Loss of Astrocyte Polarization in the Tg-ArcSwe Mouse Model of Alzheimer’s Disease

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Abstract. Aquaporin-4 (AQP4) is the predominant water channel in brain and is selectively expressed in astrocytes. Astrocytic endfoot membranes exhibit tenfold higher densities of AQP4 than non-endfoot membranes, making AQP4 an excellent marker of astrocyte polarization. Loss of astrocyte polarization is known to compromise astrocytic function and to be associated with impaired water and K\textsuperscript{+} homeostasis. Here we investigate by a combination of light and electron microscopic immunocytochemistry whether amyloid deposition is associated with a loss of astrocyte polarization, using AQP4 as a marker. We used the tg-ArcSwe mouse model of Alzheimer’s disease, as this model displays perivascular plaques as well as plaques confined to the neuropil. 3D reconstructions were done to establish the spatial relation between plaques and astrocytic endfeet, the latter known to contain the perivascular pool of AQP4. Changes in AQP4 expression emerge just after the appearance of the first plaques. Typically, there is a loss of AQP4 from endfoot membranes at sites of perivascular amyloid deposits, combined with an upregulation of AQP4 in the neuropil surrounding plaques. By electron microscopy it could be verified that the upregulation reflects an increased concentration of AQP4 in those delicate astrocytic processes that abound in synaptic regions. Thus, astrocytes exhibit a redistribution of AQP4 from endfoot membranes to non-endfoot membrane domains. The present data suggest that the development of amyloid deposits is associated with a loss of astrocyte polarization. The possible perturbation of water and K\textsuperscript{+} homeostasis could contribute to cognitive decline and seizure propensity in patients with Alzheimer’s disease.

Keywords: Aquaporin-4, cerebral amyloid angiopathy, electron microscopy, immunocytochemistry, 3D reconstruction

INTRODUCTION

Scant information is available regarding the roles of astrocytes and astrocyte endfeet in the pathogenesis and pathophysiology of Alzheimer’s disease. This is surprising given the fact that astrocytes are essential in regulating transport across the brain-blood interface and in regulating homeostatic processes critical for neuronal function.

A perturbation of astrocytic function secondary to plaque formation and cerebral amyloid angiopathy (CAA) could interfere with synaptic processing and contribute to cognitive decline. Specifically, it remains
animals and approved by the Biological Research
Health Guide for the care and use of laboratory
mates were used in this study. All animal experiments
mice) [12] and age-matched non-transgenic litter-
and Swedish (K670N, M671L) mutations (tg-ArcSwe
animals with a loss of astrocyte polarization. The term
astrocyte polarization refers to the fact that astro-
cytes are endowed with processes that differ in regard
to structure, function, and complement of membrane
molecules [1]. Most notably, astrocytic processes abut-
ting on cerebral microvessels or pia are characterized
by a high density of the water channel AQP4 which is
retained in these processes through interaction to the
dystrophin associated protein complex (DAPC) [2–4].
The same processes are also enriched in the inwardly
rectifying K\(^+\) channel Kir 4.1, as shown in quantita-
tive immunogold analyses of retinal Müller cells [5].
In contrast, astrocyte processes facing synaptic regions
are enriched in glutamate transporters while the den-
sity of AQP4 is comparatively low [6–8]. A loss of
astrocyte polarization may compromise the ability of
astrocytes to regulate volume and water transport in
the CNS and could also thwart K\(^+\) siphoning which
depends on the polarized expression of inwardly rec-
tifying K\(^+\) channels [9]. Animal models with loss of
astrocyte polarity reveal delayed potassium clearance
and increased seizure intensity [10]. Loss of astro-
cyte polarity has also been observed in human mesial
temporal lobe epilepsy (MTLE) [11].

Here we use a combination of techniques to establish
whether amyloid plaques, and perivascular amyloid
deposits in particular, are associated with a perturba-
tion of astrocyte endfeet leading to loss of astrocyte
polarization. Electron microscopy provided the reso-
lation of astrocyte endfeet leading to loss of astro-
cyte polarization. Our data indicate that the accu-
mulation of amyloid in tg-ArcSwe mice is coupled
temporally and spatially to loss of astrocyte polariza-
tion and a mislocalization and overexpression of AQP4
in non-endfoot membranes.

MATERIAL AND METHODS

Animal model and tissue preparation

Amyloid-\(\beta\) protein precursor (A\(\beta\)PP) transgenic
mice harboring hA\(\beta\)PP with the Arctic (E693G)
and Swedish (K670N, M671L) mutations (tg-ArcSwe
mice) [12] and age-matched non-transgenic litter-
mates were used in this study. All animal experiments
were in accordance with the National Institutes of
Health Guide for the care and use of laboratory
animals and approved by the Biological Research
Ethics Committee in Norway. The animals used for
immunofluorescence and electron microscopy were
deeply anesthetized with Equithesin, transcardially
perfused with 4% formaldehyde (PFA), post-fixed in
4% PFA overnight, and stored in 1/10 fixative at 4°C
until further preparations. The animals used for West-
ern blotting were decapitated, cerebral cortex dissected
out and quickly frozen on dry ice and stored at −80°C
until processed. Each age-group studied contained
between 4–8 pairs of animals (tg-ArcSwe/wt). For
electron microscopic analysis, pieces of cerebral cortex
were dissected from 500-μm-thick free floating sections under microscopy guidance
(Olympus SZX12) and embedded in Lowicryl LM20
as described [13, 14]. There are two main steps in this
procedure, cryoprotection and cryosubstitution. Cryo-
protection was done by immersing the tissues into
phosphate buffered glucose followed by increasing
concentrations (10, 20, and 30%) of glycerol prior to
plunging the tissue specimens into liquid propane at
−190°C in a liquid nitrogen-cooled cryofixation unit
KF80 (Reichert, Vienna, Austria). Cryosubstitution
was undertaken in 0.5% uranyl acetate in anhydrous
methanol at −90°C for 24 h in a cryosubstitution
unit (AFS, Reichert). The temperature was stepwise
increased to −45°C and Lowicryl HM20 was gradu-
ally substituted for methanol. Polymerization was
performed under UV light for 48 h at −45°C.

Serial section and reconstruction

3D reconstruction of serial sections was described in
our previous study [15]. In summary, serial sections of
the Lowicryl-embedded tissue were cut with a diamond
knife and placed on formvar-coated single-hole grids.
Postembedding immunocytochemistry with 6E10 (or
A\(\beta\)L) antibody was used to identify amyloid deposits,
and glutamine synthetase (GS) for astrocytic pro-
cesses. Electron micrographs were obtained digitally
from a transmission electron microscope with a cam-
era. Analysis and 3D reconstruction were performed by
Reconstruct, software specifically developed for serial
section electron microscopy [16].

Antibodies and chemicals

6E10, the monoclonal (mouse) antibodies against
amyloid-\(\beta\)-1-16 (Nordic BioSite) or polyclonal
A\(\beta\)1–42, (gift from UCI), polyclonal (rabbit) anti-
body against AQP4 (Chemicon and Sigma), polyclonal
(chicken and rabbit) antibodies against GFAP (Nordic
BioSite and Dako Cytomation), polyclonal antibody

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Table 1

<table>
<thead>
<tr>
<th>Exp</th>
<th>Combination of 1st ab</th>
<th>Combination of 2nd ab</th>
</tr>
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<tbody>
<tr>
<td>1.</td>
<td>AQP4: Millipore, rabbit, 1 : 1000</td>
<td>Alex488: donkey anti rabbit, 1 : 1000</td>
</tr>
<tr>
<td>2.</td>
<td>GFAP: Nordic Biosite, chicken, 1 : 1000</td>
<td>cy3: donkey anti chicken, 1 : 1000</td>
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<tr>
<td></td>
<td>GFAP: Nordic Biosite, chicken, 1 : 500</td>
<td>Alex488: donkey anti mouse, 1 : 1000</td>
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<tr>
<td></td>
<td>CD31: BD Biosciences, rat, 1 : 500</td>
<td>cy3: donkey anti rabbit, 1 : 1000</td>
</tr>
<tr>
<td></td>
<td>CD31: BD Biosciences, rat, 1 : 500</td>
<td>Alex488: donkey anti mouse, 1 : 1000</td>
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Table 2

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<tr>
<th>Exp</th>
<th>1st ab</th>
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<tbody>
<tr>
<td>1.</td>
<td>AQP4: Sigma, rabbit, 1 : 400</td>
<td>GAR-15 nm</td>
</tr>
<tr>
<td>2.</td>
<td>GFAP: Dako Cytomation, rabbit, 1 : 2000</td>
<td>Ap-10 or 15 nm</td>
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<tr>
<td>3.</td>
<td>6E10: Nordic BioSite, mouse, 1 : 1000</td>
<td>Abcam, 1 : 20</td>
</tr>
<tr>
<td>4.</td>
<td>GS: Sigma, rabbit, 1 : 1000 (anti-AQP4 1 : 300, 6E10 1 : 1000, anti-GS 1 : 300, anti-GFAP 1 : 2000)</td>
<td>2 h</td>
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<tr>
<td>5.</td>
<td>AQP4 and 6E10</td>
<td>Abcam and BBI, 1 : 20</td>
</tr>
<tr>
<td></td>
<td>AQP4 and GS</td>
<td>Abcam and BBI, 1 : 20</td>
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Immunocytochemistry

Light microscopy

25 μm-thick free-floating sections were cut by vibratome (Leica), pretreated in 90% formic acid, incubated in pre-incubation solution (10% NDS, 1% BSA, 0.5% Triton-X-100 in 0.01 M PBS) at 4°C overnight. After washing steps, the sections were incubated for 1 h at room temperature in the secondary antibody (shown in Table 1) diluted in 3% NDS, 1% BSA, 0.5% Triton-X-100 (anti-AQP4 1 : 300, 6E10 1 : 1000, anti-GS 1 : 300, anti-GFAP 1 : 2000). The sections were then rinsed twice by TBST before being incubated with goat anti-rabbit or anti-mouse Fab fragments coupled to gold particles (Table 2) in 2% HSA TBST for 1 h. Double labeling was achieved by incubating tissue section with both monoclonal and polyclonal antibodies or using formaldehyde vapor at 80°C for 1 h before applying the next round of immune-incubation if the antibodies were of the same type. For enhancing the contrast, uranyl acetate (Fluorochem) in double distilled water and lead citrate were used. The labeling was examined in a transmission electron microscope (TECNAI 12).

Western blot

Protein concentrations were determined with a DC protein assay (Bio-Rad). After diluting the samples in loading buffer (0.875% SDS, 5% Glycerol, 50 mM Tris-Cl pH 6.8, 0.83% β-mercaptoethanol and trace amount of bromophenol blue), 2 μg of protein was
explained by retraction of astrocyte processes as GFAP (Fig. 2C-D). Nor could the loss of perivascular AQP4 be a function of age as brain microvessels devoid of amyloid retained a strong AQP4 immunosignal (arrow, Fig. 2E-F; also see Fig. 6). The loss of perivascular AQP4 immunolabeling was significant AQP4 labeling (Fig. 2C-F; also see Fig. 6). CAA was commonly observed around brain microvessels (Fig. 2A). Specifically, the AQP4 immunosignal that vessels devoid of CAA retain their complement of AQP4 while contiguous amyloid-laden vessels have lost their perivascular AQP4 pool. AQP4 while contiguous amyloid-laden vessels have lost their complement of AQP4 while contiguous amyloid-laden vessels have lost their perivascular AQP4 pool. The changes observed in the immunofluorescence preparations were confirmed at the electron microscopic level (Figs. 3 and 4). The distinct perivascular AQP4 signal typical of wild-type animals (Fig. 3A) stood in sharp contrast to the patchy labeling in the transgenic animals. Notably, there were few particles immunopositive for AQP4 at sites where the amyloid deposits touched the astrocyte processes or the endothelial basal lamina (Fig. 3B, 4D). Figure 4 shows that vessels devoid of CAA retain their complement of AQP4 while contiguous amyloid-laden vessels have lost their perivascular AQP4 pool. Electron microscopic analysis also confirmed the light microscopic data indicating an increased neuropil labeling in the transgenic animals (compare Fig. 3A with Fig. 3C). The enhanced AQP4 immunogold in the neuropil could be attributed to an increased labeling density of GFAP immunopositive profiles, suggestive of a redistribution of labeling from perivascular astrocyte processes to astrocyte processes contacting neuronal elements in the neuropil. To corroborate the immunocytochemical data indicating increased AQP4 immunolabeling in neuropil of transgenic animals (Figs. 2 and 3), we performed a Western blot analysis. This analysis showed that the level of AQP4 protein was significantly higher in AD animals than in wild-type controls (Fig. 5). In the transgenic animals, the amount of AQP4 (recorded in frontal cortex) was higher at 9 months of age than at 16 months. Endfoot astrocytic membranes only constitute a small membrane fraction compared with the astrocytic membranes in the neuropil. Thus the Western blot signals reflect the size of the AQP4 pool in the latter membranes. Aquaporin-4 is anchored in perivascular membranes through binding to the dystrophin associated protein complex [2]. Similar to AQP4, dystrophin was lost from those vessels that accumulated amyloid deposits (Fig. 6).
Fig. 1. Electron micrograph of Aβ42 immunopositive deposit in tg-AβPP-ArcSwe mouse cortex (A). The plaque (Pl) is of considerable size with radiating fibrils. The fibrils are decorating a nearby vessel (L, vessel lumen). The plaque is associated with dystrophic neurites. To appreciate the immunogold labeling, two areas (indicated with box and star, respectively) are enlarged in B and C. In B, the fibrillar deposit is indicated by arrowheads; in C, the dystrophic neurites (dn) are clearly visible. Aβ deposits (immuno-positive for Aβ42) were found in the extracellular space around the astrocyte endfoot-endothelial complex, including pericyte processes, astrocyte endfoot processes, and endothelial cells (D). E) The 3D reconstruction shows that Aβ42 deposits touch several structures of this complex. No breakage of the endothelial lining was observed in the serial sections included in this study. The basal lamina still covers the entire abluminal surface of the capillary endothelium, separating Aβ42 deposits from the endothelial lining. The astrocytic endfoot covering is interrupted by Aβ deposits. Abbreviations: Ast, astrocyte processes; E, endothelial cell; P, pericytes. Scale bars, 0.5 μm in A-C and 0.3 μm in D.
Fig. 2. Immunoreactive Aβ aggregates (red) was first seen at 4 months in AD mice (B), and peaked at 8–16 months when severe extracellular plaque deposition was apparent (C–F) in association with infiltration of GFAP-immunoreactive astrocytes (purple; D). Deposits around the vessels were invariably associated with an increase in AQP4 immunofluorescence (green) in the neuropil (C–F), showing loss of polarity compared to the normal distribution of AQP4 (A). Small vessels covered by Aβ lost their linear labeling of AQP4. However, AQP4 immunolabeling was dense in glial membranes in close vicinity to the affected vessels, highlighted in C and D and indicated by the lines. The arrows in A, B, C, and D point to endfeet with a normal distribution of AQP4. Arrowheads point to Aβ deposits (B,C,D,E,F). Arrow in E indicates a site where AQP4 persists. All sections were counterstained with DAPI (blue) featuring the nuclei. Scale bars, 50 μm.
DISCUSSION

The present data indicate that the development of amyloid deposits in tg-ArcSwe mice is associated with a loss of astrocyte polarization. Our time course analysis suggests that loss of astrocyte polarization is a consequence rather than cause of Aβ deposition. The data are consistent with the idea that aggregation of...
Fig. 4. Close relationship between Aβ deposition and down regulation of AQP4 labeling. Sites of large amyloid deposits (CAA) are characterized by a mislocalization of AQP4 as shown by confocal microscopy (A,B,C). Asterisk indicates vessel where AQP4 is lost (AQP4, green) and arrows point to normal AQP4 distribution. Arrowhead in B and C indicate Aβ deposits around a vessel (red). D) High magnification of the close relationship between amyloid and AQP4. Where Aβ occurs (small gold particles and arrowheads), AQP4 is lost from the endfeet (big gold particles). Where amyloid is absent, the linear labeling for AQP4 is maintained (arrows). The insert represents a closer view of the relationship between these players. Abbreviations: Ast, astrocyte; L, lumen. Scale bars, LM 50 μm, EM 0.5 μm.
Loss of astrocyte polarization is likely to have significant impact on brain function. Astrocytes are endowed with specialized membrane domains that reflect the multitude of spatially restricted tasks that the astrocytes normally subserve. Potassium siphoning is an example of such a task. As originally defined [23], potassium siphoning is a special case of $K^+$ spatial buffering that helps clear excess $K^+$ from synaptic regions by redistribution to distant sites. Studies in Müller cells revealed much higher $K^+$ conductances in endfeet membranes than in membranes opposed to synaptic regions, supporting the idea that $K^+$ taken up in synaptic regions is “siphoned” through the endfeet membranes [24]. Immunocytochemical data support this idea, by demonstrating a tenfold higher density of the inwardly rectifying $K^+$ channel Kir 4.1 in endfeet membranes than in non-endfeet membrane domains [5]. The distribution of Kir 4.1 mirrored the distribution of AQP4, leading to the hypothesis that AQP4 mediated water transport accompanies $K^+$ redistribution [1]. Indeed, animals suffering a loss of AQP4 from astrocytic endfeet following targeted deletion of alpha-syntrophin show delayed clearance of $K^+$ from the extracellular space [10]. Deletion of alpha-syntrophin (which serves to anchor AQP4 in endfeet membranes) leads to a redistribution of AQP4 along the astrocytic membrane [10], mimicking the changes in AQP4 expression presently observed in the tg-ArcSwe model.

Analyses of glutamate transporters were beyond the scope of the present study. But it should be emphasized that the distribution of such transporters shows a polarity opposite to that of AQP4. Thus while the glutamate transporter EAAT1 is enriched in those astrocyte membranes that abut on excitatory synapses, it is weakly expressed in those endfeet membranes that contain high densities of AQP4 [6]. If loss of astrocyte polarity is generalized so as to also involve glutamate...
transporters, this would imply that glutamate clearance would be less efficient than normal. The changes presently observed imply that astrocytes are impaired when it comes to the homeostatic functions that they normally subserve.

It has long been known that patients with Alzheimer’s disease are predisposed to epileptic seizures [25–29]. Similarly, non-convulsive seizure activity has been demonstrated in AβPP transgenic mice [30]. As yet, no mechanisms have been identified to underpin this association. Loss of astrocyte polarity might provide the link. Thus, mice with targeted deletion of alpha-syntrophin [2] (known to anchor AQP4 to endfoot membranes) show a loss of astrocytic polarization similar to that found in the present material and exhibit increased severity of seizures compared with wild-type animals [10, 31]. Loss of astrocyte polarization (reflected as a loss of perivascular AQP4) is also found in the sclerotic hippocampi of patients with mesial temporal lobe sclerosis [11]. By extrapo-
lation, the loss of astrocyte polarization unraveled in the present animals may be indicative of a perturbed A<sub>1</sub>-<sub>2</sub> spatial buffering that in turn may predispose for hyporeactivity and epileptic seizures.

The ratio between A<sub>1</sub>B<sub>2</sub> and A<sub>1</sub>B<sub>3</sub> in the present mouse model [32] may not be representative of that in humans where A<sub>1</sub>B<sub>2</sub> may be relatively more abundant, depending, i.e., on apolipoprotein status [33–35]. This calls for caution when extrapolations are made from the present Alzheimer’s disease model to the clinical condition.

In conclusion, the present data suggest that perivascular A<sub>1</sub>B<sub>2</sub> deposits may be particularly deleterious to brain function as they by compromising endfoot function also will interfere with the homeostatic functions of astrocytes at large. Our findings introduce a new dimension to the understanding of the pathophysiology and pathogenesis of Alzheimer’s disease.

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