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Research paper

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

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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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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.

1. 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

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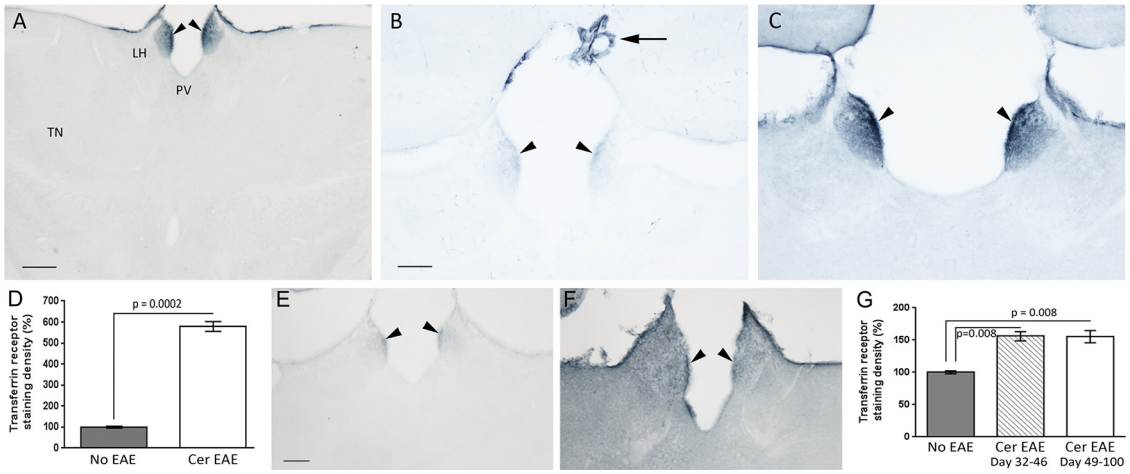

Fig. 1. Transferrin receptor immunohistochemical labeling. (A) Transferrin receptor is located at the medial habenula (arrowheads) adjacent to the 3rd ventricle. Note the low or absent staining in the lateral habenula (LH), paraventricular thalamic nucleus (PV), and other thalamic nuclei (TN). This picture is of a cerebral EAE C57BL/6 mouse at Day 66 post-encephalitogen injection. Bar = 400 μm. (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 μm. (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 μm. (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.

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.

2. Methods

2.1. 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.

2.2. 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 and 66 days post-encephalitogen injection. Tissue was processed as previously described [5].

2.3. Iron histochemistry

Sections were rinsed 2× in PBS, processed through upgraded alcohols into SafeClear (Fisher Scientific, Kalamazoo, MI) and rehydrated back to PBS. Sections were washed 2× 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 μl 4% potassium ferrocyanide trihydrate in water; 300 μl 2% triton X-100 in water; and 150 μl 0.5 N HCl). Sections were rinsed 3× in PBS, then incubated in a mixture of 10 mg 3,3′-diaminobenzidine tetrahydrochloride (Sigma Cat. #D5905): 50 ml 0.01 M Tris HCl pH 7.4: 200 μl 30% H_2O_2 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.

2.4. 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.

2.5. Image and statistical analyses

Images containing the habenula were captured using a 4× 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