Evidence of Müller Glial Dysfunction in Patients with Aquaporin-4 Immunoglobulin G—Positive Neuromyelitis Optica Spectrum Disorder

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Purpose: To compare functional and structural changes in the retina in patients with aquaporin-4 immunoglobulin G (AQP4-IgG)-positive neuromyelitis optica spectrum disorder (NMOSD) and multiple sclerosis (MS).

Design: Cross-sectional study; biochemical study of human retinas.

Participants: A total of 181 participants, including 22 consecutive patients (44 eyes) with NMOSD, 131 patients (262 eyes) with multiple sclerosis (MS), and 28 normal subjects (56 eyes). In addition, 8 eyeballs from healthy donors were used for biochemical analysis.

Methods: Full-field electroretinography (ERG) and spectral-domain OCT were performed in all the subjects. Topography of AQP4 expression and Müller glial distribution were analyzed using Western blotting and immunohistochemistry.

Main Outcome Measures: Full-field ERG parameters, including amplitudes and peak times. Tissue volume of each of the retinal layers at the fovea by OCT segmentation. Levels of AQP4 expression at different retinal regions.

Results: The b-wave amplitude was significantly reduced in patients with AQP4-IgG+ NMOSD in scotopic ERGs (compared with AQP4-IgG- subjects, patients with MS, and normal controls) but not in photopic ERGs. Further analysis showed that this b-wave change was mainly caused by reduction of the slow PII component, suggesting specific Müller cell dysfunction. We also found thinning of specific retinal layers at the fovea in patients with AQP4-IgG+ NMOSD, in the Henle fiber outer nuclear layer (HFONL) and the inner segment (IS) layer, but not in the inner nuclear layer (INL), outer plexiform layer (OPL), or outer segment (OS) layer. Furthermore, there was a significant association between foveal HFONL-IS complex thinning and scotopic b-wave amplitude reduction (P = 0.005 ~ 0.01, fixed-effects model). Western blotting demonstrated that Müller cell—specific AQP4 was expressed at a higher level at the fovea than the peripheral retina. Immunohistochemical studies revealed that the specific foveal thinning reflected the topography of AQP4 expression and Müller glial distribution in the human macula.

Conclusions: This study provides in vivo structural and functional evidence of Müller glial dysfunction in eyes of patients with AQP4-IgG+ NMOSD. Topography of retinal structural change is supported by distribution of Müller cells and patterns of AQP4 expression. The study suggests that visual electrophysiology and retinal imaging could be useful biomarkers to assess the potential retinal astrocytopathy in NMOSD. Ophthalmology 2019;126:801-810 © 2019 by the American Academy of Ophthalmology. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Supplemental material available at www.aaojournal.org.

Since the discovery of serum aquaporin-4 immunoglobulin G (AQP4-IgG) antibodies in patients with neuromyelitis optica, the disease has been distinguished from multiple sclerosis (MS) and recognized as a broadened spectrum of AQP4-IgG—mediated disorders (neuromyelitis optica spectrum disorder [NMOSD]). Differential patterns of optic nerve damage in NMOSD and MS have been described. However, the exact mechanisms of neuroinflammation, axonal loss, and demyelination in NMOSD remain unknown. Given the fact that AQP4, functioning as a mercury-insensitive water channel protein, is highly concentrated in astrocyte endfeet, NMOSD has been hypothesized to be primarily an astrocytopathy. It was shown that the binding of IgG to AQP4 water channel is associated with AQP4 internalization, reduced glutamate uptake, and impaired water flux in astrocytes, which may subsequently lead to pathological changes in NMOSD such as edema and inflammation. In addition, AQP4-IgG
can affect the interactions between astrocytes and endothelial cells at the blood—brain barrier level, causing enhanced leukocyte migration.6

However, it is difficult to directly test the theory of astrocytopathy in vivo in patients with NMOSD. Although the retina is a part of the central nervous system, it has a specific glial component: Müller cells. Compared with the brain and the spinal cord, pathological changes in the visual system are more clinically measurable.10 Reduction of the retinal thickness at the fovea has been detected by OCT in a small group of patients with AQP4-seropositive non-optic neuritis (ON) NMOSD.11 Considering the fact that the fovea is enriched in AQP4-expressing Müller cells, the results of this study might suggest a primary retinal astrocytopathy.12 However, direct evidence of the involvement of Müller glial cells in patients with NMOSD is still lacking.

Full-field electroretinography (ERG) is a widely used ophthalmic clinical test that provides a measure of the neuroretinal function.13 The cornea-positive b-wave, or PII component of the ERG, is primarily driven by ON bipolar and Müller cells. The PII signal can be further divided into a fast PII element, which is generated by bipolar cells, and a slow Müller glial response (slow PII).14 This provides a potential means of assessing Müller glial function in vivo in humans. In this study, we performed full-field ERGs in patients with AQP4-IgG+ NMOSD to assess retinal neurofunction. The same recording protocol was also used in patients with MS and normal subjects for comparison. Spectral-domain OCT coupled with the most advanced segmentation software represents state of the art technology to analyze retinal structure, including precise measurement of various retinal layers. Therefore, in the current study, foveal microstructural changes in patients with AQP4-IgG+ NMOSD were analyzed by using detailed OCT segmentation. In addition, the patterns of AQP4 expression and Müller cell distribution were assessed in postmortem human retinas. This study supports a specific Müller glial dysfunction in AQP4-IgG+NMOSD and suggests that the Müller glial changes could be associated with subclinical foveal thinning in the disease.

**Methods**

**Participants**

Consecutive AQP4-IgG+ NMOSD and relapsing-remitting patients with MS were recruited between December 2015 and March 2017 from 4 tertiary neurology and ophthalmic clinics in Sydney (Royal North Shore Hospital, Brain and Mind Centre, Inner West Neurology, and Save Sight Institute). Diagnosis of NMOSD was made as per the 2015 revised diagnostic criteria.17 All the patients with NMOSD were tested for AQP4-IgG and anti-myelin oligodendrocyte glycoprotein immunoglobulin G (MOG-IgG) antibodies using commercially available cell-based assays (EUROIMMUN).18 Patients with NMOSD who were negative for AQP4-IgG or positive for MOG-IgG were included as AQP4-IgG+ NMOSD subjects. Multiple sclerosis was diagnosed according to the McDonald criteria.19 Patients were functionally assessed using the Expanded Disability Status Scale (EDSS). Exclusion criteria included retinal, optic nerve, or other neurologic diseases (other than ON) affecting the visual system. In addition, healthy participants with similar sex and age distributions were also recruited as controls. This research study adhered to the tenets of the Declaration of Helsinki and was approved by the Human Research Ethics Committee of the University of Sydney (Sydney, Australia). An informed consent was obtained from all participants.

**Full-Field ERG**

Full-field ERG recordings were performed as per the International Society for Clinical Electrophysiology of Vision standard18 using an ESPION system (Diagnostics LLC, Lowell, MS).19 Pupils were dilated with tropicamide 1%. Dark-adapted (DA) ERG responses were recorded to white flashes with stimulus strengths of 0.01 cd m s−2 (DA 0.01), 3.0 cd m s−2 (DA 3.0), and 12.0 cd m s−2 (DA 12.0), respectively, after 20 minutes dark adaptation. For light-adapted (LA) ERGs, after patients were exposed to 30 cd m−2 background luminance for 10 minutes, single-flash cone response (LA 3.0) and 30 Hz flicker were recorded accordingly. Both eyes were recorded in all the participants.

**OCT Imaging and Segmentation**

OCT scans were performed using a Spectralis spectral-domain OCT scanner (Heidelberg Engineering, Heidelberg, Germany). A peri-papillary circular scan (diameter: 3.5 mm, manual placement of ring) and a macular radial star-like scan were obtained as previously described.20,21 All OCT images fulfilled the OSCAR-IB criteria.22 Automated retinal segmentation (Eye Explorer software version 6.9.5.0) was performed at the central macular region over a distance of 2 mm (1 mm on each side of the fovea centralis) on each of the 6 slices of the star-like scan. Because the ganglion cell inner plexiform layer can be affected by not only ON but also retrograde trans-synaptic degeneration,23 only the thicknesses of each of the middle to outer retinal layers were analyzed, including the inner nuclear layer (INL), the outer plexiform layer (OPL), the Henle fiber outer nuclear layer (HFNOL) (the 2 layers were combined for analysis because the border between the Henle fiber layer and the outer nuclear layer is not consistently identifiable on macula OCT scans), and inner segment (IS) and outer segment (OS) layers of photoreceptors. In addition, tissue volume of those layers was also calculated on the basis of averaged thickness. All tests were performed with the same device under room light conditions, and the results presented in this study were in line with the international APOSTEL criteria.24

**Western Blots of Human Retinas**

Fresh retinal punches (2-mm diameter) from different locations of the human retina were isolated for Western blot as described previously.25 Human retinas were obtained from healthy donors (Lions NSW Eye Bank) with consent and approval of the University Human Research Ethics Committee. Briefly, retinal punches were lysed in RIPA buffer (Sigma Aldrich, St. Louis, MO) with protease inhibitor for 10 minutes and then centrifuged at 12 000 g for 10 minutes. The supernatant was collected and heated with reducing buffer and NuPAGE loading buffer (Life Technologies, Carlsbad, CA) at 70°C for 10 minutes. The samples were then loaded onto NuPAGE 4-12% Bis-Tris Protein Gels (Thermo Fisher Scientific, Waltham, MA) and electrophoresed at 180V, 4°C for 70 minutes. The proteins were transferred to polyvinylidene difluoride membranes and then blocked with 5% bovine serum albumin for 1 hour. The membrane was incubated with primary antibody overnight at 4°C: AQP4 (Merck Millipore [Burlington, MA] AB3594, 1:500) or a/b tubulin (Cell Signaling [Danvers, MA], #2148, 1:1000) and then horseradish peroxidase–conjugated secondary antibody at room temperature for 2 hours on the following day. After washing, the membranes were incubated in Clarity Western ECL Substrate (Bio-Rad [Hercules, CA]) for 5 minutes and
Immunofluorescent Staining

Human macula was isolated for immunofluorescence staining as described previously. Briefly, retinal vibratome sections were blocked with 5% normal goat serum overnight. Sections were then incubated with primary antibodies, AQP4 (Merck Millipore, AB3594, 1:2000) and cellular retinaldehyde-binding protein (CRALBP; Abcam [Cambridge, UK], ab15051, 1:500), for 4 nights at 4°C. Thereafter, vibratome sections were incubated with the species-specific secondary antibodies conjugated with Alexa Fluor 488 (green) or 594 (red) ( Molecular Probes, Eugene, OR) at a 1:1000 dilution at 4°C overnight. Nuclei were stained with Hoechst (Thermo Fisher Scientific) for 15 minutes. After being mounted with VECTASHIELD antifade mounting medium (Vector Laboratories, Burlingame, CA), the immunofluorescent images of vibratome sections were captured with a ZEISS (Oberkochen, Germany) confocal laser-scanning microscope.

Statistical Analysis

Full-field ERG parameters (including amplitudes and peak times of DA rod response, mixed rod/cone response at 2 levels of flash intensity, LA cone response, and 30 Hz flicker) and thicknesses of retinal layers were compared between patient groups and healthy controls. Data from both eyes were included for the comparison between groups using the generalized estimating equation model (SPSS version 22.0, IBM, New York, NY). Age, gender, disease duration, and history of ON were all included as covariates. The correlation between ERG and OCT parameters in patients with AQP4-IgG+ NMOSD was determined by using the fixed-effects model for combined right and left analysis for P values in addition to Pearson correlation coefficient. D’Agostino-Pearson omnibus normality test was used to determine whether data were sampled from Gaussian distributions. The comparison of AQP4 expression between the macula and the peripheral retina in the Western blotting study was performed by using paired t test. GraphPad Prism (version 7.0, GraphPad Software Inc., La Jolla, CA) was used for graphs and data visualization. P < 0.05 was considered statistically significant.

Results

Müller Glial Dysfunction in Patients with AQP4-IgG+ NMOSD

In total, 22 patients with NMOSD, 131 patients with MS, and 28 healthy controls were recruited. Demographics of all the participants are shown in Table 1. Fifteen of the patients with NMOSD in this study showed positive AQP4-IgG antibodies (AQP4+), and the other 7 patients were AQP4-IgG negative (AQP4−). Three of the AQP4− patients were positive for MOG-IgG antibodies. Both eyes of the participants were analyzed, including 44 NMOSD eyes, 262 MS eyes, and 56 normal eyes in total.

We first separated the patients with NMOSD into 2 groups based on AQP4-IgG serology. No differences were observed between normal subjects and patients with AQP4− NMOSD in all the ERG parameters (Table S1, available at www.aajoournal.org). However, we found significantly reduced b-wave amplitudes in patients with AQP4+ NMOSD in all the scotopic ERGs, including DA 0.01, DA 3.0, and DA 12.0 (Fig 1A). These retinal functional changes were not associated with the history of ON or the visual acuity (P > 0.05). Also, the reduced b-wave amplitudes (averaged right and left eyes) were not associated with EDSS or disease duration (P > 0.05). In contrast, no b-wave amplitude reduction in scotopic ERGs was found in the patients with MS (Fig 1A). In addition, no changes were found in the photopic ERGs (LA 3.0 [Fig 1C] and 30 Hz flicker) in AQP4+ NMOSD (P > 0.05). As described earlier, the Müller cell response contributes to the tail of the scotopic b-wave of ERGs. The pattern of isolated scotopic b-wave loss in AQP4+ NMOSD strongly supported Müller cell dysfunction (bipolar cell dysfunction would have affected both scotopic and photopic ERGs). In the DA 0.01 ERG (the waveform of which is less influenced by the photoreceptor driven a-wave), the b-wave functional loss in AQP4+ NMOSD subjects was further demonstrated by subtracting the averaged ERG trace from these patients from that of normal subjects. We obtained a waveform (Fig 1B, blue trace) that is typical of the Müller cell–driven slow PII response.

The ERG changes in patients with MS were consistent with our previous findings. By using a larger sample size, we found that MS-related retinal dysfunction was predominantly in photopic ERGs (Table S1, available at www.aajoournal.org).

Foveal Microstructural Changes in Patients with AQP4-IgG+ NMOSD

To investigate whether subclinical foveal thinning in AQP4+ NMOSD as hypothesized by the previous study is related to Müller glial pathology, we first performed detailed retinal segmentation at the foveal to parafoveal macula region by using OCT scans (Fig 2A). Group-based analysis revealed specific tissue loss in HFONL and IS in AQP4+ NMOSD but not in the other retinal layers (INL, OPL, or OS of photoreceptors) (Fig 2B). We did not observe any thickness difference between ON and non-ON eyes in patients with AQP4+ NMOSD in all the middle to outer retinal layers. We did not include AQP4− NMOSD in the OCT analysis because of the small sample size (MOG+ NMO may represent a separate disease and relatively high intersubject variability of retinal thickness. In patients with MS, there was no difference between ON and non-ON eyes in INL, OPL, IS, or OS layers at the fovea. However, a small degree of thickening in HFONL was found in ON versus non-ON eyes in MS (tissue volume: MS-NON = 0.25 mm3 [95% confidence interval, 0.249–0.260] vs. MS-ON 0.26 mm3 [95% confidence interval, 0.259–0.270]; P = 0.001 by the generalized estimating equation model). To avoid potential effects of MS-ON on HFONL, we repeated the tissue volume analysis by excluding MS-ON eyes. We found that the reanalysis did not change the conclusion that there is HFONL thinning in AQP4+ NMOSD compared with MS (Fig S1, available at www.aajoournal.org). We further mapped the retinal thicknesses of HFONL and IS layers from the fovea centralis to the parafoveal region. As shown in Figure 2C, HFONL thinning in AQP4+ NMOSD was more evenly distributed (slightly more at the center), whereas reduction of IS thickness was found to be mainly localized at the central fovea.

Specific Foveal Thinning in OCT as a Biomarker of Müller Glial Dysfunction

We combined the volume of HFONL and IS layers at the foveal region and correlated the tissue volume of HFONL-IS complex with Müller cell dysfunction measured by the b-wave amplitude of DA ERGs. Of interest, HFONL-IS thinning in patients with AQP4+ NMOSD was found to be associated with the b-wave amplitude in DA 0.01 ERG (P = 0.01; Fig 3A). In addition, there was also a significant correlation between HFONL-IS volume and the b-wave amplitudes in DA 3.0 ERG (P = 0.005; Fig 3B), as well as in DA 12.0 ERG (P = 0.01; Fig 3C). We did not observe a significant association between HFONL-IS volume and disease duration or severity (EDSS/visual acuity). These results

imaged using the G-Box imaging system (In Vitro Technologies, Noble Park North, VIC).

You et al • Müller Glial Dysfunction in AQP4+ NMOSD

803
suggest that thinning of foveal HFONL-IS complex could be related to Müller cell dysfunction in AQP4+ NMOSD and can (together with visual electrophysiology) potentially be used as an in vivo biomarker to evaluate AQP4-related retinal glial pathology in the disease.

Table 1. Demographic Data of the Study Participants

<table>
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<th>NMOSD</th>
<th>MS</th>
<th>Control</th>
<th>P Value</th>
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<tr>
<td>n</td>
<td>22</td>
<td>131</td>
<td>28</td>
<td></td>
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<tr>
<td>Age (yrs) (mean ± SD)</td>
<td>46.91±14.53</td>
<td>43.55±10.09</td>
<td>40.79±14.04</td>
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<td>Sex (male: female)</td>
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<td>36:95</td>
<td>7:21</td>
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<td>Disease duration (yrs [median (range)]</td>
<td>3 (1–80)</td>
<td>4 (1–25)</td>
<td>-</td>
<td>0.25 (c)</td>
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<tr>
<td>EDSS [median (range)]</td>
<td>2 (0–6.5)</td>
<td>1 (0–6)</td>
<td>-</td>
<td>0.02 (c)</td>
</tr>
<tr>
<td>Eyes (n)</td>
<td>44</td>
<td>262</td>
<td>198</td>
<td>56</td>
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<tr>
<td>ON</td>
<td>27</td>
<td>64</td>
<td>-</td>
<td>-</td>
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<tr>
<td>non-ON</td>
<td>17</td>
<td>198</td>
<td>-</td>
<td>-</td>
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<tr>
<td>tRNFL (µm) (mean ± SD)</td>
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<td>49.7±14.6</td>
<td>-</td>
<td>0.05 (d)</td>
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<tr>
<td>ON</td>
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<td>49.7±14.6</td>
<td>-</td>
<td>0.05 (d)</td>
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<td>non-ON</td>
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<td>BCVA (logMAR) (mean ± SD)</td>
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<td>&lt;0.001 (d)</td>
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<td>ON</td>
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<td>-0.01±0.19</td>
<td>-</td>
<td>&lt;0.001 (d)</td>
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<td>-0.06±0.11</td>
<td>-0.07±0.08</td>
<td>&gt;0.05 (d)*</td>
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Statistics: (a) unpaired t test; (b) chi-square test; (c) Mann–Whitney test; (d) generalized estimating equation.

*NMOSD versus MS: P = 0.03; normal versus MS: P < 0.001; NMOSD versus normal: not significant.

The P values of comparisons among NMOSD, MS, and normal all > 0.05.

Next, we sought to determine whether the described foveal microstructural changes are related to patterns of AQP4 expression in the human retina.

OCT Changes Reflect Patterns of AQP4 Expression in the Human Retina

Figure 1. Comparisons of averaged scotopic electroretinograms (ERGs) from normal controls and patients with neuromyelitis optica spectrum disorder (NMOSD) and multiple sclerosis (MS). A, There was a significant b-wave amplitude reduction in aquaporin-4+ (AQP4+) patients but not in AQP4− patients and patients with MS (P values were 0.01, 0.02, and 0.04 for DA 0.01, DA 3.0, and DA 12.0 ERGs, respectively). B, The averaged b-wave trace of dark-adapted (DA) 0.01 ERG from AQP4+ patients (red) was subtracted from that of normal subjects (green) using best fitting 5th order polynomials. The obtained trace (blue) represented scotopic b-wave loss in AQP4+ NMOSD, which is typical of the Müller cell response (slow PII). C, No changes were found in photopic ERGs in AQP4+ NMOSD. Averaged traces of light-adapted (LA) 3.0 ERG from AQP4+ patients (red) and normal subjects (green) are shown.
Figure 2. Foveal microstructural changes in patients AQP4+ NMOSD by OCT segmentation. A, OCT-based automated macula segmentation. The inner nuclear layer (INL), outer plexiform layer (OPL), Henle fiber/outer nuclear layer (HFONL), and inner segment (IS) and outer segment (OS) layers of photoreceptors were identified and analyzed. The border (red arrows) between the Henle fiber layer and the outer nuclear can be seen in some retinal areas but is not consistent. Therefore, those 2 layers were combined for analysis. B, Tissue volume loss was found only in HFONL (P values were 0.03 between AQP4+ and normal, and 0.008 between AQP4+ and MS) but not in the INL, OPL, or OS. IS tissue loss was identified only at the central fovea. Dashed lines represent standard error of the mean. AQP4 = aquaporin-4; GCiPL = ganglion cell inner plexiform layer; ILM = inner limiting membrane; MS = multiple sclerosis; NMOSD = neuromyelitis optica spectrum disorder; OLM = outer limiting membrane; RNFL = retinal nerve fiber layer.

You et al • Müller Glial Dysfunction in AQP4+ NMOSD

805
and Müller cell distribution in the human retina. We first explored topographic distribution of AQP4 protein expression by using a series of 2-mm fresh retinal punches from different locations across the retina. We found that tissue punches from the fovea exhibited significantly higher levels of AQP4 expression than punches from the mid-peripheral (5–7 mm from the fovea, ~60% of the AQP4 level in the fovea) and the far-peripheral retinas (9–11 mm from fovea, ~30% of the AQP4 level in the fovea), regardless of the intersubject variations among the donors (Fig 4A, B) (demographic data of the 4 donors in Table S2, available at www.aaojournal.org). No difference in AQP4 expression was found among the superior, inferior, nasal, or temporal peripheral retinal areas.

To further examine specific patterns of AQP4 expression in different retinal layers in the AQP4-rich central macular area, we performed immunofluorescent staining on the human macular retina using CRALBP (red, a Müller cell marker) and AQP4 (green) (Fig 5). High-magnification images demonstrated strong colocalization of CRALBP and AQP4 (Fig 5B). The results are consistent with previous reports that AQP4 in the retina is predominantly expressed in Müller cells.31 More importantly, we found that AQP4 is strongly expressed in the Henle fiber layer at the fovea (Fig 5A). In contrast to the peripheral retina, Müller glia at the macula have unique cellular morphology. Macular Müller cells run vertically from the inner limiting membrane, then horizontally toward the fovea (forming the Henle fiber layer), and again vertically to the outer limiting membrane,53 enveloping photoreceptor cell bodies within the outer nuclear layer (Fig 6). The central part of the external limiting membrane is mainly formed by the endfeet of those Müller cells. In the current study, we also observed AQP4 expression by Müller cell endfeet in the outer limiting membrane microvilli at the central part of the fovea (Fig 5B). The location of AQP4 expression and alignment of Müller cells at the foveal region are demonstrated in Figure 6. This is in agreement with our OCT findings, which demonstrated that retinal thinning in patients with AQP4+ NMOSD is specifically seen in HFONL and the central foveal part of IS.

**Discussion**

This study presents in vivo electrophysiologic evidence of potential Müller cell dysfunction in patients with AQP4+ NMOSD, which was associated with thinning of specific retinal layers at the fovea. The fact that Müller glial dysfunction was seen only in AQP4+ but not in AQP4− patients supports the notion that AQP4+ NMOSD is indeed an astrocytopathy mediated by AQP4-IgG autoantibodies. Our findings are in line with previously published OCT studies in patients with NMOSD, which demonstrated foveal thinning in non-ON eyes, a possible sign of retinal astrocytopathy.11,33 However, contrary to OCT imaging, which could not establish a direct link between the morphologic changes and Müller cell pathology, full-field ERG directly records the neuroretinal responses to light, each ERG wave component representing the function of specific types of retinal cells. Therefore, this technique may provide a clinical measure of the retinal glial dysfunction in vivo. In this study, we further demonstrate that the foveal thinning in NMOSD is specifically localized in the HFONL-IS complex where AQP4 is highly expressed by Müller cells. Furthermore, HFONL-IS complex thinning was associated with Müller glial dysfunction measured by full-field ERG. Together, the findings provide in vivo evidence of a subclinical retinal glial pathology in AQP4+ NMOSD.
It is believed that the electrophysiologic response of Müller cells is mainly generated by the K+-induced flow of currents through the cell body and extracellular space. The K+ plays an important role in maintaining the electrochemical gradients across cell membranes for normal neuronal activity. Certain potassium channels participate in this process: K+ enters Müller cells via inwardly rectifying potassium channel 2.1 (Kir2.1) and leaves the cell via Kir4.1.14 Of interest, it has been recently shown that AQP4 water channel is involved in regulating potassium homeostasis under physiologic conditions. Coexpression of AQP4 and Kir4.1 has been described in the end foot membrane of astrocytes and Müller cells, suggesting the role of AQP4 in K+ clearance and special buffering. Aquaporin-4 was also found to be coupled with potassium homeostasis, and impaired K+ clearance was seen in AQP4−/− mice. In addition, significantly reduced ERG b-wave has been observed in AQP4 knockout mice. Therefore, it is likely that the Müller cell dysfunction seen in the current study might reflect a similar retinal pathology of impaired AQP4 function and potassium homeostasis in AQP4−/− subjects. In addition, intravitreally injected AQP4-IgG was found to deposit on Müller cells and lead to inflammation and retinal pathology. It is also important to note that the distinct patterns of ERG changes in AQP4+ and AQP4− patients suggest these 2 NMOSD subgroups may have different mechanisms with regard to the involvement of astrocytes.

It is well known that Müller cells in the human macula have unique morphology compared with those in the peripheral retina. Previous studies have also found a special group of cone-shaped zone of Müller cells that composes the central and inner part of the fovea. Besides the morphologic diversities, we also found that macular and peripheral Müller cells have different levels of expression of some functional proteins to adapt the microenvironment in different regions of the retina (Zhang et al, unpublished data, 2018). One recent study suggested that AQP4 is highly expressed by macular Müller cells, contributing to a specialized glymphatic system and macular edema. In this study, we performed detailed analysis of disturbance of AQP4 and Müller cells in the human retina, which may help explain the fact that the macula is more sensitive to disease conditions compared with the peripheral retina.

This study also confirmed our previous findings of INL dysfunction in MS, which is associated with retinal nerve fiber layer loss, suggesting a panretinal subclinical damage. In contrast to NMOSD, the most significant cross-sectional ERG changes in patients with MS were found in cone-driven photopic responses. This potentially can be explained by the fact that cones are mainly localized at the macula and perimacular regions, where there is more significant retinal ganglion cell and retinal nerve fiber layer loss (temporal retinal nerve fiber layer) in MS.

**Study Limitations**

The small sample size of subjects with NMOSD, because of the rare nature of the disease in white persons, remains a limitation of the study. This represents a particular problem for AQP4− patients and limits our ability to identify any potential retinal dysfunction in this disease subgroup. It was also impossible to further analyze the potential link between Müller dysfunction and serum AQP4 titer because of the small sample size and variability of AQP4 titer, particularly when some study patients were receiving plasma exchange. We did not find any association between ERG/foveal changes and disease duration/severity in NMOSD, which also could be due to the small sample size and the presence of many other potential contributing factors, such as IgG titer and treatment. By contrast, in the MS cohort with a larger sample size, ERG changes were found to be associated with disease duration and EDSS. In addition, potential foveal pathology will still need to be validated by postmortem studies in patients with NMOSD.

In conclusion, future longitudinal studies with larger sample sizes are required to validate the current findings and to determine whether this Müller glial dysfunction in NMOSD is associated with disease progression and severity. However, the results from the current study provide in vivo evidence supporting the concept of glial cell dysfunction in the retina in patients with AQP4+ NMOSD, which not only directs us toward pathophysiology of the disease but also may be relevant to future therapeutic approaches.
Figure 5. Patterns of aquaporin-4 (AQP4) expression and Müller glial distribution in the human macula. A, Representative images of immunofluorescence visualization of AQP4 (green), cellular retinaldehyde-binding protein (CRALBP) (red), and merged images with Hoechst staining and the white field image. The nerve fiber layer (NFL), ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), Henle fiber layer (HFL), outer nuclear layer (ONL), and inner segment (IS) are labeled. Aquaporin-4 is highly expressed in HFL at the central fovea (white arrow) (B). Field-enlarged image from (A) (white-dotted box) in the fovea. Note: AQP4 expressed by Müller cell endfeet in the outer limiting membrane (OLM) at the central part of the fovea (red arrows). Scale bar: 50 μm.
References


Footnotes and Financial Disclosures

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Abbreviations and Acronyms:
AQP4-IgG = aquaporin-4 immunoglobulin G; CRALBP = cellular retinaldehyde-binding protein; DA = dark-adapted; EDSS = Expanded Disability Status Scale; ERG = electroretinography; HFONL = Henle fiber outer nuclear layer; INL = inner nuclear layer; IS = inner segment; LA = light-adapted; MOG-IgG = myelin oligodendrocyte glycoprotein immunoglobulin G; MS = multiple sclerosis; NMOSD = neuromyelitis optica spectrum disorder; ON = optic neuritis; OPL = outer plexiform layer; OS = outer segment.

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