

The Classic spectrum of astrocytopathies in NMO

Astrocytic lysis

This finding was common in advanced demyelinating NMO lesions, being observed in 16/20 NMO cases, and characterized morphologically by focal loss of GFAP immunoreactivity, fragmentation of astrocytic processes, and GFAP-positive debris within macrophages.

Unexpectedly, we occasionally observed focal astrocytic lysis (Supplementary Fig. 1E, 1F), with extensive AQP4 loss (Supplementary Fig. 1C, 1D), coexisting with well-preserved myelin. We interpreted this finding to indicate that fulminant astrocytic lysis, with clearance of dead astrocytes (presumably by macrophages), preceded the development of myelin pathology (Supplementary Fig. 1G, 1H). This phenomenon supported astrocyte damage being a primary event in NMO.

“Dystrophic” morphology suggesting a degenerative astrocytic process

“Dystrophic” astrocytes were characterized by shrunken and variably-sized profiles of irregular configuration, as well as loss of stellate architecture. Some “dystrophic” astrocytes showed blunted morphology with few preserved stem branches and loss of finely branching processes (Supplementary Fig. 2) and a corresponding decrease in GFAP distribution. “Dystrophic” astrocytes were seen more often in advanced demyelinating lesions (65%, 185/286) than in non-demyelinated lesions lacking AQP4 (39%, 37/94). In addition, GFAP immunoreactivity in “dystrophic” astrocytes varied from weak to relatively strong.

Bipolar/unipolar astrocytes

Astrocytes are acknowledged to proliferate and regain stem cell potential following severe CNS injury.¹⁻³ Presumed astrocytic progenitor cells⁴ were characterized by bipolar/unipolar projections, low cytoplasmic volume, and lack of branched fine process. Ninety-one percent of NMO cases (21/23) showed bipolar/unipolar astrocytes in lesions. They were GFAP immunoreactive but lacked AQP4, AQP1 and myelin markers (PLP, MAG, and MOG) (Supplementary Fig. 3). Bipolar/unipolar astrocytes were seen mainly in white matter demyelinating lesions, especially in those lacking mature astrocytes. Polarized astrocytes have been reported in the deep cortex⁵, cerebellum (known as Bergmann cells), retina (Müller cells),

and periventricular radial glia during CNS development⁶. However, polarized glial cells found in the NMO lesions differed from these groups at the anatomical locations and expression of AQP1/AQP4.

Nuclear morphology of astrocytes in NMO lesions

Variable nuclear morphologies were seen in all lesion stages (even in non-demyelinated areas) and included multinucleation, uneven nuclear size, and mitotic figures. Multinucleated astrocytes, observed in 74% of cases (17/23), were found in 38% of active demyelinating lesions (41/109) and 19% of non-demyelinating lesions (61/323). We did not observe Creutzfeldt-Peters cells, a special type of reactive astrocyte defined by multiple fragmented nuclear inclusions (“micronuclei”) and often seen in active lesions of multiple sclerosis.

Astrocyte cytoplasmic vacuolation

Fifty-nine percent of NMO cases (13/22) exhibited cytoplasmic vacuolation in astrocytes, most commonly within demyelinating lesions and in periplaque white matter. The vacuolation varied from a few small vesicles to massive vacuolated structures (Supplementary Fig. 4). Twenty-four percent of active demyelinating lesions (40/165) showed astrocyte cytoplasmic vacuolation, while only 9% of non-demyelinating regions (38/417) showed this feature.

Astrocyte cellular hypertrophy and astrogliosis

In 87% of NMO cases (20/23), astrocytes were enlarged (hypertrophic) with increased cytoplasmic GFAP-immunoreactivity, and usually enlarged nuclear size or nuclear numbers. Hypertrophic astrocytes were found in 28% of demyelinating lesions (82/293) and 34% of periplaque non-demyelinated parenchyma (79/231). Hypertrophic astrocytes were also noted beyond lesions, at inflamed pial and periventricular CSF interfaces and perivascular regions. Typically, hypertrophic astrocytes were not evenly distributed but haphazardly arranged, in contrast to their distribution in demyelinating lesions and periplaque non-demyelinating parenchyma.⁷

Astrocytic Reactions in Relation to NMO Lesion Type

Demyelinating lesions contain severe astrocytopathies

As described in this laboratory's earlier studies,⁸ loss of AQP4-immunoreactivity was noted in active demyelinating lesions of NMO. This study noted further that astrocytes in active demyelinating lesions exhibit a wide spectrum of morphological changes: 90% had evidence of astrocytic lysis (187/208) and 75% contained "dystrophic" astrocytes (133/177). These lesions occasionally contained hypertrophic astrocytes as well (55/178); bipolar/unipolar astrocytes were seen in 73% (124/171), and 16% contained Rosenthal fibers (31/198). Except for Rosenthal fibers, all of these changes were less frequent in inactive demyelinating lesions.

Astrocytic reactions in non-demyelinating lesions with AQP4 loss

Independent of the degree of demyelination, regions of early AQP4 loss showed all types of astrocytic changes, hypertrophy, "dystrophy", lysis, Rosenthal fibers; occasional astrocytes were bipolar or unipolar. Morphological changes in regions of early AQP4 loss (Supplementary Fig. 8, 9) were less severe than in advanced demyelinating lesions.

Astrocytic reactions with respect to complement deposition

A majority of regions with astrocytic lysis (66%, 111/169) or AQP4 loss (63%, 155/248) showed complement C9neo deposition. Regions that exhibited astrocyte lysis or AQP4 loss but lacked deposits of terminal complement activation components were consistent with astrocyte injury occurring in NMO independent of assembly of the cytolytic membrane attack complex. The spectrum of sublytic astrocytopathy in C9neo-negative regions did not differ from that seen in C9neo-positive regions. Sublytic morphological changes varied from mild AQP4 loss to dystrophic astrocytes and Rosenthal fiber deposition.

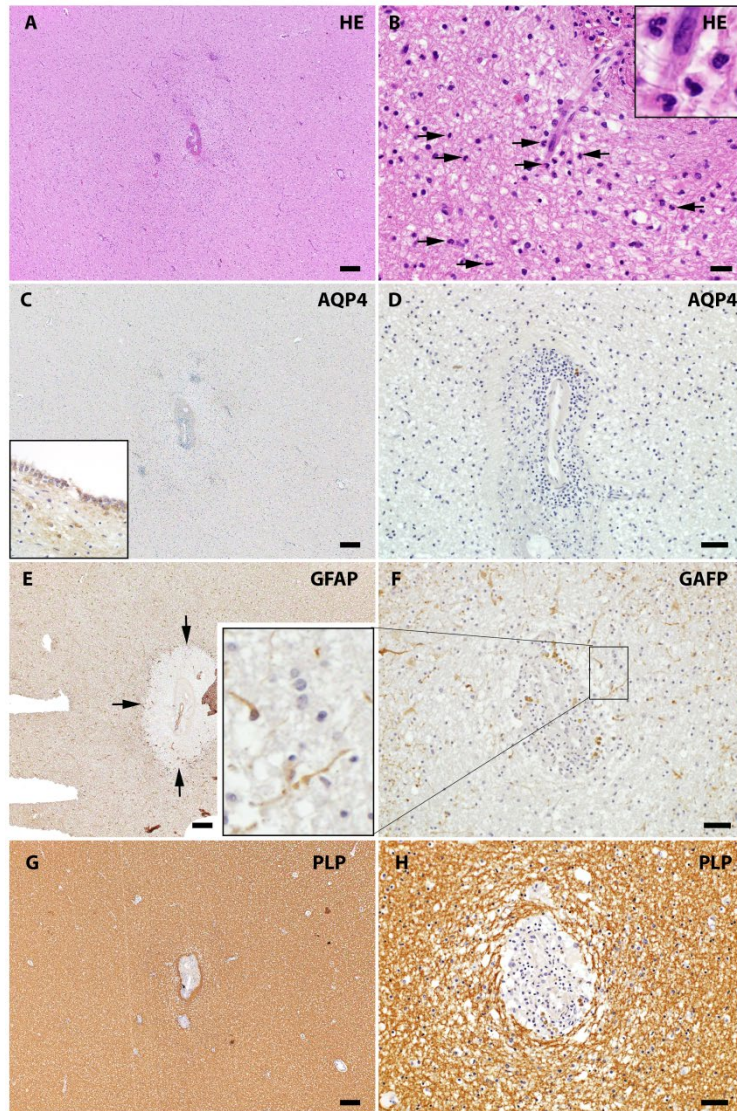
Astrocytic reactions with respect to microglial reactions

Astrocytic reaction was observed in regions with profound microglial activation. Most regions with AQP4 loss or reduction (95%, 189/200) exhibited microglial activation. Microglial/macrophage activation, highlighted by enhanced CD68 immunoreactivity, was observed in 92% (230/250) of regions with hypertrophic astrogliosis. Morphologically, most

CD68-positive cells resembled ramified microglia rather than foamy macrophages. Microglia predominated in 29% (47/162) of areas exhibiting only dystrophic astrocytes, compared to 87% (148/171) of areas exhibiting only hypertrophic astrocytes. Foamy macrophages were more prominent than ramified microglia in 71% (115/162) of areas with predominant dystrophic astrocytes.

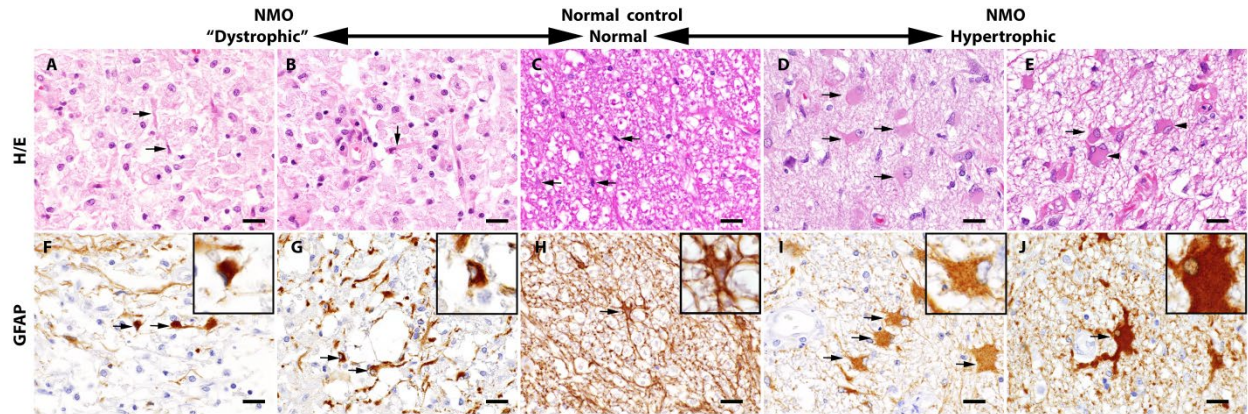
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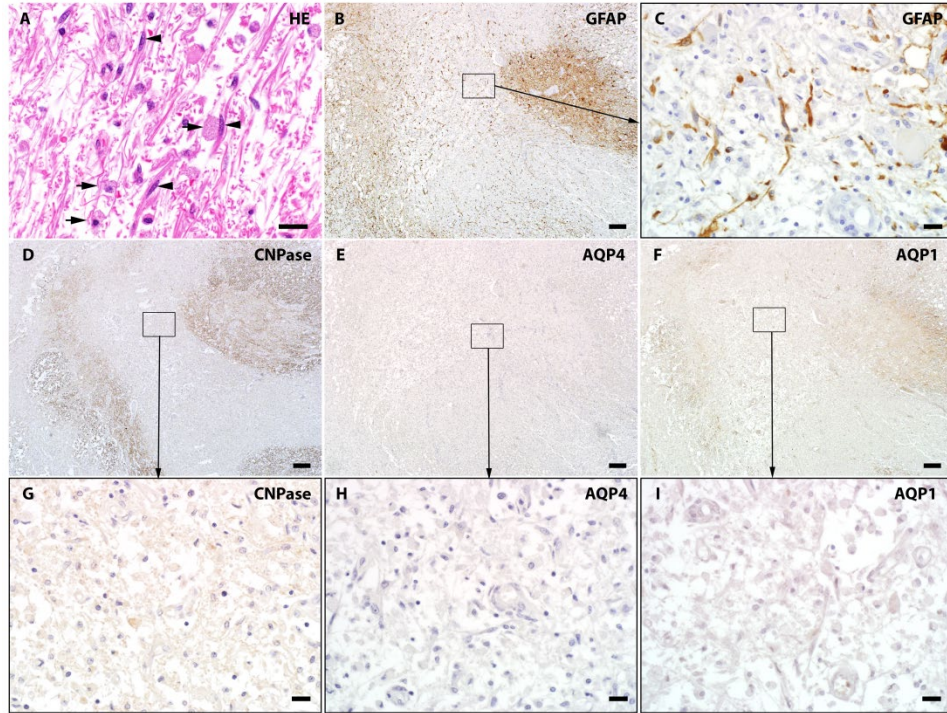
Supplementary figure 1. Astrocytic lysis in an early pre-demyelination lesion in NMO

(A) H&E stain shows relatively intact white matter in the brain tissue of a NMO patient . (B) At high power, mild tissue vacuolation with infiltrates of numerous granulocytes (arrows, enlarged view in the inset). (C and D) AQP4 immunohistochemistry highlights extensive AQP4 loss in the same region on the consecutive section. The inset in (C) shows preserved AQP4 immunoreactivity in the periventricular region on the same tissue block. (E) Immunohistochemistry shows focal perivascular GFAP loss suggesting astrocyte lysis (arrows). (F) The enlarged view of panel E indicates loss of astrocytes in the perivascular region with only a few GFAP bipolar/unipolar astrocytes present. (The framed region is enlarged as indicated.) (G) However, the PLP stain shows no demyelination. (H) The high power view of PLP reveals the intact myelin structure and mild tissue vacuolation in this region. Scale bars :200 μm (A, C, E, G), 20 μm (B). 50 μm (D, F, H)



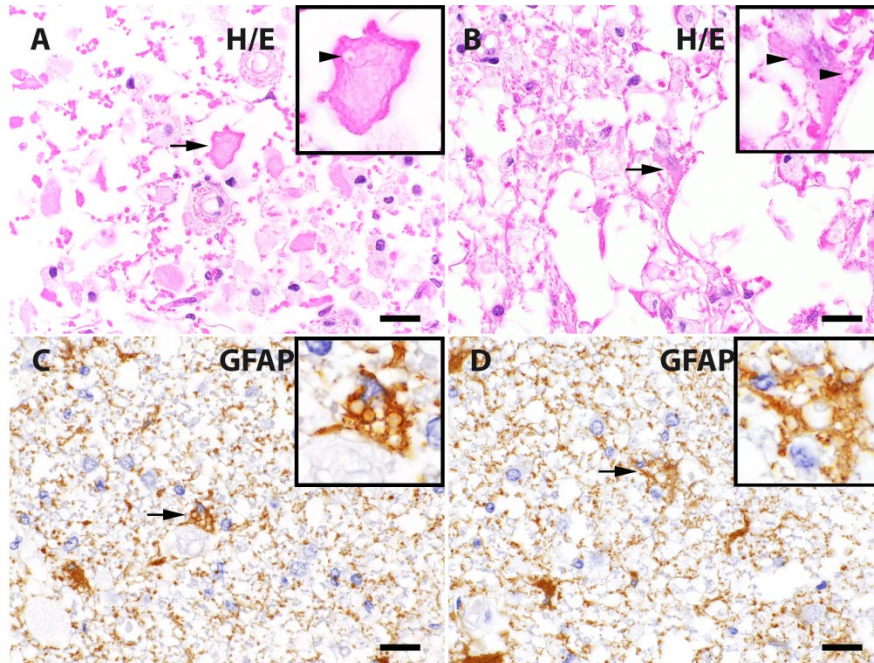
Supplementary figure 2. The variable morphological changes of astrocytic profiles in NMO

Astrocytes show variable morphologies in NMO (A, B, D, E, F, G, I and J) compared with normal CNS control (C and H) on the H&E (A-E) and GFAP (F-J) stains. “Dystrophic” astrocytes: (A) and (B) H&E stain shows some “dystrophic” astrocytes with shrunken cell bodies (arrows). GFAP immunohistochemistry (F, G) identified the “dystrophic” cells with small size and few processes as astrocytes (arrows). Normal control: (C) The astrocytes (arrows) in the normal CNS tissue are barely distinguishable on the H&E staining due to its cytoplasm staining matching the surrounding parenchyma. (H) GFAP immunohistochemistry shows the “star” shape of the astrocyte (arrow) and networks formed by astrocytic processes. Hypertrophic astrocytes: (D) and (E) H&E stain highlights the hypertrophic reactive astrocytes (arrows) with increased body sizes and eosinophilic cytoplasm in the NMO lesions. Some hypertrophic astrocytes show multiple nuclei (arrowhead). The fine processes are also preserved around the astrocytes. GFAP immunohistochemistry highlights the abundant GFAP immunoreactivities in the hypertrophic astrocytes (I) and (J). Scale bars: 20 μ m.



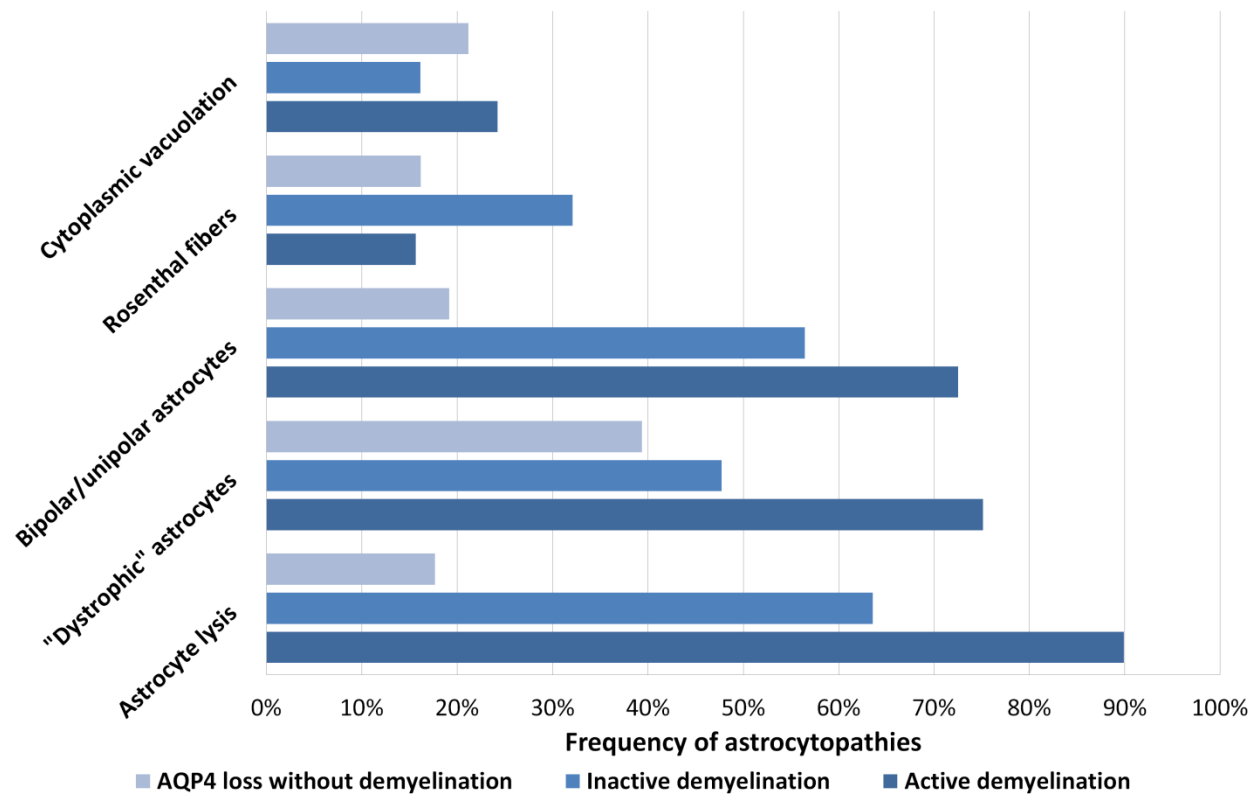
Supplementary figure 3. The bipolar/unipolar astrocytes in NMO lesions

(A) H&E stain shows an active lesion with foamy macrophages (arrows) infiltrating the spinal cord of an NMO patient. Some elongated bipolar/unipolar cells with limited cytoplasm are also noted (arrowheads). (B) Immunohistochemistry highlights decreased GFAP positive cells in the lesions suggesting loss of astrocytes. (C) The enlarged view of the framed region in B shows the elongated bipolar/unipolar cells are GFAP positive. Additional immunohistochemical studies show these bipolar/unipolar cells are negative for myelin CNPase (D and G), AQP4 (E and H), and AQP1 (F and I). Scale bars: 20 μ m (A, C, G, H, I), 200 μ m (B, D, E, and F).



Supplementary figure 4. Astrocyte vacuolation in NMO

(A) and (B) HE stains of the NMO destructive lesions show reactive astrocytes with obvious vacuolation (arrowhead). (C) and (D) GFAP immunohistochemistry highlights numerous cytoplasmic vacuoles (enlarge view in the insets) in the reactive astrocytes (arrows). Scale bars: 20 μm (A-D).



Supplementary figure 5. Comparison of different astrocytopathies and astrocyte damage in NMO lesions at different stages

Active demyelinating lesions show the highest frequency of astrocyte lysis, “dystrophy”, and bipolar/unipolar astrocytes, suggesting prominent astrocytic damage, degeneration, and regeneration in the most advanced lesion stages of NMO. Early-stage NMO lesions, exhibiting AQP4 loss but no demyelination, also show all types of astrocytopathy, but of milder degree. This suggests astrocytopathy is an early event in NMO lesion development. The extent of astrocytopathic changes in inactive demyelinated lesions is intermediate between those of active demyelination and non-demyelinated lesions with early AQP4 loss, except for Rosenthal fibers. The high frequency of Rosenthal fibers in astrocytes residing in inactive demyelinated lesions is consistent with a long-term stress reaction and upregulation and aggregation GFAP and other stress proteins in NMO. Cytoplasmic vacuolation is more frequent in lesions exhibiting active demyelination and early AQP4 loss.