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The habenula and iron metabolism in cerebral mouse models of multiple sclerosis

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Abstract

Iron accumulates in the CNS of patients with multiple sclerosis, but our understanding of the mechanism accounting for this accumulation is unclear. Mouse models of cerebral experimental autoimmune encephalomyelitis (EAE) in C57BL/6 and SJL mice were used together with a histochemical stain for iron and immunohistochemical stains for transferrin receptor, synaptophysin, iron regulatory protein 1 (IRP1) and/or IRP2 to investigate the role of disease activity on CNS iron metabolism. The expression of transferrin receptor, but not IRP1 or IRP2, increased in the medial habenula, which is adjacent to the third ventricle, in response to both types of cerebral EAE. In the habenula, the elevated expression of transferrin receptor in C57BL/6 mice with cerebral EAE was generally restricted to the medial habenula while the expression in SJL mice with cerebral EAE was more diffusely expressed. Iron levels were increased in all regions of the habenula in C57BL/6 mice with cerebral EAE, and in the medial and medial lateral but not the lateral habenula in SJL mice with cerebral EAE. Synaptophysin, which has been observed previously in endocytic vesicles together with the transferrin receptor, was concentrated at the medial habenula, but its levels did not increase with disease in C57BL/6 mice with cerebral EAE. Our results support the model that the medial habenula responds to disease activity by upregulating transferrin receptor to facilitate the movement of iron into the brain from the third ventricle, raising the possibility that a similar mechanism accounts for iron accumulation in deep gray matter structures in patients with multiple sclerosis.

Keywords

Experimental autoimmune encephalomyelitis; habenula; iron; IRP1; IRP2; multiple sclerosis; synaptophysin; transferrin receptor

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Competing interest

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Introduction

The pathological profile of multiple sclerosis (MS) includes the deposition of iron in various CNS structures. Iron deposition can occur in perivascular locations, be associated with plaque edges, and present in multiple deep gray matter (DGM) structures [1,2]. MRI studies have revealed that the accumulation of iron in DGM begins early in the disease course [3,4] and is positively associated with various measures of disease activity [1,2]. However, the mechanism of iron accumulation in DGM structures is unknown. Neuronal degeneration has been suggested to be one mechanism accounting for its enhanced detection [1]. Alternatively, the stress associated with ongoing inflammation or degeneration may promote an enhanced uptake of iron [1,2].

Since the accumulation of iron in MS occurs in cerebral structures, we wanted to utilize a model of MS that involves the cerebrum. Previously, we showed that a cerebral model of experimental autoimmune encephalomyelitis (EAE) in mice has perivascular iron deposits [5] that were associated with protein nitration [6]. Since both iron deposits [7,8] and protein nitration [9,10] have been detected in perivascular localizations in MS, it suggests the cerebral model of EAE is relevant for the study of cerebral changes that occur in MS. In the present study, we used two cerebral EAE models, i.e., relapsing remitting disease in SJL mice and chronic disease in C57BL/6 mice, to investigate possible mechanisms of iron uptake during disease.

Methods

Mice

All studies involving the use of animals were approved by the Institutional Animal Care and Use Committee of the University of Kansas Medical Center. Female SJL mice aged ~5–7 weeks and female C57BL/6 aged ~8–9 weeks old (Jackson Laboratory, Bar Harbor, ME) were used for induction of cerebral EAE. Mice were given 31M Nutrigel (ClearH2O, Portland, ME) supplementation once advanced disease developed.

Cerebral EAE induction

Cerebral EAE in SJL mice was induced as previously described [5] except that isoflurane was used as the anesthetic and no pertussis toxin injections were given. SJL mice were sacrificed up to 100 days post-encephalitogen injection, typically during a relapse. Cerebral EAE was induced in C57BL/6 mice using two subcutaneous injections, on the dorsum, of myelin oligodendrocyte glycoprotein peptide (amino acids 35–50; 150 µg total) with emulsion [Freund's incomplete adjuvant containing 375 µg *Mycobacterium tuberculosis* (Difco Laboratories, Detroit, MI)]. This was followed with an i.p. injection of pertussis toxin (PTX; 100 ng/100 µl saline; List Biological Laboratories, Campbell, CA), and additional PTX injections on day 3 and day 7 post-encephalitogen injection. C57BL/6 mice were sacrificed between 15–66 days post-encephalitogen injection. Tissue was processed as previously described [5].

Iron histochemistry

Sections were rinsed 2x in PBS, processed through upgraded alcohols into SafeClear (Fisher Scientific, Kalamazoo, MI) and rehydrated back to PBS. Sections were washed 2x in PBS, once in 1% potassium ferrocyanide trihydrate (Fisher), and incubated for 30 min on a horizontal rotating platform in 1% potassium ferrocyanide trihydrate/1% triton X-100/0.125 N HCl solution (added as follows: 150 μ l 4% potassium ferrocyanide trihydrate in water; 300 μ l 2% triton X-100 in water; and 150 μ l 0.5N HCl). Sections were rinsed 3x in PBS, then incubated in a mixture of 10 mg 3,3'-diaminobenzidine tetrahydrochloride (Sigma Cat. #D5905): 50 ml 0.01M Tris HCl pH 7.4: 200 μ l 30% H₂O₂ on a horizontal rotating platform for 15 min. Sections were rinsed in PBS followed by water, then mounted on slides in 0.2% gelatin and air-dried overnight, after which slides were coverslipped in Permount.

Immunohistochemistry

Immunohistochemical staining was performed as previously described for iNOS labeling [6] except that rat anti-transferrin receptor (1:150; AbD Serotec, Raleigh, NC), rabbit anti-synaptophysin (1:500; Sigma, St. Louis, MO), chicken anti-IRP1 (1:250, a kind gift from Dr. Elizabeth Leibold, University of Utah, Salt Lake City, UT [11]) and rabbit anti-IRP2 (1:250, also a kind gift from Dr. Elizabeth Leibold [11]) antibodies were used together with biotinylated rabbit anti-rat IgG, biotinylated goat anti-rabbit IgG, or biotinylated goat anti-chicken IgY secondary antibodies, and incubation with SG Peroxidase Substrate Kit (Vector Laboratories, Inc., Burlingame, CA) was for 10 min.

Image and statistical analyses

Images containing the habenula were captured using a 4x objective. For transferrin receptor, synaptophysin, IRP1, and IRP2, the density of the stained area in the inner third of the medial habenula, adjacent to the third ventricle, was analyzed using ImageJ (NIH), and the mean of the two hemispheres from each animal was used for statistical comparisons. For iron histochemistry, the densities of staining in three areas within each structure (medial habenula, medial lateral habenula, and lateral habenula) were averaged, and the mean from the two hemispheres for each structure from each animal was used for comparisons. Statistical evaluations were performed by the Wilcoxon two sample test with $p = 0.05$ as significant. The numbers of mice per group were as follows: SJL control mice, $n = 5$; SJL mice with cerebral EAE (early phase), $n = 5$; SJL mice with cerebral EAE (late phase), $n = 5$; C57BL/6 control mice, $n = 4$ (except for synaptophysin where $n = 3$); C57BL/6 mice with cerebral EAE, $n = 19$.

Results

Transferrin is an iron transport protein that delivers iron to cells following binding to its membrane receptors [12]. Immunohistochemical staining for the transferrin receptor revealed a high level of expression in the medial habenula of C57BL/6 mice with cerebral EAE, with the greatest levels of expression associated at or near the walls of the 3rd ventricle (Fig. 1A,C). This expression was above levels observed in control C57BL/6 mice (Fig. 1B,D) or levels found in other DGM structures (Fig. 1A). The expression of transferrin receptor was elevated in SJL mice with cerebral EAE (Fig. 1F,G), but the localization was

more diffusely expressed in the habenula (Fig. 1F) compared to that seen for C57BL/6 mice with cerebral EAE (Fig. 1A,C) or to that in control SJL mice (Fig. 1E). The elevated expression was persistent with elevated levels present in SJL mice 49–100 days after encephalitogen injection (Fig. 1F,G). The expression of the transferrin receptor was also elevated in the medial habenula in EAE mice given an intracerebral injection of saline in place of cytokines, indicating that the changes in expression levels were not dependent on the injection of cytokines (not shown).

Following binding of transferrin to its receptor, the complex undergoes clathrin-mediated endocytosis [12]. Besides facilitating the trafficking of synaptic vesicles, synaptophysin has been found to be associated with the transferrin receptor in endocytic vesicles [13,14]. Immunohistochemical staining of synaptophysin in cerebral EAE and control C57BL/6 mice revealed a high level of expression in the medial habenula with the greatest concentration adjacent to the 3rd ventricle (Fig. 2A,B), similar to the expression distribution of transferrin receptor (Fig. 1A–C). However, unlike the transferrin receptor, the expression levels of synaptophysin did not differ between cerebral EAE and control C57BL/6 mice (Fig. 2C).

Since a mechanism of expression regulation for the transferrin receptor occurs via IRP1 and IRP2 [12], their expressions were examined by immunohistochemistry in cerebral EAE. IRP1 expression was greater in the medial habenula than in surrounding structures, e.g., lateral habenula and paraventricular thalamic nucleus (PV) in C57BL/6 mice with (Fig. 2D,E depicting Day 15 post-encephalitogen injection) or without (not shown) cerebral EAE. IRP2 expression was also concentrated in the medial habenula, but it was often more concentrated in the PV than IRP1 in C57BL/6 mice with (Fig. 2G,H depicting Day 37 post-encephalitogen injection) or without (not shown) cerebral EAE, where some of its expression appeared as punctate labeling (not shown). Despite some variability, there was no clear indication of shifts in expression levels of IRP1 or IRP2 in cerebral EAE in either C57BL/6 (Fig. 2F,I) or SJL (not shown) mice relative to respective control mice.

The distribution and relative concentration of iron was investigated by iron histochemistry. In contrast to the expression of the transferrin receptor, staining density was greater in the lateral habenula than in the medial habenula (Fig. 3B); however, in response to disease in C57BL/6 mice, the concentration of iron increased in the medial, medial lateral, and lateral habenula (Fig. 3A,C,G). In SJL mice with cerebral EAE, there was a greater concentration of staining in the medial and medial lateral habenula, but not the lateral habenula, compared to control animals, and often vessel walls were stained in diseased mice (Fig. 3D–F, H).

Discussion

In normal animals, transferrin receptor expression is elevated in the medial habenula compared to most other CNS structures [15]. In the present study, we describe a further increase of the expression of transferrin receptor in the medial habenula in response to EAE. The habenula is located adjacent to the 3rd ventricle. Since transferrin receptor and synaptophysin can be colocalized in endocytic vesicles [13,14], having overlapping high concentrations at this site suggests a mechanism for enhanced endocytic trafficking of transferrin from the cerebrospinal fluid. Consistent with this view, a study that examined the

distribution of transferrin following its administration by intracerebroventricular injection found substantial binding to the medial habenula [16]. Since we observed that the upregulation of transferrin receptor expression was maintained over prolonged periods following disease initiation, this pathway could be an important mechanism accounting for the accumulation of iron that has been observed in DGM structures in MS patients.

Besides an elevated expression of transferrin receptor, staining for iron, but not IRP1 or IRP2, was increased in the habenula in cerebral EAE mice compared to control mice. The elevated levels of iron occurred in all regions of the habenula in C57BL/6 mice but only in the medial and medial lateral habenula in SJL mice with cerebral EAE. Furthermore, the elevation of transferrin receptor was largely focused to the medial habenula in C57BL/6 with cerebral EAE, but was more widely distributed in the habenula of SJL mice with cerebral EAE. Together, these findings suggest that relapsing remitting MS and chronic forms of MS may have varied mechanisms of iron accumulation in DGM, but studies on human specimens representing different forms of MS are necessary to make this determination.

Even though IRPs normally act to inversely coordinate transferrin receptor expression and iron levels [12], co-elevations of both transferrin receptor and iron have been observed previously. In a model of psychological stress in the rat [17], and in a double transgenic mouse model of Alzheimer's disease [18], increases in both transferrin receptor and iron were observed in the cortex and hippocampus. This dual increase for transferrin receptor and iron was also observed in the spinal cord of a transgenic mouse model of amyotrophic lateral sclerosis [19]. Thus, a mechanism other than IRP regulation may be mediating these increases. Inflammatory cytokines were found to induce the expression of transferrin receptor and a second iron transport protein, divalent metal transporter 1 (DMT1), while decreasing ferroportin levels, resulting in iron accumulation [20]. DMT1 expression can become activated via NFkB [20] while the binding of c-Myc to E-boxes in the transferrin receptor 1 gene can induce expression and enhance iron uptake [21,22]. Other mechanisms can also induce transferrin receptor expression. For example, insulin [23] or inflammatory stimuli [24] upregulate transferrin receptor 1 expression via activation of hypoxia inducible factor-1, which binds the transferrin receptor 1 promoter [25]. These mechanisms likely do not influence synaptophysin expression since its expression did not change in response to disease. An absence of a change in synaptophysin expression in the habenula has been observed in response to another stressor. For instance, increases in expression levels of vesicular glutamate transporter and spinophilin in the medial habenula, as well as in other structures, were observed following sensitization with D-amphetamine while synaptophysin expression remained unchanged [26].

In summary, the habenula may be an important pathway for the delivery of iron from the cerebrospinal fluid to DGM structures, and this pathway could function to increase the deposition of iron in DGM structures during MS.

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Highlights

Transferrin receptor expression is upregulated in the medial habenula, which is adjacent to the third ventricle, in cerebral mouse models of multiple sclerosis (cerebral experimental autoimmune encephalomyelitis)

Iron levels increased within the habenula of mice with cerebral experimental autoimmune encephalomyelitis

The medial habenula may facilitate the accumulation of iron in deep gray matter structures in patients with multiple sclerosis by upregulating transferrin receptor allowing for greater iron uptake by the brain from the third ventricle.

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Fig. 1.

Transferrin receptor immunohistochemical labeling. **A)** Transferrin receptor is located at the medial habenula (arrowheads) adjacent to the 3rd ventricle. Note the low or absent staining in the lateral habenula (LH), paraventricular thalamic nucleus (PV), and other thalamic nuclei (TN). This picture is of a cerebral EAE C57BL/6 mouse at Day 66 post-encephalitogen injection. Bar = 400 μ m. **B)** Transferrin receptor staining is present in the medial habenula (arrowheads) of a control C57BL/6 mouse (No EAE), but the staining is very light. Note, the abundant staining of the choroid plexus (arrow), which acts as an internal control indicating that the staining procedure was functioning properly. Bar = 200 μ m. **C)** Transferrin receptor staining of the medial habenula (arrowheads) is intense in a C57BL/6 mouse with cerebral EAE at Day 37 post-encephalitogen injection. Bar in B also relates to C. **D)** In the medial habenula, the amount of transferrin receptor staining was significantly greater in C57BL/6 mice with cerebral EAE (Cer EAE; n = 19) compared to control mice (No EAE; n = 4). **E)** Transferrin receptor staining is light in the medial habenula (arrowheads) of a SJL control mouse (No EAE). Bar = 200 μ m. **F)** Transferrin receptor staining in a SJL mouse with cerebral EAE at Day 43 post-encephalitogen injection. Although staining in the medial habenula (arrowheads) is increased relative to that in control SJL mice, the pattern of elevated staining was not as discretely expressed in the medial habenula as that observed in C57BL/6 mice with cerebral EAE (A, C). Bar in E also relates to F. **G)** In the medial habenula, transferrin receptor staining was significantly greater in SJL mice with cerebral EAE (Cer EAE) at both an early phase (Day 32–46 post-encephalitogen injection; n = 5) and a late phase (Day 49–100 post-encephalitogen injection; n = 5) compared to control mice (No EAE; n = 5). Note: G represents analysis of data generated with a different purchase of the same antibody that was used for D.

Fig. 2.

Synaptophysin, IRP1, and IRP2 immunohistochemical labeling in C57BL/6 mice. **A)** Synaptophysin staining is concentrated in the medial habenula (MH). A less concentrated amount of staining is also present in the lateral habenula (LH) and paraventricular thalamic nucleus (PV). C57BL/6 mouse with cerebral EAE, Day 37 post-encephalitogen injection. Bar = 250 μ m. **B)** A higher power view of synaptophysin staining in A reveals concentrated staining towards the ventricle side (left of picture) of the medial habenula. Bar = 50 μ m. **C)** Levels of synaptophysin did not differ appreciably between control C57BL/6 mice (No EAE; n = 3) and C57BL/6 mice with cerebral EAE (Cer EAE; n = 19). ns = not significant. **D)** IRP1 staining is denser in the medial habenula compared to that in the lateral habenula and in the paraventricular thalamic nucleus. C57BL/6 mouse with cerebral EAE, Day 15 post-encephalitogen injection. Bar in A also applies to D. **E)** A higher power view of IRP1 staining in the medial habenula in D. Bar in B also applies to E (left of picture is the 3rd ventricle). **F)** Levels of IRP1 staining were not statistically different between control C57BL/6 mice (No EAE; n = 4) and C57BL/6 mice with cerebral EAE (Cer EAE; n = 19). ns = not significant. **G)** IRP2 staining was more generalized than synaptophysin or IRP1 staining, but there was more staining in the medial habenula and periventricular thalamic nucleus compared to other structures. C57BL/6 mouse with cerebral EAE, Day 37 post-encephalitogen injection. Bar in A also applies to G. **H)** A higher power view of IRP2 staining in the medial habenula in G. Bar in B also applies to H (left of picture is the 3rd ventricle). **I)** The level of IRP2 staining was not statistically different between control C57BL/6 mice (No EAE; n = 4) and C57BL/6 mice with cerebral EAE (Cer EAE; n = 19). ns = not significant.

Fig. 3.

Iron histochemical staining in the habenula in C57BL/6 and SJL mice. **A)** The stained habenula was divided into the medial habenula (MH), the medial lateral habenula (MLH), and the lateral habenula (LH) for analyses. C57BL/6 mouse with cerebral EAE, Day 37 post-encephalitogen injection. Bar = 200 μ m, which also applies to B and C. **B)** A stained section from a control (No EAE) C57BL/6 mouse reveals the lightest staining in the MH with increased staining in the MLH and LH. **C)** A stained section from a C57BL/6 mouse with cerebral EAE (Cer EAE) at Day 22 post-encephalitogen injection. Staining in the habenula is increased compared to that in the control mouse in B. **D)** Little staining is present in the medial and medial lateral habenula, but there is concentrated staining in the lateral habenula in a control (No EAE) SJL mouse. Bar = 200 μ m, which also applies to E and F. **E)** Compared to the SJL control mouse in D, there is more staining in the medial and medial lateral habenula in a SJL mouse with cerebral EAE at Day 32 post-encephalitogen injection. Vessel staining is also apparent (arrowhead) as is an area of dense accumulation of iron (arrow). **F)** At Day 49 post-encephalitogen injection in a SJL cerebral EAE mouse, there is still elevated staining in the medial and medial lateral habenula compared to the control SJL mouse in D. Arrowheads point to vessel staining. **G)** C57BL/6 mice with cerebral EAE (Cer EAE; n = 19) had elevated levels of iron staining in the MH, MLH, and LH compared to control mice (No EAE; n = 4). **H)** SJL mice with cerebral EAE (Cer EAE) at Day 32–46 (n = 5) and Day 49–100 (n = 5) had elevated levels of staining in the MH and MLH, but not LH, compared to control (No EAE; n = 5) SJL mice. ns = not significant.