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REGULAR ARTICLE

Agrin defines polarized distribution of orthogonal arrays of particles in astrocytes

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Abstract Accumulating evidence indicates that agrin, a heparan sulphate proteoglycan of the extracellular matrix, plays a role in the organization and maintenance of the blood-brain barrier. This evidence is based on the differential effects of agrin isoforms on the expression and distribution of the water channel protein, aquaporin-4 (AQP4), on the swelling capacity of cultured astrocytes of neonatal mice and on freeze-fracture data revealing an agrin-dependent clustering of orthogonal arrays of particles (OAPs), the structural equivalent of AQP4. Here, we show that the OAP density in agrin-null mice is dramatically decreased in comparison with wild-types, by using quantitative freeze-fracture analysis of astrocytic membranes. In contrast, anti-AQP4 immunohistochemistry has revealed that the immunoreactivity of the superficial astrocytic endfeet of the agrin-null mouse is comparable with that in wild-type mice. Moreover, in vitro, wild-type and agrin-null astrocytes cultured from mouse embryos at embryonic day 19.5 differ neither in AQP4 immunoreactivity, nor in OAP density in freeze-fracture replicas. Analyses of brain tissue samples and cultured astrocytes by reverse transcription with the polymerase chain reaction have not demonstrated any difference in the level of AQP4 mRNA between wild-type astrocytes and astrocytes from agrin-null mice. Furthermore, we have been unable to detect any difference in the swelling capacity between wild-type and agrin-null astrocytes. These results clearly demonstrate, for the first time, that agrin plays a pivotal role for the clustering of OAPs in the endfoot membranes of astrocytes, whereas the mere presence of AQP4 is not sufficient for OAP clustering.

Keywords Aquaporin-4 · Astrocytes · Blood-brain barrier · Freeze-fracturing · Mouse (Agrin-null mutation; C57BL/6)

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Introduction

Astrocytes are classical glial elements mediating between the neuronal and vascular compartments. They play decisive roles in the maintenance both of the metabolic parameters of synapses and of the barrier properties of the brain microcapillary endothelial cells by forming complex glio-neuronal and glio-vascular interactions. This is accomplished by the formation of specialized glial endfeet that contact blood vessels and the subpial surface of the brain (type II processes; Reichenbach 1989). During early development, radial glia cells form the glial limiting membrane; later at around birth, they transform into astrocytes (Schmechel and Rakic 1979; Voigt 1989). The manner and timing of the replacement of radial glial endfeet by astroglial endfeet has not been investigated in detail. The region at which the endfeet of the glial cells contact the superficial or perivascular basal lamina (the *glia limitans superficialis et perivascularis*), viz. the glial membrane, becomes highly enriched in orthogonal arrays of intramembranous particles (OAPs) from the

perinatal stage onwards (Anders and Brightman 1979; Gotow and Hashimoto 1989). From the discovery of OAPs in astrocytes in the early 1970s (Dermietzel 1973) up to the middle of the 1990s, the freeze-fracture technique was the only experimental approach for the detection of OAPs (Wolburg 1995); other methods are now available but these will be discussed later (see Discussion). Today, the water channel protein aquaporin-4 (AQP4) is generally believed to be constitutive for the appearance of OAPs in freeze-fracture replicas (Rash et al. 1998). The observation that the density of OAPs is dramatically reduced in regions where the direct contact of the glial cell membrane with the basal lamina is interrupted (Rohlmann et al. 1992) suggests a mandatory role of the basal lamina for the OAP-based configuration of the endfoot membrane in astroglial and astroglia-related cells. In the present study, the large difference in OAP density between the endfoot and the non-endfoot membrane is referred to as "astroglial polarity".

The finding that, in brain tumours, OAPs decrease in number or even disappear (Neuhaus 1990; Hatton and Sang 1990), whereas AQP4 is up-regulated (Saadoun et al. 2002; Warth et al. 2004), has prompted the question as to whether AQP4 is the only molecule within OAPs. Although each OAP appears to contain AQP4, we do not know whether AQP4 molecules always occur in the form of OAPs. Interestingly, the two main splice variants of AQP4, M1 and M23 (now also called AQP4a and AQP4c, respectively: Moe et al. 2008), which exhibit different water transport capacities (Silberstein et al. 2004), elicit distinct OAP morphologies (Furman et al. 2003). Nevertheless, so far, no study has systematically investigated the correlation between M1/M23 expression and OAP occurrence in vivo.

Recently, we have published the effects of the extracellular heparan sulphate proteoglycan, agrin, on the expression and distribution of AQP4, on the swelling capacity of cultured astrocytes of neonatal mice and on freeze-fracture properties revealing an agrin-dependent clustering of OAPs in astrocytes (Noell et al. 2007). Agrin was originally identified as being required for the clustering of acetylcholine receptors at the neuromuscular junction (McMahan 1990; Bezakova and Ruegg 2003). More recently, agrin has also been described as being important for the regulation of synaptic processes within the central nervous system (CNS) and for the integrity of the blood-brain barrier (Barber and Lieth 1997; Berzin et al. 2000; Ksiazek et al. 2007). The agrin splice variant A0B0 has been reported to be restricted to the subendothelial basal lamina of CNS capillaries and meningeal cells (Stone and Nikolics 1995). This agrin variant, when present in astrocyte primary cultures, has no effect on the expression level of AQP4 or on the water transport capacity in hypotonic challenge experiments (Noell et al. 2007). However, agrin A0B0 is able to induce increased insertion of AQP4 into the membrane of cultured astrocytes. This observation is in agreement with the previous in vivo finding that, in the absence of agrin from the basal lamina, AQP4 immunoreactivity is randomly distributed across the entire surface of the cell (Warth et al. 2004). This suggests that agrin plays a role in the restriction of AQP4 molecules to the membrane of glial endfeet. In a review searching for a function of agrin in the CNS, Smith and Hilgenberg (2002) have phrased the question: "What happens to the blood-brain barrier when microvascular expression of agrin is lost?"

Accordingly, our aims have been to determine directly the events that occur in the mouse brain after the loss of agrin and to demonstrate whether agrin is responsible for the AQP4- and/or OAP-related polarity of astrocytes in vivo by using agrin-null mice. However, agrin-null mice die during the first postnatal day as a result of their inability to breathe, which is caused by dysfunctional muscle lacking sufficient clustering of acetylcholine receptors in the neuromuscular endplate. In mice, no data concerning OAP densities in astrocytes before, at and after birth are available in the literature. In the rat, Anders and Brightman (1979) have reported an increase in OAP density from 2 OAPs/μm^2 at embryonic day 20 (E20) up to 14 OAPs/μm^2 at postnatal day 3 in the superficial endfoot membranes of astrocytes. As we have found a much higher OAP density in marginal endfoot membranes of wild-type C57BL/6 mice at postnatal day 1 in comparison with that in the rat (Anders and Brightman 1979), we decided to investigate the effect of an agrin-null mutation on OAP density, anti-AQP4 immunoreactivity, AQP4 expression at the mRNA and protein levels and swelling capacity after hypotonic challenge in primary astrocytes of C57BL/6 mice.

Materials and methods

Breeding of agrin-null mice

Agrin-null mice were produced by the targeted replacement of the genomic region starting in exon 6 and including all sequences up to intron 33 by a LoxP-flanked PGK-*neo* cassette (Lin et al. 2001), thereby completely abolishing the expression of agrin. Heterozygous agrin mutant mice were bred to C57BL/6 inbred mice for at least six generations before homozygous agrin-null offspring were produced by the mating of heterozygous mice. Homozygous agrin-null pups die during and immediately after birth. For analysis or isolation of astrocytes, embryos were obtained by Cesarian section at E19.5. All littermates used for culture of astrocytes, cyto- and histochemistry and freeze-fracturing were genotyped by the polymerase chain reaction (PCR) with lysates of tail biopsies.

All animal care and experimental protocols conformed to Swiss and German legislation regulating the use of animals in research. The use of animals in this study was minimized to only those numbers necessary for quantitative and qualitative analysis.

Culture of murine brain astrocytes

Murine brain astrocytes were separately isolated from individual E19.5 embryos derived from the breeding of heterozygous agrin mice according to the method of Beyer and Raab (1998) and Ivanova et al. (2001, 2002). Genotypes of astrocytic cultures were determined by PCR analysis of tail biopsies from the individual mice used for the isolation of astrocytes. Briefly, to establish primary cortical astroglial cultures, brains were removed and the cortices were prepared and collected according to Noell et al. (2007). After removal of the meninges, the tissues were digested with 0.1% trypsin and 0.02% EDTA (Lonza, Verviers, Belgium) in phosphate-buffered saline (PBS) for 15 min at room temperature. The cell suspension was then transferred into culture medium, centrifuged (400*g*; 5 min), resuspended in culture medium, filtered through a 40-μm nylon mesh and re-centrifuged (400*g*, 5 min). Cells were seeded on poly-DL-ornithine (Sigma, Deisenhofen, Germany)-coated plastic dishes and cultured in Dulbecco's modified Eagle's medium (DMEM; Lonza, Vervier, Belgium) supplemented with 20% fetal calf serum (Lonza) and penicillin (10,000 U/ml; Lonza) and streptomycin (10,000 μg/ml; Lonza) under standard conditions up to passage two. Absence of agrin in the serum was verified by enzyme-linked immunosorbent assay (performed by H. Kalbacher, Tübingen). Cells were passaged twice after having reached confluency. The astrocytes were subsequently processed for immunocytochemistry, freeze-fracture, volume measurements and reverse transcription (RT) with PCR (RT-PCR). Cell cultures were established from all embryos at E19.5 and identified as being wild-type or agrin-null mice after genotyping.

Freeze-fracture experiments

Superficial slices of E19.5 mouse brains and monolayers of cultured cells were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 2 h at room temperature. The specimens were then cryoprotected for freeze-fracturing in 30% glycerol and quick-frozen in nitrogen-slush (-210°C). Subsequently, they were fractured in a Balzer's freeze-fracture device (BAF400D; Balzers, Liechtenstein) at 5×10^{-6}mbar and −150°C. The fracture faces were shadowed with platinum/carbon (3 nm, 45°) for contrast and carbon (30 nm, 90°) for stabilization of the replica. After removal of the cell material in 12% sodium hypochlorite, the replicas were rinsed in double-distilled water several times and mounted on Pioloform-coated copper grids. The replicas were observed by using a Zeiss EM10 electron microscope (Oberkochen, Germany). OAP densities were determined as OAPs per square micrometer at a magnification of 100,000:1 in 30 replicas of three different assays in each experiment. Statistical evaluation was performed by using the Kruskal-Wallis one-way analysis of variance (ANOVA) on ranks followed by Dunn's method for comparisons (SigmaPlot Software, Scientific Solutions, Lausanne, Switzerland).

Immunostaining

Dissected brain tissues of wild-type and agrin-null embryos were frozen in Tissue Tek O.C.T (Sakura Finetek, Heppenheim, Germany), sectioned and fixed in 4% formaldehyde for 15 min. Anti-AQP4 antibody (rabbit polyclonal 1:100; Santa Cruz, Heidelberg, Germany) was used to localize AQP4 in tissues and was detected with secondary Cy3-labelled antibodies (Dianova, Hamburg, Germany). Sections were stained with anti-zonula occludens (ZO)−1 antibody (monoclonal anti-ZO−1; Zymed, Invitrogen, Karlsruhe, Germany) and detected by Cy2 goat-anti-mouse IgG (MoBiTec, Göttingen, Germany). To control for unspecific staining or autofluorescence, the primary antibodies were omitted. Unspecific binding was blocked by incubation of the sections for 30 min in 4% normal goat serum and 1% bovine serum albumin in PBS. Primary antibodies were diluted in 0.25% Triton X−100 and 1% dimethyl sulphoxide in PBS and incubated on the samples at 4°C overnight. Following washes in PBS, specimens were mounted in Mowiol (Calbiochem, Merck, Germany). Fluorescence was visualized with a confocal laser scanning microscope (Zeiss LSM510 META, Jena, Germany) by using laser excitation at 543 nm and 488 nm with appropriate filter sets and a 40× oil objective (N.A. 1.3).

RT-PCR analysis

RT-PCR analysis for mRNAs for AQP4 isoforms M23 and M1, agrin and hypoxanthine guanine phosphoribosyl transferase (HPRT) was performed as described by Ivanova et al. (2002). The *HPRT* gene has been reported as a constitutively expressed house-keeping gene (Frericks and Esser 2008) and has been identified as the single best reference gene (De Kok et al. 2005). Total RNA was isolated by using the Peq Gold RNApure extraction kit (Peqlab, Germany) according to the manufacturer's protocol. cDNA was synthesized from 1 μg of each RNA, 1 μl dNTP (0.8 mM), 1 μl reverse transcriptase (MMLV), 5 μl 5×−buffer, and 1.5 μl hexanucleotide (10 pmol/μl; all reagents from Invitrogen) for 1 h at 37°C followed by

enzyme inactivation at 95°C for 5 min. PCR was conducted with a 1.5-µl probe from the RT reaction, 0.5 µl sense and antisense primers, 0.4 mM dNTP, 1.5 mM $MgCl_2$, and 1.5 U *Taq* polymerase (all from Invitrogen). PCR conditions were: 32 cycles (for the M23 and M1 primers), 30 cycles (for the agrin primer) or 29 cycles (for the HPRT primer) of denaturation for 1 min at 95°C, annealing for 1 min at 62°C (for the M23, M1 and agrin primers) or at 61°C (for the HPRT primer) and extension for 1 min at 72°C, followed by a final elongation step at 72°C for 3 min. Concurrent RT-PCR amplification of HPRT was carried out as an internal control for variations in the efficiencies of RNA isolation and RT. The primer sequences were as follows: HPRT sense: GCT GGT GAA AAG GAC CTC T, HPRT antisense: CAC AGG ACT AGA ACA CCT GC; AQP4 M23 sense: GGA AGG CTA GGT TGG TGA CTT C, AQP4 M23 antisense: TGG TGA CTC CCA ATC CTC CAA C; AQP4 M1 sense: CTC CCA GTG TAC TGG AGC CCG, AQP4 M1 antisense: TGG TGA CTC CCA ATC CTC CAA C; agrin sense: CGA GGG AGG CCA CTT ATG GCC, agrin antisense: GCC ATG TAG TCT GCA CGT TCT CCA ACC TTT CC.

PCR products were separated by electrophoresis on a 1.5% agarose gel and stained with ethidium bromide. For semiquantitative product analysis, gels were analysed by a UV transilluminator and densitometrically quantified by using ImageJ software (available at http://rsb.info.nih.gov/ij). Absolute optical densities (OD) were normalized to the ODs of the corresponding bands of the housekeeping gene HPRT and expressed as relative abundance in arbitrary units. Each experiment was performed at least nine times; RT-PCRs were confirmed in replicates. Prior to the semiquantitative analysis, we determined that 32 PCR cycles of the M23 and M1 primers and 30 cycles of the agrin primer were well within the linear detection range. The statistical analysis for comparison of the experimental groups was carried out by Kruskal—Wallis one-way ANOVA on ranks (SigmaPlot Software).

Cell volume measurements

For volume measurements, cells were grown on round coverslips fitting into an incubation chamber according to the method described in Noell et al. (2007). Briefly, cells were incubated (without Fungizone) in 5 µM calcein (Invitrogen) in culture medium at 37°C for 30 min. The coverslips were then mounted in a closed flow-through incubation chamber (LaCon, Staig, Germany). Cells were imaged on a confocal microscope (Zeiss LSM 510 META) via a 63× long-distance objective. The 488-nm argon laser line was used for excitation with appropriate filter sets, the laser intensity being kept low to avoid cell damage and bleaching during the experiment. To test for cellular volume changes, the following paradigm was used. To establish a base line, the chamber was perfused with isotonic Hanks' balanced salt solution (HBSS; 300 mosmol) at a flow rate of 300 µl/min. After 180 s, the solution was switched to hypotonic (200 mosmol) HBSS for an additional 180 s and finally back to isotonic buffer for at least another 200 s. For cell volume changes, the increase or decrease in fluorescence intensity were taken as a measure for cell shrinkage or swelling, respectively. Subsequent calculations were performed with the computer program Microsoft Excel. The fluorescence measurements were plotted as dF/F0, where F0 is the averaged initial fluorescence intensity and dF is the difference of the measured fluorescence to the initial fluorescence. The initial fluorescence values were compared with fluorescence minima (highest volume) and tested for statistical significance by applying the non-parametric Wilcoxon rank test (n>50 cells were measured in each experimental group).

Results

We studied the molecular, physiological and morphological properties of astroglial cells of agrin-null mice in comparison with wild-type mice in vivo and in vitro. We compared the astrocytes of both agrin-null and wild-type genotypes regarding their levels of AQP4 mRNA (RT-PCR) and AQP4 protein (immunocytochemistry), their water transport capacity and their molecular membrane morphology (freeze-fracturing).

Agrin is required for efficient OAP formation in vivo

Superficial endfeet at the surface of the brain were observed by freeze-fracturing. Because of the time-point of investigation (E19.5), the nature of these endfeet belonging to radial glia or astrocytes was not absolutely clear (see Schmechel and Rakic 1979; Voigt 1989; for problems in astroglial lineage during brain development, see Ihrie and Alvarez-Buylla 2008). However, the goal of this study was to investigate the role of agrin in the morphological constitution of the endfoot membrane. The density of OAPs in the superficial glial endfoot membranes of wild-type mice was 140/µm^2 (median; Figs. 1b, 2a, e) at E19.5. When the fracture plane was oriented appropriately, we were able to observe the direct transition from the endfoot membrane domain to the non-endfoot domain, the latter being characterized by the loss of contact with the basal lamina. In areas in which the glial membrane had "dived" into the neuropil, no OAPs were observed (see also Rohlmann et al. 1992). This polarity between endfoot and non-endfoot membrane was strikingly reduced in agrin-null mice in which the OAP-density was only 10/ µm^2 (median;

Figs. 1d, 2c, e) and the density in the non-endfoot membranes was unaltered.

In the study of astrocytes in vitro, we compared the membrane architecture of wild-type astrocytes with that of astrocytes from agrin-null embryos and investigated their OAP densities. In contrast to the results in vivo, no difference was found between astrocytes from wild-type (8 OAPs/μm^2; Fig. 2b, f) and agrin-null embryos at E19.5 (8 OAPs/μm^2; Fig. 2d, f).

In summary, our data provide the first direct evidence for a requirement of agrin during the formation of OAPs in astrocytic endfeet in vivo. Under in vitro conditions and in the absence of agrin in the medium, few OAPs are observed independently of whether the cells are cultured from wild-type or agrin-null mice.

AQP4 expression and distribution is not affected by lack of agrin

To investigate the distribution of AQP4 in vivo, cerebral brain sections of both wild-type and agrin-null embryos at E19.5 were immunostained for AQP4. In general, the superficial area of astrocytes including endfeet did not differ with regard to AQP4 immunoreactivity between the wild-type and agrin-null embryos (Fig. 3a, b). Interestingly, the anti-AQP4 immunoreactivity was not restricted to the endfeet but was recognized in astrocytes up to several micrometers from the superficial border. In order to assure that we were indeed investigating the superficial layers of the brain, we used an antibody against ZO-1 to stain cellular junctions formed by meningeal cells. Regularly and without exception, anti-ZO−1 immunoreactivity was located near the anti-AQP4 immunoreactivity of the superficial astrocytes.

Primary astrocytes established from E19.5 embryos with wild-type or agrin-null genotypes were cultured for comparable periods and to similar densities and then stained with the antibody against AQP4. In wild-type cells, as observed previously, AQP4 immunoreactivity was diffusely distributed (compare with Fig. 1A in Noell et al. 2007). Astrocytes cultured from agrin-null mice revealed an identical immunoreactivity for AQP4, consistent with the freeze-fracture in vitro data.

The expression of mRNAs coding for the AQP4 isoforms M23 and M1 was analysed in both wild-type

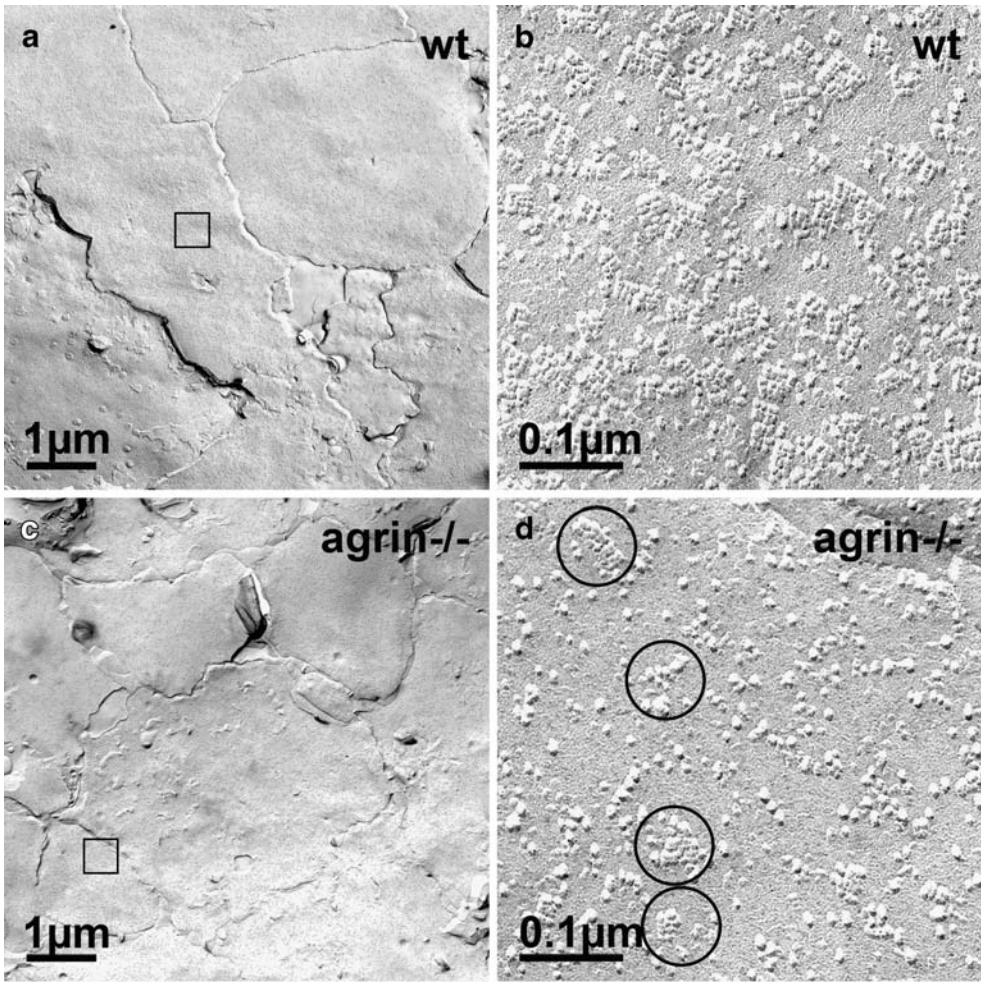

Fig. 1 Freeze-fracture replicas of superficial astroglial endfeet from wild-type (*wt*) and agrin-null (*agrin−/−*) mice at E19.5. **a**, **b** Low and high magnifications of an endfoot in wild-type mouse embryos with many OAPs. The *boxed area* in **a** is shown at higher magnification in **b**. **c**, **d** Low and high magnifications of an endfoot in an agrin-null newborn mouse with a low number of OAPs (*circles*); littermate of mouse in **a**, **b**. The *boxed area* in **c** is shown at higher magnification in **d**

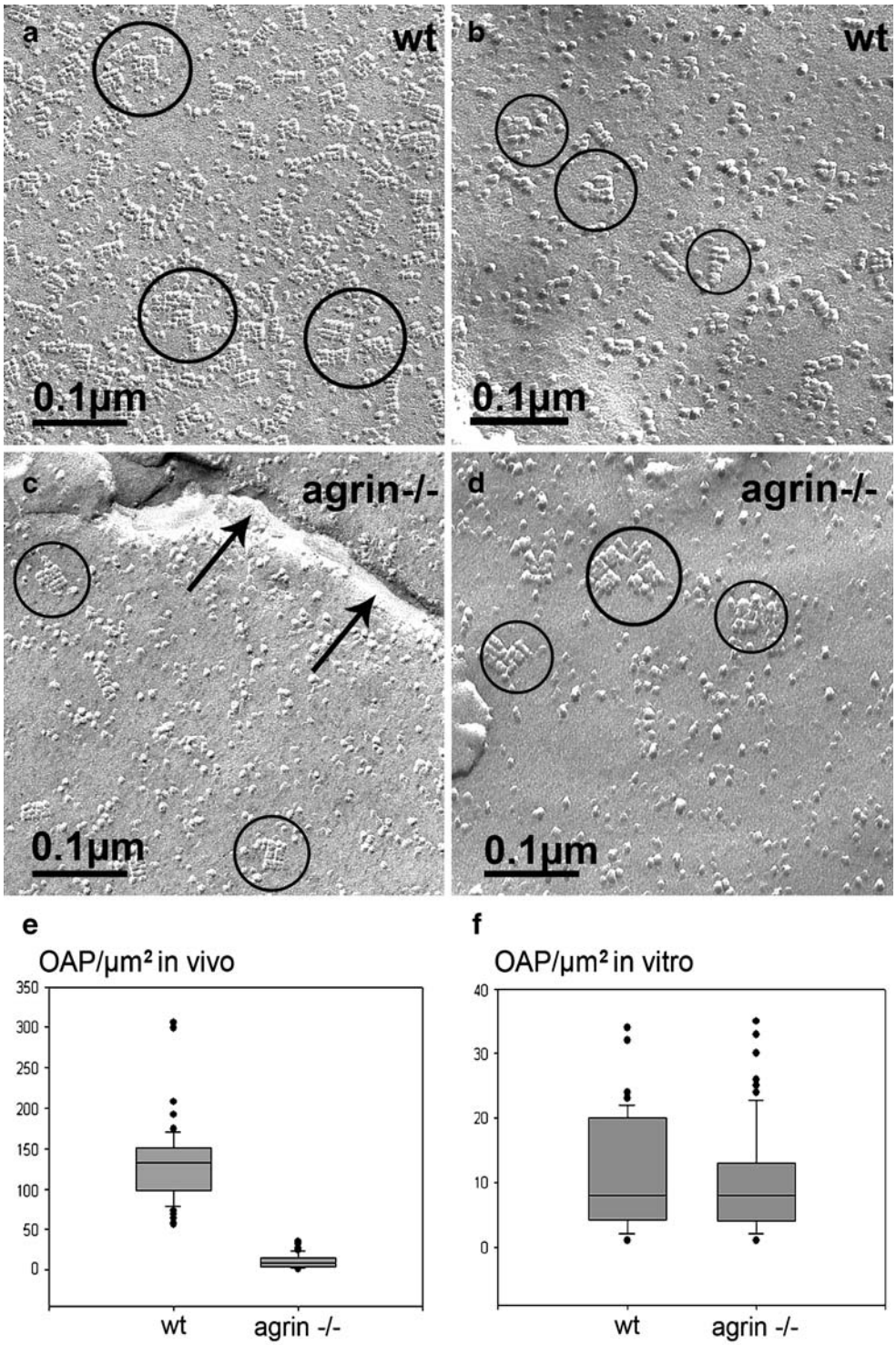

Fig. 2 Comparison between OAPs in vivo (**a**, **c**, **e**) at E19.5 and in vitro (**b**, **d**, **f**) in wild-type (*wt*, **a**, **b**) and agrin-null (*agrin −/−*, **c**, **d**) mice; freeze-fracture replicas. The wild-type and agrin-null mice are littermates. OAPs in **a–d** are *circled*. **a** An endfoot membrane revealing numerous OAPs. **c** An endfoot membrane of the agrin-null littermate showing only a few OAPs (*arrows* border between two adjacent endfeet). **e**, **f** Statistical analysis (box-blots) of the OAP densities in wild-type and agrin-null mice. The difference between wild-type and agrin-null mice in vivo is highly significant ($P<0.001$). Note the different scales in the ordinates of **e**, **f**

and agrin-null E19.5 embryos by RT-PCR (Fig. 4). No significant difference was seen between the expression level of the M1 isoform in tissue from wild-type and agrin-null embryos ($P=0.414$) or for the M23 isoform ($P=0.225$). Moreover, in astrocytic cell culture, the PCR level showed no significant difference between wild-type and agrin-null embryos for the expression of the M1 isoform ($P=0.329$) or the M23 isoform ($P=0.233$) confirming that agrin had no influence on the expression levels of AQP4 in vivo or in vitro.

Cell volume regulation is not compromised in agrin-null astrocytes

Cultured astrocytes of both wild-type and agrin-null E19.5 embryos were transferred into a flow-through chamber and

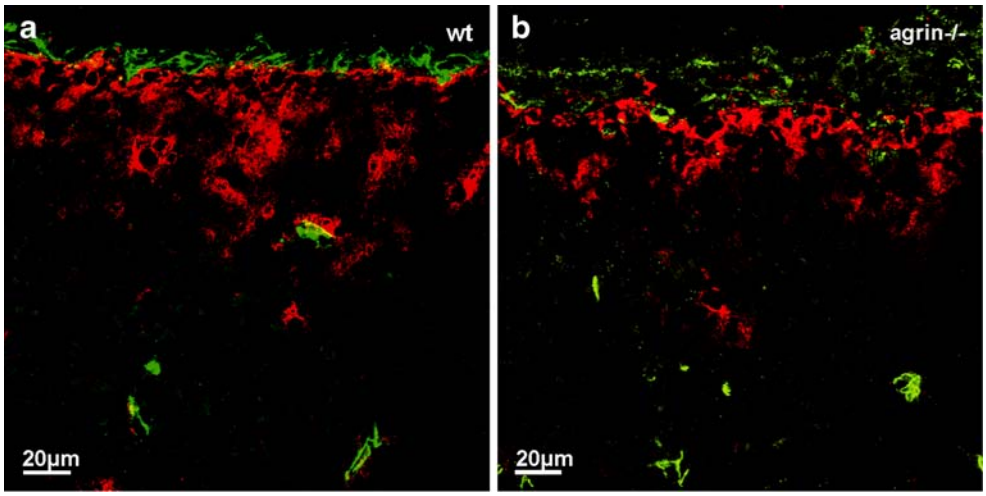

Fig. 3 Immunocytochemical staining of AQP4 (*red*) and ZO-1 (*green*) in the surface region of wild-type (*wt*, **a**) and agrin-null (*agrin−/−*, **b**) mouse brain cortex at E19.5. ZO-1 staining labels the network of meningeal cells and the endothelial cell junctions. *Red* anti-AQP4 staining does not merge with the tight-junction staining at the meningeal surface and is not restricted to the endfoot membranes of the superficial astrocytes. Anti-AQP4 immunoreactivity is also present in the upper region of the glial cells, both in wild-type and agrin-null mice

allowed to equilibrate for a few minutes at constant flow in isotonic buffer solution. As described in the previous study by Noell et al. (2007), the solution was switched to hypotonic buffer (~200 mosmol) for 180 sc and finally returned to isotonic buffer. Under normal growth conditions (controls), the calcein fluorescence intensity decreased by about 8% dF/F0 (Fig. 5), indicating a volume increase of the imaged wild-type astrocyte, and then returned to the initial intensity in isotonic condition. The identical result was observed in astrocytes from agrin-null mice. Therefore, the lack of agrin also had no effect on water transport efficiency in cultured astrocytes.

Discussion

In the present study, we show for the first time that the extracellular heparan sulphate proteoglycan agrin is responsible for the polarity in astrocytes, when defined as the uneven distribution of OAPs in the membrane. In the absence of agrin in the agrin-null mouse, only a lower number of OAPs can be found in endfoot membranes. OAPs are well-known to consist of the water channel protein AQP4 (for a review, see Wolburg et al. 2009). Therefore, one might expect that agrin is also responsible for the polarity in astrocytes, as based on the uneven distribution of AQP4. However, we have found that, independent of the presence of agrin (in both the wild-type and the agrin-null mouse), the AQP4 protein distribution detected by immunocytochemistry is different from the OAP distribution detected by freeze-fracturing: in E19.5 mouse embryos, we have observed AQP4 immunoreactivity several micrometers below the pial surface in both wild-type and agrin-null mice (Fig. 3). Investigation of the freeze-fracture morphology of these cerebral astrocytic membranes in parallel has revealed that OAPs of the

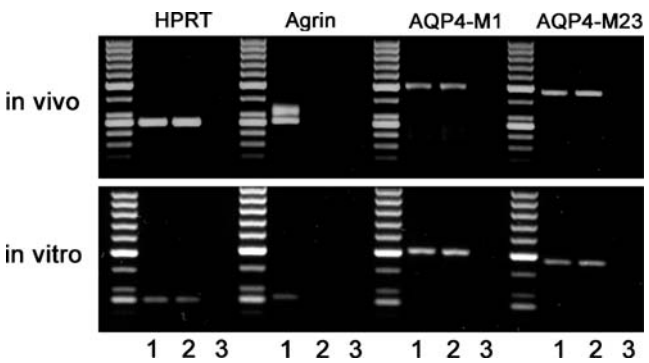

Fig. 4 PCR analysis of agrin and the AQP4 isoforms, M1 and M23, in brains of wild-type (*lanes 1*) and agrin-null embryos (*lanes 2*) at E19.5. There is no difference in the expression level of both AQP4 isoforms in wild-type and agrin-null mice (*lanes 3* negative H_2O control)

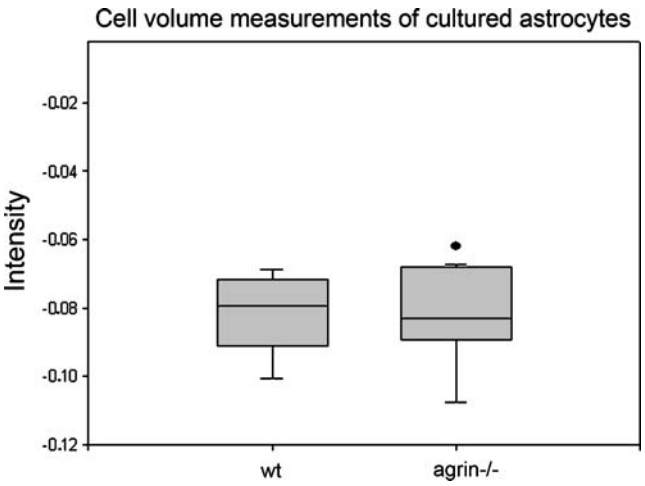

Fig. 5 Cell volume measurements of cultured astrocytes from wild-type (*wt*) and agrin-null (*agrin−/−*) mice by using the method of calcein loading and dilution on swelling upon hypotonic challenge (as performed in Noell et al. 2007). The lowest corrected fluorescence intensity values were compared between the two groups and showed no significant difference between the wild-type and the agrin-null astrocytes (Wilcoxon rank test; $P<0.001$). The *dot* above the *right column* represents a statistical anomaly

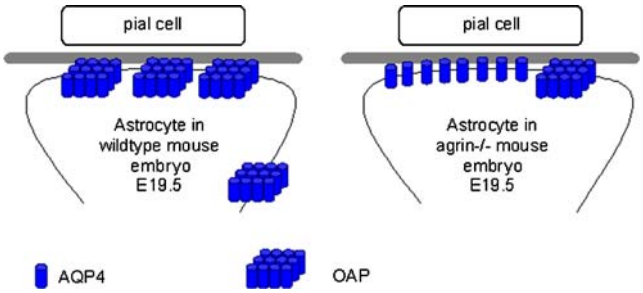

Fig. 6 Representation of the interrelationship between AQP4 and OAPs. Generally, each OAP is believed to consist of AQP4 in a consistent arrangement. However, in the agrin-null (*agrin−/−*) mouse (this study), AQP4 is expressed at levels indistinguishable from wild-type, whereas the occurrence of OAPs is extremely low. This demonstrates a role of agrin in clustering AQP4 into OAPs. The observation that a few OAPs remain in the agrin-null mice can be explained by implicating a rescuing activity by as yet unidentified proteoglycans other than agrin

wild-type embryos are restricted to endfoot membranes having contact with the basal lamina, as occurs in adults (Fig. 1; Rohlmann et al. 1992; Nielsen et al. 1997). We have not detected any OAPs in membranes distant from contacts with the basal lamina. This strongly suggests that, during embryonic development, AQP4 in early glial cells is inserted from cytoplasmic pools into membranes without forming OAPs and that OAPs are formed only in those membrane domains directly in contact with the basal lamina.

The scope of this study has not included a determination of whether these glial cells are astrocytes or still radial glial cells. In contrast to the inner glial cells of the cerebral wall, which have revealed anti-AQP4 immunoreactivity only at their perivascular endfeet, the superficial glial cells exhibit anti-AQP4 immunoreactivity that is not restricted to their subpial endfeet. This suggests that these endfeet are those of radial glial cells described to transform perinatally into multipolar astrocytes (Schmechel and Rakic 1979; Voigt 1989). From these observations, we can conclude that the polarized distribution of OAPs requires the presence of an agrin-positive basal lamina. In contrast, the localization of AQP4 protein is not affected by the absence of agrin. Moreover, the levels of both AQP4 mRNA and protein remains stable, irrespective of the presence of agrin in the brain, in vivo. Therefore, we have to presume that agrin is required to form OAPs but that agrin does not play a role in the expression of AQP4.

Hitherto, a classical role of agrin has been considered to be its participation in the differentiation of the neuromuscular junction (McMahan 1990) and in the regulation of synaptic processes in the CNS (Ksiazek et al. 2007). An additional possible role for agrin in the establishment of the blood-brain barrier became evident when agrin expression was correlated with the maturation of the blood-brain barrier (Barber and Lieth 1997; Berzin et al. 2000; Smith and Hilgenberg 2002). This was supported by the observation that a breakdown of the blood-brain barrier was accompanied by the disappearance of agrin (Rascher et al. 2002; for reviews, see Wolburg 2006; Wolburg et al. 2008, 2009). However, the fundamental question regarding the manner in which agrin-dependent astrocyte polarity influences the quality of the blood-brain barrier in terms of the permeability-restricting tight-junction proteins cannot be answered as yet.

The molecular context of this novel blood-brain barrier-related role of agrin has been elucidated by the recent observation that agrin is able to influence AQP4 in the membrane of astrocytes (Noell et al. 2007). This water channel protein is mainly responsible for water and ion homeostasis in the brain (Simard and Nedergaard 2004).

The present study contributes new aspects concerning the interrelationship between AQP4 and OAPs. The distribution of OAPs but not of AQP4 protein is altered in the agrin-null mouse. OAPs can be visualized only in the electron microscope and, as we have learnt over the last few years, AQP4 is indispensable for the formation of OAPs. This has been demonstrated by means of OAP formation after AQP4 cDNA transfection (Yang et al. 1996), fracture-labelling experiments with anti-AQP4 antibodies that label OAPs in the freeze-fracture replica (Rash et al. 1998) and the absence of OAPs in the AQP4-knockout mouse (Verbavatz et al. 1997). However, in tumour tissues, reports have contradicted a simple equivalent relationship between OAPs and AQP4, since AQP4 is upregulated (Saadoun et al. 2002; Warth et al. 2004), whereas OAPs are lost or at least reduced in number in gliomas (Hatton and Sang 1990; Neuhaus 1990). Of course, we have to differentiate between the continuously increasing restriction of AQP4 onto the endfeet during development on the one hand, and the redistribution of AQP4 under conditions of agrin loss on the other hand.

To test the effect of agrin on the polarization of the glial cells in terms of OAP formation and distribution, we have investigated the agrin-null mouse. Unfortunately, the agrin-null mouse dies immediately after separation from the maternal blood circulation after birth, when breathing becomes essential (Gautam et al. 1996). Confirmation that OAPs are present in wild-type mice at birth is essential. To our knowledge, data concerning OAPs during perinatal development have been reported only for the rat and have revealed only a few OAPs in superficial astrocyte endfoot membranes at birth, with an increasing density in the days after birth (Anders and Brightman 1979; Gotow and Hashimoto 1989). Information about OAPs in the mouse has previously been gathered only during later postnatal development (Landis and Reese 1981). The result that, in newborn wild-type mice, the OAP density in the astrocyte

superficial endfoot membranes is much higher than that in newborn rats, reaching 140/μm^2 at E19.5 (Figs. 1b, 2a, e) was thus rather unexpected. This has nevertheless enabled us to compare the endfeet from wild-type embryos and their agrin-null littermates and to show a much lower density of OAPs in the absence of agrin (Fig. 2e), which, however, is not accompanied by a reduction of AQP4 expression rate, as shown by our PCR experiments (Fig. 4).

The data presented here suggest that AQP4 alone is not sufficient for the process of OAP formation and requires the presence of agrin, presumably for clustering of the AQP4 water channel proteins to these structures. Is agrin the sole molecule involved in the aggregation of AQP4 to form OAPs? We have found a strong reduction of OAPs in the endfoot membranes of the agrin-null mouse, but not a complete disappearance of OAPs. We are tempted to speculate that proteoglycans other than agrin (e.g. versicans, syndecans, appicans: Bandtlow and Zimmermann 2000) play a similar role in the aggregation of OAPs.

No alteration of water transport capacity has been found in cultured astrocytes from the agrin-null mouse compared with wild-type cultured astrocytes. This is not unexpected, since AQP4 expression and the membrane architecture are also unchanged.

In this report, we have studied OAPs in superficial endfeet. Can we now extrapolate our findings to the blood-brain barrier and glio-vascular interactions, as we have not investigated perivascular endfeet? Here, we have primarily focused on the interrelationship between OAPs and AQP4. We have chosen to look at superficial endfeet, because vascularization of the brain is not completed at birth and the frequency of perivascular endfoot formation is too rare to be investigated by freeze-fracturing at this age in mice. The superficial OAPs most probably behave in a similar way as the perivascular OAPs. However, important differences exist between both types of endfeet, as, for example, perivascular astroglial endfeet are in intimate contact with endothelial cells and pericytes, whereas the superficial endfeet are not. We will address this point in a separate study (in preparation). Again, we have to concede that we have no idea regarding whether and how the signal of the polarized astrocyte is transduced to the barrier properties of the brain capillary endothelial cells.

Finally, we want to stress the methodological question as to whether freeze-fracture technology is still adequate for investigating OAPs. Recently, several reports have been published on the "molecular composition of square arrays" (Sorbo et al. 2008), the "expression of multiple AQP4 pools" (Nicchia et al. 2008), and "determinants of AQP4 assembly in orthogonal arrays" (Crane and Verkman 2009), without performing freeze-fracturing but by using biochemical methods or quantum-dot single-particle tracking instead. The property "orthogonal" is a morphological property that can only be visualized by a morphological method with the power of electron-microscopical resolution if the structure is a nanostructure of the OAP type. Crane et al. (2008) have reasoned that AQP4-M1 as a (morphologically) small (and highly mobile) molecule cluster and AQP4-M23 as a (morphologically) large (and relatively immobile) molecule cluster can be identified by large-range and short-range diffusion of quantum dot-labeled AQP4, respectively. Since agrin deficiency has been shown here to be linked neither to an alteration of the ratio of M1 and M23 (Fig. 4), nor to a change of AQP4 protein synthesis, the application of the quantum-dot single particle tracking experiments (Crane et al. 2008; Crane and Verkman 2009) to this system would presumably not reveal any alteration of the OAPs. In contrast, we have observed such differences (Fig. 1). This strongly suggests that the freeze-fracture method is still the only method to allow statements on the molecular architecture of the OAPs in cell membranes.

Concluding remarks

The main result of this study, as summarized in Fig. 6, is the finding that agrin is required for the formation and assembly of OAP structure. In agrin-null embryos, the astrocytic endfoot membranes are not completely devoid of OAPs but their density is much reduced when compared with those of wild-type littermates. All investigations regarding astrocyte polarity and the blood-brain barrier commonly show the correlation of a high density of AQP4-positive OAPs with a mature blood-brain barrier (Wolburg 1995; Nico et al. 2001). Our data underscore the hypothesis that the OAP-related polarity must have a causative impact on the quality of the blood-brain barrier. Our novel findings that, in astrocytes from agrin-null embryos, neither the AQP4 mRNA, nor the level and distribution of AQP4 protein are altered and that agrin exclusively affects OAP formation raise new questions. What is the mechanism of AQP4 clustering? Most importantly, what are the roles of AQP4 subunits in OAP formation and function? Answering these questions should shed light on the complex glio-vascular interactions that manage the interface between the blood and the brain.

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