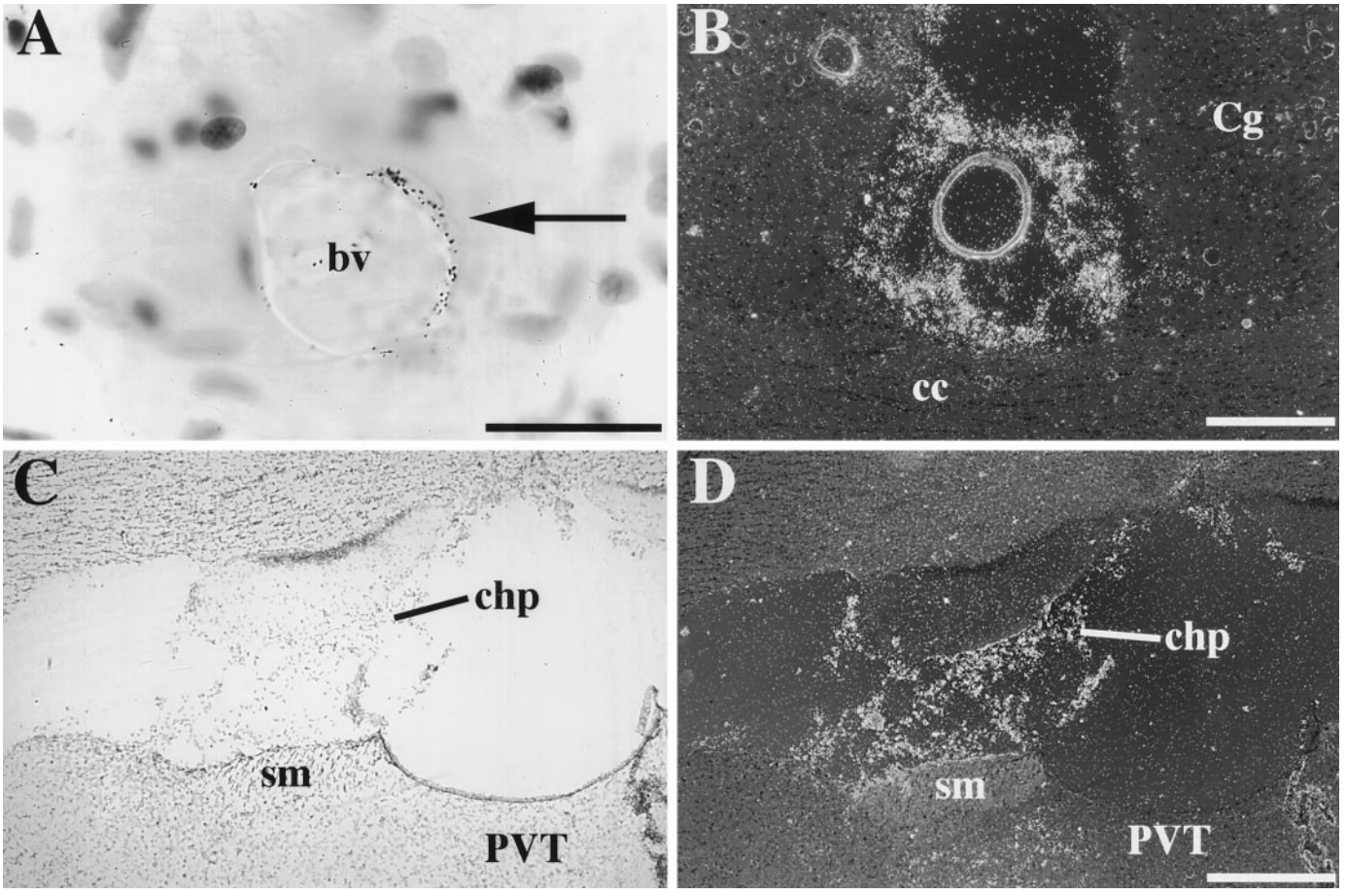

FIG. 5. *In situ* hybridization to rat brain tissues with a ^{35}S-labeled antisense probe to all forms of the leptin receptor mRNA. A, Microvessel from thalamus. bv, Brain vessel. *Scale bar* = 30 μm. B, Leptomeninges. Cg, Cingulate cortex; cc, corpus callusum. *Scale bar* = 300 μm. C, Brightfield of choroid plexus (chp). sm, Stria medullaris; PVT, paraventricular thalamus. D, Darkfield of C. *Scale bar* = 500 μm.

gold-labeled leptin or [^{125}I]leptin in combination with electron microscopy may be needed to address these questions.

An unanticipated finding of this study was that the mRNA of the long isoform of the leptin receptor is highly expressed in the cerebellum of the rat. Cerebellar expression exceeds that in other regions of the rat brain including the hypothalamus, the site where leptin is thought to act most potently to regulate body weight. Similar results have recently been reported in a study of the human brain (37, 38). The cerebellum is concerned with the coordination of somatic motor activity, the regulation of muscle tone, and mechanisms that influence and maintain equilibrium. Defects in these functions of the cerebellum have not heretofore been noted in animal models of impaired leptin function or action or in humans with lack of leptin function. Further studies are needed to clarify the biology of these long form leptin receptors in the cerebellum, including the possibility that they bind another, yet unidentified, ligand.

In summary, we have demonstrated that the mRNA encoding the leptin receptor short form is highly expressed in rat brain microvessels. These data support the hypothesis that the BBB is the major site for transport of leptin into the brain. Further studies are clearly needed to characterize this process, including efforts to demonstrate receptor-mediated transcytosis of leptin across brain capillaries. Such studies are critical, given the possibility that the BBB is a major site for leptin resistance in humans.

References

1. **Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM** 1994 Positional cloning of the mouse *obese* gene and its human homologue. Nature 372:425–432
2. **Halaas JL, Gajiwala KS, Maffei M, Cohen SL, Chait BT, Rabinowitz D, Lallone RL, Burley SK, Friedman JM** 1995 Weight-reducing effects of the plasma protein encoded by the *obese* gene. Science 269:543–546
3. **Campfield LA, Smith FJ, Guisez Y, Devos R, Burn P** 1995 Recombinant mouse OB protein: evidence for peripheral signal linking adiposity and central neural networks. Science 269:546–549
4. **Pelleymounter, MA, Cullen MJ, Baker MB, Hecht R, Winters D, Boone T, Collins F** 1995 Effects of the *obese* gene product on body weight regulation in *ob/ob* mice. Science 269:540–543
5. **Tartaglia LA, Dembski M, Weng X, Deng N, Culpepper J, Devos R, Richards GJ, Campfield LA, Clark FT, Deeds J, Muir C, Sanker S, Moriarty A, Moore K J, Smutko JS, Mays GG, Woolf EA, Monroe CA, Tepper RI** 1995 Identification and expression cloning of a leptin receptor. Cell 83:1263–1271
6. **Lee G-H, Proenca R, Montez JM, Carroll KM, Darvishzadeh JG, Lee JI, Friedman JM** 1996 Abnormal splicing of the leptin receptor in *diabetic* mice. Nature 379:632–635
7. **Ghilardi N, Ziegler S, Wiestner A, Stoffel R, Heim MH, Skoda RC** 1996 Defective STAT signaling by the leptin receptor in *diabetic* mice. Proc Natl Acad Sci USA 93:6231–6235
8. **Baumann H, Morella KK, White DW, Dembski M, Bailon PS, Kim H, Lai C-F, Tartaglia LA** 1996 The full-length leptin receptor has signaling capabilities of interleukin 6-type cytokine receptors. Proc Natl Acad Sci USA 93:8374–8378
9. **Vaisse C, Halaas JL, Horvath CM, Darnell Jr JE, Stoffel M, Friedman JM** 1996 Leptin activation of Stat3 in the hypothalamus of wild-type and *ob/ob* mice but not *db/db* mice. Nat Genet 14:95–97

10. **Stephens TW, Basinski M, Bristow PK, Bue-Valleskey JM, Burgett SG, Craft L, Hale J, Hoffmann J, Hsiung HM, Kriauciunas A, MacKellar W, Rosteck Jr PR, Schoner B, Smith D, Tinsley FC, Zhang X-Y, Heiman M** 1995 The role of neuropeptide Y in the antiobesity action of the *obese* gene product. Nature 377:530–532
11. **Jacob RJ, Dziura J, Medwick MB, Leone P, Caprio S, During M, Shulman GI, Sherwin RS** 1997 The effect of leptin is enhanced by microinjection into the ventromedial hypothalamus. Diabetes 46:150–152
12. **Ollmann MM, Wilson BD, Yang YK, Kerns JA, Chen Y, Gantz I, Barsh GS** 1997 Antagonism of central melanocortin receptors. *in vitro* and *in vivo* by agouti-related protein. Science 278:135–138
13. **Schwartz MW, Seeley RJ, Woods SC, Weigle DS, Campfield LA, Burn P, Baskin DG** 1997 Leptin increases hypothalamic pro-opiomelanocortin mRNA expression in the rostral arcuate nucleus. Diabetes 46:2119–2123
14. **Satoh N, Ogawa Y, Katsuura G, Hayase M, Tsuhi T, Imagawa K, Yoshimasa Y, Nishi S, Hosoda K, Nakao K** 1997 The arcuate nucleus as a primary site of satiety effect of leptin in rats. Neurosci Lett 224:149–152
15. **Shimabukuro M, Koyama K, Chen G, Wang MY, Trieu F, Lee Y, Newgard CB, Unger RH** 1997 Direct antidiabetic effect of leptin through triglyceride depletion of tissues. Proc Natl Acad Sci USA 94:4637–4641
16. **Muller G, Ertl J, Gerl M, Preibisch G** 1997 Leptin impairs metabolic actions of insulin in isolated rat adipocytes. J Biol Chem 272:10585–10893
17. **Zachow RJ, Magoffin DA** 1997 Direct intraovarian effects of leptin: impairment of the synergistic action of insulin-like growth factor-I on follicle-stimulating hormone-dependent 17β-estradiol production by rat ovarian granulosa cells. Endocrinology 138:847–850
18. **Mikhail AA, Beck EX, Shafer A, Barut B, Gbur JS, Zupancic TJ, Schweitzer AC, Cioffi JA, Lacaud G, Ouyang B, Keller G, Snodgrass HR** 1997 Leptin stimulates fetal and adult erythroid and myeloid development. Blood 89:1507–1512
19. **Chen H, Chatlat O, Tartaglia LA, Woolf EA, Weng X, Ellis SJ, Lakey ND, Culpepper J, Moore KJ, Breitbart RE, Duyk GM, Tepper RI, Morgenstern JP** 1996 Evidence that the diabetes gene encodes the leptin receptor: identification of a mutation in the leptin receptor gene in *db/db* mice. Cell 84:491–495
20. **Cumin F, Baum HP, Levens N** 1996 Leptin is cleared from the circulation primarily by the kidney. Int J Obes Relat Metab Disord 20:1120–1126
21. **Banks WA, Kastin AJ, Huang W, Jaspan JB, Maness LM** 1996 Leptin enters the brain by a saturable system independent of insulin. Peptides 17:305–311
22. **Golden PL, Maccagnan TJ, Pardridge WM** 1997 Human blood-brain barrier leptin receptor. J Clin Invest 99:14–18
23. **Maffei M, Halaas J, Ravussin E, Pratley RE, Lee GH, Zhang Y, Fei H, Kim S, Lallone R, Ranganathan S, Kern PA, Friedman JM** 1995 Leptin levels in human and rodent: measurement of plasma leptin and *ob* RNA in obese and weight-reduced subjects. Nat Med 1:1155–1161
24. **Considine RV, Sinha MK, Heiman ML, Kriauciunas A, Stephens TW, Nyce MR, Ohannesian JP, Marco CC, McKee LJ, Bauer TL, Caro JF** 1996 Serum immunoreactive-leptin concentrations in normal-weight and obese humans. N Engl J Med 334:292–295
25. **Caro JF, Kolaczynski JW, Nyce MR, Ohannesian JP, Opentanova I, Goldman WH, Lynn RB, Zhang PL, Sinha MK, Considine RV** 1996 Decreased cerebrospinal-fluid/serum leptin ratio in obesity: a possible mechanism for leptin resistance Lancet 348:159–161
26. **Schwartz MW, Peskind E, Raskind M, Boyko EJ, Porte Jr D** 1996 Cerebrospinal fluid leptin levels: relationship to plasma levels and to adiposity in humans. Nat Med 2:589–593
27. **Brendel K, Meezan E, Carlson EC** 1974 Isolated brain microvessels: a purified, metabolically active preparation from bovine cerebral cortex. Science 185:953–955
28. **Brecher P, Tercyak A, Gavras H, Chobanian AV** 1978 Peptidyl dipeptidase in rabbit brain microvessels. Biochim Biophys Acta 526:537–546
29. **Sussman I, Carson MP, McCall AL, Schultz V, Ruderman NB, Tornheim K** 1988 Energy state of bovine cerebral microvessels: comparison of isolation methods. Microvasc Res 35:167–178
30. **Hausamen T-U, Helger R, Rick W, Gross W** 1967 Optimal conditions for the determination of serum alkaline phosphatase by a new kinetic method. Clin Chim Acta 15:241–245
31. **Simmons DM, Arriza JL, Swanson LW** 1989 A complete protocol for *in situ* hybridization of messenger RNA's in brain and other tissues with radiolabelled single stranded RNA probes. J Histotechnol 12:169–181
32. **Brady LS, Lynn AB, Herkenham M, Gottesfeld Z** 1994 Systemic interleukin-1 induces early and late patterns of c-*fos* mRNA expression in brain. J Neurosci 14:4951–4964
33. **Chan RK, Brown ER, Ericsson A, Kovacs KJ, Sawchenko PE** 1993 A comparison of two immediate-early genes, c-*fos* and NGFI-B, as markers for functional activation in stress-related neuroendocrine circuitry. J Neurosci 13:5126–5138
34. **Broadwell RD, Banks WA** 1993 Cell biological perspective for the transcytosis of peptides and proteins through the mammalian blood-brain fluid barriers. In: The Blood-Brain Barrier. Raven Press, New York, pp 165–199
35. **Schwartz MW, Baskin DG, Bukowski TR, Kuijper JL, Foster D, Lasser G, Prunkard DE, Porte Jr D, Woods SC, Seeley RJ, Weigle DS** 1996 Specificity of leptin action on elevated blood glucose levels and hypothalamic neuropeptide Y gene expression in *ob/ob* mice. Diabetes 45:531–535
36. **Wu-Peng XS, Chua SC Jr, Okada N, Liu SM, Nicolson M, Leibel RL** 1997 Phenotype of the obese Koletsky (f) rat due to Tyr763Stop mutation in the extracellular domain of the leptin receptor (Lepr): evidence for deficient plasma-to-CSF transport of leptin in both the Zucker and Koletsky obese rat. Diabetes 46:513–518
37. **Savioz A, Charnay Y, Huguenin C, Graviou C, Greggio B, Bouras C** 1997 Expression of leptin receptor mRNA (long form splice variant) in the human cerebellum. NeuroReport 8:3123–3126
38. **Mercer JG, Moar KM, Hoggard N** 1998 Localization of leptin receptor (ObR) messenger ribonucleic acid in the rodent hindbrain. Endocrinology 139:29–34