Oligodendrocytes are damaged by neuromyelitis optica immunoglobulin G via astrocyte injury

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Devic’s neuromyelitis optica is an inflammatory demyelinating disorder normally restricted to the optic nerves and spinal cord. Since the identification of a specific autoantibody directed against aquaporin 4, neuromyelitis optica-immunoglobulin G/aquaporin 4 antibody, neuromyelitis optica has been considered an entity distinct from multiple sclerosis. Recent findings indicate that the neuromyelitis optica-immunoglobulin G/aquaporin 4 antibody has a pathogenic role through complement-dependent astrocyte toxicity. However, the link with demyelination remains elusive. Autoantibodies can act as receptor agonists/antagonists or alter antigen density in their target cells. We hypothesized that the neuromyelitis optica-immunoglobulin G/aquaporin 4 antibody impairs astrocytic function and secondarily leads to demyelination. Rat astrocytes and oligodendrocytes from primary cultures and rat optic nerves were exposed long-term (24 h) to immunoglobulin G in the absence of complement. Immunoglobulin G was purified from the serum of patients with neuromyelitis optica who were either neuromyelitis optica-immunoglobulin G/aquaporin 4 antibody positive or negative, as well as from healthy controls. Flow cytometry analysis showed a reduction of membrane aquaporin 4 and glutamate transporter type 1 on astrocytes following contact with immunoglobulin G purified from neuromyelitis optica-immunoglobulin G/aquaporin 4 antibody positive serum only. The activity of glutamine synthetase, an astrocyte enzyme converting glutamate into glutamine, decreased in parallel, indicating astrocyte dysfunction. Treatment also reduced oligodendrocytic cell processes and approximately 30% oligodendrocytes died. This deleterious effect was confirmed ex vivo; exposed optic nerves showed reduction of myelin basic protein. Immunoglobulin G from neuromyelitis optica-immunoglobulin G/aquaporin 4 antibody positive patients and from healthy controls had no similar effect. Neuromyelitis optica-immunoglobulin G/aquaporin 4 antibody did not directly injure oligodendrocytes cultured without astrocytes. A toxic bystander effect of astrocytes damaged by neuromyelitis optica-immunoglobulin G/aquaporin 4 antibody on oligodendrocytes was identified. Progressive accumulation of glutamate in the culture medium of neuromyelitis optica-immunoglobulin G/aquaporin 4-antibody-treated glial cells supported the hypothesis of a glutamate-mediated excitotoxic death of oligodendrocytes in our models. Moreover, co-treatment of glial cultures with neuromyelitis optica-immunoglobulin G/aquaporin 4 antibody and d+2-amino-5-phosphonopentanoic acid, a competitive antagonist at the N-methyl-d-aspartate/glutamate receptor, partially protected oligodendrocytes. Co-immunolabelling of oligodendrocyte markers and neuromyelitis
optica-immunoglobulin G/aquaporin 4 antibody showed that astrocytic positive processes were in close contact with oligodendrocytes and myelin in rat optic nerves and spinal cord, but far less so in other parts of the central nervous system. This suggests a bystander effect of neuromyelitis optica-immunoglobulin G-damaged astrocytes on oligodendrocytes in the nervous tissues affected by neuromyelitis optica. In conclusion, in these cell culture models we found a direct, complement-independent effect of neuromyelitis optica-immunoglobulin G/aquaporin 4 antibody on astrocytes, with secondary damage to oligodendrocytes possibly resulting from glutamate-mediated excitotoxicity. These mechanisms could add to the complement-induced damage, particularly the demyelination, seen in vivo.

Keywords: autoimmunity; aquaporin 4; demyelination; astrocyte; Devic’s disease

Abbreviations: AQP4 = aquaporin 4; CNPase = 2’,3’-cyclic nucleotide 3’-phosphodiesterase; CRMP5 = collapsin response mediator protein 5; D-AP5 = D+2-amino-5-phosphonopentanoic acid; DMEM = Dulbecco’s modified Eagle’s medium; GFAP = glial fibrillary acidic protein; GFP = green fluorescent protein; IgG = immunoglobulin G; MBP = myelin basic protein; NMDA = N-methyl-D-aspartate; NMO-IgG = neuromyelitis optica-immunoglobulin G autoantibody; PBS = phosphate buffered saline

Introduction

Devic’s neuromyelitis optica is an inflammatory demyelinating disorder of the central nervous system normally restricted to the optic nerves and the spinal cord (Devic, 1894; Wingerchuk et al., 2007). It is characterized by severe attacks of optic neuritis and myelitis leading in most cases to loss of vision, motor weakness, bladder dysfunction and sensory failure. The pathology is characterized by extensive demyelination and axonal loss in both white and grey matter (Lucchinetti et al., 2002). Devic’s neuromyelitis optica has frequently been considered a subtype of multiple sclerosis but recent clinical, epidemiological, pathological and immunological data support the view that it is a distinct entity to multiple sclerosis (O’Riordan et al., 1996; Wingerchuk et al., 1999; De Seze et al., 2002). A specific autoantibody, neuromyelitis optica-immunoglobulin G (NMO-IgG), has been identified (Lennon et al., 2004). The target antigen for NMO-IgG is a water channel protein, aquaporin 4 (Lennon et al., 2005). In the central nervous system, AQP4 is organized in heterotetramers that are expressed mainly on the plasma membrane of astrocytes and concentrated in domains that face endothelial basal membranes (Amiry-Moghaddam et al., 2003). AQP4 is not expressed in oligodendrocytes. Studies in knockout mice show that AQP4 plays a role in brain water homeostasis, astrocytic glutamate transport and neural cell excitability (Verkman et al., 2006; Ding et al., 2007; Zeng et al., 2007).

Recent findings support a direct pathogenic role for NMO-IgG/AQP4 antibodies in Devic’s neuromyelitis optica. Depositions of immunoglobulins (Ig) and complement are preferentially located in areas of high AQP4 expression and AQP4 is selectively lost in active Devic’s neuromyelitis optica lesions (Misu et al., 2007; Roemer et al., 2007). In animal models, intraperitoneal injection of human sera containing NMO-IgG/AQP4 antibodies induces neuromyelitis optica-like lesions with T cell mediated brain inflammation (Bradl et al., 2009). More recently, similar lesions were induced by intracerebral co-injection of human sera containing NMO-IgG/AQP4 antibodies and human complement (Saadoun et al., 2010) but not by injection of human sera containing NMO-IgG/AQP4 antibodies alone. In vitro, binding of NMO-IgG/AQP4 antibodies to AQP4 initiates complement activation and complement-dependent cytotoxicity on astrocytes (Hinson et al., 2007; Kinoshita et al., 2009; Sabater et al., 2009). All these findings highlight the important role of complement in the pathogenesis of Devic’s neuromyelitis optica. However, it is now well established that, besides activating the complement cascade, antibodies may also act as specific receptor agonists or antagonists, or alter antigen density in target cells (Diamond et al., 2009). It was recently shown that, in absence of complement, NMO-IgG/AQP4 antibody binding induced a reversible internalization of the AQP4-IgG complex and loss of the glutamate transporter 1/excitatory aminoacid transporter 2 (Hinson et al., 2008). We hypothesized that demyelination in Devic’s neuromyelitis optica lesions partly resulted from an indirect deleterious effect of NMO-IgG/AQP4 antibodies on oligodendrocytes mediated by impairment of astrocyte function. Alterations of astrocyte metabolism in various other experimental and pathological conditions result in oligodendrocyte cell death and demyelination (Matute et al., 2007). We therefore analysed: (i) the effect of NMO-IgG/AQP4 antibodies in the absence of complement on astrocyte function in glial cell cultures and in optic nerve preparations; (ii) the effect of NMO-IgG/AQP4 antibodies on oligodendrocyte integrity and survival in these models; and (iii) the spatial association between NMO-IgG/AQP4 antibody immunoreactive astrocytes and oligodendrocytes in optic nerves and spinal cord.

Materials and methods

Human sera and IgG isolated from patient sera

Sera were obtained from eight patients with a Devic’s neuromyelitis optica spectrum disorder (Wingerchuk et al., 2007); six sera were positive for NMO-IgG and AQP4 antibody and two were negative with both techniques. Characteristics of patients with Devic’s neuromyelitis optica are presented in Table 1. We also used control sera collected from five healthy blood donors at Etablissement Français du Sang. IgG from the eight Devic’s neuromyelitis optica and five control sera were purified on Protein-A Sepharose 4 Fast Flow™ beads.
### Table 1 Characteristics of Devic's neuromyelitis optica spectrum patients and summary of the use of NMO-IgG and control-IgG in the experiments

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Sex</th>
<th>Age at onset</th>
<th>First syndrome</th>
<th>Number of relapses</th>
<th>NMO-IgG-IIF</th>
<th>AQP4-CBA</th>
<th>AQP4-FIPA</th>
<th>CSF, oligoclonal bands</th>
<th>Brain MRI suggestive of multiple sclerosis</th>
<th>Longitudinally extensive lesion at spinal cord MRI</th>
<th>Diagnosis</th>
<th>Flow cytometry analysis</th>
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<tr>
<td>NMO-1</td>
<td>F</td>
<td>35</td>
<td>Optic neuritis</td>
<td>11</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>NMO</td>
<td>DNMO</td>
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<td>55</td>
<td>Optic neuritis</td>
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<td>Positive</td>
<td>Positive</td>
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<td>No</td>
<td>No</td>
<td>Yes</td>
<td>NMO</td>
<td>DNMO</td>
</tr>
<tr>
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<td>49</td>
<td>Myelitis</td>
<td>2</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>NMO</td>
<td>rLETM</td>
</tr>
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<td>Yes</td>
<td>NMO</td>
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<td>Positive</td>
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<td>Yes</td>
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<td>50</td>
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<tr>
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<td>Yes</td>
<td>DNMO</td>
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<td></td>
<td>Healthy donors</td>
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</tbody>
</table>

NMO-1 F 35 Optic neuritis 11 18 Positive Positive Positive No No Yes DNMO 5 4 3 2
NMO-2 F 55 Optic neuritis 5 3 Positive Positive Positive No No Yes DNMO 6 2 3 2
NMO-3 F 49 Myelitis 2 2 Positive Positive Positive No No Yes DNMO 1 1 1 1
NMO-4 F 55 Myelitis + optic neuritis 3 20 Positive Positive Positive No No Yes DNMO 3 1 2
NMO-5 M 26 Myelitis 2 1 Positive Positive Positive No No Yes DNMO 1 1 1 1
NMO-6 F 13 Myelitis + optic neuritis 3 10 Positive Positive Positive No No Yes DNMO 2 2 2

AQP4-CBA = cell-based assay for AQP4-Ab detection; IIF = indirect immunofluorescence; FIPA = fluorescent immunoprecipitation assay; rLETM = relapsing longitudinally extensive transverse myelitis; OLG = oligodendrocytes.

**Tissue preparation and glial cell cultures**

Rats (CBA/Ea; Elevage, France) received intracardiac infusion with 15 ml phosphate buffered saline (PBS) 0.1 M PH 7.4 (soil at room temperature) to eliminate red blood cells, then with 15 ml 4% paraformaldehyde fixed brain, spinal cord and optic nerve. The spinal cord was performed in 4% paraformaldehyde for 2 h at 4°C before being frozen in isopentane at −25°C and prepared as 8–10 micron sections. These sections were used for NMO-IgG immunoreactivity of 2°C immediate ultraviolet light after dehydration. They were used for experiments. NMO-IgG isolation from patients with Devic’s neuromyelitis optica and healthy donors was validated using a fluorescence-based immunoprecipitation assay routinely used in the laboratory.

In addition, total sera from the six NMO-IgG/AQP4 positive patients with Devic’s neuromyelitis optica and eight patients with multiple sclerosis (according to established criteria; McDonald et al., 2001) were used for immunostaining in 4°C paraformaldehyde fixed rat brain, spinal cord and optic nerve.

In addition, total sera from the six NMO-IgG/AQP4 positive patients with Devic’s neuromyelitis optica and eight patients with multiple sclerosis (according to established criteria; McDonald et al., 2001) were used for immunostaining in 4°C paraformaldehyde fixed rat brain, spinal cord and optic nerve. All sera were heated for 4°C before immunostaining. Primary glial cultures were obtained by mechanical disruption of 50% astrocytes and 50% oligodendrocytes as systematically determined by glial fibrillary acidic protein (GFAP) and 2°C dendrocytes [as systematically determined by glial fibrillary acidic protein (GFAP)] and prepared as 8–10 micron sections.

The medium was changed every 3 days after plating (10% foetal calf serum). Using this procedure we typically obtained a culture composed of approximately 50% astrocytes and 50% oligodendrocytes. We termed the NMO-IgG-negative serum of patients with Devic’s neuromyelitis optica and healthy donors negative for AQP4 antibodies detection by fluorescence-based immunoprecipitation and cell-based assays. We termed IgG isolated from the NMO-IgG seropositive serum of patients with Devic’s neuromyelitis optica and healthy donors as the total IgG isolated from the serum of healthy donors.

AQP4-CBA = cell-based assay for AQP4-Ab detection; IIF = indirect immunofluorescence; FIPA = fluorescent immunoprecipitation assay; rLETM = relapsing longitudinally extensive transverse myelitis; OLG = oligodendrocytes.
astrocyte culture, dissociated cells were diluted to a density of 2 \times 10^5 cells/ml in DMEM containing 1 g/l glucose and 10% foetal calf serum. In this condition, glial culture contained more than 90% GFAP positive cells. Primary oligodendrocyte culture was obtained from 15 days mixed glial culture, shoo for 18 h (rotary shaker 220rpm, 37°C, CO₂ atmosphere). The cell suspension was filtered through a 30 mm nylon mesh and plated on bacterial-grade Petri dishes for 3 h to remove attached astrocytes and microglia. The final suspension was cultured in bacterial-grade Petri dishes for 3 days in serum-free medium (DMEM:F12) containing B27 and N2 supplements (Gibco™), platelet-derived growth factor and basic fibroblast growth factor (Sigma-Aldrich), to promote oligodendroglial progenitors in gliosphere. The culture was then differentiated in CNPase positive oligodendrocytes for 2–3 days in DMEM containing 20% foetal calf serum (Supplementary Fig. 2B). Mixed glial, astrocyte and oligodendrocyte cultures were used for antibody treatment.

Isolated optic nerves were dissected from adult rats and put immediately on 30mm semi-permeable membrane inserts (Millicell-CM, Millipore) in a 6-well plate containing 1ml of culture medium (50% minimum essential medium Gibco, 25% Hank’s balanced salt solution, 25% heat-inactivated horse serum, 1% L-glutamine 200 mM and 6.6 mg/ml glucose) in each well. One day later they were used for antibody treatment.

Glial and optic nerve cultures were treated, when appropriate, with D-2-amino-5-phosphonopentanoic acid (D-AP5), a competitive N-methyl-D-aspartate (NMDA) antagonist (Tocris Cookson Inc., USA, Ref 0106).

### Treatment of glial cells and optic nerves with IgG\textsuperscript{NMO−}, IgG\textsuperscript{NMO+} and IgG\textsuperscript{Control}

Glial cell cultures were incubated for 24 h with IgG (0.2 µg/µl) purified from patients with Devic’s neuromyelitis optica or controls, then washed in PBS and fixed with cold acetone (10 min). Optic nerve cultures were treated for 24 h with purified IgG, then were extensively washed with DMEM, fixed with 4% paraformaldehyde for 5 h, stored in 15% sucrose overnight, embedded in Titecote® preparation, frozen in isopentane at –50°C and then prepared as 8–10 micron sections. The effect of IgG\textsuperscript{NMO+} on astrocyte and oligodendrocyte was evaluated on treated glial culture and optic nerve slices using immunodetection of specific markers: GFAP for astrocyte, CNPase and collapsin response mediator protein 5 (CRMP5) for oligodendrocyte (in glial culture and optic nerve, respectively). Cells were counted under fluorescence microscope (at least 10 microscopic fields counted). AQ4P and GLT1 expression at the cell surface was evaluated using flow cytometry. Glial cells were also treated with cell supernatant (24 h conditioned medium) harvested from glial culture treated with IgG\textsuperscript{NMO+}, IgG\textsuperscript{NMO−} and IgG\textsuperscript{Control} and examined for oligodendrocyte survival. The use of IgG\textsuperscript{NMO+}, IgG\textsuperscript{NMO−} and IgG\textsuperscript{Control} in the experiments is summarized in Table 1. The effect of IgG on AQ4P, GLT1 expression and CNPase/CRMP5 positive cell number was expressed in percentage of decrease related to the level in untreated culture.

### Immunodetection

**NMO-IgG immunostaining**

NMO-IgG immunostaining was performed on fixed slices of neural tissue. To avoid staining of non-organ specific autoantibody, neural slices were pre-incubated for 1.5 h in normal goat serum (10%). Patient sera were diluted (1/50) in phosphate buffer 0.1 M pH 7.4 and incubated with slices overnight (room temperature or 4°C). Three washes with PBS eliminated non-bound material. Bound human IgG was detected using Alexa-488 labelled anti-human IgG antibody directed against all human IgG subclasses (1/500 in PBS, 1.5 h at room temperature) (Molecular Probes A11013, USA). Following three washes in PBS, slides were prepared with Fluoroprep® solution and coverslides for fluorescence microscopy.

### Specific immunodetection

Specific immunodetection of astrocyte, oligodendrocyte and endothelial cell markers was similarly performed on glial cell culture and slices of neural tissue. Astrocyte was visualized using GFAP immunostaining, oligodendrocytes using CNPase and CRMP5 immunostaining (cell culture and optic nerve, respectively). Polyclonal or monoclonal sera were directed to CNPase (C5922, Sigma, USA), CRMP5 (Ricard et al., 2000), myelin basic protein (MBP, MCA70, antibody-D, Serotec), H200 neurofilament (AHP245, antibody-D, Serotec), GFAP (Z0334, Dako), Factor VIII (A0082, Dako), NMDA receptor-2B (AB1557P Chemicon International) or NMDA receptor-1 (AB9864, Millipore™) followed by fluorescein-conjugated anti-rabbit or anti-mouse IgG (Alexa 488-GAR and 488-GAM, Molecular Probes). Water channel and glutamate transporter were immunodetected using rabbit anti-AQP4 (AB2218, Millipore™) and anti-GLT1 (Dutuit et al., 2000) polyclonal antibodies. Detection of active caspase-3 by anti-cleaved caspase-3/Apoptigen antibody (H9661, Cell Signalling Technology Inc.) evidenced apoptotic cell death. Glial cells were fixed for 10 min in 4% paraformaldehyde for NMDA receptor subunit detection. MBP immunostaining intensity was evaluated using Analysis® software (10 different microscopic fields per condition, three treated and three untreated optic nerves).

### Flow cytometry

Flow cytometry was performed on untreated glial cell cultures and following 24 h IgG treatment. Cells were detached from culture dishes using enzyme solution RDB (1/100, 10 min, 37°C), then washed in DMEM, incubated for 1 h at 37°C in DMEM supplemented with 20% foetal calf serum to restore surface proteins, then blocked by pre-incubation (20 min, 4°C) with anti-CD32 antibody to avoid non-specific staining. Following cell wash in 1 ml cold PBS, cells were incubated with antibody directed against surface proteins GLT1, AQ4P (AB2218, Millipore™), O4 (FAB1326P, R&D systems), NMDA receptor-2B (AB1557P Chemicon International), NMDA receptor-1 (AB9864, Millipore™) (30 min, 4°C, diluted antibody in 50 µl), washed (1 ml cold PBS), then incubated with anti-rabbit IgG (30 min, 4°C, Alexa 488-GAR, Molecular Probes), washed again (1 ml cold PBS) then fixed (4% paraformaldehyde in cold PBS, 1 ml). Intracellular proteins GFAP (556330 BD Pharmingen™) and CNPase (C5922, Sigma, USA) were thereafter immunodetected following cell permeabilization (20 min at room temperature, 150 µl PBS, 0.2% foetal calf serum, 0.5% saponin) and washed (1 ml cold PBS). Alternatively, incubation with mouse monoclonal anti-GFAP antibody (clone G8A, Sigma USA) (30 min at 4°C, 50 µl diluted antibody in PBS 0.05% saponin) was followed by incubation with anti-mouse IgG (30 min, 4°C, Alexa 660-GAM, Molecular Probes). Fluorescence was evaluated on the cytofluorimeter (FC500 MPL, Beckman Coulter). AQ4P and GLT1 expression following IgG treatment is presented in graph as decreased positive cell number compared to untreated cultures (in percent; Fig. 1A and B).
Absorption of IgG\textsuperscript{NMO+} on aquaporin-4 molecules from transfected cells

Human embryonic kidney-293 cells were seeded in 75 cm\textsuperscript{2} flask at a density of 1 x 10\textsuperscript{5} cells and transfected to overexpress AQP4-green fluorescent protein (GFP) fusion protein or GFP as control. Human AQP4-M1 complementary DNA was obtained from Origene (Rockville, USA) and cloned into plasmid EGFP-C1 (Clontech, Saint-Germain-en-Laye, France) using the reverse primer 5’-CCGCTACCTACAGGAACAAACATT introducing a Kpn1 site at the 3’ end and the forward primer 5’-GAAGATCTAGTCAGACCCAC introducing a Bgl2 site at the 5’ end. The polymerase chain reaction product was inserted into the Bgl2 and Kpn1 sites of the EGFP-vector and the DNA sequence was verified by sequencing. Thirty-six hours after transfection, IgG\textsuperscript{NMO+} was diluted in DMEM medium and added to AQP4-GFP- and GFP-transfected cells for 1 h at 37°C, CO\textsubscript{2} atmosphere and gentle rotation. Contact with cells was performed three times. After incubations, diluted IgG was removed and centrifuged for 15 min at 12 000 rpm, and tested for AQP4 antibody titre. Adsorption on human embryonic kidney-AQP4-GFP cells decreased AQP4 antibody by ~40% (from 4490 to 2540 fluorescence units).

Glutamine synthetase activity

Brain glutamine synthase enzymatic activity was routinely measured as previously described (Boksha et al., 2000) by the transferase reaction (ADP+L-Gln+NH\textsubscript{2}OH) and was determined by measuring γ-glutamyl hydroxamate formation. Cells were treated with cyclic adenosine monophosphate (Sigma Aldrich, 100 mM, 3 h) and subsequently with glutamate (Sigma Aldrich, 1 mM, 1 h). They were further dissociated using Polytron and homogenization buffer: phosphate buffer 0.1 M, glucose 70 mM, beta-mercaptoethanol 1.4 mM, bovine serum albumin 0.02% and glycerol vol/vol. The reaction mixture was as follows: imidazole–HCl, 50 mM, NH\textsubscript{2}OH, 50 mM, γ-glutamine 100 mM, MnCl\textsubscript{2} 0.5 mM, KH\textsubscript{2}AsO\textsubscript{4} 25 mM and adenosine diphosphate 0.2 mM. After incubation at 37°C for 30 min, an equal volume of stop solution (0.37 M FeCl\textsubscript{3}, 0.3 M trichloroacetic acid, 0.6 M HCl) was added. The reaction was performed in a 96-well plate. Absorbance of the coloured complex formed by γ-glutamyl hydroxamate with iron was measured at 505 nm. Data are expressed in mole of γ-glutamyl hydroxamate per milligram protein per minute.

Glutamate quantification

Extracellular glutamate level was estimated using measure of amino acid content in glial cell medium, as reported (Touret et al., 2007) using an automatic capillary zone electrophoresis P/ACE\textsuperscript{TM} MDQ system (Beckman, USA) equipped with a ZETALIF laser induced fluorescence detector (PicoMetrics, France; Plateform facilities Neurochem, Institut Fédératif des Neurosciences, IFR 19 Inserm). Excitation was performed using a He–Cd laser (Liconix, USA) at a wavelength of 442 nm, the emission wavelength being 490 nm. Separation was carried out on a 63 cm x 50 µm fused-silica capillary (Composite Metal Services, Worcester England) with an effective length of 52 cm. The separation conditions were: an applied voltage of 25 kV, hydrodynamic sample injection (10 s at 0.65s) and a temperature between 36 and 38°C. The capillary was sequentially flushed for 30 s with 0.25 mol/l NaOH, ultrapure water and running buffer (75 mmol/l sodium borate, pH 9.20 ± 0.02, containing 10 mmol/l HP-β-CD and 70 mmol/l sodium dodecyl sulphate) between analyses. Electropherograms were acquired at 15 Hz using P/ACE\textsuperscript{TM} MDQ software.

Statistical analysis

Data are presented as mean±SD. Normality of variable distribution was first tested with the Shapiro–Wilk test and study histograms. Considering the non-normal distribution, we used a non-parametric Wilcoxon–Mann–Whitney test. Levels of significance were indicated as follows: *P<0.05, **P<0.01 and ***P<0.001. Computations were performed using SAS software version 9.1 (SAS Institute Inc., Cary, NC).

Results

Prolonged exposure to IgG\textsuperscript{NMO+} induced severe functional astrocyte changes in the glial cell culture model

For these experiments, we used either enriched astrocyte primary cultures or mixed glial cell cultures established from new-born rat cortex. In mixed glial cell cultures, oligodendrocytes grew over and in close association with astrocytes, as shown by immunodetection of the oligodendrocyte marker CNPase and astrocyte markers GFAP and AQP4 (Supplementary Fig. 2A). We compared the effects of IgG\textsuperscript{NMO+} (n = 6), IgG\textsuperscript{NMO−} (n = 2) and IgG\textsuperscript{Control} (n = 2) on these glial cells. To mimic the prolonged contact of target tissue with Devic’s neuromyelitis optica, glial cell cultures were treated for 24 h.

Following IgG treatment, astrocyte and astrocyte-oligodendrocyte cultures were examined for the expression of AQP4 and the glutamate transporter type1 (GLT1, analogous to excitatory aminoacid transporter 2 in human) and for the activity of glutamine synthetase (Fig. 1). In untreated glial cells, AQP4 was detected in cultured glial cells. To mimic the prolonged contact of target tissue with astrocytes by flow cytometry (Fig. 1A, top histogram). Treatment of primary astrocyte cultures with IgG\textsuperscript{NMO+} significantly reduced the number of AQP4-positive cells (28±11% decrease) compared to IgG\textsuperscript{Control} (8±7% decrease; P<0.05) and IgG\textsuperscript{NMO−} (11±9% decrease; P<0.05) (Fig. 1A, bottom histogram and graph). The number of GFAP positive cells was not modified whatever the IgG origin (data not shown), indicating that the AQP4-positive cell decrease did not result from astrocytic death. The glutamate transporter GLT1 was detected in cultured glial cells by GLT1 and GFAP co-labelling, and again at the surface of astrocytes, as shown by immunodetection of the oligodendrocyte marker CNPase and astrocyte markers GFAP and glial fibrillary acidic protein (GFAP) in close association with astrocytes, as shown by immunodetection of the non-parametric Wilcoxon–Mann–Whitney test. Levels of significance were indicated as follows: *P<0.05, **P<0.01 and ***P<0.001. Computations were performed using SAS software version 9.1 (SAS Institute Inc., Cary, NC).
decrease in the activity of glutamine synthetase following treatment with IgG
NMO+ (three experiments, data not shown). This observation was confirmed in astrocyte cultures (two experiments) and ascribed the glutamate homeostasis dysfunction mediated by IgG
NMO+ mainly if not totally to astrocytes. IgG
NMO+ (n = 3) specifically reduced glutamine synthetase activity in astrocytes (mean decrease 51 ± 18%) compared to IgG
NMO- (n = 2) (6 ± 15% decrease; P < 0.05) and IgG
Control (n = 2) (no decrease) (Fig. 1C,
Figure 1 Prolonged exposure to IgG
NMO+ deeply impaired astrocyte function, reducing AQP4, GLT1 expression and glutamine synthetase activity. (A) Expression of AQP4 was detected in the glial culture (astrocytes and oligodendrocytes) using immunofluorescence (scale bar = 20 μm). Nuclei are shown by 4',6-diamidino-2-phenylindole staining (blue). Co-labelling and flow cytometry detected AQP4 at the membrane of GFAP positive astrocytes (top histogram). Astrocyte cultures were exposed for 24 h to IgG
NMO+ (n = 6), IgG
NMO- (n = 2) and IgG
Control (n = 2). The number of AQP4 positive cells was significantly reduced by IgG
NMO+ compared to IgG
Control (bottom histogram, P < 0.05) and IgG
NMO- (P < 0.05) (data expressed in per cent of decrease related to level in untreated cells). Graph represents cumulative data (n = 20) of two repeated experiments. (B) Glutamate transporter GLT1 was detected in GFAP positive astrocytes using immunofluorescence (scale bar = 20 μm). Flow cytometry analysis confirmed GLT1 expression at the astrocyte membrane (co-labelling GLT1-GFAP, red line) and detected a decreased expression following treatment with IgG
NMO+ (blue line) (representative histogram). All IgG
NMO+ induced a decreased number of GLT1 positive cells compared to IgG
Control (P < 0.05) and IgG
NMO- (P < 0.05). Graph represents cumulative data (n = 20) of two repeated experiments. (C) Activity of the astrocytic catabolic enzyme of glutamate, glutamine synthetase (GS), was determined using transferase reaction, which detected γ-glutamyl hydroxamate formation expressed in M/mg protein/mn. Compared to IgG
Control (n = 3) and IgG
NMO- (n = 2), chronic exposure to IgG
NMO+ (n = 3) reduced glutamyl hydroxamate production in astrocytes (expression in decreased glutamine synthetase activity) (IgG
NMO+ versus IgG
NMO- P < 0.05).
results expressed in per cent of decrease in comparison to basal glutamine synthetase activity in untreated cultures). To conclude, the prolonged exposure to IgG<sup>NMO+</sup> profoundly injured astrocytes, resulting in functional changes.

**IgG<sup>NMO+</sup> damages oligodendrocytes in in vitro and ex vivo models**

We further investigated a possible toxic effect of altered astrocytes on oligodendrocytes following contact with IgG<sup>NMO+</sup>. Astrocyte-oligodendrocyte cultures were treated for 24 h with IgG<sup>NMO+</sup>, IgG<sup>NMO–</sup> and IgG<sup>Control</sup> then tested for the presence of oligodendrocytes using CNPase immunolabelling (13 independent experiments). Treatment with IgG<sup>NMO+</sup> modified oligodendrocyte morphology and induced the loss of CNPase positive cells whereas IgG<sup>Control</sup> had no effect (Fig. 2A). The percentage of oligodendrocytes significantly decreased after treatment with IgG<sup>NMO+</sup> (50 ± 15%) compared to IgG<sup>NMO–</sup> (15 ± 12%; P < 0.001) and IgG<sup>Control</sup> (11 ± 8%; P < 0.0001) (Fig. 2A, graph). The detection of active caspase-3 in oligodendrocytes (Fig. 2B) indicated that the lost of CNPase positive cells resulted from oligodendrocyte death, probably via an apoptotic pathway. To link IgG<sup>NMO+</sup>-induced astrocyte injury and oligodendrocyte loss, the number of CNPase positive cells was evaluated in mixed glial culture already tested for astrocytic GLT1 expression and glutamine synthetase activity. After IgG<sup>NMO+</sup> contact, the oligodendrocyte frequency followed the same decrease pattern as GLT1 and glutamine synthetase (38% CNPase positive cells in untreated versus 28–27% in IgG<sup>NMO+</sup>-treated culture) (Fig. 2C). In addition, oligodendrocyte loss was clearly reduced by adsorption of IgG<sup>NMO+</sup> on GFP-AQP4-transfected human embryonic kidney cells (30% decrease of oligodendrocyte number before adsorption versus 14% with GFP-AQP4-adsorbed IgG<sup>NMO+</sup>; 32% with GFP-adsorbed IgG<sup>NMO+</sup>) indicating the role anti-AQP4 antibody in oligodendrocyte damage.

The deleterious effect of IgG<sup>NMO+</sup> on oligodendrocyte was confirmed ex vivo (Fig. 3). Rat optic nerves were treated for 24 h with IgG<sup>NMO+</sup> (n = 6) and IgG<sup>Control</sup> (n = 3) then tested for the presence of oligodendrocytes. CRMP5 positive oligodendrocytes were uniformly distributed along GFAP positive astrocyte tracks in optic nerves treated with IgG<sup>Control</sup> (Fig. 3A). Treatment with IgG<sup>NMO+</sup> dramatically modified oligodendrocyte morphology (Fig. 3A, higher magnification). The number of oligodendrocytes was reduced by 47 ± 23 and 16 ± 28% following contact with

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**Figure 2** IgG<sup>NMO+</sup> profoundly altered oligodendrocyte in glial culture. (A) CNPase positive oligodendrocytes were examined following treatment of glial cultures for 24 h with IgG<sup>NMO+</sup> (n = 6), IgG<sup>NMO–</sup> (n = 2) and IgG<sup>Control</sup> (n = 5). Chronic exposure to IgG<sup>NMO+</sup> altered oligodendrocyte morphology (immunocytochemistry, four representative data, scale bar = 20 µm) and reduced their number (graph) (data expressed in per cent of decrease related to level in untreated cells). Graph represents cumulative data (n = 44) of nine repeated experiments (IgG<sup>NMO+</sup> versus IgG<sup>Control</sup> P < 0.0001; IgG<sup>NMO+</sup> versus IgG<sup>NMO–</sup> P < 0.01). (B) Detection of the active caspase-3 in CNPase positive cells suggested apoptotic oligodendrocyte death following treatment with IgG<sup>NMO+</sup>. (C) Oligodendrocyte loss was confirmed by flow cytometry using CNPase labelling (green histogram, three representative data). Dotted line indicates CNPase level in untreated culture.
IgG<sup>NMO</sup> and IgG<sup>Control</sup>, respectively (P < 0.01) (Fig. 3A, graph). In addition, immunofluorescence analysis of myelin detected a decrease in MBP staining in IgG<sup>NMO</sup>-treated optic nerves (647 ± 417 fluorescent relative units in untreated versus 495 ± 199 in IgG<sup>NMO</sup>-treated optic nerves, P < 0.01) (Fig. 3B, graph). These experiments showed the ability of IgG<sup>NMO</sup> to induce oligodendrocyte and myelin loss in our study models.

Finally, to address the question of a possible direct effect of IgG<sup>NMO</sup> on oligodendrocytes, we treated primary cultures enriched in oligodendrocytes with IgG<sup>NMO</sup>, IgG<sup>NMO</sup>/C0 and IgG<sup>Control</sup>. Whatever their origin, IgG had no effect on either oligodendrocyte survival or on processes (two independent experiments, not shown). Thus, oligodendrocyte loss was not directly induced by IgG<sup>NMO</sup> but rather depended on IgG<sup>NMO</sup>-induced astrocyte alteration.

IgG<sup>NMO</sup> could induce a glutamate-mediated excitotoxicity to oligodendrocytes

The loss of oligodendrocytes in optic nerves and glial cell cultures chronically exposed to IgG<sup>NMO</sup>, in association with astrocyte metabolic alteration, suggested that oligodendrocytes died through a bystander toxic effect. To validate this hypothesis, glial cultures were treated with conditioned medium harvested from astrocyte and astrocyte-oligodendrocyte cultures treated with IgG<sup>NMO</sup> (n = 5), IgG<sup>NMO</sup>/C0 (n = 2) and IgG<sup>Control</sup> (n = 2) for 24 h. The effect of conditioned media on CNPase positive oligodendrocytes was evaluated by immunofluorescence. In each experimental condition IgG<sup>NMO</sup> was more toxic than IgG<sup>NMO</sup>/C0 (P < 0.01) and IgG<sup>Control</sup> (P < 0.0001). Conditioned medium from IgG<sup>NMO</sup>-treated astrocyte and astrocyte-oligodendrocyte cultures reduced the number of oligodendrocytes to the same extent. This comforted our hypothesis of a toxic bystander effect of damaged astrocytes on oligodendrocytes. It must be noted that, without reaching significance, IgG<sup>NMO</sup>/C0 also displayed some deleterious effect.

Glutamate was suspected to be one of the toxic effectors released by astrocytes in response to IgG<sup>NMO</sup> binding, as oligodendrocytes are particularly susceptible to glutamate-mediated excitotoxicity and astrocytes can release glutamate (Parpura et al., 1994; Matute et al., 1997). To answer this question, we analysed glutamate levels in the conditioned medium harvested from untreated and IgG<sup>NMO</sup>-treated glial cells (n = 2). Extracellular glutamate was evaluated 2, 5 and 18 h post-treatment using capillary electrophoresis, as previously described (Touret et al., 2007). Glutamate progressively accumulated in the
cell medium following exposition to IgG\textsuperscript{NMO+} (mean extracellular glutamate concentration: 88.4 M/l \(10^{-8}\) at 18 h versus 6.4 in untreated cells) (Fig. 4B). We then analysed the co-expression of NR2B and NR1 subunits and O4, an oligodendrocyte surface marker, in mixed glial culture. Co-labelling detected 86% NR2B positive and 37% NR1 positive among the O4 cell population (Fig. 4C, histograms). Immunofluorescence confirmed NR2B and NR1 expression on cultured oligodendrocytes (Fig. 4C). To validate our excitotoxic death hypothesis, we investigated whether IgG\textsuperscript{NMO+}-induced oligodendrocyte loss could be limited by blocking NMDA receptors. Astrocyte-oligodendrocyte cultures were co-treated for 24 h with IgG (IgG\textsuperscript{NMO+} \(n=5\); IgG\textsuperscript{Control} \(n=2\)) and D-AP5, a competitive antagonist of NMDA receptors. Treatment with D-AP5 partially protected oligodendrocytes from the IgG\textsuperscript{NMO+}-induced toxic effect (33% decrease of CNPase positive cells with D-AP5 versus 45% without D-AP5) \((P=0.08)\) (Fig. 4D). The protective effect of D-AP5 on oligodendrocytes was also tested on optic nerve tissue from experimental allergic encephalomyelitis (EAE) and multiple sclerosis (MS) patients. Neurofilament protein and myelin basic protein expression was assessed. D-AP5 treatment prevented demyelination induced by IgG\textsuperscript{NMO+} in both EAE and MS samples.

**Figure 4** A glutamate-mediated excitotoxicity induced by IgG\textsuperscript{NMO+} could be involved in the deleterious interaction between oligodendrocytes and astrocytes. (A) Glial cultures were treated with conditioned medium harvested from astrocyte and astrocyte-oligodendrocyte cultures treated with IgG\textsuperscript{NMO+} \((n=5)\), IgG\textsuperscript{NMO-} \((n=2)\) and IgG\textsuperscript{Control} \((n=2)\). The effect of conditioned media on CNPase positive oligodendrocyte was evaluated using immunofluorescence (graph represents 24 cumulative data of two independent experiments, data expressed in per cent of decrease related to level in untreated cells). Conditioned medium from IgG\textsuperscript{NMO+}-treated astrocyte and astrocyte-oligodendrocyte cultures reduced the number of oligodendrocytes in a same extent \((P=0.1)\). In each experimental condition IgG\textsuperscript{NMO+} was more toxic than IgG\textsuperscript{NMO-} \((P<0.01)\) and IgG\textsuperscript{Control} \((P<0.0001)\). (B) Extracellular glutamate kinetic evaluation using capillary electrophoresis showed that glutamate progressively accumulated in the culture medium of cells exposed to IgG\textsuperscript{NMO+}, and not in untreated cultures. (C) Cultured glial cells expressed N-methyl-D-aspartate (NMDA) receptor subunits NR2B and NR1, as shown by flow cytometry analysis (green histogram). Co-labelling with O4 showed the presence of NR2B and NR1 on O4 positive oligodendrocytes (86% NR2B+ and 37% NR1+ in O4 population) (blue histogram). Immunofluorescence confirmed NR2B and NR1 expression in O4 positive cells. Scale bar 20 \(\mu\)m. (D) Glial cultures were co-treated for 24 h with IgG\textsuperscript{NMO+} \((n=5)\) and the competitive antagonist of NMDA receptors, D-AP5. Treatment with D-AP5 partly protected oligodendrocytes from the IgG\textsuperscript{NMO+} induced toxic effect (33% decrease versus 45% for IgG\textsuperscript{NMO+} alone) \((P=0.08)\). Graph represents cumulative data \((n=10)\) of three repeated experiments.
nerve treated with IgG\textsuperscript{NMO+} (n = 4) and IgG\textsuperscript{Control} (n = 3). Again, the decrease in the number of oligodendrocytes following IgG\textsuperscript{NMO+} contact was partly reduced by D-AP5 treatment (not shown).

Altogether, our results substantiate the hypothesis that, in our study models, IgG\textsuperscript{NMO+}-induced oligodendrocyte loss is associated with glutamate excitotoxicity.

In adult rat spinal cord and optic nerve, NMO-IgG bind astrocyte processes that closely wrap oligodendrocytes

Association of NMO-IgG-immunoreactive astrocyte processes and oligodendrocytes in tissues affected in Devic’s neuromyelitis optica would support the view of a bystander affect of damaged astrocytes on oligodendrocytes. We investigated NMO-IgG immunoreactivity in rat optic nerve and spinal cord using NMO-IgG positive sera from patients with Devic’s neuromyelitis optica (n = 6) and negative sera from patients with multiple sclerosis (n = 8) as a control. Our previous experiment showed that NMO-IgG immunoreactivity was predominantly present in the processes and soma of astrocytes on 4% paraformaldehyde fixed tissues (Supplementary Fig. 3). No staining was observed with control sera whereas all NMO-IgG positive sera from patients with Devic’s neuromyelitis optica showed similar labelling. In rat spinal cord, NMO-IgG immunoreactivity displayed a mesh-like pattern organized in long fibre network throughout the white matter (Fig. 5A; Supplementary Fig. 4) and concentrated in rim and rosette vasculocentric patterns within grey matter. Co-immunolabelling showed that reactivity for NMO-IgG, GFAP and AQP4 largely co-localized but not with neurofilament, a neuronal marker (Supplementary Fig. 3A and B). Similar distribution was observed in the optic nerve. NMO-IgG immunoreactivity organized along the astrocytic fibre network known to assemble along the nerve length (Butt and Kirvell, 1996) (Fig. 5A). Again, NMO-IgG immunoreactivity co-localized with AQP4 and GFAP but not with Factor VIII, an endothelial cell marker (Supplementary Fig. 5A and B). As previously reported for AQP4 expression (Misu et al., 2007; Roemer et al., 2007), NMO-IgG staining was prominent in the spinal cord and optic nerve in comparison to brain white matter (not shown). Supporting our hypothesis, dual labelling of NMO-IgG and CRMP5, a marker of oligodendrocyte, showed that NMO-IgG positive processes closely enfolded oligodendrocytes both in optic nerve and spinal cord.

Figure 5  NMO-IgG positive astrocyte processes closely wrapped oligodendrocytes in the rat spinal cord and the optic nerve. Longitudinal slices (8–10 micron) of adult rat spinal cord and optic nerve were fixed with 4% paraformaldehyde then incubated with sera (diluted 1/50) from NMO-IgG positive patients with Devic’s neuromyelitis optica (representative pictures). Anti-human IgG detected the binding of NMO-IgG (green). (A) NMO-IgG immunoreactivity displayed a mesh-like pattern in the spinal cord white matter. In the optic nerve, NMO-IgG positive cell processes organized in long trails in the optic nerve (arrow). (B) Rabbit anti-CRMP5 antibody, mice anti-MBP antibody and anti-IgG species were used to detect oligodendrocyte soma and myelin, respectively (red). CRMP5, MBP and NMO-IgG immunoreactivity detected positive processes around oligodendrocyte soma (CRMP5 positive) and in close contact with myelin (MBP positive) in the spinal cord white matter and the optic nerve (arrows). Scale bars = 20 μm.
cord (Fig. 5B). Co-immunolabelling with MBP confirmed the close contact of NMO-IgG positive processes with myelin tracts. These observations suggest that a bystander effect of NMO-IgG-damaged astrocytes on oligodendrocytes might occur in the nervous tissues affected by Devic’s neuromyelitis optica.

## Discussion

Our results confirm the pathogenic role of IgG isolated from neuromyelitis optica patients with NMO-IgG/AQP4 antibody (IgGNMO+). Using models based on IgGNMO+ treated primary glial cultures and optic nerve, we showed that IgGNMO+ induced alteration and death of oligodendrocytes, possibly through an excitotoxic mechanism resulting from glutamate homeostasis disruption in astrocytes. The ability of NMO-IgG to bind AQP4 positive astrocytic processes in close contact with oligodendrocytes in the spinal cord and optic nerve suggests the possibility of disruption of glutamate homeostasis in Devic’s neuromyelitis optica.

There are increasing evidences for a direct role of NMO-IgG/AQP4 antibody in the pathophysiology of Devic’s neuromyelitis optica. Most of the studies have focused on NMO-IgG/AQP4 antibody-induced alteration of astrocytes, as AQP4, the target of NMO-IgG, is mainly expressed on astrocytes in the central nervous system (Hinson et al., 2007, 2008; Vincent et al., 2008; Kinoshita et al., 2009; Sabater et al., 2009). However, Devic’s neuromyelitis optica lesions are also characterized by extensive demyelination and oligodendrocyte loss associated with axonal injury (Lucchini et al., 2002) and the link between astrocyte alterations and demyelination remains elusive. We hypothesized that lesion pathology of Devic’s neuromyelitis optica could be driven by an altered cross-talk between damaged astrocytes and oligodendrocytes, as astrocytes are crucial for oligodendrocyte survival (Belin et al., 1997; Szymocha et al., 2000). Using astrocyte-oligodendrocyte and astrocyte cultures, we showed the deleterious effect of IgGNMO+ on oligodendrocytes. These in vitro data were validated ex vivo on optic nerve, a typical white matter tract and an archetypal model tissue for studying glial cell physiology and interaction (Butt et al., 2004). Prolonged application (24h) of IgGNMO+ mimicked the suspected in situ effect of NMO-IgG/AQP4 antibody during Devic’s neuromyelitis optica attacks. All experiments were performed with purified IgG isolated from sera of patients with Devic’s neuromyelitis optica rather than total sera to avoid molecular signal other than autoantibody. It remains to be determined whether, in our study models, the NMO-IgG/AQP4 antibody itself or another autoantibody present in the purified IgG fraction are deleterious for glial cells. The minor effect of IgGNMO— and AQP4-absorbed IgGNMO+ towards oligodendrocytes, compared to the effect of IgGNMO+, argues for the involvement of anti-AQP4 antibody in glial injury. However, it is likely that, as in other autoimmune diseases, there might be more than one target auto-antigen. A study using protein microarray has identified a possible autoantibody other than AQP4 in the serum of one patient with Devic’s neuromyelitis optica (LaIive et al., 2006). Eventually, testing cells and tissues from AQP4 knockout animals should define more precisely the role of AQP4 antibodies. Finally, purified IgG were used without complement in all experiments, as our objective was to analyse the effect of the autoantibody per se. The critical role of autoantibody associated with complement in Devic’s neuromyelitis optica pathophysiology has already been well documented (Hinson et al., 2008; Sabater et al., 2009; Saadoun et al., 2010). However, autoantibody also acts as specific receptor agonist or antagonist, or alters antigen density in target cells (Diamond et al., 2009). Recent data (Hinson et al., 2008) combines with ours to suggest that, besides complement activation, antibody-directed alteration of surface AQP4 could be involved in the pathophysiology of Devic’s neuromyelitis optica. Moreover, this latter antibody function could accentuate the complement effect in Devic’s neuromyelitis optica pathophysiology as oligodendrocytes are sensitized to complement attack following disruption of glutamate homeostasis (Alberdi et al., 2006).

The loss of astrocyte driven by NMO-IgG/AQP4 antibody is most likely involved in Devic’s neuromyelitis optica pathophysiology. Staining of GFAP is lost in recent Devic’s neuromyelitis optica lesions also lacking AQP4 (Misu et al., 2007). A marked increase of GFAP and S100β in the cerebrospinal fluid during acute Devic’s neuromyelitis optica relapses has been associated with clinical severity and length of spinal lesions (Misu et al., 2009; Petzold et al., 2010). In vitro, NMO-IgG/AQP4 antibody can induce necrosis of astrocytes in a context of complement activation (Kinoshita et al., 2009; Sabater et al., 2009). Our observations suggest that, in addition, a functional alteration of astrocytes might also be implicated. In our study models, long-time exposure of glial cells to IgGNMO+, validated as containing AQP4 antibody, specifically led to a prolonged down-expression of both AQP4 and the glial glutamate transporter GLT1 at the astrocyte membrane. Such an effect has already been observed following short exposure of isolated astrocytes to NMO-IgG/AQP4 antibody (Hinson et al., 2008). Given the prominent role of GLT1 in extracellular glutamate clearance (Danbolt et al., 1992), downregulation at astrocyte membrane might be involved in the progressive increase of extracellular glutamate level detected in IgGNMO+-treated glial cells. Extracellular glutamate elevation could also result from reduced glutamine synthetase activity as pharmacological inhibition of glutamine synthetase enhances exocytotic release of glutamate from astrocytes (Ni and Parpura, 2009). We show that glutamine synthetase activity was reduced in astrocytes following exposure to IgGNMO+. The down-regulation of AQP4 in response to IgGNMO+ could also participate in astrocyte homeostasis disruption. It is generally agreed that AQP4 plays a role in brain water control (Papadopoulos et al., 2004); however, recent works have indicated that functions of AQP4 in the central nervous system are broader than expected (Verkman, 2005). In astrocytes, AQP4 has been functionally associated with the glutamate transports GLAST and GLT1 and the inward rectifying K+ channel Kir4.1. This macromolecular complex plays an important role in K+ buffering, extracellular space expansion, neuronal transmission and excitability (Binder et al., 2004; Verkman, 2005; Zeng et al., 2007), which could be impaired by NMO-IgG/AQP4 antibody. Overall, the NMO-IgG-mediated downregulation of glutamine synthetase activity, and of AQP4 and GLT1 on the astrocyte cell surface, suggests that alteration
of glutamate homeostasis in astrocytes is a potential pathogenic mechanism in Devic’s neuromyelitis optica.

Our experiments demonstrate that astrocytes, when altered by IgG\textsuperscript{NMO-}, are toxic \textit{in vitro} for oligodendrocytes and \textit{ex vivo} for myelin through a bystander effect possibly involving glutamate. This led us to propose that the white matter injury observed in Devic’s neuromyelitis optica lesions could be related to changes in astrocyte homeostasis and elevated extracellular glutamate level as a consequence. Several findings support this hypothesis. Astrocytes can release a variety of gliotransmitters, including glutamate (Parpura and Zorec, 2010), and oligodendrocytes in the spinal cord and optic nerve are highly vulnerable to this main excitotoxic transmitter (Matute, 2006; Xu et al., 2008). In optic nerve, blockade of the glutamate transporter GLT1, which is preferentially expressed on astrocyte processes close to oligodendrocytes, led to massive demyelination (Domerçq et al., 2005; Arranz et al., 2008). The role of glutamate NMDA receptors in such oligodendrocyte injury is likely. They are functionally expressed in the myelinating processes of oligodendrocytes and have higher glutamate affinity relative to \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor (Karadottir et al., 2005). Over-activation leads to disintegration of oligodendroglial processes in the optic nerve (Salter and Fern, 2005) and NMDA receptor blockade reduced damage to white matter in a model of demyelination (Wallstrom et al., 1996; Basso et al., 2008). In our study model, NMDA receptor subunits were expressed on oligodendrocytes and treatment with the competitive NMDA receptor antagonist, D-AP5 partly reduced oligodendrocyte loss after IgG\textsuperscript{NMO-} exposure. Finally, a direct toxic effect of NMO-IgG/AQP4 antibody on oligodendrocytes, although not definitely excluded, remains highly hypothetical considering that AQP4 is not expressed by oligodendrocytes. Moreover we showed that IgG\textsuperscript{NMO-} had no direct effect on enriched oligodendrocyte primary cultures, neither on morphology nor survival.

The involvement of AQP4 in the pathophysiology of Devic’s neuromyelitis optica probably depends on its location within neural tissue as several pools of AQP4 have been described (Amiry-Moghaddam et al., 2004; Nagelhus et al., 2004). AQP4 mainly accumulates in astrocyte end-feet membranes, at fluid-parenchymal interfaces and the blood–brain barrier through \(\alpha\)-syntrophin anchorage. Independently of this anchorage, astrocyte also expresses AQP4 in non-end-feet membranes within parenchyma (Costa et al., 2007). We showed here that NMO-IgG/AQP4 antibody has the ability to bind both astrocyte end-feet around blood vessels and astrocytic processes that surround oligodendrocytes in the rat spinal cord and optic nerve. The close contact of NMO-IgG/AQP4 positive processes with oligodendrocytes and myelin tracts suggest that a bystander effect of NMO-IgG-damaged astrocytes on oligodendrocytes might occur in the nervous tissues affected by Devic’s neuromyelitis optica. NMO-IgG-induced dysfunction of perivascular astrocyte foot process could trigger an initial osmotic dysregulation as NMO-IgG positive sera significantly increased blood–brain barrier permeability \textit{in vitro} (Vincent et al., 2008). Then, NMO-IgG could easily cross the damaged blood–brain barrier and reach astrocytes in contact with oligodendrocyte, leading to astrocyte, oligodendrocyte and myelin dysfunction. To conclude, NMO-IgG/AQP4 antibody is toxic for cultured oligodendrocyte possibly through an excitotoxic mechanism following disruption of glutamate homeostasis in astrocytes. These experimental data suggest that neuromyelitis optica lesions, which are initiated by AQP4-antibody binding to AQP4 and activation of the classical complement pathway, could also result from downregulation of AQP4, GLT1 and glutamine synthetase with astrocyte dysfunction and oligodendrocyte damage.

### Supplementary material

Supplementary material is available at \textit{Brain} online.

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