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### Research Article

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## Human Blood-Brain Barrier Leptin Receptor Binding and Endocytosis in Isolated Human Brain Microvessels

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### Abstract

The peripheral production of leptin by adipose tissue and its putative effect as a signal of satiety in the central nervous system suggest that leptin gains access to the regions of the brain regulating energy balance by crossing the brain capillary endothelium, which constitutes the blood-brain barrier in vivo. The present experiments characterize the binding and internalization of mouse recombinant leptin in isolated human brain capillaries, an in vitro model of the human blood-brain barrier. Incubation of <sup>125</sup>I-leptin with isolated human brain capillaries resulted in temperature-dependent binding: at 37°C, ~ 65% of radiolabeled leptin was bound per milligram of capillary protein. Two-thirds of the bound radioactivity was resistant to removal by acid wash, demonstrating endocytosis of <sup>125</sup>I-leptin into capillary cells. At 4°C, binding to isolated capillaries was reduced to ~ 23%/mg of protein, the majority of which was acid wash resistant. Binding of <sup>125</sup>I-leptin to brain capillary endothelial plasma membranes was saturable, described by a two-site binding model with a high-affinity dissociation constant of  $5.1 \pm 2.8$  nM and maximal binding capacity of  $0.34 \pm 0.16$  pmol/mg of membrane protein. Addition of porcine insulin or insulin-like growth factor at a final concentration of 100 nM had a negligible effect on leptin binding. These results provide evidence for a leptin receptor that mediates saturable, specific, temperature-dependent binding and endocytosis of leptin at the human blood-brain barrier. (*J. Clin. Invest.* 1997; 99:14–18.) Key words: obesity • OB protein • cerebrovascular • central nervous system • OB-R

### Introduction

Leptin, the product of the *ob* gene (1), is a 16-kD protein monomer that has been identified as a blood-borne factor that regulates appetite (2–4). Leptin is secreted by peripheral adi-

pocytes in vivo in response to stimuli that include food intake (5) and insulin administration (6), and appears to serve as a signal of satiety. Increases in *ob* gene mRNA expression have been observed in mice and rats after insulin administration (7), food intake (7), increased glucocorticoid levels, and decreased sympathetic neural activity (8); *ob* mRNA was decreased by fasting, but restored to normal levels upon refeeding (7, 9) or insulin administration (7). When genetically obese, leptin-deficient *ob/ob* mice received leptin either intraperitoneally or intracerebroventricularly, the mice demonstrated dose-dependent weight loss and reduction in food intake compared with control animals (2–4, 10, 11). Decreased serum glucose and insulin concentrations (10, 11) and increased metabolic rates (2) also have been observed in response to exogenous leptin administration in *ob/ob* mice. Unlike *ob/ob* mice, however, obese humans are not deficient in leptin. In fact, like *db/db* mice (5), they show increased *ob* gene expression and, consequently, elevated leptin concentrations in the blood as compared with normal-weight subjects. Evidence to date indicates that, in the absence of leptin deficiency, decreased access of leptin to its receptor site or decreased sensitivity at the receptor site may mediate obesity in humans (12–14).

Leptin mRNA has not been identified in brain tissue (15). This observation, combined with the peripheral production of leptin in adipose tissue and its likely target in the brain (1), points to the presence of a transport system that mediates delivery of circulating leptin to brain cells. To date, leptin binding sites have been identified in the mouse choroid plexus and leptomeninges (16–19). While these leptin receptors will allow the distribution of circulating leptin into cerebrospinal fluid (CSF)<sup>1</sup> within the ventricles and subarachnoid space, they allow minimal distribution of circulating leptin into brain interstitial fluid that bathes brain receptors. Direct transport of leptin from blood to brain interstitium is possible via receptor-mediated transcytosis through the brain capillary endothelium (20), which makes up the blood-brain barrier (BBB) in vivo. For example, the BBB insulin, insulin-like growth factor (IGF), and transferrin receptors mediate the BBB transport of insulin, IGFs, and transferrin (21–25). If a leptin receptor did exist at the brain capillary endothelium, this receptor would not have been detectable in previous studies, which used film autoradiography (16–19), a technique that lacks the resolution for detection of peptide binding to parenchymal capillaries.

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1. Abbreviations used in this paper: B<sub>max</sub>, binding maximum; BBB, blood-brain barrier; CSF, cerebrospinal fluid; IGF-1, human recombinant insulin-like growth factor-1; mg<sub>p</sub>, milligrams of protein; RHB, Ringer-Hepes buffer.

Therefore, the present studies assess the saturable binding and endocytosis of  $^{125}\text{I}$ -leptin by isolated human brain capillaries, an *in vitro* model of the human BBB.

## Methods

**Chemicals.** Mouse recombinant leptin was expressed in insect High Five cells using a baculovirus expression system. Leptin secreted into the growth medium was purified by ammonium sulfate precipitation, DEAE Sephacel anion exchange, and Sephacryl S100HR gel filtration chromatography. The isolated leptin decreased overnight food consumption when injected intraperitoneally into *ob/ob* mice at doses  $< 10 \mu\text{g}/\text{mouse}$ . Leptin ( $125 \mu\text{g}/\text{ml}$ ;  $7.8 \mu\text{M}$ ) was radiolabeled with iodine-125 according to the method of Bolton and Hunter (26). Briefly,  $0.31 \text{ nmol}$  ( $5 \mu\text{g}$ ) leptin and  $1 \text{ mCi}$  *N*-succinimidyl 3-(4-hydroxy, 5- $^{125}\text{I}$ iodophenyl)propionate (Bolton and Hunter reagent, specific activity  $2 \text{ mCi}/\text{nmol}$ ; Amersham, Arlington Heights, IL; final incubation concentration,  $10 \mu\text{M}$ ) were incubated with  $5 \mu\text{l}$   $0.5 \text{ M}$  borate buffer over ice for 1 h. The reaction was quenched by the addition of  $0.05 \text{ M}$  glycine.  $^{125}\text{I}$ -Labeled leptin was separated from unbound reagent over an exocellulose desalting column (GF5 KwikSep Excellulose plastic desalting column; Pierce Chemical Co., Rockford, IL) and washed with a buffer consisting of  $0.01 \text{ M}$   $\text{Na}_2\text{HPO}_4$ ,  $0.5 \text{ M}$   $\text{NaCl}$ , and  $0.2\%$  gelatin (from bovine bone; J.T. Baker Chemical Company, Phillipsburg, NJ).  $^{125}\text{I}$ -Leptin synthesized according to this procedure had a specific activity of  $161 \mu\text{Ci}/\text{nmol}$  ( $10 \mu\text{Ci}/\mu\text{g}$ ), was  $93\%$  pure as assessed by  $10\%$  TCA precipitation analysis, and was used without further purification. The purity of  $^{125}\text{I}$ -radiolabeled peptides as analyzed by  $10\%$  TCA precipitation analysis was well-correlated with HPLC and gel filtration analysis of purity in previous investigations in this laboratory (21, 27, 28). Recombinant human insulin-like growth factor-1 (IGF-1) was obtained from R & D Systems, Inc. (Minneapolis, MN). Crystalline porcine insulin was purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of analytical grade or better and were obtained from commercial sources.

**Isolated human brain capillaries and capillary membranes.** Human brains were obtained from one male and one female (male: 65 yr, weight  $44.3 \text{ kg}$ , height  $135 \text{ cm}$ ; female: 64 yr, weight  $52.8 \text{ kg}$ , height  $169 \text{ cm}$ ) at 20–24 h postmortem from the UCLA Division of Neuropathology (Dr. Harry V. Vinters). Capillaries were isolated by mechanical homogenization as described previously (27). The final capillary pellet was suspended in a cryopreservation buffer ( $0.02 \text{ M}$  Tris,  $0.25 \text{ M}$  sucrose, and  $2 \text{ mM}$  dithiothreitol) and stored at  $-70^\circ\text{C}$  under liquid nitrogen. Human brain capillary membranes were isolated from the capillaries of the female donor on the same day as the capillary isolation according to the method of Lidinsky and Drewes (29). Both capillaries and plasma membranes isolated by these techniques maintain biochemical characteristics of the intact BBB, as characterized previously in this laboratory for the human BBB insulin receptor (21).

**Uptake of  $^{125}\text{I}$ -leptin into isolated human brain capillaries.** Isolated human brain microvessels from the male donor were thawed at room temperature and resuspended in Ringer-Hepes buffer (RHB;  $141 \text{ mM}$   $\text{NaCl}$ ,  $4 \text{ mM}$   $\text{KCl}$ ,  $2.8 \text{ mM}$   $\text{CaCl}_2$ ,  $10 \text{ mM}$  Hepes,  $\text{pH}$  7.4) with  $0.1\%$  HSA. The microvessels were incubated with  $^{125}\text{I}$ -leptin at a final concentration of  $2.2 \mu\text{Ci}/\text{ml}$  ( $13.7 \text{ nM}$ ) for up to 120 min. Incubations were performed in triplicate in  $0.45 \text{ ml}$  of RHB with  $0.1\%$  HSA in plastic  $12 \times 75 \text{ mm}$  test tubes. At the end of the incubation,  $400 \mu\text{l}$  was transferred to small Beckman microfuge tubes and centrifuged at  $10,000 \text{ g}$  for 45 s. The supernatant was removed and reserved for TCA precipitation analysis. The microvessel pellet was placed in  $0.5 \text{ ml}$   $1 \text{ N}$   $\text{NaOH}$  for measurement of iodine-125 by gamma counting (Gamma 5500 gamma counter; Beckman Instruments, Inc., Fullerton, CA), then heated at  $60^\circ\text{C}$  for 30 min to solubilize the capillary protein. Protein concentration was determined using the bicinchoninic acid protein assay (Pierce Chemical Co.).

The internalization of  $^{125}\text{I}$ -leptin was determined with an acid wash technique as described previously (30). This technique, as used

with  $^{125}\text{I}$ insulin, removed completely the cell surface-bound radioactivity without damaging cell integrity.  $^{125}\text{I}$ -Leptin was incubated with isolated microvessels as described above. At the end of the incubation, the microvessels were centrifuged and incubated in  $450 \mu\text{l}$  ice-cold acid wash solution ( $0.12 \text{ M}$   $\text{NaCl}$ ,  $0.02 \text{ M}$  sodium barbital,  $0.028 \text{ M}$  sodium acetate,  $\text{pH}$  3) over ice for 6 min to remove  $^{125}\text{I}$ -leptin bound to the outer capillary wall.  $400 \mu\text{l}$  was pipetted to a small Beckman microfuge tube and centrifuged at  $10,000 \text{ g}$  for 45 s. The capillary pellet was analyzed for iodine-125 and protein content as described above.

**Leptin binding to plasma membranes.** Because of the rapid internalization of leptin into intact human brain microvessels, brain capillary endothelial plasma membranes were used to determine the affinity of leptin binding at the human BBB. Human plasma membranes ( $115 \mu\text{g}$  of membrane protein per tube) were incubated with  $0.02 \mu\text{Ci}$  ( $0.625 \text{ nM}$ )  $^{125}\text{I}$ -leptin and varying concentrations of unlabeled leptin (from  $0.625$  to  $200 \text{ nM}$ ) in RHB with  $0.1\%$  HSA overnight at  $4^\circ\text{C}$ . Alternatively, to determine the specificity of leptin binding, membranes were incubated with  $0.02 \mu\text{Ci}$  of  $^{125}\text{I}$ -leptin and porcine insulin or IGF-1 (final concentration  $100 \text{ nM}$ ). All incubations were performed in triplicate. The next morning, the contents of each incubation tube were combined with  $4 \text{ ml}$  ice-cold PBS ( $0.01 \text{ M}$ ,  $\text{pH}$  7.4) and filtered immediately by rapid vacuum filtration through preconditioned Whatman GF-D borosilicate glass microfiber filters ( $0.68 \text{ mm}$  thickness, particle retention up to  $2.7 \mu\text{m}$ ; Whatman LabSales, Hillsboro, OR). Filters were preconditioned by soaking for 1 h in ice-cold PBS with  $5\%$  HSA to minimize nonspecific binding to the filter. After the initial filtration, the incubation tube was washed once with  $4 \text{ ml}$  ice-cold PBS, and this solution was used to wash the filter. Radioactivity remaining on filters was determined by gamma counting.

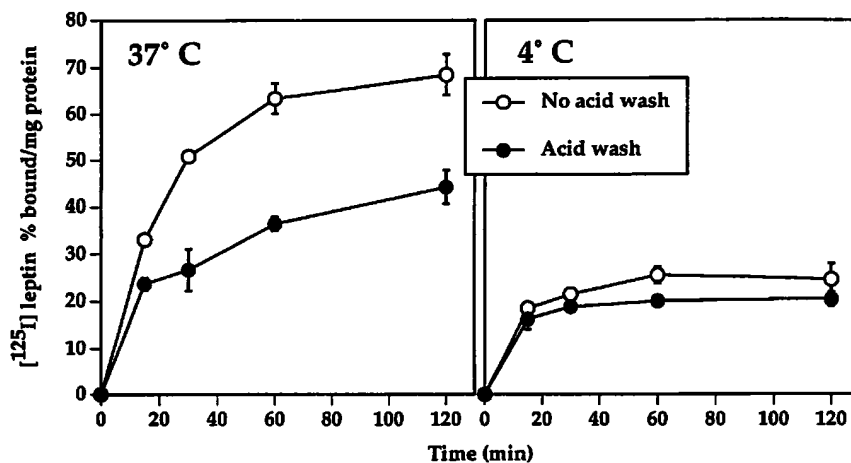
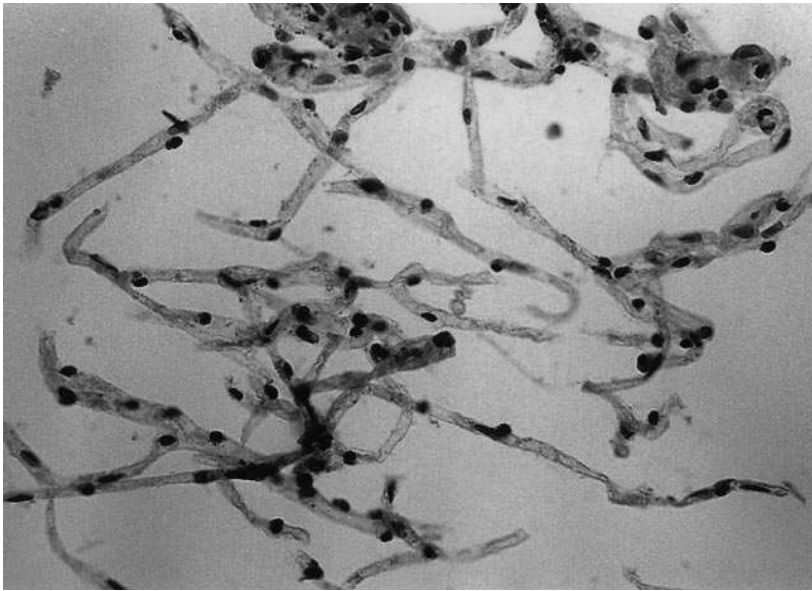
**Data analysis.** All measurements were performed in triplicate. Parameters governing the binding of  $^{125}\text{I}$ -leptin to human capillary plasma membranes were estimated by nonlinear least-squares regression using the software program BMDP (BMDP Statistical Software, Los Angeles, CA), with initial parameter estimates determined by graphical analysis according to the Scatchard method (31).

## Results

After cryopreservation, human brain microvessels were intact (Fig. 1, *top*). At  $37^\circ\text{C}$  (Fig. 1, *bottom left*), leptin binding to isolated capillaries increased over the course of the 120-min incubation, with maximum binding at 120 min of  $68.6 \pm 4.3\%$  (mean  $\pm$  SE,  $n = 3$ ) binding of  $^{125}\text{I}$ -leptin per milligram of capillary protein ( $\text{mg}_\text{p}$ ). The majority of  $^{125}\text{I}$ -leptin binding was resistant to removal by acid wash, indicating incorporation into the capillary membrane or endocytosis at physiologic temperature. As with total binding, acid-resistant binding increased over the incubation time, with  $44.3 \pm 3.6\%$  bound per  $\text{mg}_\text{p}$  at 120 min. The temperature dependence of leptin binding and internalization was evident (Fig. 1, *bottom right*). Over the same incubation period at  $4^\circ\text{C}$ , binding of leptin increased only marginally between 30 and 120 min. At 120 min, total binding was  $24.5 \pm 3.3\%$  per  $\text{mg}_\text{p}$ . This binding was almost entirely acid wash resistant, with  $20.4 \pm 1.5\%$  bound per  $\text{mg}_\text{p}$  at 120 min.

A small degree of metabolism or degradation of  $^{125}\text{I}$ -leptin to non-acid-precipitable products by the isolated capillaries was noted. The TCA-soluble medium radioactivity increased 6 and  $0\%$ , respectively, at  $37$  and  $4^\circ\text{C}$  after a 120-min incubation.

Binding of  $^{125}\text{I}$ -leptin to isolated plasma membranes from the human BBB was saturable in the presence of increasing concentrations of nonradiolabeled leptin (Fig. 2, *inset*). Scatchard analysis of the binding data (Fig. 2, *main graph*) indicated the presence of two independent leptin binding sites. The parameters governing the binding of leptin at the BBB,



**Figure 1.** (Top) Light photomicrograph of capillaries isolated from human autopsy brain (stained with 0.5% toluidine blue). Capillaries were intact after thawing. (Bottom) At 37°C (left), total binding of  $^{125}\text{I}$ -leptin to isolated capillaries (open circles) increased continuously over the incubation period, as did acid-resistant binding (filled circles). At the end of the incubation at 37°C, acid-resistant binding represented approximately two-thirds of total  $^{125}\text{I}$ -leptin binding, indicating endocytosis of leptin into isolated human brain capillaries. At 4°C (right), acid-resistant and non-acid-resistant binding were nearly superimposable. Each data point represents the mean ( $\pm$ SE) of three observations.

estimated by nonlinear regression analysis, indicated a high-affinity binding site with a dissociation constant ( $K_d$ ) of  $5.1 \pm 2.8$  nM (estimate  $\pm$ SD) and binding maximum ( $B_{\text{max}}$ ) of  $0.34 \pm 0.16$  pmol/mg<sub>p</sub>. The second, low-affinity and high-capacity class of binding sites had an estimated dissociation constant ( $K_d$ ) of  $743 \pm 226$  nM and binding maximum ( $B_{\text{max}}$ ) of  $26 \pm 5$  pmol/mg<sub>p</sub>. Specific binding of  $^{125}\text{I}$ -leptin was not altered by either insulin or IGF-1. Specific binding of leptin was  $5.7 \pm 0.01$ ,  $6.2 \pm 0.4$ , and  $4.0 \pm 0.7\%$  per mg<sub>p</sub> in the presence of buffer, 100 nM insulin, and 100 nM IGF-1, respectively.

## Discussion

The results of the present experiments demonstrated that leptin was bound and internalized by isolated human brain capillaries. Both binding and endocytosis were temperature dependent (Fig. 1). Endocytosis was monitored in these experiments with isolated microvessels with the acid wash technique (30). The movement of peptide into a postmembrane space that is resistant to acid wash is not abolished by cold temperature (32) and is independent of ATP (33). Since incubation at 4°C suppressed receptor binding to a greater extent than peptide movement into the acid wash-resistant compartment (Fig. 1),

we measured the saturation of receptor binding in isolated plasma membranes (Fig. 2), where endocytosis is absent. Binding to human brain microvessel membranes was specific and was inhibited competitively by increasing concentrations of leptin, but was not altered by the presence of insulin or IGF-1 (Fig. 2). The behavior of leptin in this in vitro model system was similar to that previously documented for insulin, IGFs, and transferrin in the same human BBB model (Table I).

These studies indicate that a leptin receptor functions at the brain capillary endothelium, which comprises the BBB in vivo and which segregates blood from brain interstitial fluid. Therefore, the BBB leptin receptor functions in parallel with the previously reported leptin receptor at the choroid plexus epithelium (16–19), which comprises the blood-CSF barrier in vivo and segregates blood from CSF. The choroid plexus leptin receptor may mediate the uptake of circulating leptin into CSF. Leptin is present in the CSF of humans and the CSF/plasma ratio for leptin is inversely related to the plasma leptin concentration, suggesting the presence of a saturable transport system for leptin at the choroid plexus (14). Similarly, recent studies provide evidence for saturable transport of  $^{125}\text{I}$ -leptin into brain in vivo in the mouse (36). However, Banks et al. (36) demonstrated that  $^{125}\text{I}$ -leptin is degraded rapidly after intrave-

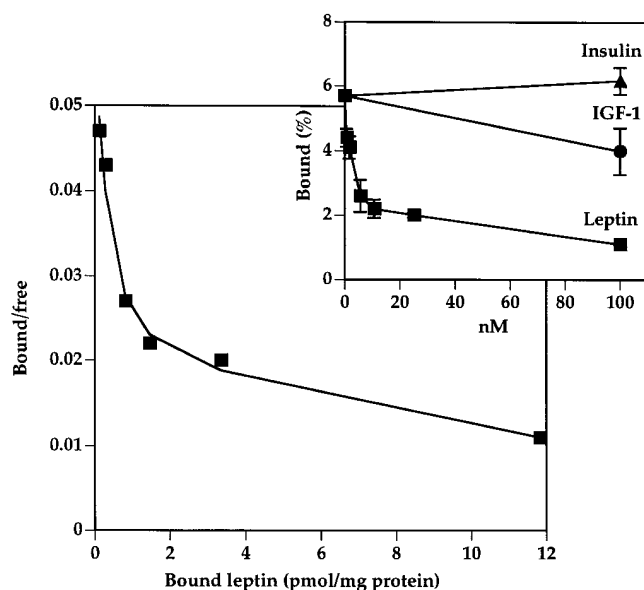


Figure 2. (Inset)  $^{125}\text{I}$ -Leptin binding to human brain capillary plasma membranes was saturable [data points represent the mean ( $\pm$ SE) of three observations]. Specificity of leptin binding was reflected by the minimal influence of 100 nM porcine insulin or human IGF-1. (Main graph) Scatchard analysis of the binding data showed the presence of two independent classes of binding sites. Nonlinear least-squares regression analysis was used to estimate the dissociation constant ( $K_d^1$ ) and binding capacity ( $B_{\max}^1$ ) of the high-affinity site as  $5.1 \pm 2.8$  nM and  $0.34 \pm 0.16$  pmol/mg<sub>p</sub>, respectively (estimate  $\pm$ SD). For the low-affinity, high-capacity sites,  $K_d^2$  was estimated as  $743 \pm 226$  nM, and  $B_{\max}^2 = 26 \pm 5$  pmol/mg<sub>p</sub>. The solid line represents the binding curve predicted by fitting a two-site binding model to the actual data (filled boxes).

nous injection in mice: 20 min after administration,  $\sim 72\%$  of radioactivity in serum and 60% of radioactivity in the brain represented intact peptide. Similar results were observed in this laboratory after intravenous administration to rats, with 70% (serum) and 50% (brain) intact radiolabeled leptin present 30 min after administration (Golden, P.L., and W.M. Pardridge, unpublished observations). The interpretation of data pertaining to the transport of metabolically labile peptide into brain after intravenous injection of the peptide is problematical owing to the rapid conversion of radiolabeled peptide into low molecular weight radioactive metabolites, which also may be transported into brain. To demonstrate saturable transport of [ $^{125}\text{I}$ ]insulin into brain in vivo, it was necessary to perform transport studies after intraarterial infusion in suck-

Table I. Peptide Binding Parameters for Isolated Human Brain Capillary Receptors

| Peptide (reference) | $K_d$         | $B_{\max}$           |
|---------------------|---------------|----------------------|
|                     | nM            | pmol/mg <sub>p</sub> |
| Leptin (this study) | $5.1 \pm 2.8$ | $0.34 \pm 0.16$      |
| IGF-2 (34)          | $1.1 \pm 0.1$ | $0.21 \pm 0.01$      |
| IGF-1 (34)          | $2.1 \pm 0.4$ | $0.17 \pm 0.02$      |
| Insulin (21)        | $1.2 \pm 0.5$ | $0.17 \pm 0.08$      |
| Transferrin (35)    | $5.6 \pm 1.4$ | $0.10 \pm 0.02$      |

ling animals, in which peripheral insulinases are downregulated (22).

The presence of specific receptors for leptin at the brain capillary endothelium, at the choroid plexus endothelium, and on brain cells (16, 37, 38) is consistent with recent studies showing that multiple mRNA splice variants exist for the leptin receptor in the brain (38, 39). These receptors may be differentially regulated. For example, leptin does not inhibit food intake in the *db/db* mouse, yet there is no apparent functional impairment in the choroid plexus leptin receptor in this species (19). Therefore, it is important in future studies to determine the activity of the BBB leptin receptor in obesity models, since the leptin receptor at the BBB may play a principal role in regulating the distribution of circulating leptin to brain cells.

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## References

- Zhang, Y., R. Proenca, M. Maffei, M. Barone, L. Leopold, and J.M. Friedman. 1994. Positional cloning of the mouse *obese* gene and its human homologue. *Nature (Lond.)* 372:425-431.
- Pelleymounter, M.A., M.J. Cullen, M.B. Baker, R. Hecht, D. Winters, T. Boone, and F. Collins. 1995. Effects of the *obese* gene product on body weight regulation in *ob/ob* mice. *Science (Wash. DC)* 269:540-543.
- Campfield, C.A., F.J. Smith, Y. Guisez, R. Devas, and P. Burn. 1995. Recombinant mouse OB protein: evidence for a peripheral signal linking adiposity and central neural networks. *Science (Wash. DC)* 269:546-549.
- Halaas, J.L., K.S. Gajiwala, M. Maffei, S.L. Cohen, B.T. Chait, D. Rabinowitz, R.L. Lallone, S.K. Burley, and J.M. Friedman. 1995. Weight-reducing effects of the plasma protein encoded by the *obese* gene. *Science (Wash. DC)* 269:543-546.
- Frederich, R.C., B. Löllman, A. Hamann, A. Napolitano-Rosen, B.B. Kahn, B.B. Lowell, and J.S. Flier. 1995. Expression of *ob* mRNA and its encoded protein in rodents. Impact of nutrition and obesity. *J. Clin. Invest.* 96:1658-1663.
- Leroy, P., S. Dessolin, P. Villageois, B.C. Moon, J.M. Friedman, G. Ailhaud, and C. Dani. 1996. Expression of *ob* gene in adipose cells: regulation by insulin. *J. Biol. Chem.* 271:2365-2368.
- Saladin, R., P. De Vos, M. Guerre-Millo, A. Leturque, J. Girard, B. Staels, and J. Auwerx. 1995. Transient increase in *obese* gene expression after food intake or insulin administration. *Nature (Lond.)* 377:527-529.
- Slieker, L.J., K.W. Sloop, P.L. Surface, A. Kriaucinas, F. LaQuier, J. Manetta, J. Bue-Vallesky, and T.W. Stephens. 1996. Regulation of expression of *ob* mRNA and protein by glucocorticoids and cAMP. *J. Biol. Chem.* 271:5301-5304.
- Becker, D.J., L.N. Ongemba, V. Brichard, J.-C. Henquin, and S.M. Brichard. 1995. Diet- and diabetes-induced changes in rat adipose tissue. *FEBS Lett.* 371:324-328.
- Weigle, D.S., T.R. Bukowski, D.C. Foster, S. Holderman, J.M. Kramer, G. Lasser, C.E. Lofton-Day, D.E. Prunkard, C. Raymond, and J.L. Kuijper. 1995. Recombinant *ob* protein reduces feeding and body weight in the *ob/ob* mouse. *J. Clin. Invest.* 96:2065-2070.
- Schwartz, M.W., D.G. Baskin, T.R. Bukowski, J.L. Kuijper, D. Foster, G. Lasser, D.E. Prunkard, D. Porte, Jr., S.C. Woods, R.J. Seeley, and D.S. Weigle. 1996. Specificity of leptin action on elevated blood glucose levels and hypothalamic neuropeptide Y gene expression in *ob/ob* mice. *Diabetes* 45:531-535.
- McGregor, G.P., J.F. Desaga, K. Ehlenz, A. Fischer, F. Heese, A. Hegele, C. Lämmer, C. Peiser, and R.E. Lang. 1996. Radioimmunological measurement of leptin in plasma of obese and diabetic human subjects. *Endocrinology* 137:1501-1504.
- Considine, R.V., M.K. Sinha, M.L. Heiman, A. Kriaucinas, T.W. Stephens, M.R. Nyce, J.P. Ohannesian, C.C. Marco, L.J. McKee, T.L. Bauer, and J.F. Caro. 1996. Serum immunoreactive-leptin concentrations in normal-weight and obese humans. *N. Engl. J. Med.* 334:292-295.
- Schwartz, M.W., E. Peskind, M. Raskind, E.J. Boyko, and D. Porte, Jr. 1996. Cerebrospinal fluid leptin levels: relationship to plasma levels and to adi-

posity in humans. *Nat. Med.* 2:589–593.

15. Masuzaki, H., Y. Ogawa, N. Isse, N. Satoh, T. Okazaki, M. Shigemoto, K. Mori, N. Tamura, K. Hosoda, Y. Yoshimasa, et al. 1995. Human obese gene expression: adipocyte-specific expression and regional differences in the adipose tissue. *Diabetes*. 44:855–858.
16. Tartaglia, L., A.M. Dembski, X. Weng, N. Deng, J. Culpepper, R. Devos, G.J. Richards, L.A. Campfield, F.T. Clark, J. Deeds, et al. 1995. Identification and expression cloning of a leptin receptor, OB-R. *Cell*. 83:1263–1271.
17. Lynn, R.B., G.-Y. Cao, R.V. Considine, T.M. Hyde, and J.F. Caro. 1996. Autoradiographic localization of leptin binding in the choroid plexus of *ob/ob* and *db/db* mice. *Biochem. Biophys. Res. Commun.* 219:884–889.
18. Malik, K.F., and W.S. Young III. 1996. Localization of binding sites in the central nervous system for leptin (OB protein) in normal, obese (*ob/ob*), and diabetic (*db/db*) C57BL/6J mice. *Endocrinology*. 137:1497–1500.
19. Devos, R., J.G. Richards, L.A. Campfield, L.A. Tartaglia, Y. Guisez, J. Van der Heyden, J. Tavernier, G. Plaetinck, and P. Burn. 1996. OB protein binds specifically to the choroid plexus of mice and rats. *Proc. Natl. Acad. Sci. USA*. 93:5668–5673.
20. Pardridge, W.M. 1986. Receptor-mediated peptide transport through the blood-brain barrier. *Endocr. Rev.* 7:314–330.
21. Pardridge, W.M., J. Eisenberg, and J. Yang. 1985. Human blood-brain barrier insulin receptor. *J. Neurochem.* 44:1771–1778.
22. Duffy, K.R., and W.M. Pardridge. 1987. Blood-brain barrier transcytosis of insulin in developing rabbits. *Brain Res.* 420:32–38.
23. Reinhardt, R.R., and C.A. Bondy. 1994. Insulin-like growth factors cross the blood-brain barrier. *Endocrinology*. 135:1753–1761.
24. Fishman, J.B., J.B. Rubin, J.V. Handrahan, J.V. Connor, and R.E. Fine. 1987. Receptor-mediated transcytosis of transferrin across the blood-brain barrier. *J. Neurosci. Res.* 18:299–304.
25. Bickel, U., Y.-S. Kang, T. Yoshikawa, and W.M. Pardridge. 1994. In vivo demonstration of subcellular localization of anti-transferrin receptor monoclonal antibody-colloidal gold conjugate within brain capillary endothelium. *J. Histochem. Cytochem.* 42:1493–1497.
26. Bolton, A.E., and W.M. Hunter. 1973. The labelling of proteins to high specific radioactivities by conjugation to a  $^{125}\text{I}$ -containing acylating agent. *Biochem. J.* 133:529–539.
27. Pardridge, W.M., J. Eisenberg, and T. Yamada. 1985. Rapid sequestration and degradation of somatostatin analogues by isolated brain microvessels. *J. Neurochem.* 44:1178–1184.
28. Frank, H.J.L., W.M. Pardridge, W.L. Morris, R.G. Rosenfeld, and T.B. Choi. 1986. Binding and internalization of insulin and insulin-like growth factors by isolated brain microvessels. *Diabetes*. 35:654–661.
29. Lidinsky, W.A., and L.R. Drewes. 1983. Characterization of the blood-brain barrier: protein composition of the capillary endothelial cell membrane. *J. Neurochem.* 41:1341–1348.
30. Olefsky, J.M., and M. Kao. 1982. Surface binding and rates of internalization of  $^{125}\text{I}$ -insulin in adipocytes and IM-9 lymphocytes. *J. Biol. Chem.* 257:8667–8673.
31. Feldman, H.A. 1972. Mathematical theory of complex ligand-binding systems of equilibrium: some methods for parameter fitting. *Anal. Biochem.* 48:317–338.
32. Carpentier, J.L., M. Fehlmann, E. van Obberghen, P. Gorden, and L. Orci. 1985. Redistribution of  $^{125}\text{I}$ -insulin on the surface of rat hepatocytes as a function of dissociation time. *Diabetes*. 34:1002–1007.
33. Moore, M.S., D.T. Mahaffey, F.M. Brodsky, and R.G.W. Anderson. 1987. Assembly of clathrin-coated pits onto purified plasma membranes. *Science (Wash. DC)*. 236:558–563.
34. Duffy, K.R., W.M. Pardridge, and R.G. Rosenfeld. 1988. Human blood-brain barrier insulin-like growth factor receptor. *Metab. Clin. Exp.* 37:136–140.
35. Pardridge, W.M., J. Eisenberg, and J. Yang. 1987. Human blood-brain barrier transferrin receptor. *Metab. Clin. Exp.* 36:892–895.
36. Banks, W.A., A.J. Kastin, W. Huan, J.B. Jaskan, and L.M. Maness. 1996. Leptin enters the brain by a saturable system independent of insulin. *Pep-tides*. 17:305–311.
37. Stephens, T.W., M. Basinski, P.K. Bristow, J.M. Bue-Valleskey, S.G. Burgett, L. Craft, J. Hale, J. Hoffmann, H.M. Hsiung, A. Kriauciunas, et al. 1995. The role of neuropeptide Y in the antiobesity action of the *obese* gene product. *Nature (Lond.)*. 377:530–532.
38. Chen, H., O. Charlat, L.A. Tartaglia, E.A. Woolf, X. Weng, S.J. Ellis, N.D. Lakey, J. Culpepper, K.J. Moore, R.E. Breitbart, et al. 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.
39. Lee, G.-H., R. Proenca, J.M. Montez, K.M. Carroll, J.G. Darvishzadeh, J.I. Lee, and J.M. Friedman. 1996. Abnormal splicing of the leptin receptor in diabetic mice. *Nature (Lond.)*. 379:632–635.