Oligodendrocyte Gap Junction Loss and Disconnection From Reactive Astrocytes in Multiple Sclerosis Gray Matter

Kyriaki Markoullis, PhD, Irene Sargiannidou, PhD, Natasa Schiza, MSc, Federico Roncaroli, MD, Richard Reynolds, PhD, and Kleopas A. Kleopa, MD

Abstract

Gap junctions are essential for glial cell function and have been increasingly implicated in multiple sclerosis (MS). Because increasing cortical abnormalities correlate with disease progression and cognitive dysfunction, we examined the expression of oligodendrocytic connexin32 (Cx32) and Cx47 and their astrocytic partners Cx30 and Cx43 in cortical lesions and normal-appearing gray matter (NAGM) in MS patients. Postmortem brain tissue samples from 9 MS cases were compared with 10 controls using real-time polymerase chain reaction, immunoblot, and immunohistochemical analyses. Connexin32 and Cx47 gap junction formation in oligodendrocytes was reduced within lesions, whereas Cx32 loss also extended to NAGM. In contrast, astrocytic Cx30 expression was increased within cortical lesions, whereas Cx43 was elevated in both lesions and NAGM. Diffuse microglial activation and marked astrogliotic changes accompanied these connexin abnormalities. Increased expression of Cx43 correlated with inflammatory load (r = 0.828, p = 0.042), whereas Cx32 expression correlated with longer disease duration and, therefore, milder course (r = 0.825, p = 0.043). Thus, there is a loss of intramyelin and intercellular oligodendrocyte gap junctions in MS gray matter lesions and NAGM, whereas interastrocytic gap junctions are increased, reflecting astrogliosis. These changes correlate with inflammation and disease duration and suggest that disconnection of oligodendrocytes from reactive astrocytes may play a role in failed remyelination and disease progression.

Key Words: Astrocytes, Connexins, Demyelination, Gray matter, Multiple sclerosis, Oligodendrocytes.

INTRODUCTION

Gray matter (GM) or cortical pathology has recently emerged as a major contributor to disease progression and related disability in multiple sclerosis (MS). Cortical pathology is characterized by well-defined demyelinated lesions with little or no immune cell infiltration accompanied by neuronal, axonal, and oligodendrocyte loss (1–5). Extensive GM demyelination is seen in secondary progressive MS and primary progressive MS but also occurs in relapsing-remitting MS (6–10) and at the earliest disease stages (11). Widespread GM pathology has been associated with cognitive dysfunction, memory problems, irreversible disabilities, and epileptic seizures (12–18). Unlike relapsing-remitting MS, secondary progressive MS is refractory to immunomodulatory treatments, indicating that accumulating axonal loss and neurodegeneration play key roles in clinical progression (19–21). However, relatively little is known about the underlying mechanisms leading to cortical pathology and clinical progression.

Glia cells play important roles in brain function and are directly relevant to MS pathogenesis. Both astrocytes and oligodendrocytes are connected through gap junctions (GJs), which are formed by opposing hexamers of connexins. Oligodendrocytes express connexin32 (Cx32), Cx47, and Cx31.3 (the rodent ortholog is Cx29) (22–24) and are coupled to other oligodendrocytes with homotypic (O/O) GJs formed mainly by Cx47 and sometimes by Cx32—and to astrocytes (A/A) via Cx43 or Cx30 homotypic GJs, as well as with oligodendrocytes (25). The major partner of Cx47 at O/A heterotypic GJs is Cx43, whereas Cx30 is coupled with Cx32.

Gial GJs mediate important intercellular and intracellular communication that is vital for myelinating cells. GJC2/Cx47 mutations cause a hypomyelinating leukodystrophy known as Pelizaeus-Merzbacher–like disease (33), whereas GJB1/Cx32 mutations cause peripheral neuropathy and chronic or acute transient encephalopathy syndromes, often induced by conditions of metabolic stress (34–36). Even mutations affecting astrocytic Cx43 cause demyelination as part of the ocudentodigital dysplasia syndrome (37), emphasizing that O/A connectivity is crucial for oligodendrocytes. The importance of glial GJs to CNS myelin integrity has been confirmed experimentally in the respective animal models of these disorders (38–43).

We recently demonstrated extensive alterations of glial GJ formation in MS white matter (WM) lesions and...
normal-appearing WM (44), as well as in the WM of the exper-
imental autoimmune encephalomyelitis mouse model of
the disease (45). We found widespread disruption of
oligodendro-

cyte GJs and loss of O/A channels in the setting of chronic
inflammation and astrogliosis favoring increased A/A con-
nectivity, which may represent a relevant mechanism contrib-
uting to MS progression. The aim of this study was to ex-
amine the alterations of oligodendrocytic connexins and their astrocytic
partners at O/A GJs in cortical MS lesions and normal-appearing
gray matter (NAGM). We found extensive disruption of O/A and
O/O GJs along with distinctly elevated A/A GJs associated with
astrogliosis, and that these alterations correlate with inflamma-
tion and disease outcome.

**MATERIALS AND METHODS**

**Human Brain Tissue Samples**

Postmortem human brain samples were provided by the
UK MS Society Tissue Bank at Imperial College (http://www.ukmsstimetissuebank.imperial.ac.uk/) and collected with fully in-
formed donor consent after approval by the UK National Ethics
Committee (08/MRE09/31). Tissues were processed and anal-
alyzed as previously described (44). Briefly, neuropathologic
analysis was performed on 10-μm cryosections obtained from
brain tissue blocks of 2 × 2 × 1 cm³ from 9 MS (mean ± SD
age, 60.1 ± 11.4 years) and 10 age-matched non-neurological
controls (66.1 ± 15.02 years; unpaired t-test, p = 0.34) (Table)
that had been frozen unfixed or fixed (in 4% paraformaldehyde
for a minimum of 4 hours, cryoprotected in 30% sucrose/
phosphate-buffered saline [PBS]) and stored at −80 °C. All cases
were processed for Luxol fast blue (LFB)/hematoxylin staining,
as recently described (4), as well as for immunohistochemistry
for ionized calcium binding adapter molecule 1 (Iba1) and myelin
oligodendrocyte glycoprotein (MOG) or myelin proteolipid pro-
tein (PLP) to assess inflammation and demyelination.

**Characterization and Classification of MS Lesions**

Cortical lesions were classified according to published
criteria (4) and defined as follows: i) leuocortical or Type I
lesions involve the deeper GM layers and neighboring sub-
cortical WM, sparing the superficial layers of the cortex; ii)
intracortical or Type II lesions are usually small and are con-
 fined to the GM; and iii) subpial or Type III lesions involve
predominantly the subpial GM and extend into the cortex down
to Layers 3 to 5. Cortical lesions are characterized by decreased
or absent myelin, as seen with stains for LFB (Figs. 1A–C) and
MOG or PLP, and contain activated microglia (Figs. 1D–I),
whereas macrophages and infiltrating lymphocytes are largely
absent. Normal-appearing gray matter was defined as GM with
apparently normal myelination and not near lesions or perilesional
tissue (Fig. 1G).

**RNA Extraction, Reverse Transcription, and
Real-Time Polymerase Chain Reaction**

Total RNA was isolated from non-MS control GM (n = 6
cases), MS NAGM (n = 6 cases), and MS cortical lesions (n = 5
lesions from 4 cases) in immunohistochemically characterized
snap-frozen brain tissue blocks using the RNeasy Lipid Tissue Mini
Kit (Qiagen, Hilden, Germany), according to the manufacturer’s
instructions. In all cases, RNA quality was checked. RNA was
subjected to reverse transcription (RT)–polymerase chain reaction
(PCR) using the TaqMan RT-PCR reagents and the GeneAmp
PCR System (Applied Biosystems, Singapore, Singapore). The
expression levels of genes encoding Cx32 (GJA1), Cx47 (GJC2),
Cx43 (GJA1), Cx30 (GJB6), PLP, and Iba1 were examined
by quantitative real-time PCR analysis using a 7900HT Real-
Time PCR System and the appropriate predesigned TaqMan
Gene Expression Assays (Applied Biosystems): for Cx32
Hs00702141_s1, Cx47 Hs00954083_m1, Cx43 Hs00748445_s1,
Cx30 Hs00272726_s1, PLP Hs00166914_m1, and Iba1
Hs00741549_g1. Glyceraldehyde 3-phosphate dehydrogenase
(GAPDH) (Hu GAPD 20×) was used as the “housekeeping”
control gene. A second housekeeping gene, β2 (Hs99999901_s1),
was used with similar results (data not shown). Polymerase chain
reaction runs were performed according to the manufacturer’s in-
structions using previously described settings (44). Samples were
loaded and run in triplicate. Expression levels for each connexin
in MS samples were calculated as fold induction value ($2^{-ΔΔCt}$)
relative to normal cases. Expression levels in cortical lesions were
also calculated ($2^{-ΔΔCt}$) relative to NAGM.

**Immunoblot Analysis**

Samples of 5 non-MS GM and 4 MS NAGM were col-
lected from characterized snap-frozen tissue blocks and processed
as previously described (44). Briefly, brain tissues were lysed,
and their proteins were fractionated by 12% sodium dodecyl
sulfate–polyacrylamide gel electrophoresis (Bio-Rad, Hercules,
CA) and transferred to a Hybond-C extra membrane. Membranes
were blocked with 5% nonfat milk/Tris-buffered saline with
Tween 20 for 1 hour at room temperature and incubated with
rabbit anti-Cx47 (diluted 1:5000) (46), anti-Cx32 (number 918;
1:5000) (47), or anti-Cx30 (1:3000; Invitrogen, Billerica, MA)
(24) antibodies and with mouse monoclonal antibodies against
Cx43 (1:2000; Chemicon, Temecula, CA), myelin basic protein
(MBP) (1:2000; Abcam, Cambridge, United Kingdom), or
GAPDH (1:4000; Santa Cruz Biotechnology, Santa Cruz, CA)
in 5% milk/Tris-buffered saline with Tween 20 at 4 °C over-
night. To reduce nonspecific binding, we preincubated the Cx32
antiserum with Gjb1-null tissue lysate (40). Blots were washed
and incubated with anti-rabbit (1:10000) or anti-mouse (1:5000)
horseradish peroxidase–conjugated secondary antibodies (Jackson
ImmunoResearch Laboratories, West Grove, PA) and visual-
ized with enhanced chemiluminescence (ECL Plus; Amersham,
Piscataway, NJ). Band optic intensity was measured with Tinascan
version 2.07d and normalized for loading with GAPDH bands.

**Immunohistochemistry**

For double immunofluorescent labeling, 10-μm sections of
snap-frozen or paraformaldehyde-fixed tissues from 6 non-MS
and 6 MS cases were permeabilized in cold methanol, washed in
PBS, blocked in 2% solution of normal horse or goat serum
(Sigma, St Louis, MO) in PBS containing 0.5% Triton X-100
(NGS-Tx or MHS-Tx buffer) for 20 minutes, and incubated
overnight at 4 °C with the following primary antibodies di-
luted in blocking solution: mouse monoclonal antibodies against
MOG (1:200; Dr. Sara Piddlesden, Cardiff, United Kingdom),
Cx32 (1:50; Zymed-Invitrogen), Cx47 (1:200; Zymed-Invitrogen),

© 2014 American Association of Neuropathologists, Inc.

Copyright © 2014 by the American Association of Neuropathologists, Inc. Unauthorized reproduction of this article is prohibited.
<table>
<thead>
<tr>
<th>Case</th>
<th>Age, years/Sex</th>
<th>Cause of Death</th>
<th>Disease Course/Duration</th>
<th>PMD, hours</th>
<th>Type and Number of Tissue Samples Examined*</th>
<th>Method Used</th>
<th>Localization of Tissue Samples Examined (Gyrus)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS58</td>
<td>51/F</td>
<td>Not known</td>
<td>SPMS/21</td>
<td>15</td>
<td>1 Fixed block; 2 unfixed blocks (NAGM, 1 GML)</td>
<td>IHC; RT-PCR</td>
<td>Inferior frontal, inferior temporal, cingulate</td>
</tr>
<tr>
<td>MS60</td>
<td>55/M</td>
<td>Aspiration pneumonia</td>
<td>SPMS/43</td>
<td>16</td>
<td>1 Unfixed block (NAGM)</td>
<td>WB</td>
<td>Inferior frontal</td>
</tr>
<tr>
<td>MS80</td>
<td>71/F</td>
<td>Heart failure</td>
<td>RRMS-SPMS/35</td>
<td>24</td>
<td>3 Unfixed blocks (NAGM, 2 GML)</td>
<td>RT-PCR, WB</td>
<td>Middle frontal, precentral, superior temporal</td>
</tr>
<tr>
<td>MS82</td>
<td>49/F</td>
<td>Aspiration pneumonia</td>
<td>SPMS/30</td>
<td>9</td>
<td>1 Fixed block</td>
<td>IHC</td>
<td>Occipitotemporal</td>
</tr>
<tr>
<td>MS88</td>
<td>54/F</td>
<td>Bronchopneumonia</td>
<td>SPMS/27</td>
<td>22</td>
<td>3 Fixed blocks; 2 unfixed blocks (NAGM, 1 GML)</td>
<td>IHC; RT-PCR</td>
<td>Cingulate, inferior temporal, middle-inferior frontal, occipital</td>
</tr>
<tr>
<td>MS100</td>
<td>46/M</td>
<td>Pneumonia</td>
<td>SPMS/8</td>
<td>7</td>
<td>1 Fixed block; 1 unfixed block (GML)</td>
<td>IHC; RT-PCR</td>
<td>Superior frontal, postcentral</td>
</tr>
<tr>
<td>MS105</td>
<td>73/M</td>
<td>Pneumonia</td>
<td>SPMS/46</td>
<td>8</td>
<td>3 Fixed blocks; 1 unfixed block (NAGM)</td>
<td>IHC; RT-PCR</td>
<td>Postcentral, frontal pole, superior frontal, inferior temporal</td>
</tr>
<tr>
<td>MS125</td>
<td>76/F</td>
<td>Not known</td>
<td>SPMS/30</td>
<td>13</td>
<td>2 Fixed blocks; 1 unfixed block (NAGM)</td>
<td>IHC; WB, RT-PCR</td>
<td>Precentral, middle frontal, middle-inferior temporal</td>
</tr>
<tr>
<td>MS313</td>
<td>66/M</td>
<td>Gastrointestinal bleeding from ulcer</td>
<td>PPMS/29</td>
<td>16</td>
<td>1 Unfixed block (NAGM)</td>
<td>WB, RT-PCR</td>
<td>Superior temporal</td>
</tr>
<tr>
<td>C10</td>
<td>73/M</td>
<td>Cardiogenic shock</td>
<td>NA</td>
<td>21</td>
<td>1 Unfixed block (GM)</td>
<td>RT-PCR</td>
<td>Inferior frontal</td>
</tr>
<tr>
<td>C14</td>
<td>64/M</td>
<td>Cardiac failure, myocardial infarction</td>
<td>NA</td>
<td>18</td>
<td>2 Fixed blocks</td>
<td>IHC</td>
<td>Superior frontal, inferior temporal</td>
</tr>
<tr>
<td>C16</td>
<td>92/M</td>
<td>Congestive cardiac failure</td>
<td>NA</td>
<td>13</td>
<td>2 Fixed blocks</td>
<td>IHC</td>
<td>Superior temporal, postcentral</td>
</tr>
<tr>
<td>C22</td>
<td>69/F</td>
<td>Lung cancer</td>
<td>NA</td>
<td>33</td>
<td>1 Fixed block; 1 unfixed block (GM)</td>
<td>IHC; RT-PCR, WB</td>
<td>Superior frontal, inferior temporal</td>
</tr>
<tr>
<td>C25</td>
<td>35/M</td>
<td>Tongue carcinoma</td>
<td>NA</td>
<td>22</td>
<td>1 Unfixed block (GM)</td>
<td>WB</td>
<td>Superior temporal</td>
</tr>
<tr>
<td>C26</td>
<td>78/F</td>
<td>Myeloid leukemia</td>
<td>NA</td>
<td>33</td>
<td>1 Fixed block; 1 unfixed block (GM)</td>
<td>IHC; WB, RT-PCR</td>
<td>Middle-inferior temporal, postcentral</td>
</tr>
<tr>
<td>C28</td>
<td>60/F</td>
<td>Ovarian cancer</td>
<td>NA</td>
<td>13</td>
<td>1 Fixed block; 1 unfixed block (GM)</td>
<td>IHC; RT-PCR</td>
<td>Precentral, middle frontal</td>
</tr>
<tr>
<td>C36</td>
<td>68/M</td>
<td>Cor pulmonale, heart failure</td>
<td>NA</td>
<td>30</td>
<td>2 Fixed blocks</td>
<td>IHC</td>
<td>Precentral, postcentral</td>
</tr>
<tr>
<td>C41</td>
<td>54/M</td>
<td>Lung cancer</td>
<td>NA</td>
<td>20</td>
<td>1 Unfixed block (GM)</td>
<td>WB, RT-PCR</td>
<td>Middle frontal</td>
</tr>
<tr>
<td>C51</td>
<td>68/M</td>
<td>Ischemic heart disease</td>
<td>NA</td>
<td>24</td>
<td>1 Unfixed block (GM)</td>
<td>WB, RT-PCR</td>
<td>Middle frontal</td>
</tr>
</tbody>
</table>

*All samples were characterized by Iba1/MOG immunolabeling and LFB/hematoxylin staining.
F, female; GML, GM lesion; IHC, immunohistochemistry; M, male; NA, not applicable; PMD, postmortem delay; PPMS, primary progressive MS; RRMS, relapsing-remitting MS; SPMS, secondary progressive MS; WB, Western blot.
or glial fibrillary acidic protein (GFAP) (1:400; Sigma); rabbit antisera against Iba1 (1:500; Biocare Medical), Cx43 (1:50; Cell Signaling Technology), Cx30 (1:500; Zymed-Invitrogen), Olig2 (1:500; Millipore, Billerica, MA), aspartoacylase (ASPA) (1:200; Abcam), CD3 (1:100; Abcam), CD3 (1:100; Abcam), and MBP (1:200; Sigma); or rat monoclonal antibodies against PLP (1:10; Reynolds Laboratory) and CD68 (1:100; Alexa Fluor). After washes, sections were incubated with biotinylated anti-mouse or anti-rabbit secondary antibodies (1:200; Vector Laboratories, Burlingame, CA) for 1 hour at room temperature then with streptavidin fluorescein isothiocyanate (1:800) and appropriate DyLight rhodamine-conjugated AffiniPure F(ab’)2 secondary antibodies (Jackson ImmunoResearch Laboratories) and counterstained with DAPI. After treatment with an autofluorescence eliminator reagent (Chemicon) for 10 minutes, slides were mounted with Dako Fluorescent Mounting Medium. Images were photographed under a Zeiss fluorescence microscope with a digital camera using the Zeiss Axiovision software (Carl Zeiss Microlmaging) with comparable exposure times to allow comparison between groups.

Quantification of GJ Plaque Formation

Gap junction plaques formed by oligodendrocytic Cx47 and astrocytic Cx43 and Cx30 were defined as individual concentrations of connexin immunoreactivity measuring between 0.1 and 1 μm². The number of GJ plaques was measured in 70,000-μm² rectangles within demyelinated lesions (L) and in NAGM (showing normal myelin immunoreactivity and not bordering on lesions) (Fig. 1G) using the ImagePro Plus software (Media Cybernetics, Bethesda, MD) to examine the gradient of GJ pathology. Gap junction counts for each connexin were obtained from 5 MS cases (7 NAGM areas, 15 lesions) and 5 control cases (17 GM areas). In addition, we counted GJ plaques in available intracortical and leucocortical lesions and in different cortical layers of control and MS NAGM for each area of interest; we averaged the counts from 4 different adjacent fields. Immunoreactivity to Iba1 and GFAP (percentage of total GM area measured) was quantified in the same fields to assess the degree of inflammation and astrogliosis, respectively. Connexin32 GJ plaque counts were not performed because specific GM staining was obtained in only 2 MS cases, hamstringing statistical analysis.

Statistical Analysis

Data are expressed as mean ± SE and analyzed by Excel and Minitab 15 software. Differences in connexin quantification by immunohistochemistry, immunoblot, and real-time PCR between MS and non-MS control brains were examined with Student t-test when normal distribution of values was proven. Correlation between connexin expression levels and clinicopathologic features of MS cases (inflammation and disease duration) was examined with Pearson correlation coefficient (r). Values of p < 0.05 were considered significant.

RESULTS

MS Tissue Classification and Pathologic Features

Multiple sclerosis brain tissue blocks (n = 23; 11 fixed and 12 unfixed from 9 MS cases) were characterized using LFB/hematoxylin staining (Figs. 1A–C) and immunostaining for myelin proteins (MOG or PLP) and microglia (Iba1) (Figs. 1D–G). We identified all types of cortical lesions (leucocortical or Type I, n = 3; intracortical or Type II, n = 5; subpial or Type III, n = 19). Lesion distribution diagrams of all characterized tissue blocks were constructed (44) and used as a guide for obtaining samples for RNA extraction and real-time PCR analysis and for obtaining protein samples for immunoblot analysis. Subpial or Type III cortical lesions were chosen for GJ plaque counts because they were more numerous; however, we additionally counted GJ plaques in available intracortical and leucocortical lesions (n = 3 cases each) for comparison. In view of evidence for a gradient of inflammation from the pial surface to the deeper cortical layers (48), GJ counts were also performed in cortical layers (Layers 1–2, 3–4, and 5–6) from 5 control and 5 MS (NAGM) cases to examine possible gradients in glial GJ pathology. Quantification of the degree of microglial/macrophage activation as an indication of neuroinflammation, assessed by Iba1 immunoreactivity, confirmed higher levels in MS tissue compared with non-MS control tissue. The presence of highly activated microglia with phagocytic capability was also demonstrated with CD68 immunoreactivity in MS NAGM and perilesional areas (Figs. 1H, I), which was absent in control GM (data not shown). No significant differences in neuroinflammation levels were observed between

© 2014 American Association of Neuropathologists, Inc.
MS NAGM and cortical lesions (Fig. 1J). Immunostaining with anti-CD3 antibody showed no significant T-cell infiltrates in the MS GM; only sections with preserved leptomeninges showed some meningeal CD3-immunoreactive cells (Figure, Supplemental Digital Content 1, http://links.lww.com/NEN/A635).

Alterations of Glial Connexin Gene Expression in MS Tissues
Expression of GJB1/Cx32, GJC2/Cx47, GJA1/Cx43, and GJB6/Cx30 was examined in non-MS control GM, MS NAGM, and demyelinated cortical lesions (Fig. 1K). Compared with control GM, no significant changes in MS NAGM were found. Within cortical lesions, only oligodendrocytic Cx32 showed reduced messenger RNA expression levels. Proteolipid protein expression was preserved in NAGM and reduced in lesions, whereas Iba1 expression was increased in cortical lesions. Similar changes with significantly reduced expression of both oligodendrocytic Cx32 and Cx47 were observed when cortical lesions were compared with NAGM (data not shown).

Disruption of Oligodendrocytic GJs in NAGM and Cortical Lesions
Immunostaining of fixed brain sections for Cx47 in combination with the oligodendrocyte cell marker ASPA was performed to assess Cx47 expression and the number of formed GJ plaques (Figs. 2A–C). Compared with non-MS control GM, Cx47 showed a similar expression in MS NAGM even though GJ plaques appeared smaller (Fig. 2B); on the contrary, perilesional areas and cortical lesions showed significantly reduced Cx47 immunoreactivity with loss of oligodendrocytes (Fig. 2C). Double staining for Cx47 and the oligodendrocyte precursor cell (OPC) marker Olig2 showed that the number of Olig2-positive OPCs in NAGM was higher than that in non-MS controls (Fig. 2H), as also shown in our previous study of MS WM (44). Oligodendrocyte precursor cells in MS NAGM expressed increasing levels of Cx47 depending on their maturity (Figs. 2D–F). Oligodendrocyte precursor cells were also present in cortical lesions but in smaller numbers and with lower Cx47 immunoreactivity. Counts of Cx47 GJ plaques confirmed the significant reduction in lesions and perilesional areas, with no significant change in NAGM (Fig. 2G). The reduction of Cx47 GJ plaques was similar in all types of MS lesions and was significant compared with the corresponding cortical layers in control cases (Figure, Supplemental Digital Content 2, http://links.lww.com/NEN/A636). Consistent with immunostaining results, immunoblot analysis of NAGM samples showed similar Cx47 protein levels compared with non-MS controls (Figs. 2I–J).

The expression of Cx32 was evaluated qualitatively in immunostained sections followed by immunoblot analysis of NAGM samples. Compared with control GM, where Cx32 was normally localized to oligodendrocyte cell bodies and also along medium-sized myelinated fibers (Figs. 3A–E, G), Cx32 expression was markedly reduced in both MS NAGM and cortical lesions. Connexin32 GJ plaques were lost even along apparently normally myelinated fibers, and the few remaining Cx32 GJ plaques were restricted around oligodendrocyte somata (Figs. 3F, H–I). Immunoblot analysis showed significantly reduced Cx32 protein levels in NAGM compared with non-MS controls (Figs. 3J–K), even though MBP levels were preserved (Figs. 3L–M), corroborating the immunohistochemical observations indicating selective loss of Cx32 in NAGM fibers.

Astrogliosis and Elevation of Astrocytic GJ Proteins in MS GM
Given the substantial changes in oligodendrocytic connexins, we then asked how their astrocytic partners at O/A GJs are affected in MS GM. We first examined astrocyte morphology and quantified astrogliosis in NAGM and lesions by immunostaining for GFAP in combination with PLP for myelin. Extensive reactive astrogliotic changes were found in and around MS GM lesions, with increased thickness and density of astrocytic processes compared with control GM (Figs. 4A–F). Astrocytic processes lining the glia limitans and perivascular end feet were intact and even more prominent in MS cortex compared with controls (Figs. 4G–J). Quantification showed that GFAP immunoreactivity was significantly increased in both cortical lesions and NAGM compared with non-MS control GM (Fig. 4K).

Immunostaining for the main astrocytic partner at O/A GJs (Cx43 in combination with the astrocyte marker GFAP) revealed increased expression of Cx43 along with GFAP immunoreactivity in NAGM and cortical lesions compared with controls (Figs. 5A–C). Connexin43 GJ plaques normally show a concentration around oligodendrocytes in control GM; however, the expression was diffusely increased in MS GM. Connexin43 GJ plaque counts were significantly increased in both NAGM and cortical lesions compared with control GM (Fig. 5G). The increase in Cx43 GJ plaque numbers was most prominent in superficial cortical layers 1 and 2 of NAGM compared with the same layers of the control cortex (Figure, Supplemental Digital Content 2, http://links.lww.com/NEN/A636), indicating a gradient of astrocyte pathology with more connectivity alterations near the brain surface.

Connexin30, the other major astrocytic connexin that, in contrast to Cx43, is almost restricted to GM, showed increased expression mainly within cortical lesions and to a lesser degree in NAGM and perilesional areas compared with controls (Figs. 5D–F). Colocalization of Cx30 with activated astrocytes was evident in GM lesions (Fig. 5F). Connexin30 GJ plaque counts confirmed a significant increase specifically within GM lesions compared with control GM (Fig. 5G), which was most pronounced in subpial lesions compared with superficial layers of control or MS NAGM (Figure, Supplemental Digital Content 2, http://links.lww.com/NEN/A636). Immunoblot analysis of Cx43 and Cx30 protein levels in NAGM showed increased Cx43, but not Cx30, levels, in agreement with the immunostaining results (Figs. 5H–I).

Disrupted O/A Connectivity in MS GM
We further examined how O/A GJs may be affected in MS GM by double immunostaining for Cx47 and Cx32 in combination with their astrocytic partners Cx43 and Cx30,
FIGURE 2. Reduced Cx47 GJ plaques in MS GM lesions and expression in recruited OPCs. (A–C) Double immunolabeling for Cx47 (green) and oligodendrocyte marker ASPA (red) shows Cx47 GJ plaques (arrowheads) at cell bodies and proximal processes of mature oligodendrocytes in control GM (A). In MS NAGM (B), Cx47-expressing cells are frequently ASPA-negative (double arrowheads), and ASPA-positive oligodendrocytes appear reduced. Connexin47 GJ plaques are markedly reduced within GM lesions and mostly expressed in ASPA-negative cells (C). (D–F) Double staining for Cx47 (green) and the OPC marker Olig2 (red) shows very few Olig2-positive OPCs that mostly do not express Cx47 in control GM (D), in contrast to mature oligodendrocytes with weak or no Olig2 expression (arrowheads), whereas in NAGM (E), Olig2-positive OPCs expressing Cx47 are increased. Some strongly Olig2-positive OPCs, likely representing earlier stages of OPC differentiation (arrows in E, F) with minimal Cx47 expression, are found in NAGM and lesions (F). High-magnification insets with separate and merged channels are shown under the overview images. Cell nuclei are stained with DAPI (blue). (G) Quantification of Cx47 plaques (n = 5 control and 5 MS cases) reveals a significant reduction in Cx47 GJ plaque numbers in cortical lesions (L) compared with NAGM and control GM (C). (H) Quantification of Olig2-positive OPCs confirms increased numbers in MS versus control GM. (I, J) Immunoblot analysis (I) of control (C1–C3) and MS NAGM samples (MS1–MS3); GAPDH blot for loading control is shown underneath. Quantification of normalized band optic density (OD) (J) confirms unchanged Cx47 protein levels in MS NAGM (lesions not examined). Scale bars = (A–F) 20 μm; insets, 10 μm.
FIGURE 3. Loss of Cx32 GJs in MS GM. Double immunolabeling, as indicated for Cx32 (green) and microglia marker Iba1 (red) (A, B, E, F), or single labeling (C, D) demonstrates the normal expression of Cx32 in control GM, forming GJ plaques diffusely along medium-sized myelinated fibers (arrowheads) (A–C, E) and around oligodendrocyte cell bodies (double arrowheads) (B, D). This expression pattern is disrupted, along with marked microglia activation in MS NAGM (F). Double labeling with anti-myelin PLP (red) shows expression of Cx32 (green) along myelinated fibers in control GM (G), which is diminished in MS NAGM despite apparently normal myelination (H) and is lost in GM lesions (I). Higher-magnification insets with separate and merged channels are shown in (G) to (I). Cell nuclei are stained with DAPI (blue). (J–M) Immunoblot analysis (J) of control (C1, C2, C3) and MS NAGM (MS1, MS2, MS2) samples. Glyceraldehyde 3-phosphate dehydrogenase blot for loading control and MBP blot for myelin control are shown underneath. Quantification of band-normalized optic density (OD) (K) confirms the immunostaining results, showing significantly reduced Cx32 levels in MS NAGM (K), whereas MBP levels are unchanged (L). Connexin32-to-MBP ratio measured by immunoblot (M) is significantly reduced, confirming selective Cx32 loss in MS NAGM. Scale bars = (A, C, E, F) 20 μm; (B, D) 10 μm; (G–I) 50 μm; insets, 20 μm.
respectively, to show the degree of colocalization in MS compared with control GM. In control GM, Cx43 immunoreactivity was mostly overlapping with Cx47-surrounding oligodendrocytes, indicating that most Cx43-formed GJs were heterotypic O/A channels; however, the reverse pattern was seen in NAGM and lesions, with most Cx43 GJs having no overlap with Cx47 GJ plaques, likely representing mostly A/A channels (Figs. 6A–C). Measurement of the Cx43-to-Cx47 GJ plaque ratio showed a significant increase in both NAGM and lesions compared with control GM (Fig. 6D), confirming widespread disruption of O/A and increase in A/A GJs in MS cortex. Similarly, the loss of Cx32 GJ plaques in cortical lesions and NAGM was accompanied by an increase in Cx30 GJs especially in lesions and lack of any colocalization with Cx32, indicating that these are mostly A/A in the setting of reactive astroglisis (Figs. 6E–G).

**FIGURE 4.** Extensive astroglisis in MS GM lesions and NAGM. Double immunostaining for astrocyte marker GFAP (green) and myelin protein PLP (red) shows, at lower magnification (A–C) and higher magnification (D–F), that compared with control GM (A, D), in NAGM (B, E) with intact myelination, there are increasingly activated astrocytes with hypertrophic cell bodies and processes. Within lesions, astroglisis is even more prominent with diffusely hypertrophic astrocytic processes, whereas PLP immunoreactivity is lost (C, F). Higher-magnification insets with separate and merged channels are shown in (D) to (F). Gial fibrillary acidic protein staining also shows that astrocytic processes lining the glia limitans (arrowheads in G, I), as well as perivascular end feet (arrowheads in H, J), are intact and even more prominent in chronic MS cortex (I, J) compared with controls (G, H). Cell nuclei are stained with DAPI (blue). (K) Quantification of GFAP immunoreactivity in multiple areas from control GM (n = 4 cases) compared with MS NAGM and GM lesions (n = 3 cases) confirms significant astroglisis in both NAGM and lesions [L]. Scale bars = (A–C) 100 μm; (D–F) 50 μm; insets, 20 μm; (G, I) 50 μm; (H, J) 20 μm.
Correlation of GJ Protein Expression With MS Clinicopathologic Features

Finally, we investigated possible correlations between clinicopathologic features of MS cases (n = 6) and glial connexin expression changes in MS NAGM revealed by real-time PCR (Fig. 7). Astrocytic Cx43 expression correlated strongly with inflammatory load assessed by Iba1 expression ($r = 0.828$, $p = 0.042$), whereas the expression of oligodendrocytic Cx32 and Cx47 and astrocytic Cx30 did not significantly correlate with Iba1 expression. When disease duration was examined, there was a significant correlation with Cx32 expression despite the small group number and a possible outlier ($r = 0.825$, $p = 0.043$), but not

![Figure 5](image_url)

**FIGURE 5.** Distinct elevation of astrocytic connexins in MS GM. Double labeling for GFAP (red) and Cx43 (A–C) or Cx30 (D–F) (both green) demonstrates that, compared with control GM (A, D), there is increased formation of Cx43 GJ plaques diffusely in NAGM (B) and less so in lesions (C). In contrast, Cx30 is increased within lesions (F), colocalizing with hypertrophic astrocyte processes and less so in NAGM (E). Higher-magnification insets with separate and merged channels are shown under overview panels. Cell nuclei are stained with DAPI (blue). (G) Cx43 and Cx30 GJ plaque counts (n = 5 control and 5 MS cases) confirms the increase in Cx43 GJs in NAGM and lesions, whereas Cx30 shows increased GJ numbers only in lesions (L) versus control GM (C). (H) Immunoblot analysis of Cx43 and Cx30 levels in MS NAGM (lesions were not examined) and quantitative analysis of GAPDH-normalized band optic density (OD) confirms increased levels of Cx43 (I) and unchanged levels of Cx30 (J) in MS NAGM, in keeping with the immunostaining results. Scale bars = (A–F) 20 μm; insets, 10 μm.
with the expression of the other connexins. Thus, Cx43 expression increases with inflammation in MS GM, and higher Cx32 expression may be associated with longer disease duration, suggesting less myelin pathology and an overall milder pathology.

**DISCUSSION**

Understanding of GM pathology is crucial for advancing MS treatments and limiting progression of the disease associated with disability. Multiple sclerosis GM is characterized by more diffuse pathologic alterations than the classic WM lesions and a lack of infiltrating immune cells. We hypothesize that changes in GJ connectivity that forms the functionally interacting pan-glial network could be an important aspect of this diffuse pathology (49,50). Our study shows marked alterations in glial GJs in MS NAGM and cortical lesions, which could have implications for the development and maintenance of tissue damage.

We describe the loss of oligodendrocytic connexins and a parallel increase in astrocytic connexins. Widespread loss of O/O and O/A GJs is accompanied by an increase in A/A connections in a pattern that is distinct from WM pathology, indicating a different molecular profile of GM astrogliosis. Some of these changes correlate with microglial activation and disease duration, both of which are linked to severity of progression in MS.

Gray matter demyelination was extensive in the MS cases that we examined, as already described in other studies (6–11). Thus, despite the finding of numerous OPCs in GM lesions, remyelination also seems to fail, although some reports suggest that it may be more successful than in the WM (51). Furthermore, we found extensive reactive astrogliosis in both GM lesions and NAGM, in contrast to previous studies finding little evidence of GM astrogliosis (52,53). Although astrogliosis was not as extensive as in WM lesions, it was prominent throughout most cortical lesions, and microglial activation was comparable.

**FIGURE 6.** Disruption of heterotypic oligodendrocyte/astrocyte (O/A) GJs in MS GM. (A–C) Immunostaining for oligodendrocytic Cx47 (red) and astrocytic Cx43 (green) normally forming most of O/A GJs and showing colocalization around oligodendrocyte cell bodies in control GM (A) reveals a diffuse increase in Cx43 GJ plaques in NAGM that mostly do not colocalize with Cx47 (B), whereas the loss of Cx47 GJ plaques in the lesions is accompanied by increased Cx43 expression (C). (D) Quantification shows that the Cx43-to-Cx47 GJ plaque number ratio in control and MS cases (n = 5 each) is significantly increased in MS NAGM and even more in lesions [L] compared with control GM [C]. (E–G) Double staining for Cx32 (green) normally expressed at GM oligodendrocyte cell bodies colocalizing with astrocytic Cx30 (red) (E) shows progressive loss of Cx32 GJs in NAGM (F) and even more in lesions (G). Connexin30 is preserved in NAGM and increased within lesions, not colocalizing with Cx32. Higher-magnification insets with separate and merged channels are shown under overview panels. Cell nuclei are stained with DAPI (blue). Scale bars = (A–G) 30 μm; insets, 10 μm.
to the WM. Thus, even though GM may initially have a higher capacity for repair, this may be progressively lost in chronic MS and especially with ongoing inflammation (4). As we have shown in MS WM (44), OPCs in MS GM express Cx47 but seem to have reduced connectivity to astrocytes, as evidenced by the loss of Cx43/Cx47 colocalization and reduction of the Cx47-to-Cx43 GJ ratio, which is associated with increasing astrogliosis. This may limit their potential for differentiation and achievement of the myelinating stage, as has been demonstrated in Cx43/Cx30 double knockout (KO) mice with loss of OPCs (54). Furthermore, impaired O/A GJ formation by Cx47 caused by lack of interaction with Cx43 (55) is likely to disrupt OPC differentiation in the reactive astrogliotic and inflammatory conditions prevalent in the MS brain, providing a possible explanation for incomplete remyelination. Interestingly, we found that changes in astrocyte connexins follow a gradient and are most prominent in subpial layers of MS cortex, likely associated with the gradient of inflammation arising from the meninges, as shown in previous studies (48).

Loss of cortical O/A connections, as demonstrated here, is likely to have a negative impact on oligodendrocyte survival and function. Through O/A GJs, astrocytes provide an important outlet for restoring K+ levels and osmotic gradients in the periaxonal spaces of myelinated fibers (56). The importance of O/A GJs is underscored by the fact that mutations in Cx43 that disrupt most A/O GJs also lead to demyelination (37). Furthermore, experimental models with deficient O/A GJs show severe demyelination (38,54,55). Although loss of O/A GJs may, in part, be attributable to oligodendrocyte loss especially in lesions, the extent of oligodendrocyte GJ pathology in perilesions and NAGM indicates that loss of O/A connectivity occurs and could be an important factor in failed remyelination and axonal loss. Interestingly, a rapidly progressive MS course with active demyelinating lesions was recently found to be associated with loss of Cx43 and disruption of O/A GJs (57), supporting the role of glial GJ changes in MS progression. In contrast, we found increased astrocytic GJ expression in MS GM, likely reflecting long-term astrogliotic changes; none of the cases studied were known to have a rapidly progressive course.

Although Cx32 is less prevalent in rodent and human GM than in WM (23,24), we found extensive loss of Cx32 GJs at oligodendrocyte cell bodies and along medium-sized myelinated fibers in MS NAGM. We cannot exclude that Cx32 loss may have been caused by incomplete remyelination, but the normal MBP levels in immunoblot samples argue against the possibility that reduced Cx32 levels are caused by shadow plaques. The proximal Cx32 GJs likely represent O/A channels with astrocytic Cx30 (25,39), and their loss may further compromise the homeostasis and viability of oligodendrocytes when combined with loss of Cx47/Cx43 GJs, similar to the profound changes in Cx32/Cx47 double KO mice (39-47,51–59). Furthermore, loss of distal Cx32 GJs along the myelin sheath occurs even in the absence of demyelination and may be detrimental to myelin homeostasis and to the axon itself. Such impact of Cx32 loss has been shown in Cx32 KO peripheral nerves with early axonal pathology preceding demyelination, including neurofilament dephosphorylation, reduced axon caliber, and slowdown of axonal transport (60). Moreover, induction of experimental autoimmune encephalomyelitis in Cx32 KO mice resulted in an exacerbated clinical course with more extensive demyelination and axonal loss (45). Loss of GJs in the myelin sheath may also disrupt axon-glial signaling through Ca2+ and second messengers (61), impair ionic homeostasis, and limit energy supply to the axon itself (49,56,62), causing further neuronal dysfunction and accelerating axonal loss. In particular, disrupted K+ homeostasis in

FIGURE 7. Changes in glial connexin expression correlate with microglial activation and disease duration in MS patients. Diagrams show the correlation of Cx32, Cx47, Cx30, and Cx43 expression in MS NAGM versus control GM (values represent log expression obtained from real-time PCR data) with either Iba1 expression (log expression) (A-D) or disease duration in years (E-H). Pearson correlation coefficients are shown in each diagram, along with significant p values (all other p values > 0.05). Connexin43 correlates positively with Iba1 expression, reflecting microglia activation (C), whereas Cx32 expression shows a significantly positive correlation with disease duration.
functionally active myelinated axons (50,56) of the cortex is likely to worsen mitochondrial dysfunction, as mitochondria are involved in ion buffering (63) owing to higher metabolic demand. Similarly increased vulnerability has been shown in the hippocampus of Cx32 KO mice under ischemic conditions (64).

Some of the Cx32 GJ loss in still myelinated fibers may be directly caused by paranodal and juxtaparanodal disruption, which is known to occur in MS (65,66) and experimental autoimmune encephalomyelitis (67). However, in contrast to peripheral myelinated fibers, Cx32 forms GJs throughout the length of the central myelin sheaths in both human and rodent CNS and, as shown in Figure 3, it is not restricted to paranodal areas, perhaps owing to the lack of incisurahkike structures in CNS myelinated fibers (68). The selective disruption of myelin GJs in NAGM (shown here) and in normal-appearing WM (44) may also be caused by the inflammatory environment in MS parenchyma and the effects of cytokines and inflammatory cells prevalent in the adjacent leptomeninges (4,11,69–72). Similar loss of Cx32 GJs occurs in inflamed peripheral nerves (73,74) and liver (75). Increased Cx43 expression and, at the same time, loss of Cx32 reflecting a reactive astrocyte phenotype have also been reported in hippocampal tissue from patients with mesial temporal lobe epilepsy (76), further supporting the link between inflammation, astrogliosis, and loss of oligodendrocyte GJs even in the absence of demyelination. We studied mostly subpial demyelinating lesions in the present study, which could be closely associated with diffuse or organized meningeal inflammation (27,71), but we could not assess any possible correlation between glial GJ alterations and meningeal inflammation because the meninges in most of our tissue samples had been removed at the time of dissection.

Astrocyte connexins show very distinct changes in MS cortex, with prominently increased Cx30 expression in astroglial cortical lesions, whereas Cx43 is increased mostly in NAGM more than in lesions. This is in contrast to the WM, where astroglial lesions in lesions is accompanied by increased Cx43 expression, whereas Cx30 is not significantly expressed in control or MS WM (44). In addition to the fact that WM astrocytes do not normally express Cx30, there are more differences between WM and GM astrocytes not only at the morphologic level but also at the molecular level, including differential response to inflammation and demyelination for the expression of glutamate receptors and transporters (77). How these distinct patterns of astroglial changes may affect the progression of MS pathology in the GM versus the WM remains to be determined. However, important regulatory roles of astrocytes, including maintenance of CNS homeostasis (49,78) and synaptic regulation (79,80), are likely to be disrupted by these changes, with negative effects not only on oligodendrocytes but also on axons and neurons.

Our study confirms that GJ pathology in both oligodendrocytes and astrocytes is widespread in MS NAGM and is not limited to lesions. This is not surprising given the diffuse activation of microglia, which are present at similar levels inside and around cortical lesions. This association of GJ pathology with chronic neuroinflammation is also demonstrated by the significant correlation between Cx43 expression and extent of microglial activation in NAGM. Furthermore, even with a relatively small number of cases studied and a possible outlier, we found a significant correlation between Cx32 expression and disease duration, suggesting that preservation of GJs in myelinated fibers is associated with less pathology and better disease outcome. Thus, the pathologic changes in GM glial GJs emerge as another factor that may contribute to MS progression, in addition to recently described alterations in MS NAGM, including oxidative stress and mitochondrial dysfunction in neurons that could lead to axonal loss and neurodegeneration (14,27,81–83).

In conclusion, we demonstrate widespread disruption of oligodendrocyte GJs in MS. Loss of cortical oligodendrocyte and myelin GJs and disconnection from reactive astrocytes in the setting of chronic neuroinflammation and astrogliosis are likely to contribute to further demyelination, failed remyelination, and axonal loss, accelerating disease progression and associated disability. The therapeutic potential of modulating astrocytic and oligodendrocytic connexins in MS or other demyelinating disorders remains to be clarified.

REFERENCES

20. Stadelmann C. Multiple sclerosis as a neurodegenerative disease: Pathology, mechanisms and therapeutic implications. Curr Opin Neurol 2011;24; 224–29
82. Nave KA. Myelination and support of axonal integrity by glia. Nature 2010;468:244–52