Ferritin: a novel mechanism for delivery of iron to the brain and other organs

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Ferritin has been established as the primary mechanism for cellular iron delivery, despite suggestive evidence for additional iron delivery mechanisms. In this study we examined ferritin, considered an iron storage protein, as a possible delivery protein. Ferritin consists of H- and L-subunits, and we demonstrated iron uptake by ferritin into multiple organs and that the uptake of iron is greater when the iron is delivered via H-ferritin compared with L-ferritin. The delivery of iron via H-ferritin but not L-ferritin was significantly decreased in mice with compromised iron storage compared with control, indicating that a feedback mechanism exists for H-ferritin iron delivery. To further evaluate the mechanism of ferritin iron delivery into the brain, we used a cell culture model of the blood-brain barrier to demonstrate that ferritin is transported across endothelial cells. There are receptors that prefer H-ferritin on the endothelial cells in culture and on rat brain microvasculature. These studies identify H-ferritin as an iron transport protein that is found in high concentrations in serum. Therefore, in this study we tested the hypothesis that ferritin could deliver iron to the brain and that the delivery was receptor mediated.

Ferritin is composed of 24 subunits of heavy (H)- or light (L)-chain peptides that are present in different ratios in various organs (27), and in the brain, the ratio of subunits is cell-type specific (12). Functionally, the ferritin subunits differ. The H-subunit contains ferroxidase activity and is responsible for converting soluble ferrous iron to the storable ferric form. The L-subunit does not have ferroxidase activity and, as a result, stores iron at a low rate compared with the H-subunit (27). Genetic manipulation studies have revealed the relative importance of H-ferritin by showing that the absence of the H-ferritin gene is embryonically lethal (17, 40). Although ferritin is considered a cytoplasmic iron storage protein, novel functions for ferritin or members of the ferritin family have recently been discovered, including a role in the cell nucleus (9, 39) and mitochondria (25). In this study we examined the ability of the different ferritin subunits to deliver iron to various organs, with particular emphasis on the brain.

MATERIALS AND METHODS

Ferritin Preparation

All the experiments reported used recombinant human H-ferritin or horse spleen ferritin. The latter was obtained commercially (Sigma, St. Louis, MO) and was chosen because it consists of 90:10 L- to H-ferritin subunits. 5’-His tagged H-ferritin was prepared by cloning H-ferritin into isopropylthiogalactoside-inducible pET30 a (+)/BL21(DE3) vector. After the cells were grown, the protein was purified with a nickel protein filter column to a final concentration of 2.8 mg/mL. The purity of the protein sample was verified by Western blotting. There were no nonferritin contaminants detected.

Cell Culture and Preparation of Endothelial Cell Monolayer

We used bovine retinal endothelial cells (BRECs) as an in vitro model of the BBB to test the hypothesis that ferritin can be transported across a layer of endothelial cells and to begin to address the mechanism of ferritin transport across the BBB. This well-studied model has been shown to posses all of the necessary characteristics and attributes of a blood-neural barrier (2, 10, 44). Cow eyes were obtained from a local abattoir, and the bovine retinal endothelial cells (BRECs) were isolated and processed according to a previously published procedure (2, 10). BRECs were grown in MCDB-131 medium (Sigma) supplemented with 10% FBS, 10 ng/ml EGF, 0.2

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mg/ml ENDO GRO (VEC Technologies, Rensselaer, NY), 0.09 mg/ml heparin, antibiotic/antimycotic solution (GIBCO, Rockville, MD), and tylosin antibiotic (Sigma). The cells are initially cultured in flasks until they reached at least 80% confluence. Subsequently, the BREC were gently trypsinized and grown to confluence on Costar Transwell 0.4-μm porous filters (Coming, Acton, MA). Fibronectin was added at a concentration of 1 μg/cm² to promote adherence to the filter. The cells were then washed and stepped to serum-free, EGF-free MCDB-131 medium supplemented with 100 nM hydrocortisone for 72 h. The addition of hydrocortisone to these cell cultures promoted the formation of tight junctions, as has been shown previously (2, 23).

Ferritin Transport in the BBB Model

Prepurified transferrin was purchased and resuspended to a final concentration of 2.5 mg/ml. About 250 μg of recombinant human (r)H-chain ferritin, spleen ferritin (Sigma), and transferrin were labeled with fluorescein isothiocyanate (FITC; Pierce Biotechnology) in 100 mM carbonate/bicarbonate buffer, pH 9.0. Removal of excess or hydrolyzed FITC was achieved by passage through a 5-ml G-25 desalting column. The FITC-conjugated H-ferritin, spleen ferritin, and transferrin were concentrated, and buffer was exchanged with PBS in a Centriprep concentrator (10,000 mol wt cut off; Amicon, Beverly, MA). Transferrin was included as a positive transport control in the BREC model (6).

Rate of flux across confluent BREC monolayer was determined as described previously (6, 10) with some modifications. Briefly, BREC were grown to confluence in a Transwell apparatus (2) before the addition of 140 μg of either FITC-labeled rH-ferritin, spleen ferritin, or transferrin to the top (apical) chamber. Transport of the tracer is determined by sampling from 100-μl aliquots collected at various times from the bottom (basal) chamber (15, 30, 45, 60, 120, 180, and 240 min) following addition of the tracer to the apical chamber. The aliquots from the basal chamber are then analyzed for fluorescence in a spectrofluorometer (Spectramax Gemini; Molecular Devices). The rate of flux across BREC monolayer was obtained using the equation (Bf/Tt) × (Vb/A) = (Flux) × t, where the rate of flux is the slope (cm/s) from the plots of bottom chamber fluorescence per unit amount of top chamber fluorescence (Bf/Tt) vs. time (t). In this equation, Bf, indicative of the amount of tracer transported across the monolayer, is normalized to the volume of the basal chamber (Vb) and also to the surface area available for transport (A). The concentration of Tf in the top chamber did not change significantly over the 4 h that the experiment was performed and thus is considered constant for calculating flux. The amount of fluorescence in the top chamber was obtained from a 100-μl aliquot at the end of the transport assay (4 h).

As a control for paracellular flux and assurance of the production of tight junctions, rhodamine isothiocyanate (RITC) dextran (70 kDa) was added simultaneously to the apical chamber for all experiments as a control. The level of paracellular transport by RITC dextran was measured in the same monolayer as FITC-ferritin, except that the RITC is detected at a different wavelength. The different fluorescent properties allow for simultaneous analysis of the protein of interest and the dextran control in each experiment. Dextran is not internalized at an appreciable level by endothelial cells (34), thus any accumulation of dextran in the basal chamber is due to paracellular transport. None of the conditions affected the rate of flux of dextran, which was minimal in each condition.

To demonstrate that the fluorescence detected in the basal chamber was FITC-labeled H-ferritin, the basal media from randomly selected experiments were collected and concentrated from 1.5 ml to 100 μl using a Centriprep concentrator. The concentrated media were subjected to SDS-PAGE, and an image of the resulting gel was obtained under fluorescence at 462 nm. Subsequently, the proteins in the gel were transferred, and an immunoblot analysis was performed with an antibody against rH-ferritin. The results demonstrated that the FITC was associated with H-ferritin.

Determination of Transport Mechanism

To determine whether pinocytosis contributed significantly to the transport of ferritin, we added 50 μg/ml filipin to the apical chambers of the Transwell apparatus for 30 min before the ferritin and dextran were added. The addition of filipin has been shown to inhibit the action of nonspecific transport via pinocytosis (38). To determine whether H-ferritin uptake occurs via clathrin-dependent endocytosis, we performed studies in potassium-deficient medium (100 mM NaCl-50 mM HEPES). The cells were incubated in potassium-deficient medium for 10 min before the addition of ferritin. Intracellular potassium depletion inhibits receptor-mediated endocytotic processes occurring through clathrin-coated pits (24, 33). These latter experiments could only be performed for 1 h before the potassium depletion altered the integrity of the cell-to-cell junctions as indicated by an increase in dextran transport. Each treatment condition (or standard) was performed a minimum of six times. Throughout the experiments, the cultures were visually assessed to ensure that the experimental treatments and manipulations did not affect cell viability. As in the baseline experiments, dextran-labeled RITC was included as an indicator of the integrity of the tight junctions. Differences between the means for FITC-H-ferritin under the different conditions were analyzed using one-way analysis of variance. For those measurements with significantly different means, a Bonferroni post hoc comparison was performed to analyze pairwise differences. The level of significance was set at P < 0.05.

Binding Experiments

The binding experiments on BREC cell homogenates were performed in duplicate on the fourth passage of the BREC cells by using 125I-labeled rH-ferritin or horse spleen ferritin. The specific activity for both was ~340,000 cpm/pmol. To establish the total, specific, and nonspecific binding, we added a range of concentrations of 125I-H-ferritin with or without a 1,000-fold molar excess of unlabeled H-ferritin to 100 μg of total protein of the BREC cell homogenate. The binding buffer consisted of 50 mM Tris-HCl, pH 7.4, and 0.1% BSA. Incubations were carried out at 22°C for 2 h. The binding was terminated by the addition of 3 ml of ice-cold 50 mM Tris-HCl. With the use of a cell harvester, the bound radioactivity was isolated by rapid filtration and washing over Whatman glass fiber C filters, which were previously coated with 5% nonfat dry milk and 0.1 mg/ml horse spleen ferritin.

Equilibrium competition binding assays were performed in which the increasing concentration of unlabeled H-ferritin was incubated with 25 μg of protein of BREC homogemate at 22°C with 0.4 nM 125I-H-ferritin for 120 min in the same binding buffer described above. Termination of binding, isolation of membranes, and calculations of specific binding were performed as described above.

Rat Brain Microvessel Preparation

Six adult rats were used for each microvessel preparation. The rats were anesthetized with a lethal dose of pentobarbital sodium (100 mg/kg body wt) and then decapitated. The brain was removed and placed in a petri dish on ice. The cerebellum and the meninges were removed, and 5 volumes of microvascular buffer (1 × MVB: 1 × salt, 1 × HEPES, 0.5% BSA, and 5 mM glucose) was added with protease inhibitors as previously reported (42). The brains were gently homogenized with 20 strokes using a glass-Teflon homogenizer (0.25-mm clearance), and the homogenate was centrifuged at 1,000 g for 10 min at 4°C. The supernatant was discarded, and the pellet was resuspended in 5 volumes/rat of 17% dextran (1:1 ratio of 1 × salt and 1 × HEPES with dextran), followed by vortexing and then centrifugation at 3,000 g at 4°C. The microvessels are collected from the wall of the tube and
resuspended in 20 ml of 1 × MVB buffer. The microvessels were filtered through a 120-μm mesh. The microvessel preparation was further purified by adherence to glass beads (Sigma) supported on a 40-μm mesh. The beads were washed with buffer that had protease inhibitors added. The beads were rinsed in 5 ml of MVB, and then the microvessels were pelleted by centrifugation at 1,000 g at 4°C for 15 min. The microvessels were resuspended in 1 ml of HES+ (10 mM HEPES, 1 mM EDTA, 250 mM sucrose, pH 7.4, and protease inhibitor cocktail; Sigma), and a total protein concentration was determined. The samples were stored at −80°C until use.

Ferritin Binding on Microvessels

The binding suspension consisted of 50 mM Tris·HCl, pH 7.4, 0.1% BSA, and 20 μg of membrane protein preparation with or without the addition of 1 μM unlabeled H-ferritin in a final volume of 250 μl. Binding was terminated by the addition of 3 ml of ice-cold 50 mM Tris·HCl. Bound radioactivity was isolated by rapid filtration over Whatman glass-fiber C filters that had been previously coated in a solution of 5% nonfat dried milk (Blotto) with 0.1 mg/ml horse spleen ferritin. This combination was determined empirically to reduce the nonspecific binding of radiolabeled protein to the filters to 1–3% of the total counts added. The filters were washed five times with 3 ml of ice-cold 50 mM Tris containing 200 mM NaCl. The filters were being counted in a Micromedic 4/200 Plus automatic gamma counter. Specific binding was calculated by subtracting binding in the presence of excess unlabeled H-ferritin (nonspecific binding) from binding without excess unlabeled H-ferritin present (total binding).

Saturation analysis. Each binding experiment was performed in duplicate. Increasing concentrations of 125I-labeled H-ferritin were added to binding suspensions consisting of the same binding buffer described previously with 20 μg of membrane protein preparation with or without the addition of 1 μM unlabeled H-ferritin in a final volume of 250 μl. A 120-min incubation at 22°C binding was terminated, total, nonspecific, and specific bindings were calculated as described previously.

Competition assays. Increasing concentrations of unlabeled competitors (H-ferritin and horse spleen ferritin) were incubated for 60 min at 22°C with 100 μg of membrane protein in the presence of 0.4 nM 125I-labeled H-ferritin in the same binding buffer described previously. Binding, termination of binding, isolation of membranes, and calculations of specific binding were performed as described above. The competition experiments were performed in duplicate.

In Vivo Uptake Studies

Recombinant H and spleen ferritin (1.2 mg) were incubated in 40 μl of 1 mM nitritoltriacetic acid, pH 6.0, 0.5 μl of ferrous ammonium sulfate, 2 μl of 0.5 M sodium bicarbonate, and 40 μCi of 59FeCl for 4 h at 37°C. After incubation, ferritin was dialyzed in a 10,000 MW cartridge in 1× PBS for 24 h to remove any unbound 59Fe. The specific activity was 12 nCi/g for H-ferritin and 24.7 nCi/g for spleen ferritin. Radiolabeled protein (3.4 μg/gram wt) was injected (n = 3) into the tail vein of female Sprague-Dawley rats (~350 g). After 48 h, the rats were decapitated and the organs removed immediately. Each organ was dissected and rinsed thoroughly in 0.1 M PBS. For the brain, the cerebrum was removed from the cerebellum and bisected, and the meninges were dissected clear of the brain. One gram of tissue (wet weight) from each organ was used to determine the iron uptake.

H-ferritin-deficient mice were evaluated as an experimental model to determine whether potentially compromised iron management in an organ could influence ferritin iron delivery. A similar approach was used to investigate ferritin uptake in control and H-ferritin-deficient mice as described above for the rats, except that the mice were injected intraperitoneally. The specific activity in the mouse studies for H-ferritin was 17.7 mCi/g for H-ferritin and 93 mCi/g for spleen ferritin. Ferritin was injected and allowed to circulate in the bloodstream for 48 h until the mice were killed and the organs removed.

The amount of radioactivity in each organ was determined on a Canberra sodium iodide-based single-channel analyzer well counter system for 1 min. The gamma counts per minute (cpm) were subtracted from background counts, divided by the efficiency of the counter, and then divided by the disintegration counts per minute to calculate radioactivity (μCi). To calculate the percentage of radioactivity in each organ, organ values were divided by the total amount injected and then multiplied by 100%.

RESULTS

In Vivo Uptake of Ferritin

The possibility that iron bound to ferritin could be taken up by different organs in rats was determined by injecting H-ferritin or spleen ferritin containing radiolabeled 59Fe into the tail vein of adult rats. The uptake of iron from H-ferritin was significantly greater than that for spleen ferritin in the brain, heart, kidney, muscle, and lung (Fig. 1A). The amount of 59Fe was two times higher in the brain when it was presented bound to H-ferritin than to spleen ferritin (P < 0.005) (Fig. 1B). Only the liver had significantly higher uptake of iron from spleen ferritin compared with H-ferritin (P < 0.05).

To determine the influence of potential alterations in iron storage capacity within various organs on H-ferritin and spleen ferritin delivery of iron to various organs, we investigated the uptake of 59Fe from these proteins in a mouse line that is deficient in H-ferritin (39). Iron delivery by H-ferritin was decreased in the spleen, lung, and muscle (P < 0.05) in the H-ferritin-compromised mice compared with littermate controls (Fig. 2A). A similar finding was observed in brain (Fig. 2B). Spleen ferritin uptake was unaltered in any organ in the iron storage-compromised mice (Fig. 3, A and B).

Transport of Ferritin

Although serum ferritin could have unrestricted access to systemic organs, to be effective for delivering iron to the brain it would have to cross an endothelial cell barrier (BBB). To begin to investigate the possibility that ferritin transcytosis was possible, we utilized a cell culture model of the BBB. H-ferritin, but not spleen ferritin, was transported across the BREC cell monolayer in significant amounts (Fig. 4A). The rate of FITC-labeled H-ferritin that was transported across the BREC cell monolayer was five times more than that of the control for RITC-labeled dextran (P < 0.001). Dextran is a control for substances that are not transported and for paracellular transport, as discussed in MATERIALS AND METHODS. The rate of transport of spleen ferritin was similar to the level seen in the dextran control. To determine the mechanism by which H-ferritin is transcytosed, we performed the transport assays in a potassium-free medium to block the formation of clathrin-coated vesicles. The absence of clathrin coat formation was associated with an 80% (P < 0.001) decrease in the rate of H-ferritin transport. In contrast, filipin pretreatment of the BREC, to block pinocytosis, resulted in no significant decrease in rate of transport. A dextran control was included include in each experimental condition, and the rate of transport did not change from that shown for the untreated condition (data not shown). Transferrin transport was included as a positive control and was detected as previously reported (5).
The specific activities of fluorescently labeled transferrin and ferritin were different, so no conclusions can be made about the relative rates of transport for these two proteins.

To demonstrate the fluorescence detected in the basal chamber (and that remaining in the apical chamber), the basal medium was collected, concentrated, and subjected to SDS-PAGE and immunoblotting. The results demonstrate that a single fluorescent band was present in the basal medium, and subsequent immunoblotting revealed this fluorescent band was associated with H-ferritin (Fig. 4B). No fluorescence could be detected in the medium that was associated with ferritin (data not shown).

**Ferritin Binding Analysis**

To more thoroughly evaluate the mechanism of iron delivery via ferritin in the brain, we performed binding studies to determine whether the transport of ferritin in the BREC model was receptor mediated. In addition, to expand the evaluation of ferritin binding to an in vivo system, microvasculature was isolated from rat brains (RBMVs). Ferritin binding to BREC and RBMVs was performed by utilizing a saturation experiment as well as a competition experiment. $K_d$ and maximum binding ($B_{max}$) values were obtained from both the approaches using nonlinear regression in Prism 4.0 (GraphPad).
Saturation curves. Various concentrations (1, 2, 3, 5, 7, and 10 nM) of $^{125}$I-labeled ferritin (rH-ferritin or spleen ferritin) were incubated with 100 μg of the tissue (BRECs or RBMVs). Total and nonspecific binding (in the presence of 1,000 nM unlabeled ferritin) were determined by performing the assay on Whatman filters. To obtain the $K_d$ and $B_{max}$ values from such a binding assay, we performed nonlinear global regression for one-site binding. With this method, both total and nonspecific binding were plotted against the concentration of labeled ferritin. The resulting plots were fitted to the following equations:

\[
\text{nonspecific} = NS \times X \\
\text{total} = \text{specific} + \text{nonspecific},
\]

where specific = $B_{max} \times X/(K_d + X)$; NS is the nonspecific binding constant, and X is the concentration of the ferritin being assayed. In the global approach, specific binding is not derived from the total and nonspecific binding data. Instead, the values of $K_d$ and $B_{max}$ are obtained by sharing the nonspecific binding constant between the two data sets (total and nonspecific). The data from this regression analysis are shown in Fig. 5, A and B. Only the rH-ferritin had significant saturable binding to either the BRECs or the RBMVs. The $K_d$ and $B_{max}$ values for the RBMVs are 7.9 ± 1.6 nM and 572.6 ± 64.0 fmol/mg protein, respectively. For the BRECs, the $K_d$ is 2.7 ± 0.9 nM and the $B_{max}$ is 465.7 ± 63.1 fmol/mg protein. The $R^2$ value for the curve fit is >0.8 for both BRECs and RBMVs.

Competition curves. Various concentrations of cold ferritin (0.03, 0.1, 0.3, 1, 3, 10, 30, 100, 300, and 1,000 nM) were incubated with 100 μg of BRECs or RBMV tissue along with 0.4 nM of radiolabeled ferritin. Total binding was obtained by performing the assay on Whatman filters. The total binding (fmol/mg protein) was then plotted against log [concentration (nM)]. These plots were then fit to the one-site competition equation total = bottom + (top - bottom)/(1 + 10 (X - log [EC50])). These data are shown in Fig. 6, A and B. The results show that H-ferritin, but not spleen ferritin, could effectively compete for the binding sites. The $K_d$ and $B_{max}$ values from the competition curves for the BRECs are 2.0 nM and 235.1 fmol/mg protein. The corresponding values for RBMVs are 3.4 nM and 304.6 fmol/mg protein. The $R^2$ value for the fit is >0.95 for both BRECs and RBMVs. The values generated by the two different curves are within acceptable ranges.

DISCUSSION

The results of this study reveal that ferritin can deliver iron to multiple organs, including the brain. Furthermore, the amount of iron delivered by ferritin is enhanced when the iron is delivered via H-ferritin instead of L-ferritin for most organs except the liver. The amount of H-ferritin iron that is taken up by cells can be altered when iron storage capacity is compromised.
mised, as demonstrated in the H-ferritin-deficient mice, whereas iron delivery by L-ferritin is not significantly affected in this model. These latter results suggest a feedback system for H-ferritin. Thus we have identified a novel transport system for iron delivery to the brain and one that could be highly significant given the amount of iron (up to 4,500 atoms; Ref. 19) that can be housed in a single molecule of ferritin compared with transferrin (maximum of 2 Fe atoms). The identification of a nontransferrin-dependent iron delivery system to the brain is consistent with our previous reports showing iron delivery to the brain in the absence of serum transferrin (30).

In the brain, in addition to binding to a receptor, ferritin must be transcytosed across the BBB. In this study we demonstrated that ferritin can be transported across a cell culture model of the BBB. The transport of ferritin in this cell culture model is clathrin dependent and receptor mediated and strongly favors the H-subunit. The preference for H-ferritin binding is consistent with the transport data. Binding of ferritin was also demonstrated on microvasculature from the rat brain, and this binding, similar to the cell culture model, also strongly favored the H-subunit. The binding and transport data are consistent with the iron uptake data that revealed increased delivery to the brain if the iron was associated with H-ferritin relative to spleen (L-rich) ferritin. The uptake of ferritin, the differences in iron delivery between H- and L-ferritin, and the changes in H-ferritin uptake in the H-ferritin-compromised mice all suggest the possibility of a ferritin receptor, and binding for H-ferritin was directly demonstrated on two different sources of endothelial cells.

The data suggest that the mechanism by which ferritin is transported across the BBB is clathrin dependent, and not pinocytosed, is similar to that previously identified using the same system for transferrin (5). The mechanism for iron delivery to the brain and regulation of those mechanisms is central to understanding how iron may accumulate or fail to reach normal levels and thus underlie or contribute to a variety of neurological disorders (43). The studies of transcytosis of iron bound to transferrin have provided conflicting results. Some studies show that iron entering the brain is bound to transferrin (18), and other studies show iron transport that is not associated with transferrin (35). We have provided evidence that there are both transferrin and non-transferrin-dependent systems for iron delivery to the brain (5) and that the

Fig. 3. A: $^{59}$Fe uptake in various systemic organs in H-ferritin-deficient (−/+), and wild-type (+/+), mice, delivered via spleen ferritin. Mice were injected intraperitoneally with spleen ferritin containing $^{59}$Fe. After 48 h, the mice were killed and the organs removed. The amount of radioactivity was determined in 0.1 g of each organ, and the percentage of total injected radioactivity was determined. Data are means ± SE from 3 animals. None of the differences reached statistical significance. B: $^{59}$Fe uptake in the brain in H-ferritin-deficient (+/−) vs. wild-type (+/+), mice, delivered via spleen ferritin. These data are from the brains of mice used to generate the data in A. The amount of radioactivity reported is the percentage of the total injected into the animals. Data are means ± SE from 3 animals.
preference for one pathway over another appears to be dependent on the iron status of the endothelial cells. The iron status of the endothelial cells forming the BBB and how this iron status impacts on the regulation of transferrin receptors for transferrin-mediated uptake of iron has been largely ignored in studies on the mechanisms of brain iron uptake (6). The expression of transferrin receptors in cells is well known to be regulated by the intracellular iron status of the cell (1), and endothelial cells have the same iron regulatory mechanism for transferrin receptors as other cells (36). Endothelial cells of the brain also express the divalent metal transport protein, whose function is to mediate iron release from endosomes within the cell and contain relatively high amounts of ferritin (7), indicating the existence of iron stores. The concept that brain endothelial cells have their own considerable iron requirement and regulate their own iron uptake is consistent with the high concentration of mitochondria in these cells and thus high iron requirement (37). Therefore, at least some of the iron delivered to these cells by transferrin should be retained within the endothelial cells. The mechanism by which iron is released from ferritin is not well understood, and some have proposed that degradation of ferritin is required to release iron (19). Therefore, ferritin may be more likely to be transcytosed with a high amount of its iron content intact and may be less likely to share its iron with endothelial cells than transferrin. The mechanism for regulating ferritin delivered iron to the brain is unknown at this time, but the decrease in H-ferritin delivered iron in the H-ferritin-deficient mice strongly suggests that such a regulatory mechanism exists.

Although the function of ferritin as an iron storage protein is well established, the concept that ferritin could be actively secreted by cells and possibly deliver iron has not been extensively studied. Ferritin levels in the blood fluctuate widely under even normal conditions. Ferritin mRNA is bound to polyribosomes (29) that are attached to the endoplasmic reticulum in rat liver cells, which would support a secretory role for ferritin. However, the function of ferritin as an iron storage protein is well established, and the concept that ferritin is actively secreted by cells and possibly delivers iron has not been extensively studied. Ferritin levels in the blood fluctuate widely under even normal conditions.
pathway for ferritin. Direct secretion of ferritin (both subunits) has been demonstrated in differentiated rat hepatoma cells (41), and the release of H-ferritin but not L-ferritin from microglial cells in culture has been shown (45). The source of ferritin for the receptors on the BBB and other organs is presumably from the serum. That ferritin is present in serum is well established, but serum ferritin is traditionally considered to be predominantly made of the L-subunits. We have shown, however, that the binding of ferritin, transport of ferritin, and delivery of iron to the brain all strongly favor H-rich ferritin. Thus the physiological significance of our finding based on the relatively small amounts of H-ferritin in the serum suggests that ferritin could be secondary to transferrin as a source of iron for the brain. It must be remembered, however, that a mole of ferritin can deliver 2,000 times more iron than a mole of transferrin. Furthermore, there are conditions under which H-ferritin is elevated in the plasma, such as inflammation and in association with some cancers (16). Therefore, chronic inflammatory conditions could increase brain iron status, a concept consistent with iron accumulation in a number of neurodegenerative diseases, including Alzheimer’s and Parkinson’s diseases. In addition, neuroferritinopathies have been identified in which serum ferritin levels are elevated and abnormal ferritin accumulation is reported in the brain (26).

The observations that H-ferritin can be transported to the brain may also be significant to prion disease. A recent study has demonstrated that prions attach to ferritin (32). This group has suggested that humans consume contaminated beef and that prions attached to the bovine ferritin are transported across the gut and into the bloodstream. Our data suggest that prions could enter the brain using the delivery system identified in this study for ferritin. Finally, the ability of the brain to acquire iron via ferritin may be relevant to recent reports that intravenous iron supplements have been used to treat restless legs syndrome, a neurological disorder linked to iron deficiency (14, 15). It is possible that the intravenous iron supplements selectively increase H-ferritin, and this concept is currently under investigation.

The possible contribution of ferritin-mediated iron delivery to brain iron homeostasis and the possible contributions to neuro-
logical disorders clearly indicate the importance of the findings in this study. Furthermore, it is equally clear that additional research into understanding the mechanisms of iron uptake into the brain and the regulation of those mechanisms is warranted.

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