Partial saturation and regional variation in the blood-to-brain transport of leptin in normal weight mice

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Banks, William A., Cecilia M. Clever, and Catherine L. Farrell. Partial saturation and regional variation in the blood-to-brain transport of leptin in normal weight mice. Am J Physiol Endocrinol Metab 278: E1158–E1165, 2000.—Impaired blood-brain barrier transport of leptin into the arcuate nucleus has been suggested to underlie obesity in humans and outbred aging mice. Here, we used a brain perfusion method in mice to measure transport rates and kinetic parameters for leptin at vascular concentrations between 0.15 and 130 ng/ml. Transport into whole brain was partially saturated at all concentrations, not only those seen in obesity. Leptin entered all regions of the brain, not only the hypothalamus, with entry and saturation rates differing among the brain regions. The value of the Michaelis-Menten constant of the transporter approximates normal serum levels and the maximum velocity value varies significantly among brain regions. These results suggest an important role for low serum levels signaling starvation status to the brain and show that the levels of leptin seen in obesity greatly saturate the transporter. Differences in regional uptake and saturation provide a mechanism by which leptin can control events mediated at the arcuate nucleus and other regions of the central nervous system with different regional thresholds for optimal function.

LEPTIN IS A 16-KDA PROTEIN secreted by adipocytes (46) that crosses the blood-brain barrier (BBB) by a saturable transport system (4) to act within the brain to control body weight (10, 26, 32). In humans, obesity is accompanied by elevated leptin levels, suggesting that obesity is caused by a resistance to leptin (14, 23, 35). That this resistance is caused by an impaired transport of leptin across the BBB is suggested by the findings that the increases in cerebrospinal fluid (CSF) levels become attenuated when serum levels reach the obese range (11, 35), that CSF/serum ratios for leptin decrease with increasing serum levels of leptin (11, 31, 35), that obese rodents that are resistant to leptin given peripherally can still respond to leptin given directly into the brain (25, 43), and that the concentration of peripherally administered leptin that inhibits the leptin transporter produces serum levels similar to those seen with obesity (4). This impaired transport has been assumed to be caused by a saturation of the transporter. However, a recent study has shown that obese mice have a defect in leptin transport not explained by partial saturation (3).

Most work has concentrated on the ability of leptin to regulate body weight by acting on receptors in the arcuate nucleus. However, leptin receptors are located in many extrahypothalamic areas of the brain, including the hippocampus, olfactory bulbs, thalamus, cerebellum, and cerebral cortex (15, 18, 19, 24, 27, 38). Identification of binding sites in both brain endothelium (7, 22) and the choroid plexus (16, 30, 41) and autoradiographic evidence showing that leptin enters many brain regions (4) demonstrate that serum leptin is able to reach those receptors. Leptin has effects on thermogenesis (28), appetite (33), lipid metabolism (33), angiogenesis (8), immune function (29), reproduction (12), sexual behavior (44), insulin sensitivity (13), serum glucose levels (34), and neurohormone levels (21, 36). It has been suggested that subpopulations of neurons within the arcuate nucleus may mediate these different effects of leptin (20); however, it is also possible that these various effects are mediated through neurons located in different brain regions.

Leptin levels increase linearly with increasing adiposity, so that an obese human or mouse with a serum leptin level of 30 ng/ml will have about three times the amount of fat as a normal-weight animal with a level of 10 ng/ml (11, 14, 33). Levels of 1 ng/ml are seen only in very thin mice and in humans with diseases such as anorexia nervosa (31, 33). Therefore, the effect of serum leptin levels of 1–30 ng/ml on the leptin transporter is of great interest. We used a brain perfusion method that allowed us to vary the vascular concentration of leptin from 0.15 to 130 ng/ml, bracketing the range of leptin values seen in thin and obese animals. We used the brain perfusion method in normal-weight mice to determine the concentration at which serum leptin levels would begin to saturate the transporter, whether leptin transport varies among brain regions, and what the kinetic parameters of leptin transport are for whole brain and brain regions.

METHODS

Iodination

Recombinant murine leptin was radiiodinated by incubation of 10 µg leptin (Amgen, Thousand Oaks, CA) with 2 mCi...
and Enzymobead reagent (Bio-Rad, Richmond, CA) for 24 h at 4°C. Radioactively labeled leptin (I-Lep) was purified on a Sephadex G10 column. The purified I-Lep with a specific activity of ~103 Ci/g was collected and stored at 4°C until used.

**Brain Perfusion**

All studies were approved by the local Animal Care and Use Committee and were performed in a facility approved by the Association for the Assessment and Accreditation of Laboratory Animal Care. Adult male ICR mice (n = 25; Charles River Laboratories, Wilmington, MA), 20–25 g, were anesthetized intraperitoneally with urethane (4.0 g/kg). I-Lep was diluted in Zlokovic’s (47) buffer (7.19 g/l NaCl, 0.3 g/l KCl, 0.28 g/l CaCl₂, 2.1 g/l NaHCO₃, 0.16 g/l KH₂PO₄, 0.17 g/l anhydrous MgCl₂, 0.99 g/l D-glucose, and 10 g/l BSA added on the day of perfusion) to varying concentrations between 0.15 and 130 ng/ml. The thorax was opened, the heart was exposed, both jugulars were severed, and the descending thoracic aorta was clamped. A 26-gauge butterfly needle was inserted into the left ventricle of the heart, and the buffer containing the various concentrations of I-Lep was infused at a rate of 2 ml/min for 5 min. This rate of perfusion quickly fills the brain’s vascular space without disrupting the BBB (37). An injection check of 10 µl of the I-Lep buffer solution was taken before and after perfusion, so exact concentration of I-Lep in ng/ml could be calculated. After perfusion, the needle was removed and the mouse was decapitated. The brain was removed and dissected into 10 regions (frontal, parietal, and occipital cortices, hippocampus, hypothalamus, thalamus, striatum, midbrain, pons-medulla, and cerebellum). Each part was weighed before its level of radioactivity was determined in a gamma counter.

**Brain region/perfusion (BRP) ratios** were calculated by dividing the counts per minute per region of brain by the weight of that region in grams and by the counts per minute in a microliter of perfusion fluid to yield units of microliters per gram. Values for whole brain were calculated after adding the counts per minute and weights for all regions.

**Inhibition of BBB I-Lep Transporter**

To determine the vascular concentration of leptin that is associated with inhibition of the BBB transporter, whole brain/perfusion or BRP ratios were plotted against the log of the concentration of leptin in the perfusion fluid. To determine whether there were differences among brain regions of the concentration at which leptin inhibited the BBB transporter, the slope of this relation with its standard error was calculated with Prism 3.0 (GraphPad, San Diego, CA) and compared by analysis of variance followed by Duncan’s range test. The y-intercept of this relation with its standard error was used to calculate the amount of I-Lep that had entered the brain with a perfusion concentration of 1 ng/ml. Similarly, brain concentrations were determined from the intercept at the perfusion concentrations of 10 and 30 ng/ml by subtracting 1.0 and 1.477 from the log concentration of leptin in the perfusion fluid.

**Kinetics of I-Lep Transporter**

The Michaelis-Menten constant (Kₘ) and maximum velocity (Vₘₐₓ) values were calculated for the BBB leptin transporter in whole brain and in brain regions. The BRP was multiplied by the respective concentration of I-Lep in the perfusion and plotted against the perfusion concentration. The slope of the nonsaturable portion of this relation was calculated and subtracted from total uptake by brain to yield a curve plotting saturable uptake vs. perfusion concentration. The Kₘ and Vₘₐₓ were calculated with the use of nonlinear hyperbola models (Prism 3.0) with statistical comparison of one- and two-site models. The Vₘₐₓ was expressed in units of picograms of I-Lep per gram that had been taken up during the 5-min perfusion period and Kₘ in units of nanograms per milliliter.

**RESULTS**

Figure 1 shows that a statistically significant (r = 0.920, P < 0.001, n = 25) inverse relation existed between whole brain/perfusion ratios and the log concentration of I-Lep in the perfusion [y = (–36.95)x + 88.87]. This relation was linear throughout the I-Lep concentration, showing that the leptin BBB transporter is partially inhibited at all vascular concentrations.

Analysis for individual brain regions showed that in all cases, a statistically significant relation (P < 0.001) existed between BRP ratios and the log concentration of I-Lep in the perfusion. Figure 2A shows the results for the hypothalamus, which had the steepest slope, and the frontal cortex, which had the most shallow slope. Figure 2B shows the values for the (–) slopes of these relations with their standard errors. A steeper slope indicates that the BBB leptin transporter of the brain region is more readily saturated by I-Lep; that is, a given level of leptin in the perfusion produced a higher degree of saturation of the transporter. ANOVA showed differences among the regions [F(10, 256) = 5.37, P < 0.001]. Duncan’s range test showed that the frontal cortex was less readily saturated (P < 0.05) and the hypothalamus more readily saturated (P < 0.001) than whole brain. Table 1 summarizes other differences among the brain regions.

Figure 3A shows the concentration of I-Lep (in µ/l/g) for whole brain and the various brain regions when the perfusion concentration was 1 ng/ml, a value which would be seen only in very thin mice and humans (31, 33). Because at this concentration a microliter contains 1 pg of I-Lep, the units can also be expressed in picograms of I-Lep per gram of brain region. At this concentration, brain regions contained differing amounts of leptin [F(10, 256) = 8.36, P < 0.001]. For
example, the frontal cortex contained less ($P < 0.01$) and the hippocampus ($P < 0.01$) and the hypothalamus ($P < 0.001$) more I-Lep than whole brain. Table 2 summarizes other differences among the brain regions.

Figure 3B shows the 1 ng/ml values expressed as a percentage of the whole brain values as well as percent values for the perfusion concentrations of 10 and 30 ng/ml, concentrations typically seen in normal and obese mice and humans (11, 14, 31, 33) after correction for vascular space.

Figure 4A shows an example of the calculation of specific and nonspecific uptake with results for whole brain used. Figure 4B shows the specific transport for whole brain derived from the calculations of Fig. 4A with a $V_{\text{max}}$ of 1.42 ± 0.15 ng/g and a $K_m$ of 15.6 ± 5.2 ng/ml. The results for specific transport for whole brain and for every brain region fitted a one-site model better than a two-site model. Figure 5 shows the $V_{\text{max}}$ (A) and $K_m$ (B) for brain regions. ANOVA showed differences among brain regions for $V_{\text{max}}$ [$F(10, 256) = 5.22, P < 0.001$] but not for $K_m$. Duncan's multiple range test showed that the $V_{\text{max}}$ for whole brain was significantly less than that for hippocampus ($P < 0.001$), pons-medulla ($P < 0.01$), and occipital cortex ($P < 0.05$). Other differences among brain regions for $V_{\text{max}}$ are shown in Table 3.

**DISCUSSION**

These studies resulted in five major findings: 1) the leptin transporter for whole brain is partially saturated at all levels typically measured in serum, not just at levels seen in obesity; 2) leptin enters all regions of the brain, not just the hypothalamus; 3) entry and saturation rates differ among the brain regions; 4) the amount of leptin needed to inhibit the transporter is near the level found in the blood of normal-weight mice and humans; 5) the maximal amount of leptin transported into the CNS varies significantly among brain regions.

The implications of each of these findings are discussed below, but taken together, they suggest that serum leptin could exert effects throughout the CNS and that the level at which serum leptin would exert maximal effects varies among brain regions.

Previous studies have shown that leptin crosses the BBB by a saturable transport system, and they have used HPLC and acid precipitation to characterize the in vivo stability of I-Lep in brain and serum after intravenous injection (4). Those studies failed to show degradation products in either brain or serum for the first 5 min after injection; therefore, that time was selected here as the perfusion time. Capillary depletion was also previously used to demonstrate that leptin completely crosses the brain endothelial cell to enter the parenchymal space of the brain (4).

![Fig. 2. A: brain region/perfusion ratios vs. log(perfusion concentration) for hypothalamus and frontal cortex. Slope for whole brain is indicated by dashed line. Hypothalamus had the highest brain/perfusion ratios and the steepest saturation slope, whole brain the lowest. B: slopes for all brain regions. W. Brain, whole brain. The greater the slope the more readily the regional leptin transporter was saturated. *Values statistically different ($P < 0.05$) from whole brain; other statistical differences are shown in Table 1.](http://ajpendo.physiology.org/)

![Fig. 3B: slopes for all brain regions. W. Brain, whole brain. The greater the slope the more readily the regional leptin transporter was saturated. *Values statistically different ($P < 0.05$) from whole brain; other statistical differences are shown in Table 1.](http://ajpendo.physiology.org/)

![Table 1. Comparison of regional differences among slopes for relation between brain region/perfusion and log (perfusion concentration)](http://ajpendo.physiology.org/)
obese mice, possibly caused by an inability to upregulate the transporter in the face of increasing serum leptin levels (3). Figure 1 demonstrates that, even at levels considered to be physiological (10 ng/ml or even pathologically low (31, 33), the transporter is partially saturated. Unlike receptors, transport systems for regulatory molecules are most efficient in ranges where they are not partially saturated, so that an increase in blood is mirrored by a proportionate increase in CNS levels. These results suggest that the transporter is so constructed that it is most efficient when leptin levels are very low. In fact, at levels below 1 ng/ml (log 0), ratios approach 0.15 ml/g. To achieve this ratio in 5 min, the influx constant must be $3 \times 10^{-2} \text{ml} \cdot \text{g}^{-1} \cdot \text{min}^{-1} [0.15 \text{ ml/g} / 5 \text{ min} = 0.03 \text{ ml} \cdot \text{g}^{-1} \cdot \text{min}^{-1}]$. This is a very rapid rate of transport across the BBB for a protein, being 100 times faster than the rate measured for intravenously administered leptin (4) or the similarly sized cytokines (6). Only the thinnest mice (33) and humans with diseases such as anorexia nervosa (31) have serum levels this low and CSF/serum ratios this high. The amount of additional leptin that enters the brain with increasing blood levels is greatly

Table 2. Comparison among brain regions of leptin concentration at 1 ng/ml perfusion

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*P < 0.05; †P < 0.001.
reduced when serum levels enter the normal and obese range. This suggests that the hemoecephalic signal (the information conveyed by the passage of regulatory substances between the blood and the CNS) provided by the blood-to-brain transport of leptin is most efficiently conveyed at blood levels seen only with starvation. This is consistent with conjectures that the capability of low levels of leptin to signal starvation to the CNS is as significant as elevated leptin levels signaling obesity (20, 40). In particular, low levels of leptin may be important in brain maturation and the initiation of reproduction (1, 2). Leptin may be considered one of the hormones, such as thyroid hormones, whose neuroendocrine axis is changed with starvation.

Leptin entered all regions of the brain. Although leptin receptors are found in many regions of the CNS, most studies and hypotheses have emphasized the role of the arcuate nucleus as the site of leptin action. The transport of leptin into the arcuate nucleus and, as shown in Fig. 2, that into the hypothalamus as a whole, is indeed very high (4). However, we found here that every region in the brain receives leptin derived from the serum. Others have found that leptin can activate early genes in brain areas outside the arcuate nucleus (17), and we have shown that leptin likely exerts its effects on reproduction through the brain and not the gonads (5). It may be that some of the many functions being ascribed to leptin, such as thermogenesis, appetite regulation, lipid metabolism, reproduction, sexual behavior, insulin sensitivity, serum glucose levels, and alteration of neurohormone levels, are mediated at different regions of the brain.

The inverse linear relation between the log concentration of I-Lep infused into the brain vasculature and the brain/perfusion ratio found for whole brain was also statistically significant among brain regions for \( V_{\text{max}} \) but not for \( K_m \). *Values statistically different \( (P \leq 0.05) \) from whole brain; other statistical differences are shown in Table 3.

### Table 3. Comparison of regional differences in \( V_{\text{max}} \)

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* \( P \leq 0.05; \uparrow \) \( P \leq 0.001 \).
seen in every region of the brain. Figure 2A compares the two extreme examples of the hypothalamus and frontal cortex with whole brain values. Saturation is quantified by the slope of the relation between log concentration and the brain region/perfusion ratio. A steeper slope shows that a given degree of saturation occurs at a lower perfusion concentration (Fig. 2B). The hypothalamus, for example, saturated more readily than any other region of the brain, and the frontal cortex saturated the least readily (Table 1). This variation in saturation results in different amounts of leptin entering the brain as a function of both brain region and vascular concentration. Figure 3A shows the brain region/perfusion ratios in units of microliters per gram and regional brain concentrations in picograms per gram for the perfusion concentration of 1 ng/ml (or 1 pg/µl). At this perfusion concentration, the hypothalamus contained more leptin than any other brain region, whereas the frontal cortex contained the least (Table 2). The brain interstitial fluid space comprises 12–22% of brain weight (9), and so the concentration of leptin in that compartment can be roughly estimated by multiplying the concentration in brain tissue by five- to eightfold.

Because regions saturate differentially with increasing perfusion concentrations, the relative amounts of leptin contained within various regions of brain compared with whole brain varied (Fig. 3B). For example, whereas at 1 ng/ml the hypothalamus contained a higher concentration of leptin than any other region (almost twice that in whole brain and about four times the concentration in frontal cortex), it contained less leptin than the hippocampus, cerebellum, midbrain, pons-medulla, or occipital cortex when perfusion concentrations were in the obse range of 30 ng/ml. This difference in uptake and saturation suggests that the optimal level at which serum leptin conveys its homeocephalic signal to the brain varies with brain region and centrally induced function and is consistent with the postulate that different thresholds could exist for different actions of leptin (20). For example, transport into the cortex would provide a pathway for integration of the higher cortical centers with body weight regulation.

Because with infusion the concentration of I-Lep is held steady and greatly exceeds that which is transported, it is possible to calculate the $K_m$ and $V_{max}$. The method used and illustrated in Fig. 4A corrects for nonspecific or nonsaturable uptake, which includes the vascular compartment. For whole brain and for each brain region, statistical analysis showed that the best fit curve was that for a single binding site. This suggests that the majority of binding sites expressed by brain endothelial cells is for a transporter with a single affinity and that signal transduction receptors with differing affinities are not expressed, at least in comparable quantities. This is consistent with brain endothelial cells containing mostly mRNA for the short form of the leptin receptor (7), which has been assumed to be the transporter. It should be noted that, because our studies used brain tissue and did not analyze cerebrospinal fluid, these conclusions most likely apply to the endothelium and not to the choroid plexus.

For the whole brain, the $V_{max}$ was $1.43 \pm 0.15$ ng/g with a $K_m$ of 15.59 $\pm$ 5.16 ng/ml (Fig. 4). The $K_m$ corresponds closely to the dissociation constant value measured in various transfected cell lines (41, 42, 45) of 0.3–0.7 nM (5–12 ng/ml) but indicates an affinity $\sim$10 times higher than that measured for brain microvessels in vitro (22). This $K_m$ approximates the serum concentration of leptin seen in normal-weight mice and humans and so suggests that, at what is considered to be normal or ideal body weight, the leptin transporter is significantly saturated.

These values for $K_m$ and $V_{max}$ also varied among brain regions (Fig. 5A and Table 3). The area with the highest $V_{max}$ was the hippocampus (2.65 $\pm$ 0.37 ng/g), over twice as high as and statistically different from (Table 3) the frontal cortex (1.24 $\pm$ 0.14 ng/g). The $K_m$ values also varied by over twofold, but the higher error terms prevented any statistically significant differences (Fig. 5B). These results again emphasize a regional difference in the transport of leptin into the brain that could form the basis for a differential control of the centrally mediated functions of leptin.

It should be noted that this is the first study in which $K_m$ and $V_{max}$ have been measured in the mouse, although other aspects of BBB transport have been determined with this method. The effect of anesthesia on leptin transport is unknown, and the $K_m$ and $V_{max}$ may differ in humans and in obese or thin subjects. The effect of iodination may affect binding to the transporter. The perfusion method has the advantage of negating the effect of circulating endogenous levels of leptin and leptin binding proteins, an effect which enhances the accuracy of measurement (39) but has the theoretical disadvantage of removing potential circulating allosteric regulators or transporter cofactors. The rate of uptake of leptin is too slow to be flow dependent, and so altering the perfusion rate should not affect calculations of $K_m$ or $V_{max}$.

In conclusion, these results show that leptin enters areas throughout the brain by a system that is partially saturated at endogenous blood levels of leptin. The results suggest an important role for low serum levels signaling starvation status to the brain and show that the levels of leptin seen in obesity greatly saturate the transporter. Differences in regional uptake and saturation provide a mechanism by which leptin can control events mediated at the arcuate nucleus and other regions of the central nervous system with different regional thresholds for optimal function.

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