Advanced Review

Aquaporin-4 orthogonal arrays of particles from a physiological and pathophysiological point of view

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Aquaporin-4 (AQP4) is the neuromuscular water channel that is also expressed at the basolateral membranes of other cell types in kidney, stomach, and lung. In skeletal muscle, AQP4 is found at the sarcolemma of fast-twitch fibers and its function is strictly correlated with the glycolytic metabolism. In the central nervous system, AQP4 is expressed at the basolateral membranes of ependymal cells, and is highly concentrated at the glial end-foot processes surrounding blood vessels and forming the glia limitans, as well as at the nonend-foot glial processes of the granule cell layer in the cerebellum. AQP4 plasma membrane organization is different from other aquaporins (AQPs). AQP4 is expressed as two major polypeptides called M1 and M23. These two isoforms form heterotetramers appearing in the plasma membrane as intramembrane particles (IMPs) observable by freeze-fracture electron microscopy. Such tetrameric organization is common to all other AQPs. In the case of AQP4, however, multiple IMPs further aggregate to form structures called orthogonal arrays of particles (OAPs). The relative abundance of M23 and M1 *in vivo* is the major determinant for the formation of OAPs of different sizes. The function of AQP4 aggregation into OAPs under normal conditions is still not completely understood. Interestingly, there are several reports indicating that OAPs are involved in different neuromuscular diseases. In particular, the OAP-related diseases that have attracted more attention are Duchenne muscular distrophy and, more recently, neuromyelitis optica, the two pathological conditions in which OAPs are involved in completely different ways.

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AQUAPORIN STRUCTURE: FROM THE FUNCTIONAL TETRAMERIC UNIT TO SUPERMOLECULAR AGGREGATIONS INTO ORTHOGONAL ARRAYS OF PARTICLES (OAPs)

All aquaporins (AQPs) are organized in tetramers in which each monomer is a functional unit for water transport. Depending on the size of the water pore, they can allow a selective flux of water molecules or be more permissive to permit the passage of small neutral solutes such as urea and glycerol. The absence of proton or ion permeation through AQPs has been attributed to the presence of steric barriers combined with electrostatic interactions and hydrogen bonds.[1]

A typical difference between aquaporin-4 (AQP4) and other AQP structures is in the tetrameric composition and organization in the plasma

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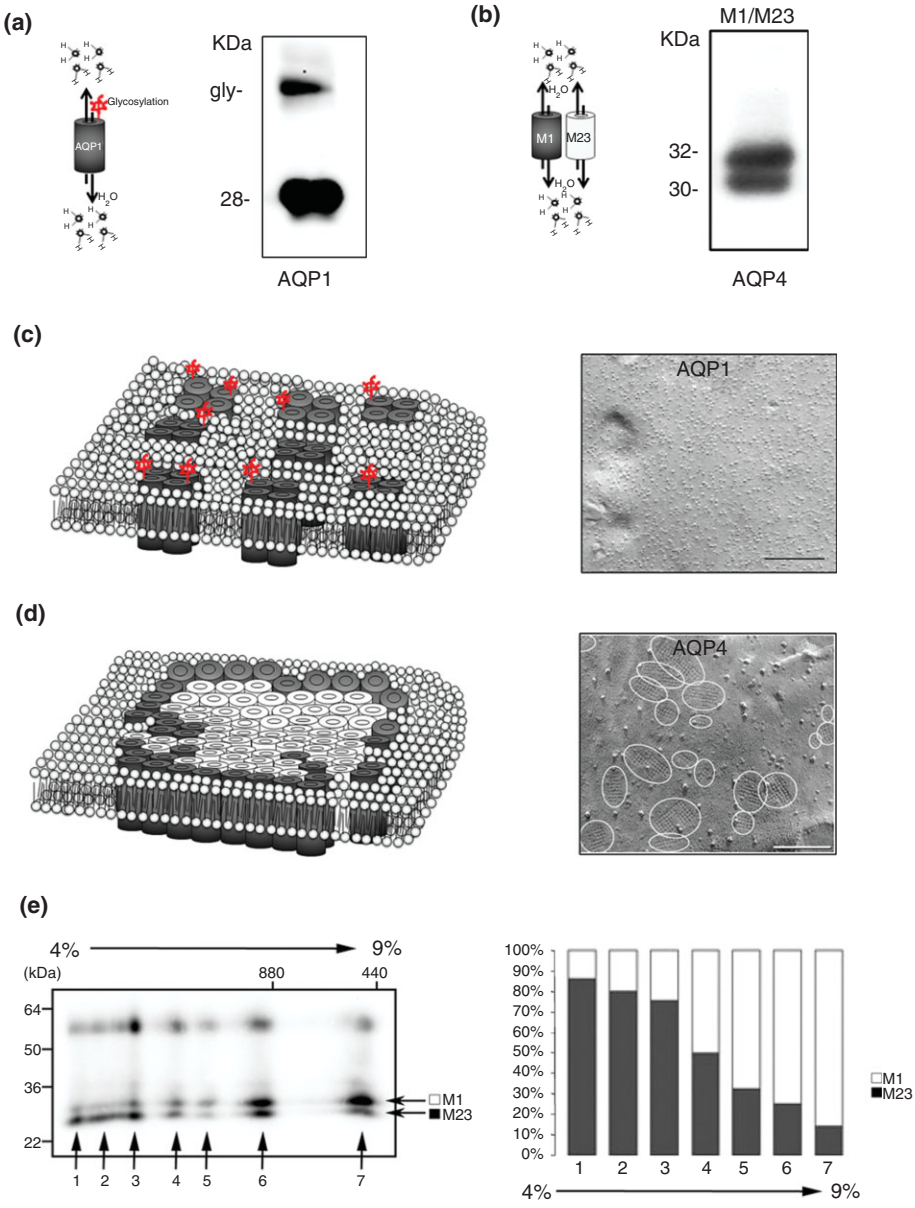


FIGURE 1 | From Aquaporins (AQP) tetrameric organization to AQP4 orthogonal arrays of particles (OAPs). (a) AQP1 exists as a 28 kDa glycosylated (gly) isoform whereas AQP4 (b) exists as two major isoforms, called M23 and M1, of 30 and 32 kDa respectively. AQP4 is not glycosylated. (c) AQP1, like all AQPs, is organized in tetramers in which each monomer is a functional unit for water transport and forms homotetramers, made of the same isoform. By freeze-fracture electron microscopy (FFEM), AQPs appear as intra-membrane particles (IMPs), as shown for AQP1 in the image on the right. (d) AQP4 is unique as it forms heterotetramers made of the two major isoforms of 30 and 32 kDa. As shown in the image on the right, AQP4-IMPs are able to further aggregate into higher order structures called orthogonal arrays of particles (OAPs) or square arrays (white circles). (e) OAPs of different sizes can be biochemically separated by BN/SDS-PAGE. Using a 4–9% gradient gel in the first dimension, performed under native conditions, seven different OAP pools can be visualized. The densitometric analysis of M1 and M23 bands, performed for each of the seven pools, shows that the smaller the size of the OAP pool, the higher is the M1/M23 ratio. (The FFEM images: Reprinted with permission from Ref 2. Copyright 2012 Springer for AQP1 in (c) and Reprinted with permission from Ref 3. Copyright 2012 National Academy of Science for AQP4. The images in (e): Reprinted with permission from Ref 4. Copyright 2012 John Wiley and Sons)

membrane (Figure 1). All AQPs form homotetramers made of the same AQP isoform (Figure 1(a) and (c)) except AQP4 which forms heterotetramers made of two major polypeptides of 30 and 32 kDa, called M23 and M1, respectively (Figure 1(b) and (d)). They are thus named according to their origin from two in-frame translation-initiating methionines.[5–8] By freeze-fracture electron microscopy (FFEM) analysis,

AQP homotetramers appear as intramembrane particles (IMPs) as shown for AQP1 tetramers in Figure 1(c). The formation of IMPs is a common feature of all AQPs, including AQP4 whose IMPs, however, are able to further aggregate into higher order structures called orthogonal arrays of particles (OAPs) or square arrays[9] (Figure 1(d). There is abundant literature showing the expression ratio of M1 and M23 isoforms as the major determinant of the size of *in vivo* OAPs.[4] When the long AQP4-M1 form is expressed alone, no stable OAPs are visible. Formation of stable and large OAPs requires the expression of AQP4-M23,[10,3,11] whereas co-expression of the two isoforms leads to OAPs of different sizes, depending on the ratio of the two isoforms (Fig 1(e)).

M1 AND M23 RNA AND PROTEIN RATIO: LEAKY SCANNING AND REINITIATION MECHANISMS

Although it has been demonstrated that hydrophobic intermolecular interactions at the N-terminus of AQP4 are necessary for OAP formation,[12] the molecular mechanisms controlling the expression of the two isoforms of AQP4 to generate OAPs of different sizes still remain unclear.

Recently, our lab has focused on the mechanisms underlying the control of the M1/M23 ratio, starting from the evidence that different tissues express different M1/M23 protein ratios.[11] The AQP4 gene codifies for two different transcripts (M1 and M23) by alternative splicing and/or alternative transcription start sites.[6] It has been demonstrated by several authors that if M23 is the predominant AQP4 isoform in terms of protein in the brain, M1 mRNA is much more abundant than M23 mRNA.[6,13] We have found two different translational mechanisms able to explain these discrepancies. In particular, we found that a large proportion of M23 protein derives from a leaky scanning mechanism (LSM) of M1 mRNA.[11] Furthermore, M1 mRNA 5′UTR analysis showed another mechanism able to modify the M1/M23 ratio through an out-of-frame upstream open reading frame (uORF), and a reinitiation mechanism.[14] LSM and reinitiation mechanisms are able to explain the discrepancies between the relative AQP4-M1 and AQP4-M23 mRNA and protein levels. They may also be tissue-specific and finely regulated, as reported for other genes. These translational control mechanisms could be crucial for M1/M23 protein ratio modulation, and consequently OAP assembly in different tissues, in both physiological and pathological conditions.

TECHNIQUES AVAILABLE TO STUDY OAP STRUCTURES

The function of AQP4-OAPs of conferring a higher level of plasma membrane stability can be deduced from their localization in microdomains not delimited by tight junctions, such as the glial perivascular processes. Moreover, the higher concentration of AQP4 water channels achieved with its aggregation into OAPs allows us to assume that OAPs represent a biochemical optimization necessary to confer a higher water permeability on such plasma membrane micro domains (Figure 2(a)). The observation and study of OAPs have been difficult for years and this is the reason why direct evidence of these functions of OAPs is still unavailable. For most of the last 40 years, OAPs have been studied exclusively by FFEM (Figure 1(d)), a technique that is difficult, time-consuming, and requires very expensive instrumentation and highly qualified researchers. Moreover, the FFEM observations are performed on fixed tissues and therefore dynamic studies of OAPs are not possible. Recently, other techniques have been proposed as alternatives to FFEM for a different analysis of OAPs, such as blue native/Sodium Dodecyl Sulfate polyacrilamide gel electrophoresis (SDS-PAGE) (BN/SDS PAGE)[4], allowing biochemical separation and visualization of OAPs (Figure 1(e)), and several high resolution microscopy techniques such as single particle tracking of Qdots on AQP4,[12,15] total internal reflection fluorescence microscopy (TIRFM),[15,16] and most recently photo-activated localization microscopy (PALM) and stochastic optical reconstruction microscopy (STORM), allowing the detection of single fluorescent OAPs at the nanoscale.[17]

The use of BN/SDS PAGE has helped us to demonstrate the existence of several independent AQP4 plasma membrane pools, corresponding to OAPs of different sizes characterized by a different M1 and M23 ratio[18] (Figure 1(e)). BN/SDS-PAGE also allows the identification of potential protein partners for AQP4 and which AQP4 pool is affected in some pathological conditions. For example, the absence of dystrophin in the cell selectively affects the largest OAP pool leaving the smaller ones unaltered.[4]

TIRF, PALM, and STORM share the common characteristic of a live microscopy approach combined with improved optical resolution able to reach the nanoscale (PALM and STORM) and therefore they have become key techniques to study the dynamics of OAP assembly and the diffusion characteristics depending on fluorescent M1 and M23 in live cells.[12,15]

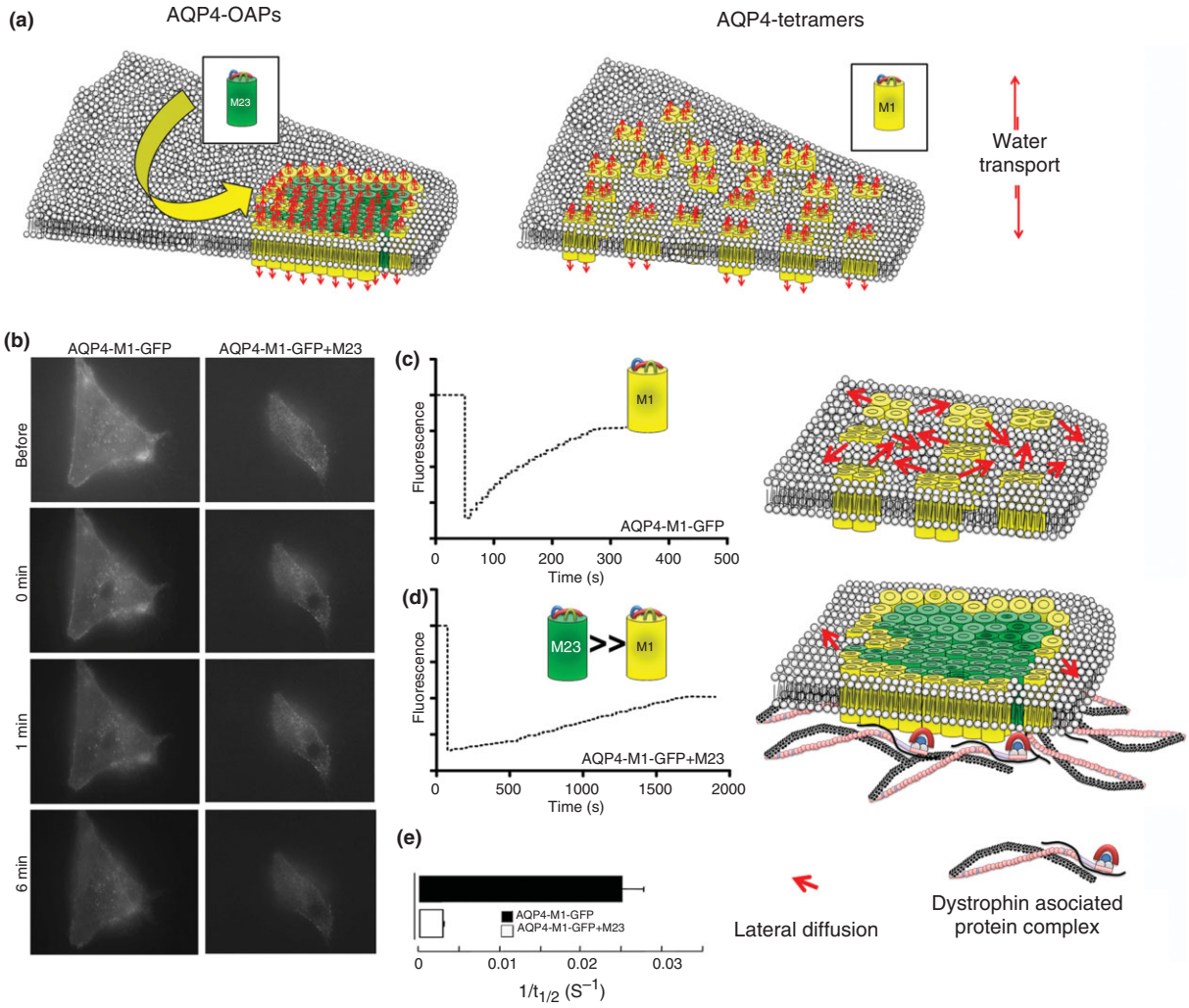


FIGURE 2 | The importance of aquaporin-4 orthogonal arrays of particles (AQP4-OAPs) in cell physiology. (a) Schematic drawing showing that AQP4 aggregation into OAPs could serve to enhance the water permeability per surface unit. (b) FRAP experiment performed on cells transfected with the M1 isoform, organized into AQP4 tetramers (c), or M23 isoform able to aggregate into very large OAPs (d). The recovery of fluorescence in the bleached area takes 5 min for M1 tetramers (c), whereas almost no recovery is shown for 'immobile' M23 (d). (e) Lateral diffusion data are shown as reciprocal half-times (t1/2) for fluorescence recovery (mean +/− SE of separate sets of measurements, n = 3–7. The schematic drawing in (d) shows that large OAPs may form macro-molecular complexes, with the dystrophin associated protein complex. (The FRAP images (b) and the fluorescence recovery analysis (c–e): Reprinted with permission from Ref 11. Copyright 2012 The American Society for Biochemistry and Molecular Biology)

AQP4-OAPS IN PHYSIOLOGY

The presence of OAPs on the plasma membrane of skeletal muscle fibers,[19,20] astrocytes,[21–23] and other cells has been known since the 1970s when these well-organized square arrays were visualized by FFEM. Twenty years later it was discovered that AQP4 water channels represent the molecular identity of OAPs when AQP4 and OAP expressions were found to overlap in astrocytes, trachea, sarcolemma, gastric parietal cells, and kidney principal cells and that, more directly, OAPs appear when CHO cells are transfected with AQP4 cDNA[10] and they are absent in AQP4 KO mice.[24]

Under normal conditions OAP function and the significance of OAPs of different sizes are still not completely understood in detail. Independently from the debated similar[7] or different[25] single channel water permeability of M1 and M23 isoforms, AQP4 aggregation of itself into OAPs could serve to enhance water permeability (Figure 2(a)) in those cellular domains, such as the perivascular one, where this physiological need is correlated to the fluid shifts occurring. Moreover, the aggregation into super-molecular well-ordered structures confers a higher level of plasma membrane stability to AQP4 water channels, which need to be confined in microdomains

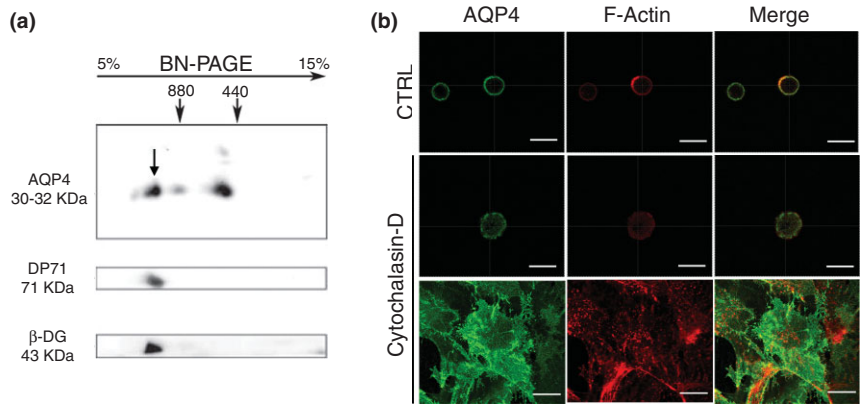


FIGURE 3 | Possible aquaporin-4 (AQP4) interacting proteins: dystrophin associated proteins (DAPs) and actin cytoskeleton. (a) BN/SDS-PAGE followed by immunoblot analysis showing that the glial dystrophin isoform (DP71), β-dystroglycan (β-DG), and AQP4 colocalize in the largest pool of orthogonal arrays of particles (OAPs) (arrow). (b) The possible interaction between AQP4 and actin cytoskeleton in cultured astrocytes seems to depend on the cell adhesion phase and is therefore correlated with rapid versus slow changes in cell morphology. Immediately after plating (round cells), F-actin and AQP4 colocalize in Control (CTRL) conditions and cytochalasin-D treatment completely impairs AQP4 plasma membrane localization. In contrast, after 1 day, when astrocytes become flat with a fibroblast-like shape, cytochalasin-D treatment does not seem to affect AQP4 localization. (The BN-PAGE analysis: Reprinted with permission from Ref 4. Copyright 2012 John Wiley and Sons). (The images in (b): Reprinted with permission from Ref 26. Copyright 2012 John Wiley and Sons)

not delimited by tight junctions such as the glial processes at the level of the blood–brain barrier (BBB), the glial limitans, or also those forming the dense network at the granular cell layer in the cerebellum. Figure 2(b)–(e) shows an Fluorescence recovery after photobleaching (FRAP) experiment performed on cells transfected with the M1 isoform (AQP4 tetramers) or M1 + M23 isoforms (AQP4-OAPs). The recovery of the fluorescence in the bleached area takes about 5 min for M1 tetramers (Figure 2(a) and (c)), whereas only a very small recovery is shown for M23 OAPs after about 30 min (Figure 2(a) and (d)).

OAP stability raises the issue that no interactions are probably needed to anchor AQP4 to the plasma membrane in cellular domains not delimited by tight junctions, such as the case for astrocyte foot processes, where the larger OAPs are in fact expressed. We have proposed that OAPs of different sizes may correspond to different AQP4 macromolecular complexes associated with different proteins,[18] as can be assumed from BN/SDS-PAGE analysis of AQP4 and the dystrophin associated protein (DAP) complexes (Figure 3(a)). In particular, the protein complex formed by AQP4, dystrophin, and β-dystroglycan would correspond to the largest pool of OAPs.[4] This hypothesis has been supported by other data available in the literature showing the absence of perivascular AQP4 in syntrophin null mice[27,28] and the strong AQP4 reduction in mouse models lacking dystrophin[29–32]. However, although it has been demonstrated that α-syntrophin is required for the anchoring of AQP4 in the plasma membrane, presumably because of the interaction of its postsynaptic density, disc-large, *ZO-1* (PDZ) domain with the C-terminal of AQP4,[27,33] it must be highlighted that this interaction occurs when a cross-linkage pretreatment is provided and no direct interaction has been demonstrated between AQP4 and α-syntrophin in studies performed on transfected cells.[27]

Supported later by other laboratories,[34] we also proposed an interaction between AQP4 and actin cytoskeleton based on the profound actin rearrangement depending on AQP4 RNA interference (RNAi) in primary cultured astrocytes.[35] Moreover, depolymerization of F-actin cytoskeleton, performed by cytochalasin-D,[26] indicated that AQP4 and F-actin interaction is transient and strictly related to the changes in cell morphology occurring during the astrocyte adhesion phase, when the cell undergoes important morphological changes going from a round to a final flat and fibroblast-like shape (Figure 3(b)).

In conclusion, the general idea that emerges from the literature at the moment is that OAPs are AQP4 aggregates that are important for reducing the mobility of the water channel in the plasma membrane. Whether this is sufficient to anchor the protein to the plasma membrane or whether AQP4 needs to interact with protein complexes such as the dystrophin-glycoprotein complex (DGC) or the actin cytoskeleton to be confined in microdomains, such as the perivascular glial processes, is still an open question. The absence of or a reduction in AQP4 in cells lacking dystrophin or syntrophin, as well as

AQP4 and actin-associated altered expression, is not enough to indicate direct physical interaction. They could be secondary phenomena due to altered plasma membrane protein patterns.

AQP4-OAPs IN PATHOLOGY

Despite the role of AQP4 aggregation into OAPs under normal conditions not having been completely elucidated, there are several reports starting from the 1970s, indicating that OAPs made of AQP4 water channels are involved in different neuromuscular diseases.[36,37] In particular, the OAP-related diseases that have attracted more attention are Duchenne muscular distrophy (DMD) and, more recently, neuromyelitis optica (NMO), the two pathological conditions in which OAPs are involved in completely different ways. In DMD patients, skeletal muscle fibers and brain astrocytes progressively lose OAPs. In NMO, AQP4-OAPs, and not AQP4 tetramers, become the molecular target of the autoantibodies typical of this autoimmune channelopathy.[16]

AQP4-OAPs and Duchenne Muscular Dystrophy

Alteration of the normal OAP membrane density has been reported in various pathological states such as epilepsy, brain ischemia, and muscular dystrophy. FFEM studies aimed at documenting the structural alterations characteristic of dystrophic skeletal muscle fibers show a significant decrease in OAP density in DMD plasma membrane[38] and in other human muscular dystrophies, such as the Fukuyama[39] and the facioscapulohumeral types.[37] The same OAP reduction has been found in the animal model of muscular dystrophy, the *mdx* mouse,[40] suggesting that OAP depletion is a common feature of the dystrophic fibers. After the discovery that OAPs are made of AQP4 protein, our laboratory focused on the analysis of AQP4 expression in the skeletal muscle of the *mdx* mouse and human dystrophic biopsies. The *mdx* mouse in particular offers the opportunity to look for AQP4 altered expression in the brain as a consequence of the absence of dystrophin. Remarkably, *mdx* mice manifest a reduction in AQP4 expression in muscle and brain[31] and more importantly, this reduction is associated with swollen perivascular astrocyte processes (Figure 4). AQP4 neuromuscular reduction is age-related and due to premature protein degradation, as the AQP4 mRNA content was unchanged between *mdx* mice and controls. Moreover, a study performed on several human skeletal muscle biopsies of patients affected by DMD and by its milder form Becker muscular distrophy (BMD), due to a different mutation in the dystrophin gene, showed that: (1) AQP4 stability in the sarcolemma does not always decrease when α-syntrophin is strongly reduced and (2) AQP4 expression is not altered in other forms of muscular dystrophy, indicating that AQP4 reduction is not a general phenomenon related to the dystrophic processes, but it is a peculiarity of those muscular dystrophies caused by the absence/reduction of dystrophin. The hypothesis we made was that a deficiency in dystrophin would cause a reduction in other DAPs, such as the dystroglycan complex, and as a consequence in AQP4. The absence of AQP4 is most likely the cause of the decreased osmotic

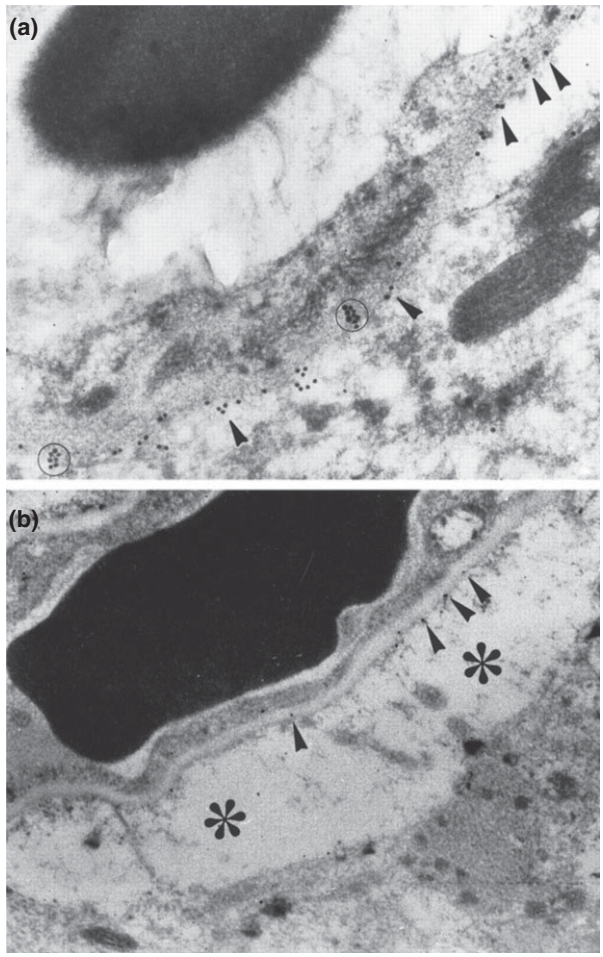


FIGURE 4 | Aquaporin-4 (AQP4) reduction and astrocyte swelling in *mdx* mice. Immunogold experiments performed in the brain of *mdx* mice (b) and relative control (a) with anti-AQP4 antibodies visualized by gold particles indicated by the arrows. Note that the number of gold particles is strongly reduced in *mdx* perivascular astrocytes. Moreover, this reduction is associated with swollen perivascular astrocyte (asterisks). (Reprinted with permission from Ref 31. Copyright 2012 The Faseb Journal)

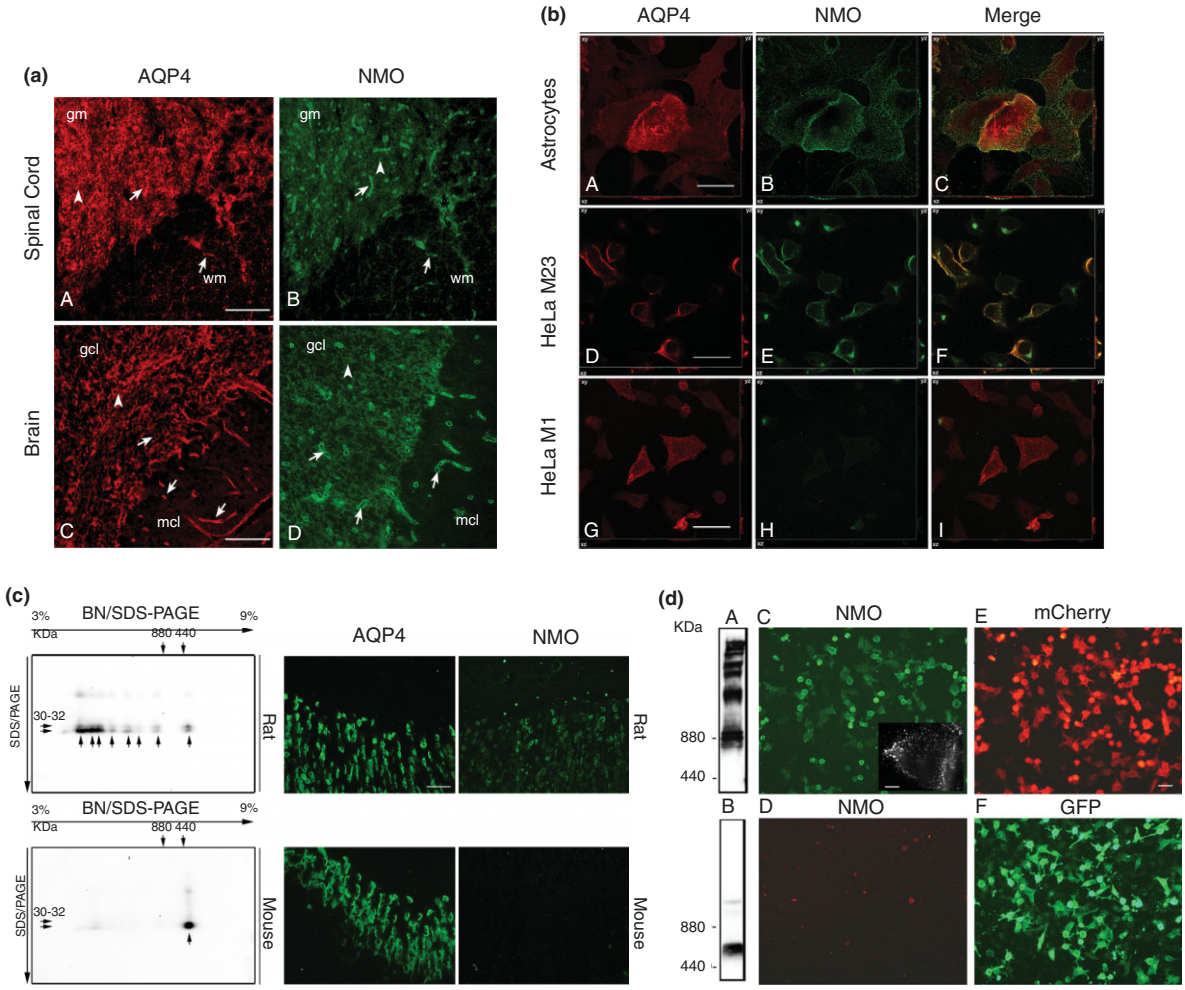


FIGURE 5 | Indication that orthogonal arrays of particles (OAPs) are the target for neuromyelitis optica (NMO) autoantibodies. (a) NMO labeling in spinal cord and brain sections is much stronger at the perivascular astrocytes, characterized by the largest OAPs (arrows) more visible in the white matter (wm) and in the molecular cell layer (mcl). In contrast, the staining is very weak at the astrocyte process network (arrowheads) in the granular cell layer (gcl) and gray matter (gm). (b) Experiments performed on astrocyte primary cultures and HeLa cells stably transfected with the M1 or M23-AQP4 isoform showing that NMO–IgGs selectively recognize OAP expressing cells (b). (c) BN/SDS-PAGE and immunofluorescence experiments showing that NMO–IgGs selectively recognize AQP4-OAPs in rat stomach whereas they do not recognize AQP4-tetramers in mouse stomach. (d) Immunofluorescence experiments performed on fluorescent AQP4 fusion proteins, mCherry C-terminus-tagged AQP4 (able to form OAPs) and *green fluorescent protein* (GFP)-N-terminus tagged AQP4 (unable to form OAPs). NMO–IgGs only recognize mCherry-AQP4. (The images in panels (a)–(c): Reprinted with permission from Ref 16. Copyright 2012 John Wiley and Sons. The images in panel (d): Reprinted with permission from Ref 45. Copyright 2012 The American Society for Biochemistry and Molecular Biology)

stability characteristic of *mdx* fibers compared with normal muscle fibers.[41] This hypothesis is supported by the interaction between AQP4 and α-syntrophin, a protein belonging to the DAP complex, through the C-terminal PDZ (PSD-95, disc-large, ZO1)-binding motif, later reported by the other authors[27,42], as mentioned in the previous paragraph. It has also been proposed that the brain, the changes in osmotic equilibrium due to altered AQP4 expression would cause cellular disturbances, therefore contributing to the cognitive impairments which characterize some Duchenne patients.

OAPs and Neuromyelitis Optica (NMO)

Since the recent discovery of their link to NMO,[16] OAPs have been attracting the interest of the ongoing scientific literature. NMO is an inflammatory autoimmune demyelinating disease of the central nervous system (CNS) characterized by the presence of autoantibodies (NMO–IgG) against AQP4 in the patients' serum.[43] However, AQP4 needs to be organized into OAPs to be recognized by NMO-IgG,[16] suggesting the need to unravel OAP structure and function in order to identify the NMO IgG epitope, which is essential for diagnostic and therapeutic purposes. Although

AQP4-OAPs are expressed in different tissues,[11] only the spinal cord and optic nerve are damaged in NMO.[44] The reason might lie in a tissue-specific molecular structure of the NMO–IgG epitope.

The first indication that NMO–IgG specifically recognizes AQP4 only if it is organized into OAPs came from immunofluorescence experiments performed on CNS tissues, highlighting the very strong NMO–IgG labeling at the level of the cerebellum and spinal cord perivascular glial processes, characterized by the largest OAPs, versus the very weak staining at the astrocyte process network in the granular cell layer and gray matter (Figure 5(a)). However, the direct proof that AQP4-OAPs, and not AQP4 tetramers, are the target for NMO–IgG came from experiments performed on astrocytes and HeLa cells stably transfected with the M1 or M23-AQP4 isoform. These experiments showed that NMO–IgG does not recognize cells transfected with M1-AQP4 (Figure 5(b)). Two alternative systems in which AQP4 tetramers and AQP4-OAPs could be compared were also explored to further confirm this conclusion. The first one was the rat stomach in which AQP4 forms OAPs used in parallel with the mouse stomach where AQP4 does not form OAPs.[25] As expected, immunofluorescence experiments showed that NMO serum only recognizes rat and not mouse stomach[16] (Figure 5(c)). The second one was based on the use of two M23 fusion proteins tagged at the N-terminus or at the C-terminus. As hydrophobic interactions between the N-terminal region of the M23 isoform are crucial for OAP formation,[12] addition of a fluorescent tag to the N-terminus prevents OAP formation. NMO–IgGs were only able to bind C-terminus tagged M23 and not the N-terminus tagged one (Figure 5(d)) confirming that the NMO–IgG epitope is generated by AQP4 aggregation into OAPs.

Another important issue to be considered therefore, to focus on the potential tissues targeted by NMO IgG, was whether the structural epitope was common to other AQPs able to assemble into supramolecular structures, such as AQP0[46] and AQPcic.[47] By using cells transfected with these two AQPs it was concluded that the NMO–IgG epitope is characteristic of AQP4 organized into OAPs,[16] although these AQPs, especially AQP0 and AQP4, share several conserved amino acids at the extracellular loops.

A study focused on NMO–IgG epitope mapping was published, starting from the obvious evidence of a complex conformational extracellular epitope absent in the lens AQP0 and the insect AQPcic. On the basis of previous crystallography studies,[48] the major differences were identified at loop A, from residue 61 to 64, at loop C, from residue 137 to 156, and at loop E, from residue 225 to 230. NMO–IgG binding was tested on transiently transfected HeLa cells by immunofluorescence and immunoprecipitation using 11 NMO patient sera.[45] On the basis of the results obtained, two different conformational epitopes and three classes of NMO sera (Figure 6) were classified with a different behavior against AQP4 mutants (for the detailed study see Ref 45).

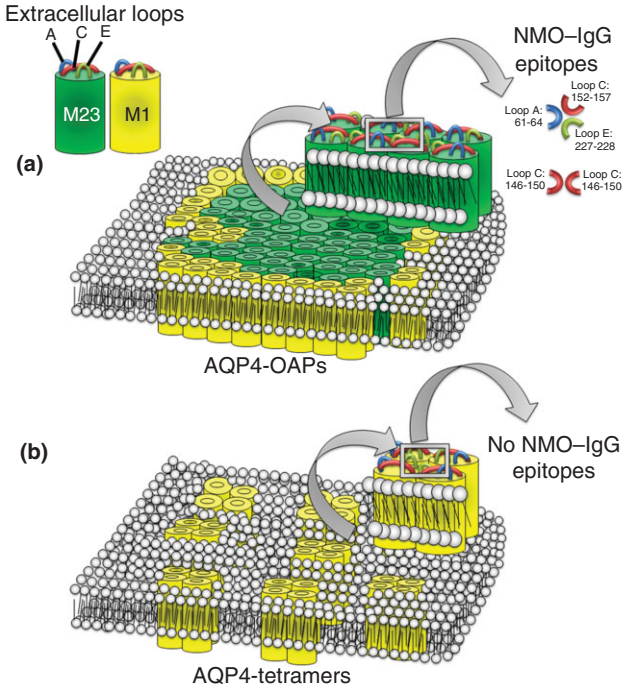
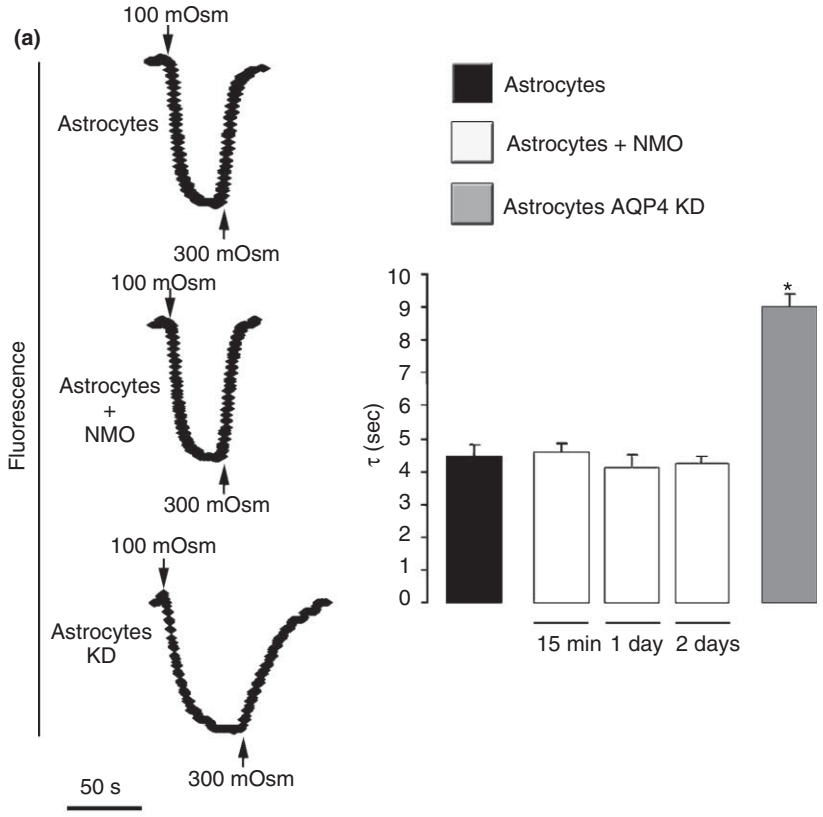


FIGURE 6 | Neuromyelitis optica (NMO–IgG epitope structures. Drawings of an aquaporin-4 orthogonal arrays of particle (AQP4-OAP) (a) and AQP4 tetramer (b). When AQP4 organizes into OAPs the extracellular loops of each tetramer interact and create at least two different NMO–IgG epitopes.

The evidence that OAPs, and not AQP4, are the target for NMO–IgG,[16] although slow to be accepted by the scientific community working on NMO,[45,49,50] is no longer debated and this needs to be highlighted in view of future developments both in molecular medicine and in the field of NMO diagnosis. Moreover, given the polyclonal origin of NMO–IgG and the demonstration that NMO patients can be subcategorized into at least three different groups,[45] we believe that caution is necessary when drawing conclusions from any results obtained with engineered monoclonal antibodies[50] not representative of the real situation from an immunological point of view.

AQP4 is the CNS water channel devoted to the regulation of the brain water balance.[51] The most obvious hypothesis was an impaired water balance in NMO patients due to the direct action of NMO–IgG



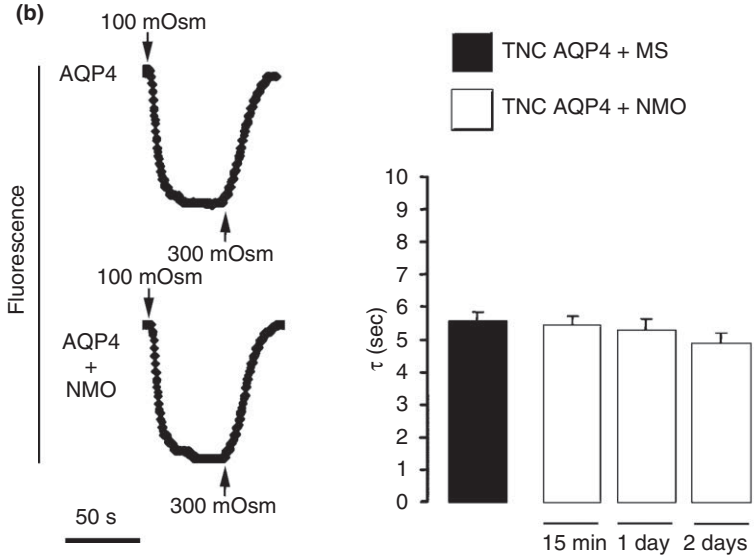


FIGURE 7 | Neuromyelitis optica (NMO)–IgG does not block aquaporin-4 (AQP4) water transport. (a) Primary cultured astrocytes were incubated with Multiple Sclerosis (MS) and NeuroMyelitis Optica (MNO) sera for 15 min, 1 and 2 days at a dilution of 1:500 in culture medium. No significant differences were found between the conditions. The cell swelling was significantly slowed only in AQP4 siRNA-treated astrocytes (astrocytes AQP4 KD) on day 6 of RNA interference (the histograms show the mean +/− SE (standard error) of separate sets of measurements, $n = 3–7$, $^*P < 0.01$). (b) TNC-1 cells transfected with M23-AQP4 isoform were treated as described in (a) for astrocytes. Typical swelling kinetics (left) and histogram (right) of the time constant (s) of the exponential curve fitting after hypotonic shock. No significant differences in cell swelling kinetics were found between the different conditions. (Reprinted with permission from Ref 16. Copyright 2012 John Wiley and Sons)

autoantibodies on AQP4 function and therefore on AQP4-mediated water transport. Three laboratories in the last few years have performed this kind of investigation.[16,52,53]

Nicchia et al. first reported that NMO–IgG does not affect AQP4 function[16] in experiments performed on primary cultured astrocytes, endogenously expressing AQP4, and on glioma cells transfected with AQP4-M23 isoform. In both cases water permeability was measured by TIRF assay with calcein as a cytosolic marker. Cells were treated with four pooled NMO sera for 15 min, 1 day, and 2 days, and then the osmotic response was analyzed (Figure 7(a) and (b)). No differences were found between cells treated with NMO versus control sera, indicating that AQP4-mediated water transport is not involved directly in

NMO pathogenesis. Although Hinson et al.[53] found that NMO–IgG binding inhibits water influx through AQP4 using the time-to-lysis assay of oocyte osmosis, Verkman's lab has confirmed the absence of the NMO–IgG effect on AQP4-mediated water transport using stopped-flow light scattering on plasma membrane vesicles isolated from AQP4-expressing cells.[52] This approach is also considered very powerful in detecting changes in water permeability because the use of plasma membrane vesicles, instead of entire cells, avoids internalization effects. Rossi et al.[52] showed that exposure of AQP4 to high concentrations of NMO–IgG from six different NMO patients did not reduce M1-AQP4 or M23-AQP4 water permeability.

In conclusion, NMO–IgG binding to AQP4 water channels does not impair water transport as shown by two different aquaporin laboratories, using different powerful and appropriate techniques. Moreover, this is in agreement with an inter-tetramer epitope in view of the large size of NMO–IgG compared with AQP4 tetramers. We believe that other mechanisms may occur to explain the pathogenic effect of NMO–IgG, including complement activation, endocytosis, and degradation of AQP4 protein but these mechanisms are still debated.[52,54,55,56]

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