

Ephrin A Receptors and Ligands in Lesions and Normal-Appearing White Matter in Multiple Sclerosis

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Complexes of the tyrosine kinase ephrin ligands (ephrins) and their receptors (Ephs) provide critical cell recognition signals in CNS development. Complementary ephrin/Eph expression gradients present topographic guidance cues that may either stimulate or repulse axon growth. Some ephrin/Ephs are upregulated in adult CNS injury models. To assess their involvement in multiple sclerosis (MS), ephrin A1-5 and Eph A1-8 expression was analyzed in CNS tissues using immunohistochemistry. Control samples showed distinct expression patterns for each ephrin/Eph on different cell types. Perivascular mononuclear inflammatory cells, reactive astrocytes and macrophages expressed ephrin A1-4, Eph A1, -A3, -A4, -A6 and -A7 in active MS lesions. Axonal ephrin A1 and Eph A3, -A4, and -A7 expression was increased in active lesions and was greater in normal-appearing white matter (NAWM) adjacent to active lesions than within or adjacent to chronic MS lesions, in contralateral NAWM, or in control samples. As in development, therefore, there are temporally dynamic, lesion-associated axonal ephrin/Eph A expression gradients in the CNS of MS patients. These results indicate that ephrin/Eph As are useful cell markers in human CNS tissue samples; they likely are involved in the immunopathogenesis of active lesions and in neurodegeneration in MS NAWM; and they represent potential therapeutic targets in MS.

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INTRODUCTION

Ephrins are a large family of membrane-bound tyrosine kinase signaling proteins consisting of ligands (ephrins) that interact with complementary receptors (Ephs) (11, 28). Ephrin complexes function in the establishment, maintenance and remodeling of cellular organization in the developing nervous, vascular and immune systems. Nine ephrin ligands and at least 15 Eph receptors have been identified to date (see <http://cbweb.med.harvard.edu/eph-nomenclature>). Eph A receptors preferentially bind to glycosylphosphatidyl inositol-anchored ephrin A ligands and Eph B receptors bind to transmembrane ephrin B ligands. The binding interactions, however, are promiscuous as there are multiple pairings both within and between the A and B families (27, 70). Contact-dependent ephrin/Eph binding can result in bidirectional signaling events that affect downstream pathways including integrin-mediated cell and extracellular matrix attachment and actin cytoskeleton organization. Therefore, ephrin/Ephs

interactions affect cell shape, adhesion and movement critical to many normal and pathologic processes (7, 19, 24, 29, 33, 37, 48, 50).

Ephrins play complex roles in different regions and at different stages of vertebrate CNS development (4, 10, 13-16, 18, 31, 43, 76, 77). In particular, they establish intercellular communication after neurogenesis by regulating cell migration and axon pathfinding through complementary expression gradients that may be either attractive or repulsive for axon growth (26, 30, 62, 79). Some ephrins and Ephs continue to be expressed in the adult mammalian CNS in which they have been implicated in synaptic plasticity and in neural stem cell migration, proliferation and function (9, 20, 36, 57, 73). Ephrin/Ephs also have immunologic functions, eg, in thymic development, T- and B-cell signaling, immunoregulation and costimulation (1, 42, 45, 55, 56, 72, 74, 75).

Alterations in ephrin/Eph mRNA and protein expression have been found both

in focal lesions and in more remote tissue regions in various vertebrate CNS injury models (6, 32, 34, 44, 53, 54, 63, 68, 69, 71). In lower vertebrates this expression has been linked to neuronal regeneration whereas in rodents it has been linked to the failure of reparative processes after injury. Ephrin/Eph genes are also expressed in CNS and non-CNS neoplasms (8, 17, 25, 51, 58). To date there has been no systematic analysis of eph/Eph protein expression in a human CNS disease.

The immunologic functions and re-expression in CNS injury models of ephrin/Ephs suggested the hypothesis that aberrant ephrin/Eph expression is involved in the immunopathogenesis of lesions and in neuronal injury in multiple sclerosis (MS). Gene microarray analyses of MS-affected CNS tissues have identified differential expression of some ephrin/Eph genes, particularly ephrin A1, EphA1 and Eph B6, but consistent patterns have not yet emerged from this approach (21, 39, 40, 49, 64). In the present study, ephrin A and Eph A expression in MS lesions, normal-appearing white matter (NAWM) and controls was analyzed and axonal expression was compared to that of β amyloid precursor protein (β -APP), a marker of injured axons (35, 52). Complex expression patterns in the adult human CNS and within frank MS plaques were identified. Furthermore, in MS NAWM, the analysis demonstrated that there are lesion-associated axonal expression gradients of certain ephrin/Ephs analogous to those that occur in development and in CNS injury models. The re-expression in MS-affected CNS tissues of members of this family of molecules that have been most extensively studied in CNS development indicates their likely roles in lesion pathogenesis, axon degeneration and

	Gender	Mean age (range)	Disease duration	Mean post-mortem time (range)	No. CNS paraffin blocks analyzed
MS autopsies	5 male; 5 female	52.6 years (12-74 years)	4 months-10+ years	7.4 hours (3.5-20.5 hours)	49 [†]
Acute inflammatory/demyelinating cerebral WM lesion biopsies	3 female	45.7 years (35-51 years)	? days-6 months	N/A	5
Non-MS/OND* autopsies (cerebrum)	5 male; 2 female	77 years (56-96 years)	N/A	10.4 hours (4-22 hours)	8
Normal autopsy	1 male	24 years	N/A	7 hours	1
Normal cerebral cortex biopsy	1 female	39 years	N/A	N/A	2

Table 1. Demyelinating disease and control cases.

*OND=other neurological diseases. Neuropathologic diagnoses for OND patients included 3 cases of Alzheimer disease, and one case each of amyloid angiopathy, incidental Lewy bodies, traumatic myelomalacia, remote cerebral infarcts, acute meningitis, and diffuse acute hypoxic-ischemic encephalopathy.

[†]41 cerebral hemisphere samples; 8 spinal cord samples

Antigen	Species [†]	Source [†]
<i>ephrins</i>		
ephrin A1 (V-18)	R	SC
ephrin A2 (L-20)	R	SC
ephrin A3 (K-19)	R	SC
ephrin A4 (P-20)	R	SC
ephrin A5 (C-19)	G	SC
<i>Ephs</i>		
Eph A1 (G-18)	R	SC
Eph A2/Eck	Mouse IgG ₁	U
Eph A3 (C-19)	R	SC
Eph A4 (A-20)	R	SC
Eph A 5	Mouse IgG ₁	R&D
Eph A6 (G-18)	G	SC
Eph A7 (A-18)	R	SC
Eph A7 (C-19)	R	SC
Eph A8 (R-19)	G	SC
β-APP	Mouse IgG ₁	C

Table 2. Antibodies.

[†]Polyclonal Abs raised in rabbits (R) or goats (G)

[†]C=Chemicon International, Temecula, Calif; R&D=R&D Systems, Minneapolis, Minn; SC=Santa Cruz Biotechnology, Santa Cruz, Calif; U=Upstate Biotechnology, Lake Placid, NY;

the failure of repair. Moreover, the expression patterns identified point to ephrin/Eph signaling pathways as potential specific therapeutic targets in MS.

MATERIALS AND METHODS

Human CNS tissue samples. Paraffin and frozen CNS tissue sample blocks were obtained from the Department of Pathology, Stanford University Medical Center, Stanford, Calif, the Veterans Affairs Health Care System, Palo Alto, Calif, the University of California Davis Medical Center, Sacramento, and the UCLA Human Brain and Spinal Fluid Resource Center, Los Angeles,

Calif. Autopsy samples were from patients with the clinical and neuropathologic diagnosis of MS and other neurologic diseases (OND) and normal controls. Brain biopsy samples had been obtained from patients with temporal lobe epilepsy (neuropathologically normal cortex and white matter) and acute inflammatory/demyelinating lesions (67). Demographic and other available clinical data, postmortem intervals and numbers of paraffin blocks analyzed are summarized in Table 1. Additional cryosection samples were used for initial antibody (Ab) screening and confirmation of reactivities but paraffin sections were used most extensively for the analyses. All HIPAA regulations and institutional policies regarding patient information confidentiality were followed.

Immunohistochemistry. Reactivities of the Abs listed in Table 2 were tested using immunoperoxidase staining on six μm thick serial sections of routinely fixed, paraffin-embedded sections. Six additional commercial antibodies to ephrin/Eph As were tested but were found unsuitable for these studies. Briefly, the slides were baked for 2 hours at 60°C, deparaffinized, rehydrated to 70% ethanol and washed in phosphate-buffered saline pH 7.4 (PBS), 3× for 10 minutes. They were then washed twice with distilled water and immersed in 10 mM citric acid monohydrate, pH 6.0 in microwave staining dishes (Shandon-Lipshaw, Pittsburgh, Pa). The dishes were covered with a loose fitting lid and heated in a microwave for 15 minutes. The slides were then cooled in the uncovered dishes at room temperature (RT) for at least 30 minutes and rinsed (2×10 minutes each) in distilled water and PBS. The slides were then incu-

bated in 10% normal serum matched to the species of the secondary Ab for 10 min at RT. They were then incubated at 2°C to 8°C overnight with primary polyclonal or monoclonal Ab diluted in PBS to optimal concentrations that had been determined in initial testing. On the following day the slides were incubated sequentially in 0.3 % H₂O₂ for 10 minutes in PBS, biotinylated secondary anti-immunoglobulin Ab for the appropriate species for 30 minutes and streptavidin-biotin horseradish peroxidase complex reagents (Vectastain Elite kit, Vector Laboratories, Burlingame, Calif) for 45 minutes with PBS washes between each step. Immunoperoxidase reaction product was visualized with 3,3′ diaminobenzidine (Sigma Chemical Company, St. Louis, Mo). The slides were then fixed in neutral buffered formalin and counterstained with hematoxylin. Negative controls included the substitution of the primary Ab with either PBS and the use of irrelevant Abs in place of anti-ephrin/Eph Abs.

Analysis of controls and MS plaques. Initial examination of normal and OND control samples demonstrated complex staining patterns on different CNS cell populations in leptomeninges, and gray and white matter for each anti-ephrin/Eph Ab. The presence or absence of staining of vascular smooth muscle, microvascular endothelial cells, microglial and cerebral cortical neurons was determined in each stained slide of the control samples and in each sample from MS patients that contained normal-appearing gray matter.

MS plaques were initially classified based on examination of hematoxylin and eosin stain (H&E), Luxol fast blue-PAS or Klüver-Barrera stains for myelin and Biel-

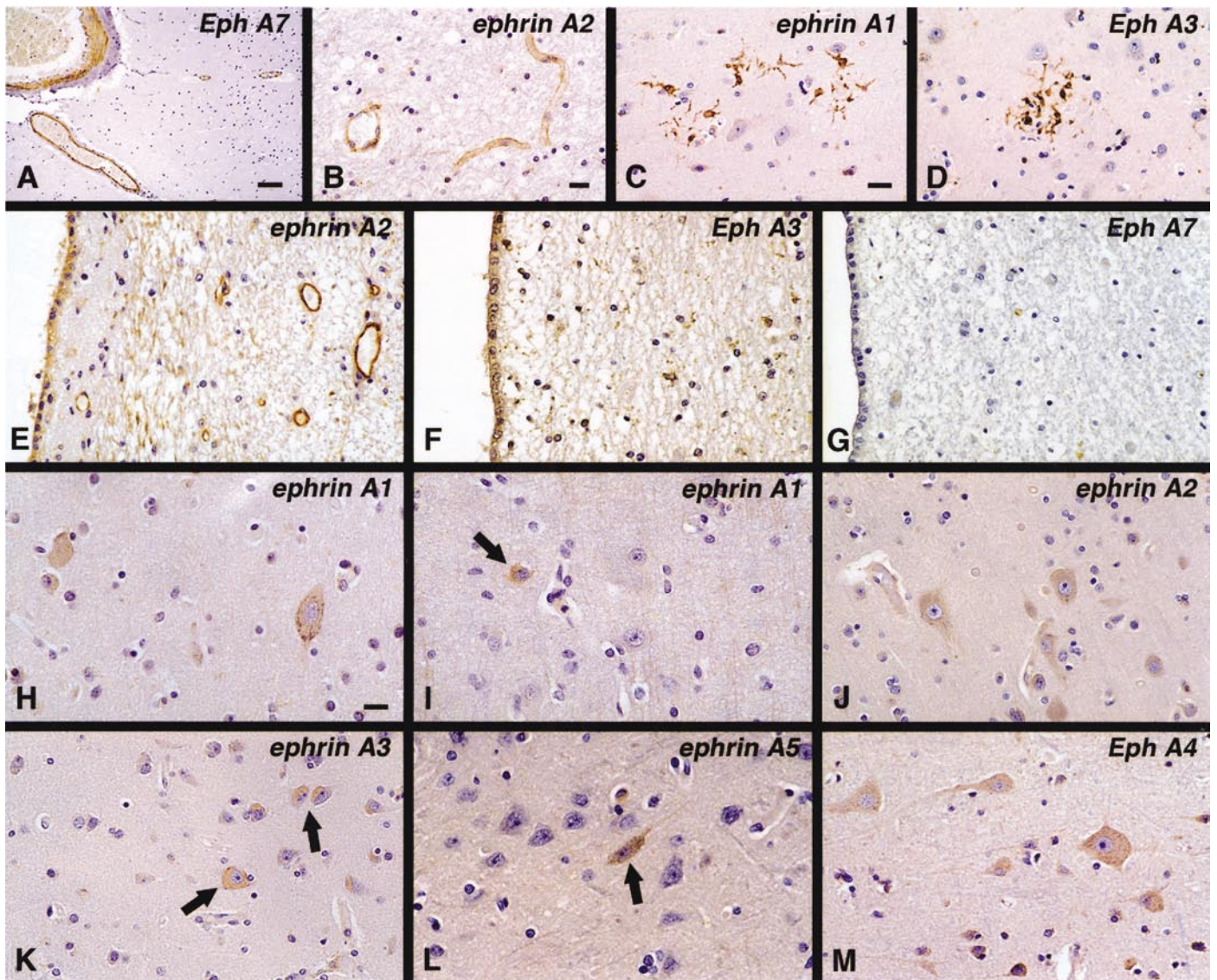


Figure 1. Representative examples of ephrin/Eph expression in normal and OND control samples. Eph A7 is expressed on smooth muscle of large meningeal (upper left of figure) and intraparenchymal arteries (A). Capillary endothelial cells express ephrin A2 (B). Reactive microglia associated with neuritic plaques in Alzheimer disease express ephrin A1 (C) and Eph A3 (D). Ependymal cells adjacent to a lateral ventricle express ephrin A2 (E) and Eph A3 (F), but not Eph A7 (G). Microvascular endothelial cells also express ephrin A2 in E. Most cerebral cortical neuron cell bodies show staining for ephrin A1 (H), ephrin A2 (J), ephrin A3 (K) (arrows) and Eph A4 (M). In some cortical regions expression of ephrin A1 (I) and ephrin A5 (L) was limited to neuron subpopulations (arrows in I and L). Other neurons in these fields are not stained. Scale bar in A = 550 μ m; scale bar in B for B, E-G = 100 μ m; scale bar in C for C, D = 50 μ m; scale bar in H for H-M = 100 μ m.

schowsky preparations for axons on sections from each paraffin block. Additional Holzer stains and glial fibrillary acidic protein (GFAP) immunostains had also been performed on several of the archival samples. Seventeen lesions were classified as “active plaques” based on the presence of perivascular and parenchymal mononuclear cell infiltrates and large numbers of lipid-laden macrophages. Samples from 3 stereotactic biopsies of acute inflammatory demyelinating lesions (Table 1) were included in this group. Fifteen hypocellular demyelinated plaques with partial or near complete axon loss were classified as “chronic inactive

plaques.” The presence of immunostaining of perivascular mononuclear cells, foamy macrophages and reactive astrocytes was determined in samples of active lesions. The presence of axonal ephrin/Eph staining was determined in samples of active and inactive plaques.

Semiquantitative analysis of axonal ephrin/Eph in control and MS NAWM. To assess the influence of proximity to active and inactive MS lesions, ephrin/Eph axon expression in NAWM in samples containing plaques and in one or two samples of NAWM immediately adjacent to the plaque

were analyzed. When intact coronal slices of cerebral hemispheres were available, 9 additional NAWM samples from hemispheres contralateral to those with grossly evident plaques at the same anatomic levels as the plaque and the periplaque samples were also analyzed. This additional control sampling permitted assessment of expression within MS brain in NAWM matched to plaque-affected areas. Gray/white matter junctions and plaque borders in each sample were determined by gross examination of slides stained for myelin; outlines were then marked on the immunostained slides. Stained axons were counted within active

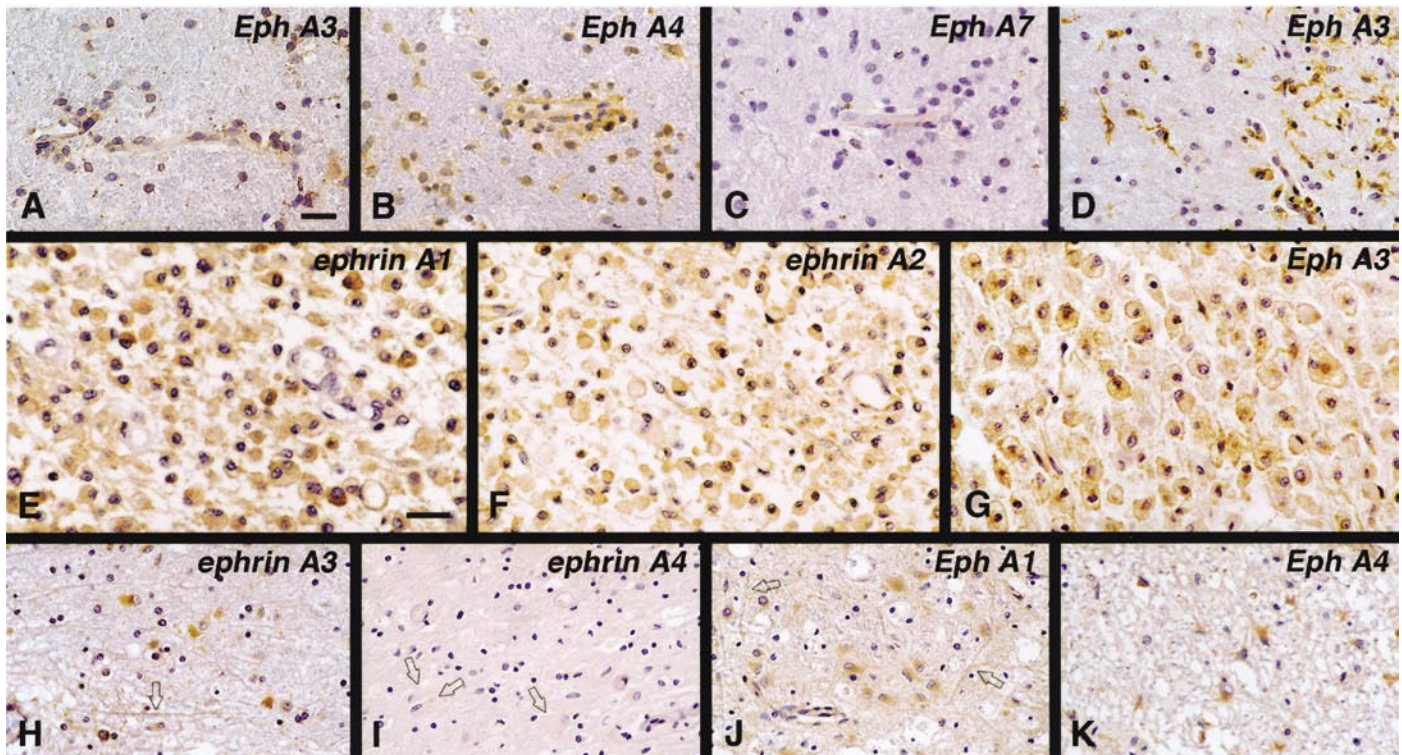


Figure 2. Ephrin/Ephs in acute inflammatory/demyelinating and active MS lesions. Perivascular mononuclear inflammatory cells express Eph A3 (A) and Eph A4 (B), but not Eph A7 (C). Panels A–C are from the same biopsy sample of an acute inflammatory/demyelinating lesion. Microglia at the edge of an active MS plaque express Eph A3 (D). The plaque center is to the right side of this field. Foamy macrophages in active MS plaque centers show surface and variable cytoplasmic staining for ephrin A1 (E), ephrin A2 (F) and Eph A3 (G). Reactive astrocytes in an active plaque express ephrin A3 (H). A prominent swollen axon in the field (open arrow) does not stain for ephrin A3. In a field adjacent to (H), there is no staining for ephrin A4 (I). Unstained gemistocytic astrocytes are indicated by open arrows. Reactive astrocytes in active plaques express Eph A1 (J) and Eph A4 (K). Axons in these fields are also not stained (open arrows in J). Scale bar in A for A–D, H–K = 20 μ m. Scale bar in E for E–G = 20 μ m.

plaques, in the MS NAWM regions and in control samples. Because of the difficulty of obtaining accurate counts of stained axons in cross section, eg, in spinal cord tracts, only longitudinal profiles in cerebral hemisphere NAWM samples were counted. Since axons are variably and often markedly depleted in inactive plaques, immunostained axons were not counted in chronic inactive plaques.

Statistics. The significance of differences in stained axon densities for anti-ephrin/Eph and anti- β -APP Abs in the three NAWM regions in MS samples, ie, adjacent to active plaques, adjacent to chronic plaques, and in opposite hemispheres, was determined by ANOVA. A *P* value of less than 0.5 for each Ab analyzed was considered significant.

RESULTS

Normal and OND controls and MS normal-appearing gray matter. In control and OND samples, each ephrin/Eph Ab reacted with specific CNS cell populations

although there were variations in the extent of staining among these samples and in the normal-appearing gray matter included in thirty-five MS samples. Nevertheless, several Abs gave consistent staining results in nearly all of these samples. Smooth muscle cells in large meningeal arteries and smaller intraparenchymal arterioles were consistently positive for Eph A7 (Figure 1A). Microvascular endothelial cells invariably expressed ephrin A2 (Figure 1B, E). In the Alzheimer disease OND control cases, there was prominent and distinct immunolabeling of reactive microglia in neuritic plaques with the anti-ephrin A1 and anti-Eph A3 Abs (Figure 1C, D). The Ab to Eph A3 also stained small numbers of cells that were morphologically consistent with rod and ramified forms of microglia in gray and white matter (not shown). Ependymal cells bordering lateral ventricles and in obliterated central canals in spinal cord were stained with some Abs to ephrin/Ephs, eg, ephrin A2 and Eph A3, but not others (Figure 1E–G). Assessment of staining of normal astrocytes in white matter was difficult because distinct cellular outlines were not evident

and in some samples there was diffuse non-specific staining of myelin (not shown). Distinct axon staining was not seen in normal control white matter for any of the ephrin/Ephs. Axon staining in OND white matter is discussed below. Oligodendrocyte ephrin/Eph expression in controls and MS will be addressed elsewhere (in preparation).

Cerebral cortical neuron cell bodies were frequently uniformly positive in control samples stained for ephrin A1, -A2 and -A3 and Eph A4 (Figure 1H, J, K, M). For some of the ephrin/Ephs, however, focal cortical areas showed staining of only a subset of cortical neurons (Figure 1I, L). Spinal anterior horn, basal ganglia and thalamic neuron cell bodies showed uniform staining for the same ephrin/Ephs that were consistently expressed in cerebral cortical neurons (not shown). There were no clear differences in neuron cell body staining patterns between control and MS normal-appearing gray matter samples.

In summary, for each ephrin/Eph there were distinct and in some cases complex expression patterns on vascular, glial and

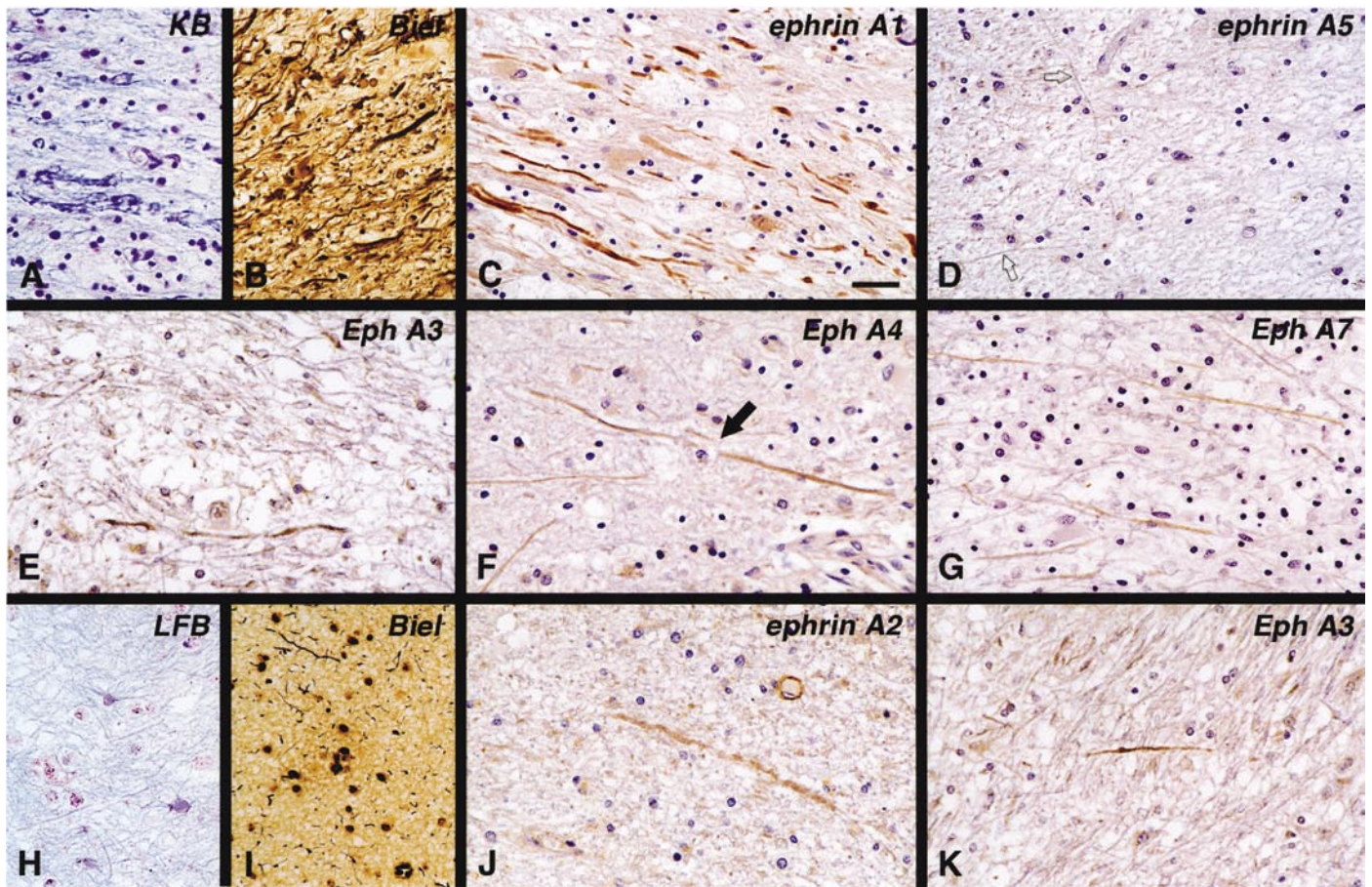


Figure 3. Aberrant axonal ephrin/Eph expression in active and chronic inactive MS lesions. Active demyelination (A) and injured axons (B) are demonstrated with Klüver-Barrera stain and Bielschowsky silver impregnation (Biel), respectively. Many dystrophic axons and axon segments in the same sample shown in (A) and (B) express ephrin A1 (C) but not ephrin A5 (D) (open arrows). Gemistocytic astrocytes are also stained for ephrin A1 (C). Variable numbers of mostly dystrophic axon segments express Eph A3 (E), Eph A4 (F) and Eph A7 (G) in active lesions. Prominent foamy macrophages can be seen in these fields indicating ongoing demyelination (arrow in F). There is less expression in chronic inactive lesions characterized by demyelination in Luxol fast blue (LFB) stain (H) and axon depletion in a Bielschowsky preparation (I). Single dystrophic axons express ephrin A2 (J) and Eph A3 (K) in chronic inactive lesions. Scale bar in C for all panels = 20 μ m.

neuronal populations in normal and OND control samples and in MS normal-appearing gray matter. The proportions of samples with positive staining of different populations are summarized in Table 3.

Acute inflammatory/demyelinating lesions and MS plaques. In acute inflammatory-demyelinating biopsy and active MS plaque samples, mononuclear inflammatory cells in perivascular cuffs variably expressed ephrin A1-3 and Eph A3 and -A4, but not other ephrin/Ephs (Figure 2A-C). Many of the stained cells were small lymphocytes. Other than foamy macrophages, however, it was often not possible to distinguish distinct mononuclear cell types. Consistent with results in controls, there was prominent staining of reactive microglia adjacent to MS plaques for ephrin A1 and Eph A3 (Figure 2D). Foamy macrophages within active plaques also showed surface and cy-

	Vascular smooth muscle	Endothelial cells	Microglia	Ependymal cells	Cerebral cortical neuron cell bodies
ephrin A1	-	-	+	+	++
ephrin A2	+	++	-	+	++
ephrin A3	+	±	-	±	++
ephrin A4	-	-	-	-	±
ephrin A5	-	-	-	±	+
Eph A1	-	+	-	-	+
Eph A2/eck	+	-	-	-	-
Eph A3	-	±	++	±	±
Eph A4	+	-	-	±	++
Eph A5	-	+	-	-	-
Eph A6	-	-	-	+	+
Eph A7	++	±	-	-	-
Eph A8	±	-	±	±	-

Table 3. Ephrin/Ephs in controls and MS normal-appearing gray matter.

++ = staining in >90% of samples; + = staining in 20% to 90% of samples; ± = staining in 0% to 20% of samples; - = negative.

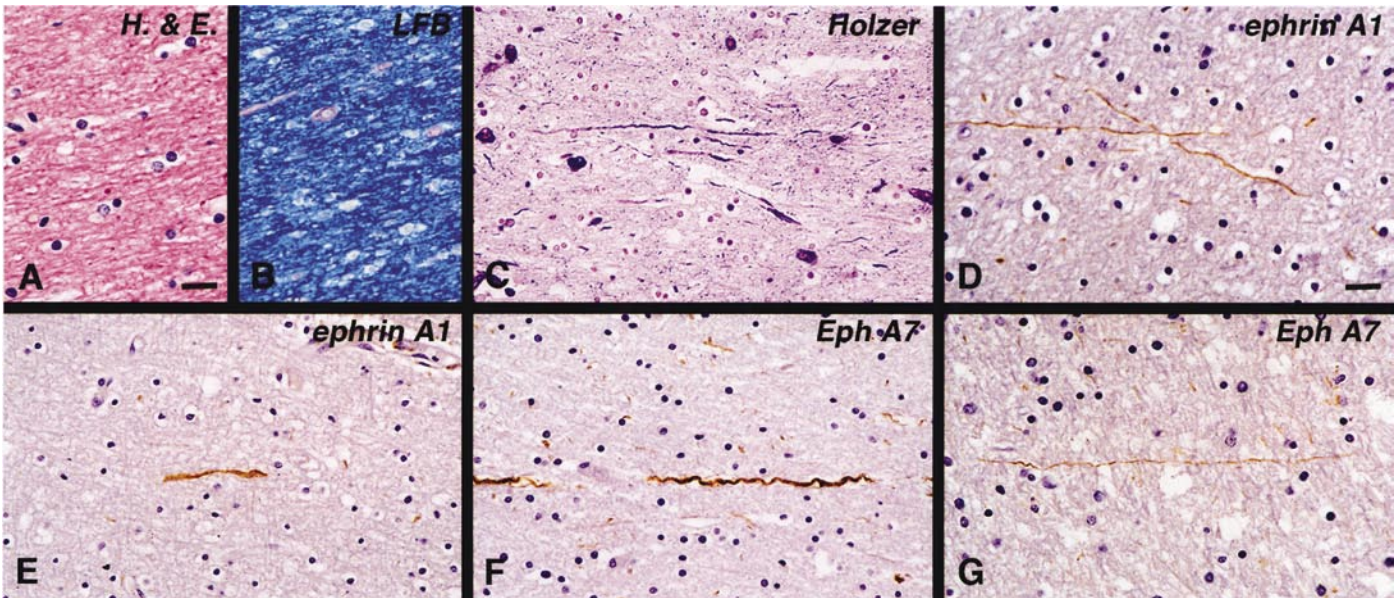


Figure 4. Axon ephrin/Ephs in NAWM adjacent to active and chronic MS plaques. Intact myelin is demonstrated in H&E. (A) and Luxol fast blue-PAS (LFB)(B) stains. A Holzer stain (C) highlights several dystrophic axons. Normal-appearing thin axons (D) and a dystrophic axon segment (E) in NAWM near an active plaque express ephrin A1. The vacuolated background indicates edema. A large dystrophic axon in MS NAWM adjacent to an active lesion expresses Eph A7 (F). This axon is swollen and shows wavy contraction, similar to the axon in the Holzer stain. A long thin normal-appearing axon segment expresses Eph A7 in NAWM adjacent to a chronic inactive plaque (G). Scale bar in A for A-C, E, F = 20 μ m; scale bar in D for D, G = 15 μ m.

	Perivascular mononuclear inflammatory cells	Perivascular/parenchymal foamy macrophages	Reactive astrocytes	Axons in active lesions	Axons in chronic inactive lesions
ephrin A1	±	+	++	++	+
ephrin A2	±	±	+	++	±
ephrin A3	±	±	++	-	-
ephrin A4	-	±	-	-	-
ephrin A5	-	-	-	±	-
Eph A1	±	-	++	±	-
Eph A2	-	-	-	-	-
Eph A3	+	++	+	+	±
Eph A4	+	±	+	+	±
Eph A5	-	-	-	±	-
Eph A6	-	±	-	-	-
Eph A7	-	±	+	+	±
Eph A8	-	-	±	-	-

Table 4. Ephrin/Eph in demyelinating disease samples.

++=staining in >90% of samples; +=staining in 20% to 90% of samples; ±=staining in 0% to 20% of samples; -=negative.

toplasmic staining of other ephrin/Ephs, including ephrin A1 and -A2 and Eph A3 (Figure 2E-G). Gemistocytic astrocytes both within and adjacent to active plaques expressed ephrin A1-A3 and Eph A1, -A3 and -A4 (Figure 2E-K). The proportions of samples with staining of these populations in demyelinating disease samples are summarized in Table 4. Inflammatory and glial ephrin/Eph expression was greatest in active lesions and was decreased or absent in chronic inactive lesions.

Normal-appearing thin axons and focally swollen, dystrophic-appearing axons and axon fragments in active lesions also showed specific expression of ephrin A1 -A2, and -A5 and Eph A3, -A4 and -A7 (Figure 3A-G; Table 4). Many of these axons were surrounded by loose irregular myelin and had foamy macrophages immediately adjacent to them indicating that they were undergoing active demyelination. Fewer chronic inactive plaque samples contained stained axons and the extent of staining within plaques with stained axons was less than in

active plaques (Table 4). Most of the ephrin/Eph-positive axons in chronic lesions were dystrophic (Figure 3H-K).

MS and OND NAWM. In routine H&E and myelin stains, NAWM adjacent to MS lesions was either indistinguishable from NAWM in normal and OND control samples (Figure 4A, B) or showed subtle abnormalities such as edema and vesiculation of myelin. Some axon abnormalities, eg, swelling and wavy contours, could also be identified in these areas in Bielschowsky preparations and Holzer-stained sections (Figure 4C). In immunostained samples of MS NAWM, both normal-appearing and abnormal axon segments were stained with Abs to several ephrin/Ephs (Figure 4D-F).

Initial analysis suggested that axonal ephrin/Eph expression in MS NAWM was more prominent in samples immediately adjacent to active lesions than in NAWM adjacent to chronic lesions (Figure 4G). Therefore, semiquantitative analysis was performed on MS and control samples stained for the ephrin/Ephs that had shown axonal staining in active MS lesions (Table 5). In NAWM samples adjacent to active lesions, the density of positively stained axons for ephrin A1 and Eph A3, Eph A4 and Eph A7 were equal to or considerably greater than the corresponding densities in the adjacent plaques. Positively stained axons were more numerous in NAWM adja-

	Stained axons/mm ² ± SE (no. samples counted) [†]					
	Active MS plaques	NAWM adjacent to active MS plaques	NAWM adjacent to chronic MS plaques	NAWM in hemisphere opposite to MS plaques	OND control WM	Normal control WM
ephrin A1	2.1 ± 1.1 (9)	10.2 ± 8.6 (8)	0.2 ± 0.1 (16)	0.2 ± 0.2 (9)	2.2 ± 1.1 (14)	0
ephrin A2	0.4 ± 0.2 (9)	0.1 ± 0.1 (10)	0.1 ± 0.1 (12)	0.0 ± 0.2 (8)	0.1 ± 0.1 (13)	0,0
ephrin A5	0.1 ± 0.01 (4)	0.1 ± 0.1 (5)	0.1 ± 0.1 (13)	0.1 ± 0.1 (8)	0.2 ± 0.2 (11)	ND [‡]
Eph A1	0.1 ± 0.1 (5)	0.2 ± 0.1 (7)	0.0 ± 0.0 (12)	0.0 ± 0.0 (7)	0.2 ± 0.1 (4)	ND
Eph A3	1.3 ± 0.8 (9)	1.0 ± 0.6 (8)[†]	0.0 ± 0.0 (15)[†]	0.0 ± 0.0 (7)[†]	0.1 ± 0.1 (7)	ND
Eph A4	0.3 ± 0.1 (10)	0.9 ± 0.5 (6)[‡]	0.2 ± 0.1 (13)[‡]	0.0 ± 0.0 (8)[‡]	0.2 ± 0.1 (10)	0
Eph A7	2.2 ± 0.9 (8)	3.3 ± 0.8 (10)[§]	0.5 ± 0.2 (17)[§]	0.2 ± 0.1 (7)[§]	1.1 ± 0.6 (12)	0
β-APP	1.2 ± 0.3 (4)	0.2 ± 0.2 (4)	0.1 ± 0.1 (10)	0.0 ± 0.0 (3)	0.0 ± 0.0 (7)	0

Table 5. White matter axonal ephrin/Eph and β-APP expression

[†]To determine the association of axon staining density with location within MS NAWM, analysis of variance (ANOVA) among the different MS NAWM sample groups was performed for each ephrin/Eph and the β-APP Ab. Significant differences related to location are in bold type.; [†]P=0.034; [‡]P=0.044; [§]P=0.001 ; [‡]ND=not done.

cent to active lesions than in NAWM adjacent to chronic plaques or in NAWM from hemispheres contralateral to the plaques. Therefore, proximity to active lesions within MS brain samples determined the extent of axon ephrin/Eph expression in NAWM. Differences among MS NAWM areas were statistically significant in sections stained for Eph A3, Eph A4 and Eph A7 (shown in bold in Table 5). There was a similar trend in sections stained for ephrin A1, but a very high value in NAWM adjacent to an active lesion (74.8 stained axons/mm²) resulted in high mean and group error that precluded statistical significance.

Axon staining densities in white matter of normal and OND controls were lower than those in MS samples for each of the ephrin/Ephs analyzed with 2 exceptions in the OND samples, ie, ephrin A1 and Eph A7 (2.2 ± 1.1 and 1.1 ± 0.6, respectively). For both Abs, this was due to high axon staining densities in the cerebral white matter of one of the OND patients who had both acute leptomeningitis and hypoxic-ischemic encephalopathy. This suggests that upregulation of these ephrin/Ephs may have been related to diffuse acute hypoxic white matter injury in that patient.

The densities of β-APP-positive and ephrin-Eph-positive axons in active lesions were similar (Table 5) but the staining patterns differed. As in the study of Kuhlmann and colleagues (35), in active MS lesions the Ab to β-APP predominantly stained axon retraction bulbs, in contrast to the longer axonal segment staining of ephrin/

Ephs (Figures 3C, E-G). Furthermore, in MS NAWM, there were few β-APP-positive axon retraction bulbs and no β-APP staining of long axon segments (Table 5).

DISCUSSION

Ephrin/Eph expression in the normal adult human CNS. Complex ephrin/Eph A expression patterns on different cell populations were identified in normal CNS samples. The neuronal and glial staining patterns observed are generally similar to those in studies of ephrin/Eph mRNA and protein in normal adult rodent CNS (22, 38, 71). However, immunostaining of mesenchymally-derived tissues, ie, Eph A7 on vascular smooth muscle and ephrin A2 on endothelial cells, was unexpected. In an immunohistochemical study of goldfish brain using the same Ab as in the present study, ephrin A2 was found to be limited to neurons (32); EphA7 expression on brain vasculature also has not previously been demonstrated. Species variations likely contribute to these apparent disparities but there currently is little other information on human CNS ephrin/Eph cellular localization.

Cerebral cortical neurons expressed most of the ephrin/Ephs but the relative lack of uniformity observed among the samples (Table 3) may have resulted from regional variations and ephrin/Eph expression gradients similar to those identified in normal adult rodents (46, 73). Variations in expression among neuron subpopulations within

cortical regions, eg for ephrin A1 and -A5 (Figure 1I, L), likely reflect plasticity of ephrin gene expression and are consistent with diverse roles of different ephrins in specific neuronal connections (10, 77). Since the control CNS samples analyzed were selected primarily for comparison to samples containing MS lesions and were predominantly from cerebral hemispheres, the present study does not represent a comprehensive neuroanatomic survey of normal human adult CNS ephrin/Eph expression. Nevertheless, since at least one neuronal or non-neuronal cell population was stained with each reagent in the majority of samples, there were internal positive controls that validated both the differences identified in expression patterns among the ephrin/Ephs and the aberrant expression identified in demyelinating disease samples. Additionally, the results suggest the potential applicability in future studies of anti-ephrin/Eph A Abs for immunohistochemical identification of specific cell populations in archival samples of paraffin-embedded CNS samples.

Altered ephrin/Eph expression in CNS injury models. Ephrin/Eph expression alterations in adult vertebrate CNS injury models likely represent attempts to recapitulate developmental programs that promote neuron survival, neurite outgrowth and reorganization, eg, through re-expression of guidance cues for axon pathfinding (18, 27, 32, 34, 54, 69, 76, 79). On the other hand, these alterations might simultane-

ously also inhibit neuron regrowth through repulsive signaling and the formation of glial scars (6). Focal injuries such as optic nerve and spinal cord transection can result in ephrin/Eph alterations not only within the lesion but on different cell populations that are connected but are located in more distant sites (34, 44, 53, 68). Therefore, in CNS injury aberrant expression can be due to anatomically remote rather than direct local effects. For ephrin/Eph signaling to occur, both ligand and receptor must be expressed and there must be direct cell-cell contacts. Furthermore, because there are multiple potential binding interactions between each ephrin/Eph, specific signaling events cannot be inferred based on the identification of upregulated expression of a single ephrin or Eph. Moreover, in development ephrin/Ephs provide signaling cues in combination and undergo dramatic changes over time. For all of these reasons, therefore, the precise functional implications of specific expression alterations may not be directly extrapolated from CNS development to human neuropathology. Because of the high degree of conservation of ephrin/Ephs among vertebrates, however, alterations identified in the adult human CNS that are similar to those in injury models probably indicate common cellular and molecular mechanisms.

Ephrin/Ephs in the immunopathogenesis of active MS lesions. Active MS lesions display a range of histologic patterns that likely reflect different pathways of injury (3, 41, 59). Although subclassification of active lesions was not feasible in the present study, the results suggest that numerous dynamic ephrin/Eph A interactions contribute to immunopathologic processes in active inflammatory/demyelinating lesions. In particular, perivascular mononuclear cell expression of ephrin A1-A3 and Eph A1, A3, A4 suggest their involvement in signaling pathways affecting immune regulation, antigen recognition, inhibition of chemotaxis (55) and cytokine production (72). Ephrin/Eph interactions may also affect T-cell adhesion to extracellular matrix molecules such as fibronectin, both within perivascular inflammatory cuffs and as they migrate within the parenchyma (58, 61).

Within and surrounding active lesions, ephrin A1-3 and Eph A1, -3, -4, -7 and -8 were identified on reactive astrocytes. Most

studies to date have addressed astrocyte ephrin/Eph B expression in the adult CNS (6, 9, 44). In a spinal cord injury model, however, Willson et al demonstrated co-expression of Eph A 3 through -8 with astrocyte GFAP (71). Murai et al showed the critical importance of astrocyte ephrin A3 in maintaining neuronal dendritic spines in the adult CNS (46); and in an editorial accompanying that study, Thompson suggested that under pathological conditions with astrocyte swelling and gliosis, interference with astrocyte ephrin A3/neuronal Eph A4 binding could result in decreased dendritic size (65). Altered astrocyte ephrin/Eph A expression might, therefore, relate to similar disturbances of functional glial/neuronal interactions in MS.

Expression of Eph A2, -A4, -A7, -B1, -B2 and -B3 by non-CNS dendritic cells has been implicated in their localization, trafficking and antigen presentation (1, 12, 74) but microglial expression has not previously been addressed. In the present study, small numbers of EphA 3-positive rod microglial cells were observed in gray and white matter in normal control samples and more numerous reactive microglia were stained for ephrin A1 and Eph A3 in neuritic plaques in Alzheimer disease and surrounding MS plaques. Foamy macrophages within active MS lesions showed even broader ephrin/Eph expression (Figure 2E-G; Table 4). These observations suggest that different levels of ephrin/Eph expression likely are associated with different pathologic functions, ie, immunologic and phagocytic, of microglia/macrophages in MS and other conditions.

Axonal ephrin/Ephs in MS plaques. Recent morphologic studies have emphasized the early occurrence and functional implications of axonal injury in MS (23, 66). The failure of neuron regeneration and growth in plaques and in NAWM is a major obstacle to endogenous repair and restoration of neurologic function in MS patients. The present study demonstrates prominent expression of ephrin A1 and Eph A3, -A4 and -A7 but not other ephrin/Ephs on dystrophic and normal-appearing axons both within and in NAWM adjacent to active MS lesions. In addition to the upregulation on glia discussed above, very similar specific patterns of upregulation of Eph A3, -A4 and -A7 mRNA and Eph A3, -A4,

-A6 and -A8 immunoreactivity on axon bundles were shown in the rat spinal cord injury model (71) suggesting that there are common injury response mechanisms. The marked upregulation of ephrin A1, an immediate early response induced by tumor necrosis factor- α (72), in active MS lesions and particularly in the hypoxic-ischemic white matter of the OND control case is also consistent with the emerging recognition of common pathogenetic patterns in hypoxic injury and MS lesions (2, 21).

It is not clear, however, whether enhanced ephrin/Eph expression is a direct consequence of injury or evidence of regeneration or both, ie, whether this expression can be considered beneficial or harmful. Enhanced expression in lesions may imply that some topographic guidance information is available to regenerated axons for re-establishing organization and connections, but enhanced repulsion signals might simultaneously inhibit regrowth. In chronic inactive lesions, decreased levels of ephrin/Eph expression were likely due to both downregulation and axonal loss. This reflects a decline both in the active immunopathologic injury and in the potential for regeneration/repair over time.

Axonal ephrin/Eph expression gradients in MS NAWM. Axonal expression of certain ephrin/Ephs was greater in MS NAWM adjacent to active lesions than in NAWM adjacent to chronic lesions, in NAWM in contralateral hemispheres of the same MS brains or in controls (Table 5). This suggests that there are lesion-associated expression gradients of specific ephrin/Ephs in MS NAWM which are affected both by the proximity to and the level of active immune-mediated injury occurring within MS plaques. Enhanced ephrin/Eph expression was often observed on swollen, injured-appearing axons (Figure 4E, F), but it was also identified on thin normal-appearing axons (Figure 4D, G). Therefore, the increased expression likely correlates not only with ongoing injury but also might indicate more subtle dysfunction of axons in white matter which both on gross inspection and on microscopic examination of Luxol fast blue stains appears normal. These observations are consistent with the current view that, in addition to demyelination and axon injury in plaques, subtle neurodegen-

erative processes in NAWM contribute to clinical progression in MS patients.

Similar levels of axon staining for β -APP and the upregulated ephrin/Ephs were seen in active lesions (Table 5) but the staining patterns differed. β -APP staining on axon retraction bulbs likely is due to accumulations resulting from disruption of axoplasmic flow (35). By contrast, upregulated ephrin/Eph axonal staining was more often observed in longer axonal segments. The greater stained axon densities of ephrin A1, Eph A1, -A4 and -A7 in the MS NAWM adjacent to active plaques than in active lesions likely is partly attributable to some net axon loss in the active lesions but also contrasts sharply with the absence of β -APP staining in MS NAWM (Table 5). These differences suggest that ephrin/Eph axonal upregulation likely involves mechanisms distinct from the disrupted axonal transport that leads to β -APP accumulation within active plaques. For example, focal upregulation of ephrin/Ephs might be due to post-injury intra-axonal protein synthesis (78). Indeed, local, RNA-based regulation of translation and cell surface Eph expression in restricted subregions of axons has been shown for Eph A2 (5). Such local regulation of synthesis and expression might account for the partial discordance between Eph A mRNA and protein expression reported in CNS injury models and could also contribute to the lack of uniformity in ephrin/Eph gene expression results in microarray analyses on MS tissues that have been performed to date.

Potential future therapeutic implications. Ephrin/Ephs have been specifically targeted in vivo with synthetic peptides and chimera proteins to affect migration, proliferation and function of neural crest and adult embryonic stem cells (9, 20, 47). These studies suggest the possibility of similar approaches for therapies of human CNS diseases, ie, both to enhance endogenous repair and to maximize the efficacy of exogenous cell therapies. However, even though the ephrin/Eph expression patterns identified in the present study are generally consistent with those observed in rodent CNS development and injury models, most of the eph/Eph expression gradients in animals involve much smaller areas than those encompassed by human MS plaques and NAWM. Furthermore, there are additional

cellular and molecular barriers such as gliosis and an inhibitory extracellular matrix in MS plaques and in adjacent white matter (60) that may impede the specific cell/cell contacts necessary for the ephrin/Eph complexes to function as they do in development and in injury models. An additional level of complexity may also arise from immune cell/CNS resident cell ephrin/Eph interactions about which essentially nothing is currently known. The present results also imply the need for targeting multiple ephrin/Eph interactions and indicate that there may be narrow temporal and regional therapeutic windows, particularly for overcoming axonal injury and loss. A better understanding of their complex roles in CNS development and in response to injury will be needed to optimize the microenvironment in lesions and in NAWM for therapeutic manipulation of ephrin/Ephs in MS and other human CNS diseases.

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