Neuromyelitis optica (NMO) is an autoimmune, relapsing inflammatory disease of the central nervous system (CNS) with a predilection for optic neuritis and myelitis associated with long spinal cord lesions. Other brain structures with high levels of aquaporin 4 (AQP4) immunoreactivity are also preferentially targeted. A pathogenic autoantibody specific for extracellular epitopes of the transmembrane CNS-predominant water channel protein, AQP4, is detectable in most patients. This antibody reproduces many aspects of NMO-specific pathologic changes when injected directly into the CNS in the presence of human complement or when injected intravenously in the setting of a disrupted blood-brain barrier resulting from prior induction of experimental autoimmune encephalomyelitis or injection of a Freund adjuvant. The relative predilection of this disease for the optic nerve and spinal cord remains poorly understood, as are the reasons why other AQP4-expressing tissues are not targeted.

Aquaporin 4 protein is expressed in humans as 2 isoforms that result from alternative transcription initiation and alternative splicing of the same gene encoded on chromosome 18. M23, the shorter isoform, lacks 22 N-terminus amino acid residues of the longer M1 isoform. The basic unit of AQP4 assembly is a heterotetramer. Heterotetramers with a high proportion of M23 isoform aggregate as supramolecular complexes that lead to formation of orthogonal arrays of...
AQP4 Expression and Tissue Susceptibility to NMO

Mice, rats, and humans. We studied tissues obtained at autopsy and evaluated supramolecular aggregation in tissues of AQP4 protein isoform-specific expression nor supramolecular aggregation was analyzed in that study.

We measured mRNA and protein expression of AQP4 and evaluated supramolecular aggregation in tissues of mice, rats, and humans. We studied tissues obtained at autopsy from control individuals and from 1 patient with NMO.

### Methods

#### Tissue Sources

We evaluated tissues from 3 BL6 mice, 3 Lewis rats, and 2 human controls. Human tissue was obtained from autopsies performed at Mayo Clinic, Rochester, Minnesota, as approved by the Mayo Clinic institutional review board, with an interval from death to harvesting of approximately 24 hours; written informed consent was not required for analysis of autopsy samples. Tissues were flash frozen in liquid nitrogen and stored at −80°C. We also evaluated 5 additional human optic nerves from controls that were collected within 12 hours of death and stored at −80°C.

#### Quantitative PCR

We analyzed mouse and human tissues using quantitative PCR. Total RNA was isolated from approximately 30 mg of pulverized tissue using an RNA purification kit (RNeasy; Qiagen). After purification, the RNA samples were stored at −80°C for future analysis. Mouse tissue was analyzed by reverse transcription–polymerase chain reaction (RT-PCR). The following mouse AQP4 isoforms were used: forward primers for M1 (510-bp product), 5′CTCCCAGTGTACTGGAGCCCG3′; and for M23 (460–base pair [bp] product), 5′GGAAGGCTAGGTTGGTGACTTC3′ and for hpRT1 (94-bp product), 5′GCACCTG-GCAAAAACATGCA3′ and 5′GGGTCTTTTACCCAGAAAGCT3′. Threshold cycle (Ct) values in each reaction were determined in quadruplicate; variations in readings were required to be less than 0.6 Ct. The comparative Ct method (2^−ΔΔCt method) was used to determine the relative expression of AQP4 to HPRT1 transcripts.

#### Protein Analysis

Frozen tissues were pulverized and homogenized in native polyacrylamide gel sample buffer (Invitrogen) containing 10% n-dodecyl-β-D-maltoside to a final concentration of 0.75%. Lysates were centrifuged at 500g for 10 minutes at 4°C. Supernatants were spun at 20 000g for 30 minutes at 4°C and frozen in aliquots at −80°C. Samples for denaturing electrophoresis were added to 2× Laemmlı sodium dodecyl sulfate (SDS)–containing loading buffer, incubated at 4°C overnight, and loaded onto an SDS-containing 18% polyacrylamide gel (Bio-Rad) without preheating. Proteins isolated from HEK293 cells transfected separately with human M1 or M23 AQP4 isoform pcDNA, a commercial cloning vector (Invitrogen), were loaded as molecular weight controls. Loading of protein on gels was optimized to visualize bands corresponding to the M1 and M23 AQP4 isoforms and to distinguish between them; to control for variations in the amount of protein loaded, the intensities of the AQP4 and β-actin control bands were compared visually and by using densitometry for semiquantitative analysis. To evaluate the supramolecular aggregation of AQP4, blue native polyacrylamide gel electrophoresis (BN-PAGE), a nondenaturing electrophoresis technique established for this purpose, was used as previously described. Briefly, protein supernatant was mixed in a 1:3 ratio with sample buffer and G-250 (a detergent; Coomassie ratio, 4:1) and loaded onto a BN-PAGE gel (Bio-Rad) without preheating. Proteins were blotted onto polyvinylidene fluoride membranes (Bio-Rad) using a blot module (XCell; Invitrogen) according to the manufacturer’s protocol. Membranes were blocked in Tris-buffered saline and Tween 20 (TBST) with 5% nonfat dry milk and incubated with a 1:1000 dilution of rabbit anti-AQP4 (Santa Cruz Biotechnology) primary antibody at 4°C overnight. Blots were then washed in TBST and incubated with goat anti-rabbit IgG horseradish peroxidase (Southern Biotech) secondary antibody, washed, and exposed to a chemiluminescent substrate (SuperSignal ECL; Thermo Scientific).
Results

Comparison of AQP4 Expression Between Tissues in Rodents and Human Controls

AQP4 Messenger RNA Expression
We evaluated tissues from 3 BL6 mice. The mean RNA expression values for the M1 and M23 isoforms were highest in optic nerve and spinal cord tissues followed by brainstem and brain tissues. The kidney, a non-CNS AQP4-expressing tissue, had much lower expression than the CNS tissues did, and the liver, as expected, had no detectable expression (Figure 1A).

We evaluated tissues from 2 human autopsies. The TaqMan assay yielded higher values for AQP4 transcripts than the SYBR-Green assay did, but ranked expression results were strongly concordant using both methods. Messenger RNA (mRNA) levels for total AQP4 were highest in optic nerve and spinal cord tissues followed by other CNS tissues; non-CNS tissues had much lower expression, and liver tissue showed the lowest expression (Figure 1B).

AQP4 Protein Expression
Immunoreactive AQP4 M1 and M23 protein per unit of tissue weight varied considerably between the tissues of all species, but the relative expression was consistent in the NMO-relevant tissues analyzed across species (Figure 2); AQP4 was most abundant relative to β-actin, the comparator protein, in optic nerve tissue from each species. Spinal cord tissue also showed high levels of expression but lower expression than the optic nerve. Expression was much lower in kidney tissue; however, kidney tissue could not be distinguished from liver tissue, which is established not to express AQP4.

Central nervous system tissue had adequate amounts of immunoreactive AQP4 for evaluation of supramolecular aggregation when we used BN-PAGE (Figure 3). Gels were loaded so as to adequately visualize higher-order bands, which were the bands of particular interest; this necessitated overloading of protein of low aggregate size. In all the species evaluated, the highest ratio of large to small AQP4 aggregates was found in optic nerve tissue, and the lowest ratio was found in tissue from the cerebral hemisphere. The relative abundance of highest-order aggregates to lowest-order aggregates was also evaluated by using 2-dimensional BN-PAGE, which confirmed an excess of higher-order aggregates in optic nerve tissue and showed that these aggregates had a high proportion of the M23 isoform, particularly in the optic nerve, as expected (Figure 4).

Comparison of Patient With NMO vs Controls
We compared total AQP4 mRNA levels in the optic nerve of a patient with NMO with those of 5 control optic nerves using Quantitative polymerase chain reaction analysis of AQP4 mRNA expression in 3 BL6 mice using SYBR-Green-based (Invitrogen) chemical analysis (A) and 2 human control tissue samples using SYBR-Green-based and gene expression assay (TaqMan; ABI) chemical analysis (B). Tissues analyzed were liver (L), kidney (K), muscle (M), frontal lobe (Br), brainstem (BS), hypothalamus (HyT), spinal cord (SC), and optic nerve (ON). Results are expressed as a mean ratio of the samples tested relative to a reference gene, hypoxanthine guanine phosphoribosyl transferase 1 (HPRT). In mouse tissue, M1 and M23 AQP4 isoforms were evaluated separately; in human tissue, total AQP4 (M1 and M23 isoforms) mRNA was evaluated. SYBR-Green-based and TaqMan chemical analysis methods show quantitatively different but similar relative levels of expression.
the SYBR-Green method (Figure 5A). The NMO sample showed intermediate numbers of transcripts compared with the controls. The relative amounts of total AQP4 protein and the relative amount of M23 vs M1 expression as determined by SDS-PAGE did not differ between the control and NMO tissues, nor did the distribution of higher-order aggregates (Figure 5B-D).

Discussion

Formation of OAPs is required for binding of NMO-IgG.15,16 Mutations that disrupt the formation of supramolecular aggregates and OAP formation abrogate NMO-IgG binding.16 Hydrophobic interactions of 7 amino acids downstream of methionine 23 in AQP4 M23, especially Ala25 and Phe26, are critical to supramolecular aggregation and OAP formation. These residues interact with Cys17 to Ser21 of AQP4 M1 to disrupt the formation of OAPs. The interaction of these M1 and M23 residues may represent nonspecific blocking effects that do not depend on interactions of specific residues.16 Supramolecular aggregates are generated after AQP4 tetramers are transported to the cell membrane.17 Extracellular proteins, including agrin and laminin, are also critical to orthogonal array formation,16 as are elements of the dystrophin cytoskeleton that anchor AQP4 in the membrane. Interference with the interaction of M1 and M23 AQP4 isoforms or of AQP4 molecules with their
cytoskeletal or extracellular anchors may disrupt the formation of OAPs. Given the critical role of the relative expression of M1 and M23 isoforms to supramolecular aggregation and OAP formation, an analysis of AQP4 isoform expression and supramolecular aggregation is relevant to understanding the tissue specificity of NMO-IgG targeting.

Aquaporin 4 is highly expressed in the CNS and is also expressed in some non-CNS tissues, especially skeletal muscle, crypts of the gastric mucosa, renal medulla (distal renal collecting tubule), lung, and placenta. Aquaporin 4 is concentrated in the CNS at astrocyte end-feet as a result of anchoring by the end-foot–concentrated dystrophin cytoskeleton. Messenger RNA of the M1 protein isoform has been reported by others as the predominant AQP4 transcript detected in the human and rat CNS. However, M23 is the dominant protein isoform expressed in mammalian tissues. The relative proportion of M23 to M1 varies between tissues, the highest proportion of M23 being in CNS tissues. The reason for the apparent dissociation between RNA transcript and protein isoform ratios is not entirely clear and may involve transcriptional, translational, and posttranslational mechanisms. The transcriptional activity of the M23 isoform is greater than that of the M1 isoform in choline acetyltransferase assays of promoters of the 2 isoforms. The differences are exaggerated in glioblastoma vs kidney cell lines, possibly explaining the tendency for relatively greater M23 expression in the CNS. Translation of M23 has been described as “leaky” because the Kozak sequence of the native M1 isoform translation start sequence deviates from what is considered optimal. This deviation results in generation of M23 proteins from M1 as well as from M23 transcripts. Finally, the disproportionate representation of the shorter M23 isoform in large molecular aggregates may be attributable to differential posttranslational turnover of the 2 different isoforms because larger aggregates may
represent a pool of AQP4 less prone to turnover; after exposure to NMO-IgG, M23 aggregates are not modulated from the surface of astrocytes as readily as M1 isoforms.23

Neuromyelitis optica clinically targets the CNS selectively; no renal abnormalities have been recognized despite expression of both isoforms in the kidney. Myositis is a rare occurrence.24 Although some patients with NMO develop pathologic changes outside the optic nerve and spinal cord, it is unusual for this to occur in early phases of NMO. Severe necrotizing pathologic features are characteristic of optic nerve and spinal cord lesions but less so of lesions in the area postrema, a common initial site of pathologic manifestation, perhaps owing to relative deficiency of the blood-brain barrier and high AQP4 expression. Area postrema lesions may be nonnecrotizing, sparing myelin and astrocytes.25

Saini et al12 studied differential tissue expression of M1 and M23 using real-time PCR methods and RACE to differentiate 2 apparently different molecular species of M23: a genomic- encoded form with an M23-specific untranslated region sequence and a second mRNA alternative splice variant. The authors concluded that M1 isoform mRNA was the predominant isoform expressed in the optic nerve and spinal cord and suggested that the proportion of M1 may be key to NMO pathophysiologic changes. The mechanism underlying the putative association of high levels of M1 mRNA in the optic nerve with pathogenesis of NMO were not explored, but the authors intimated that an epitope related to the M1 isoform might be important. The M1-specific isoform sequence encodes an intracellular domain of AQP4. Different from the rat and mouse M23-specific sequence, the human M23-specific sequence within the untranslated region of M23 is very short (15 bases) and AT rich (66.7%), restricting primer design; by contrast, the rat M23-specific sequence is much longer (105 bases).22 We quantitated the M1 and M23 isoforms of AQP4 transcripts by quantitative PCR in mouse tissue but were unable to do so in human tissue because of the short M23-unique transcript sequence. However, NMO is an autoantibody-mediated disease; therefore, protein expression is more relevant than RNA expression to the pathogenesis of the disease. In this study, we assessed M1 and M23 AQP4 protein expression in NMO-relevant tissues.
We report 2 key findings: (1) total AQP4 mRNA and protein per unit of tissue weight were greater in the optic nerve and spinal cord relative to other CNS and non-CNS tissues and (2) the distribution of supramolecular aggregate species of AQP4 differed, with the proportion of the largest aggregates highest in the optic nerve and, to a lesser extent, in the spinal cord. These observations may be relevant to why NMO selectively targets the optic nerve and spinal cord and spares non-CNS AQP4-expressing tissues, such as the kidney. The relative amounts of AQP4 and control proteins were evaluated in whole-tissue lysates, and the results were not skewed by the immunoprecipitation of AQP4 protein. Expression of total AQP4 mRNA and of protein products of both principal isoforms of AQP4 relative to control genes and proteins varied similarly in the 3 species evaluated. In CNS tissues, AQP4 was most highly expressed in the optic nerve and spinal cord at the mRNA and protein levels, which are the tissues most frequently and severely targeted in NMO. Furthermore, we found that the distribution of supramolecular aggregates of AQP4 was skewed toward those of the greatest molecular size in the optic nerve and spinal cord compared with other tissues. Aggregates of different sizes were observed in all the tissues studied, consistent with expression of both isoforms of AQP4 in all the CNS and extra-CNS tissues. We found no clear differences in mRNA or protein isoform expression between a single patient with NMO whose tissues we had an opportunity to study and multiple human control tissues; however, the fact that only a single NMO case was studied is a notable limitation of this study. Thus, extrapolating from this case, the key difference between patients with NMO and controls is the presence of NMO-IgG autoantibody in serum and not the molecular expression of AQP4, at least as measured with the techniques used in this study.

Our study did not distinguish white from gray matter in the spinal cord; immunostaining performed in other studies suggests that expression is higher in central gray matter than in white matter and that NMO lesions tend to be central in the spinal cord, although they involve both gray and white matter. However, the optic nerve, a white matter tract, had the highest expression of AQP4 per unit of tissue weight, and the differences between the tissues we studied are not explained by the relative proportion of gray vs white matter.

Some of the differences between CNS tissues and the differences between non-CNS tissues and CNS tissues could relate to the density of AQP4-expressing cells rather than to differences in the amount of AQP4 expressed per cell; comparators other than β-actin that are sensitive to astrocyte density may be of value in future studies. However, the differences between the optic nerve and spinal cord compared with the frontal lobe were large and unlikely to be explained by the density of astrocytes alone.

Other factors, such as differences in the inflammatory microenvironment or differences in proteins that mitigate complement-mediated pathologic changes, including complement inhibitory proteins (eg, CD59), and regional differences in blood-brain barrier characteristics could also contribute to differential pathologic changes in the CNS or in certain structures, such as the optic nerve and spinal cord. Further studies of the expression and aggregation of AQP4 may provide additional insights into the relative susceptibility of tissues to NMO pathologic changes.