ABSENCE OF GLIAL ALPHA-DYSTROBREVIN CAUSES ABNORMALITIES OF THE BLOOD-BRAIN BARRIER AND PROGRESSIVE BRAIN EDEMA.

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Running Head: Functional blood-brain barrier requires glial α-dystrobrevin

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Background
Functional blood-brain barrier requires interactions between endothelia and astrocytes but molecules involved in these contacts are not known.

Results
Absence of glial alpha-dystrobrevin protein causes leaky blood-brain barrier, water retention and progressive brain edema.

Conclusion
Glial alpha-dystrobrevin is essential for endothelium-astrocyte interactions required for blood-brain barrier functions.

Significance
Pathologies altering alpha-dystrobrevin might lead to blood-brain barrier abnormalities.

The blood-brain barrier (BBB) plays a key role in maintaining brain functionality. Although mammalian BBB is formed by endothelial cells, its function requires interactions between endotheliocytes and glia. To understand the molecular mechanisms involved in these interactions is currently a major challenge. We show here that α-dystrobrevin (α-DB), a protein contributing to dystrophin-associated protein (DAP) scaffolds in astrocytic endfeet is essential for the formation and functioning of BBB. Absence of α-DB in null brains resulted in abnormal brain capillary permeability, progressively escalating brain edema and damage of the neurovascular unit. Analyses in situ and in 2D and 3D in vitro models of BBB containing α-DB-null astrocytes demonstrated these abnormalities to be associated with loss of aquaporin-4 water and Kir4.1 potassium channels from glial endfeet, formation of intracellular vacuoles in α-DB-null astrocytes and defects of the astrocyte-endothelial interactions. These caused deregulation of tight-junction proteins in the endothelia. Importantly, α-DB but not dystrophins showed continuous expression throughout development in BBB models. Thus, α-DB emerges as a central organizer of DAP in glial endfeet and a rare example of a glial protein with a role in maintaining BBB function. Its abnormalities might therefore lead to BBB dysfunction.

The blood-brain barrier (BBB) has a crucial role in maintaining the specific microenvironment required for proper brain functioning. While BBB relies on the properties of brain endothelial cells (BEC) (1, 2), recent studies indicate that BEC cooperate with pericytes, neurones and astrocytes (3, 4) within functional “glio- and neurovascular units”. These interactions shape the unique barrier properties and then regulate the cerebral blood flow and barrier performance (5).

We now have a considerable knowledge of the molecular components of physical and metabolic barriers as well as the diverse transport systems controlling BBB permeability (1, 2). In contrast, very little is known about the specific interactions between the endothelium
and glia within gliovascular units. The astrocytic perivascular endfeet are closely apposed to the abluminal endothelial surface and are separated by but also cooperate in the formation and maintenance of the basement membrane (BM) composed of different extracellular matrix (ECM) proteins. Numerous observations indicate that both direct astrocyte interactions and astrocyte-derived factors are essential for the development and/or maintenance of BBB properties in BEC (2, 6, 7). However, our understanding of the exact molecules and mechanisms employed by astrocytes to influence BBB during ontogenic development and influencing its maturation and permeability is far less comprehensive.

Matrix adhesion receptors, particularly dystroglycan (a dystrophin-associated protein, DAP) and the integrin families (8) are required for a proper interplay between ECM and cells. DAPs anchored via dystrophins are differentially expressed in BEC, neurons and glia, show affinity for various ECM components (e.g., laminin, agrin) but also form heteromeric interactions with cell-surface proteins (e.g. neurexins). Such multi-functionality is particularly applicable to complex interactions, like those required at BBB. Indeed, absence of DAP in dystrophic mouse brains results in BBB perturbations (9, 10). We have previously demonstrated a highly specific sub-cellular distribution of α-dystrobrevin (α-DB), a dystrophin-related DAP member, in cells forming blood-tissue barriers and in glial endfeet (11). Developmental expression of this protein coincides with the induction of specific differentiation processes, including the functional maturation of BBB (12). The α-dystrobrevin and its homologue β-DB show differential localisation in the brain (13, 14). Dystrobrevins, by complex network of interactions with DAPs, e.g. dystrophins and syntrophins and with dysbindin, syncoilin, and beta-synemin (desmuslin) provide anchorage for a further set of proteins, including various cytoskeleton components, receptors and channels (15, 16). While dystrophin absence causes disruption of the entire DAP complex, we have shown here that it is α-DB in glial endfeet that is essential for the formation and function of BBB. Its absence caused leaky blood vessels and progressively developing brain edema in α-DB-null (ADB) brains coinciding with abnormalities in astrocyte-BEC assembly involving dysregulation of specific DAPs, Kir4.1 and AQP4 channels. Thus, α-dystrobrevin emerges as a very rare example of a structural glial protein crucial for endothelial BBB functioning.

**EXPERIMENTAL PROCEDURES**

**Animals**- Adult C57Bl/6 control, mdxβgeo (10) dystrophin null and alpha-dystrobrevin knockout (ADB) mice were used (17). Animals were maintained in a 12 hours light/dark cycle and fed normal diet and water ad libitum. All procedures were performed with permission of the local Animal Health and Welfare Committees and in accordance with the UK Home Office guidelines.

**Antibodies**- Alpha-dystrobrevin (α-DB) antibodies have been described previously (11, 12). Mouse anti-α-DB (clone 23; BD Biosciences), rabbit anti-α-DB-1 (α1-CTFP; gift from D.J. Blake), rabbit anti-α/β-dystrobrevin (gift from T.C. Petrucci), mouse anti-α-dystroglycan (clone VIA4-1; Millipore), mouse anti-β-dystroglycan (MANDAG2 ; DSHB), mouse anti-dystrophin (MANDRA1; DSHB), mouse anti-dystrophin (MANDRA1; DSHB), mouse anti-utrophin (MANCHO3; DSHB), mouse anti-syntrophin (clone 1351; Affinity BioReagents), rabbit anti-aquaporin-4 (AB2218, Millipore and AQP-004, Alomone), rabbit anti-Kir4.1 (Alomone), rabbit anti-laminin (Sigma), rabbit anti-glial fibrillary acidic protein (GFAP; Abcam), rabbit anti-occludin and rabbit anti-ZO-1 (Life Technologies) and rabbit anti-actin (Sigma-Aldrich) were also used. For permeability studies rabbit anti-fibrinogen (Calbiochem), rabbit anti-albumin (Dako) and Cy3-conjugated donkey anti-mouse IgG (Fab fragment; Jackson Immunoresearch) were used. DyLight595/Fluorescein-conjugated GS-IB4 isolectin (Vector Lab) were used as endothelial markers.

**Blood-brain barrier permeability study**- 3 month old mice were injected with 4% Evans blue in saline (0.1 mL/10 g body weight) either i.p. or anesthetized and injected i.c. Following dye
circulation period mice were perfused with phosphate buffer saline (PBS; pH 7.4) and 4% paraformaldehyde (PFA) and 15% saturated picric acid in 1x PBS and brains post-fixed at 4°C overnight using the same fixative. Brains from non-perfused mice were also analyzed, for comparison (18). 50-100 µm thick vibratome sections were cut (VT1000S Leica), fixed and either analysed for Evans blue fluorescence or stained for fibrinogen, albumin and IgG, as described below. Morphometric analyses of immunoreactive blood vessels were performed using Axiovision Rel. 4.6 software (Axio Imager, Carl Zeiss), as described (19,20). Random areas of confocal images were acquired and the numbers of immunoreactive blood vessels within the area counted. Statistical analysis was done with OriginPro 8.1 software (Northampton, USA) using one way analysis of variance (ANOVA) followed by Tukey or Games-Howell post-hoc test. All measurements were expressed as means ± SEM. p value < 0.05 were considered as significant.

Brain water content measurement. 3-months old mice were killed and their brains rapidly removed. Isolated hemispheres were weighed (wet mass) then dried in a vacuum oven (VT6025; Thermo Fisher Scientific) for 24 h at 80°C and -1,000 mbar and percent brain water content was calculated: (wet mass – dry mass) × 100/(wet mass) as described (21).

Cell co-culture in vitro models- Postnatal day 0-2 brains from ADB or wild-type mouse pups were isolated and meninges carefully removed (22). Cerebral cortices were dissected and dissociated in Dulbecco’s modified eagle medium (DMEM) containing 0.25% trypsin (Sigma-Aldrich) and 1 mg/mL DNase1 (Roche) for 1 hr with gentle agitation. Dissociated tissue was washed 3 times in the astroglia culture medium (DMEM containing 2mM L-glutamine, 10% FBS, 1 mM sodium pyruvate, 1% non-essential amino acids and 100 units/mL penicillin and 0.1 mg/mL streptomycin) at 37°C with 5% CO₂. Only bEnd3 cells from passages 31-35 were used for this study. All culture media were changed every 3 days. For laminin-induced clustering cells were treated for 24 h with 20 mM or 40 mM laminin-1, (L2020; Sigma-Aldrich) (28).

For 2D cultures ADB/wild-type cortical astrocytes were seeded into 25 cm² culture flasks or on coverslips and grown in the astroglia medium and in some cases with added 1 mM dBcAMP and 1% horse serum (29). After establishing a glial monolayer bed, 1 x 10⁵ bEnd3 cells were added and grown in the endothelial medium. BBB co-cultures were kept at 37°C with 5% CO₂ and analysed at 0 (+2 hr), 1, 2, 4, 6 and 8 days. Bright-field images were acquired using ColorView II digital camera mounted on the IX71 inverted microscope (Olympus). Morphometric measurements of the length and area of glial columns were taken using its integrated analySIS software (Olympus).

3D co-cultures were prepared as described (30,31,32), with few modifications. Briefly, ADB or wild-type cortical astrocytes and bEnd3 cells were pooled in DMEM to obtain a combined density of 1 x 10⁶ cells/mL (1:1 astrocyte:endothelial cells ratio). Cells were gently spun down (5 min at 1,000 rpm) to replace DMEM with 4 mg/mL Matrigel™ (BD Biosciences) in the endothelial culture medium. Droplets of the Matrigel™ dual-cell suspension were spotted into a 6-well plate and solidified for 15 min at 37°C. These droplets were overlaid with the endothelial culture medium and incubated at 37°C with 5% CO₂ for a week. Droplets were then detached, cryo-sectioned (cryostat CM3050 S, Leica) and immunostained as described below.

ECIS monitoring of blood-brain barrier formation. ECIS arrays (8W10E+; 8-well chambers, Applied Biophysics) were prepared
and used as described before (33). 2 x 10^5 primary astrocytes/cm² (isolated as described above) were seeded into each chamber and maintained for 7 days in astroglial culture medium until monolayer was established. For electrode coating with endogenous extracellular matrix (34), astrocytes were lysed sequentially in: sterile water (30 min at 37°C), 1% Nonidet-P40 (5 min at 37°C) followed by 1% Nonidet-P40 and 1% sodium deoxycholate in sterile water for 1 min at room temperature (RT). Subsequently, 2 x 10^7 bEnd3 cells/cm² were seeded into each chamber containing either live wild-type/ADB astrocytes (direct cell-cell contact) or their respective ECM and cultured in endothelial culture medium. Barrier integrity and cell resistance were monitored at 2,000 Hz frequency using ECIS® Zθ (Applied Biophysics) for 168 h at 37°C with 5% CO₂.

**Immunolocalisation and fluorescence confocal imaging** - Vibratome sections or cell cultures were post-fixed with 4% PFA in 1x PBS on ice for 30 min. Samples were blocked in 10% normal donkey serum (Biosera) in 1x PBS containing 0.5% Triton X-100 (PBST) for 3 hr and incubated overnight at 4°C with specific antibodies (see above). In some cases 10 µg/mL DyLight 594/Fluorescein-conjugated GS-IB4 isolecitin (Vector Laboratories) was used to visualise brain endothelial cells. Secondary antibody incubation was with 8 µg/mL Alexa 488-conjugated goat anti-rabbit IgG, 8 µg/mL Alexa 594-conjugated goat anti-rabbit IgG (Fab² fragment; Life Technologies), or 6 µg/mL Cy3-conjugated donkey anti-mouse IgG (monovalent Fab fragment; Jackson Immunoresearch). 5 µg/mL Hoechst 33342 (Life Technologies) was used for nuclear counterstaining. After each incubation step, samples were washed three times in PBST for 10 min. Samples were mounted in FluorPreserve™ anti-fading reagent (Calbiochem) and sections were examined with a confocal laser-scanning microscope (LSM710; Zeiss, Oberkochen, Germany) using either a Plan Apochromatic 63x DIC oil objective (NA1.4) or a Plan Apochromatic 100x DIC oil objective (NA1.46). Z-stacks were used for routine evaluation of the labelling. All images represent a single optical section. Images from WT and ADB tissue sections were acquired under identical microscope settings using sequential acquisition of the different channels to avoid cross-talk between fluorophores. The pinholes for each channel were adjusted to one airy unit.

All images were processed identically using the software Zen2009 Light Edition (Zeiss, Oberkochen, Germany). Only brightness and contrast were adjusted for the whole frame, and no part of a frame was enhanced or modified in any way. Images were exported into Adobe Photoshop for figure arrangement. In negative controls, primary antibodies were omitted while the rest of the procedure done exactly as described.

**Transmission electron microscopy** - Perfused brains were fixed in 4% paraformaldehyde and 15% saturated picric acid in 1x PBS at 4°C for 24h, postfixed in a mixture of 1% osmium tetroxide (OsO₄), 0.8% potassium ferricyanide K₄[Fe(CN)₆] and the material was processed for transmission electron microscopy using standard protocols and analyzed in JEM 1011 (JEOL).

**Histological staining** - Perfusion fixed brains (as described above) from 18 month old wild-type/ADB mice were processed into paraffin and sectioned (5 µm) using RM2235 microtome (Leica). Sections were deparaffinised, incubated in Haematoxylin QS (H-3404; Vector Laboratories) and then in alcoholic Eosin Y (Sigma-Aldrich) for 5 min each. Sections were differentiated, dehydrated in graded series of ethanol and mounted in DPX.

**Protein extraction, SDS-PAGE and Blue native analysis** - Brains or cells were homogenized in ice cold lysis buffer containing 0.15 M sodium chloride, 50 mM sodium fluoride, 10 mM HEPES (pH7.5), 5 mM EGTA, 5 mM EDTA, 1% Triton X-100 and protease inhibitors 2 mM sodium orthovanadate, 1 mM PMSF, 1 mM Pefabloc, 1 mM benzamidine, 10 µg/mL aprotinin, Complete™ Mini inhibitor cocktail tablet (Roche). After incubation on ice for 30 min on a shaking platform, the homogenates were centrifuged at 200 x g for 5 min at 4°C. Protein supernatants were collected and quantified using the Bicinchoninic acid kit (Sigma-Aldrich). SDS-PAGE: Solubilized proteins (30 µg) were mixed with Laemml sample buffer (Biorad) and 2.5 % β-
mercaptoethanol), heated to 95°C for 5 min, separated on 8-10% SDS-polyacrylamide gels and electroblotted onto Hybond-P PVDF membranes (GE Healthcare). Blue native analyses were performed according to (35). Brains were homogenized in ice-cold lysis buffer containing 0.32 M sucrose, 50 mM HEPES (pH7.5), 2 mM EDTA and protease inhibitors 2 mM sodium orthovanadate, 1 mM PMSF, 1 mM benzamidine, Complete™ Mini inhibitor cocktail tablet (Roche). After 1 h incubation on ice, the homogenates were centrifuged at 1,000 x g for 15 min at 4°C to remove the nuclear pellets. Supernatants were further centrifuged at 164,000 x g for 30 min at 4°C using ultracentrifuge (XL-90; Beckman Coulter) with Ti70.1 rotor and the crude membrane pellets were resuspended in the same lysis buffer but without sucrose. Cells were lysed in 1x NativePAGE sample buffer supplemented with 1% dodecyl-b-D-maltoside (DDM) (Life Technologies) for 15 min on ice. The lysates were centrifuged at 23,000 x g for 30 min at 4°C. Protein samples were quantified using the Bicinchoninic acid kit (Sigma-Aldrich).

Solubilized proteins (20 µg) were mixed with Coomassie G-250 (4:1), resolved on NativePAGE Novex 3-12% Bis-Tris gels (Life Technologies) alongside a molecular weight ladder NativeMARK (LC0725; Life Technologies) and electroblotted onto Hybond-P PVDF membranes (GE Healthcare). Immunoblotting- All blots were incubated with a blocking solution containing 5% non-fat milk powder in PBST (1x PBS/0.05% Tween 20) for 1 hr at room temperature and incubated with a specific antibody in the same blocking solution overnight at 4°C. Following 3 washes with PBST, membranes were incubated with appropriate hors eradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (1:5,000, Sigma-Aldrich) at room temperature for 1 hr and signal visualized using the UptiLight HS Chemiluminescence Substrate kit (Cheshire Sciences) and GBOX Chemi XT 16 gel imaging system (Syngene). For negative controls primary antibodies were omitted and the rest of the protocol performed as described above.

Statistical analysis- was done with OriginPro 8.1 software (Northampton, USA) using one way analysis of variance (ANOVA) followed by Tukey post-hoc test. All measurements were expressed as means ± SEM. p value < 0.001 was considered as highly significant.

RESULTS

Leaky BBB in α-dystrobrevin knockout brains. To investigate the impact of α-DB absence on BBB integrity and function, we compared numbers of blood vessels showing the Evans blue (EB) extravasation and blood proteins leakage into the brain tissues in wild-type (WT) and α-dystrobrevin null (ADB) mice. There were few blood vessels with detectable EB, fibrinogen, albumin or IgG staining around or outside the capillary lumen in the wild-type brains. In contrast, analysis of ADB brains showed abundant perivascular extravasations of both injected EB and blood-borne proteins into the brain. Mdxgeo brains used as a positive control (10) confirmed BBB permeability (Fig.1). Detailed morphometric analyses (19,20) of vessels leaky for fibrinogen and IgG showed a highly statistically significant (p<0.001) increase in numbers of such events in ADB brains (Fig.1D).

Abnormalities in endothelia-glia interactions caused by absence of α-dystrobrevin in astrocytes. To dissect the mechanism(s) responsible for the leaky BBB in the absence of α-DB, two- and three-dimensional co-culture systems were used. Such systems, involving interaction of just brain endothelial cells (BEC) and astrocytes from normal or ADB mice allow for direct comparisons of changes in molecular interplay between BEC and glia caused by the absence of α-DB. As described (36), BEC added to confluent cultures of astrocytes penetrated under the astrocytic monolayer and caused its rearrangement into glial islands interconnected by thick multicellular glial columns. With time, the size of the islands decreased while the columns elongated and the complexity of this network of columns increased (Fig. 2A). In the presence of wild-type astrocytes, such arrangements were highly reproducible and persisted for over two weeks in culture. However, when ADB glia was used, this glial
lattice framework was significantly less complex and the columns were thinner and fragile, with many discontinuities appearing (Fig. 2B). Surviving columns were extended and sustained by their larger than usual width (Fig. 2). Morphometric measurements confirmed that the BEC/ADB glia columns were indeed fewer than those formed by BEC/WT astrocytes. ADB columns were also significantly longer (albeit brittle) and thus areas in between lattices were larger (Fig. 2C). Electric cell-substrate impedance sensing was used to assess the functional impact of ADB glia in this BBB model. Both direct cell-cell interactions and ECM produced by astrocytes significantly increases impedance (34). While we found no alteration of the relative values in BEC cultured on ECMs produced by either the wild type or ADB glia (Fig. 2D), there was a dramatic difference in BEC co-cultured with WT vs. ADB astrocytes (i.e. with a direct cell-cell contact) (Fig. 2E).

Subsequently, BEC/astrocyte co-cultures were analysed using immunofluorescence with confocal microscopy. Z-stacks taken through 2D cultures at the points of interface between Kir4.1-positive glial columns and endothelial cells showed that ADB astrocytes contained large intracellular vacuoles scattered throughout the cytoplasm (Fig. 3). This abnormality was not present in wild-type cells. Vacuolation could be indicative of a defect in water and/or ion transport. Considering that AQP4 and Kir4.1 channels involved in water/potassium homeostasis are known to be anchored in the cell membrane via specific interactions with DAP (16), we have studied these two proteins. Fluorescence signals for both AQP4 and Kir4.1 in ADB astrocytes were more diffuse and lost from cell membranes (Fig. 3B), which coincided with a lack of co-localisation with DAP complexes. Indeed, the lack of co-localisation of beta dystroglycan (used as a membrane-associated marker of DAP) and its extracellular ligand – laminin revealed disruption of the very DAP complexes in ADB astrocytes (Fig. 3C&D). We have then analysed capillary formation in three dimensional co-culture systems (Fig. 4). In this well-established model, BEC and wild-type astrocytes formed well delineated tubular structures with astrocyte contact points being immunopositive for DAP. This regular arrangement was largely lost when ADB glia was used, with cells growing in large, dense clusters (Fig.4).

Alterations of both glial and endothelial proteins in the absence of α-dystrobrevin. To understand the molecular mechanisms underlying these morphological and functional abnormalities occurring in the absence of α-DB, we have analysed changes in expression of specific proteins in developing and fully established co-cultures (Fig. 5). First, to unequivocally identify cells producing specific proteins we compared samples from astrocytes and endotheliocytes cultured separately (Fig. 5A). Western blotting of proteins from wild type astrocytes confirmed significant expression of dystrophin Dp71 but only traces of utrophin (not shown) were found. There was a significant expression of DAP proteins: dystrobrevins (predominantly α-DB), α- and β-dystroglycans, lower levels of syntrophins, laminin, Kir4.1 and traces of AQP4. Moreover, astrocytes expressed ZO-1 but no tight junction protein occludin. ADB astrocytes expressed no α-DB but had significantly higher levels of β-DB (Fig. 5A). As expected, BEC cultures expressed tight junction markers ZO-1 and occludin, significant amounts of laminin and low levels of DAPs such as β-DB, dystroglycans and syntrophins. Moreover, BEC expressed low levels of Kir4.1 but no α-DB, Dp71 or AQP4 (Fig. 5).

Next, protein samples were taken from co-cultures at time 0 (2 hrs post-addition of BEC to astrocytes) and 1, 2, 4, 6 and 8 days in co-culture. Western blot analyses showed that there were very significant differences in both temporal expression patterns and in levels of expression of specific DAP-associated proteins as well as endothelial BBB markers between wild-type and ADB co-cultures. Specifically, glial Kir4.1 potassium channel and endothelial occludin were expressed from the earliest stages in wild-type cultures but in ADB the onset of their expressions was delayed by 2 to 4 days. Expression of AQP4 was also delayed, first appearing at day 6 in wild-type but at day 8 in ADB samples (Fig. 5A). Moreover,
expression levels of these three proteins as well as of laminin were significantly reduced in ADB co-cultures at all time-points analysed (Fig. 5A). Interestingly, time course analysis showed that expression of the Dp71 dystrophin isoform decreased gradually with days in culture, with a dramatic drop between days 4 and 6 and disappearance at day 8 of culture in both wild-type and ADB samples (Fig. 5A). Nevertheless, Dp71 levels were reduced at all time-points in ADB co-cultures. As expected, expression patterns of specific dystrophin-associated proteins were also altered, with over-expression of β-DB in the absence of its α-DB and lower levels of α- and β-dystroglycans (Fig. 5A). Moreover, AQP4 multimerizes into orthogonal arrays of particles (OAP), a supramolecular assembly which is important for polarization of AQP4 subunits and water movement through pores (37). BN-PAGE analyses (Fig. 5B) revealed that ADB astrocytes failed to form OAP. As expected, in wild-type cell cultures, these assemblies were formed both in co-cultures with BEC and following pre-treatment with laminin (28).

Absence of α-dystrobrevin results in alterations of AQP4 and Kir4.1 and water retention in brains in situ. Considering the altered expressions of AQP4 and Kir4.1 in ADB astrocytes, we examined distributions of these proteins in brains in situ. Immunofluorescent confocal analyses of cellular localisations of these proteins revealed very specific alterations (Fig. 6A & B). In normal brains, AQP4 and Kir4.1 were clearly located to the astrocytic endfeet, in close contact with blood vessel endothelia. However, in α-DB deficient brains both were dislodged from endfeet and instead, increased diffused signal was found in astrocyte cell bodies (Fig. 6A & B). Western blotting showed that these differences were caused by mislocalization of AQP4 and Kir4.1 rather than changes in expression as there was no significant decrease of protein levels in ADB brains (Fig. 6C). It was also the case for syntrophin – the DAP protein tethering AQP4 and Kir4.1 to dystrobrevin (Fig. 6C). Moreover, this dislocation of water/ion channels coincided with significantly increased baseline water content in ADB mice hemispheres than in WT controls (p<0.005) despite the total brain mass being higher in WT brains (Fig. 6D).

Glial vacuolation, progressive brain edema and neurovascular unit damage in the absence of α-DB. Taking into account the AQP4 and Kir4.1 abnormalities, increased permeability of ADB capillaries and brain water retention we have examined young and old age-matched brains morphologically. While analysis of H&E stained sections of 3 month old brains (used in all previous experiments) confirmed no discernible differences, electron microscopy revealed some glial endfeet vacuolation in ADB samples. Furthermore, H&E and EM in aged (18 month old) ADB samples revealed spongiform degeneration and glial endfeet edema. Tissue spongiosis was severe, particularly in the Ammon’s horn the dentate gyrus and in the cerebellum, affecting brain parenchyma while neurons being preserved (Fig. 7A). In the EM, tissue edema was not restricted to astrocyte endfeet but extending beyond those, destroying neighboring neuropil and distorting the capillary lumen (Fig. 7B). Endothelial cell showed signs of damage with numerous pinocytotic vesicles in the cytoplasm, microvilli on the luminal surface and tight junction abnormalities. The latter could be linked to thickened basement membranes and apparent hyperplasia (multilayered endothelium) and therefore decreased vessel lumens. Neurons and pericytes of ADB brains also showed signs of damage and thus α-DB deficiency affects the entire neurovascular unit. Importantly, analysis of 18 month old dystrophic (mdx<sup>geo</sup>) brains revealed similar abnormalities (manuscript in preparation). In contrast, WT ultrastructure, albeit showing features typical for aged brains, was otherwise normal.

DISCUSSION

While BBB relies on the properties of brain endothelial cells, growing data indicate that glia and pericytes of the neurovascular unit are essential for BBB function in BEC. These interactions involve secreted factors (38) and extracellular matrix and cell-cell interactions. Dystrophin absence results in leaky BBB (this work Fig. 1 and Ref. 9). However, the mechanism of this abnormality is unclear,
especially as dystrophin is not expressed in BEC forming the BBB. This indicates an indirect effect of the dystrophin absence and points to abnormality occurring specifically in astrocytic glial endfeet: Astrocytes, unlike brain BEC and pericytes, contain a specific set of dystrophin-associated proteins. The results of this study show that one member of glial DAP; α-dystrobrevin is vital for proper BBB formation and function. Its absence in ADB knockout mice, even in the presence of dystrophins, causes profound blood-brain barrier abnormality. This is an important result as it uncovers one of the still poorly understood molecular mechanisms functioning at the cell-cell interface between endothelia and glia. In this context, α-dystrobrevin emerges as a rare example of a glial protein with a specific role in maintaining BBB function. Bearing in mind that α-DB is an entirely intracellular protein, the mechanisms that could explain its influence over endothelial cells must include indirect interactions. Dystrobrevin links dystrophin/utrophin, dystroglycan and the syntrophin families of proteins. This interaction is reciprocal as proper localization of α-dystrobrevin requires α-syntrophin (39,40). Resulting DAP scaffolds interact with ECM components (e.g. laminin) and serve to anchor a further set of proteins, including receptors and channels (41,42). We have shown that α-DB absence produced rearrangements of DAP components resulting in a secondary decrease or loss of their interacting proteins. Morphological disarrangements that we observed in ADB astrocyte-BEC co-cultures could be caused by abnormalities in cell attachments due to decreased levels of laminin and dystroglycan, in a mechanism analogous to dystrophic muscle damage. However, as ADB astrocytes in vitro and in vivo contained large intracellular vacuoles, this could indicate defects in water and/or ion transport. Therefore, a different mechanism could be envisaged: laminin, dystroglycan and syntrophin have a role in Kir4.1 potassium and AQP4 water channel aggregation and localisation at glial endfeet (42,43) and these two proteins are important for ion and water transport (see below). Our data advance our understanding of these astrocyte specialisations by demonstrating that α-DB is a key scaffolding protein for AQP4 and Kir4.1 at glial endfeet. Consequently, morphological malformations in co-cultures in vitro, increased BBB permeability and progressive brain edema in ADB brains in vivo could result from abnormalities of water and ion homeostasis in astrocytes and at the glia-BEC interface. Astrocytic perivascular endfeet cover the vessel wall completely (45) and thus must play a part in the exchange of water and solutes between blood and brain. The presence of AQP4 and Kir4.1 at glial endfeet is of vital importance for ion and water homeostasis. Active neurones release K+ and water. K+ is taken up by glial processes surrounding neurones and then released at the distant perivascular endfeet via the potassium spatial buffer mechanism (2). The net ion gain results in osmotic water uptake and the colocalisation of AQP4 water channels in endfeet is essential for redistribution of excess water (46,47). Disruptions of glial endfeet assembly impair these functions and could lead to osmotic opening of tight junctions in response to changes in cell volume (48). If that were the case, any disruption to the DAP complex should produce alterations of BBB functions. Indeed, mdx dystrophic mice, α-syntrophin and, as shown here, α-dystrobrevin-null mice all show such abnormalities (10,49,50).

The blood-brain barrier in the higher vertebrates is formed by endothelial cells. However, in lower organisms (invertebrates up to the elasmobranch fish) it is created by glia. Evidently, during evolution the BBB shifted from the glial to endothelial compartment (51). It is, therefore not surprising that intricate interactions of BEC with glia influence the BBB properties in mammals (2,7,52). Moreover, there is increasing evidence that dysfunctions at the abluminal surface of BEC in contact with astrocytes (1,2) and endfeet abnormalities (53) may contribute to BBB damage in several neuropathologies. Specifically, redistribution of AQP4 in perivascular endfeet coincides with increased BBB permeability in glioblastomas or with brain edema (54). However, we only begin to understand the exact molecular mechanisms functioning at the interface between endothelial and glial cells. Our data provide a new insight into these interactions and introduce α-
dystrobrevin and the dystrophin-associated protein complex as important players in this process. It needs to be confirmed whether these abnormalities occur in human brains. As dystrophinopathies lead to premature death of young adults due to muscle failure, no data on aged dystrophic brains exist. However, the impact of α-DB mutations might be greater in humans than what has been observed here in mice. Due to lineage-specific mutations in the murids (55), the mouse brain has fewer than half of the isoforms found in the human brain (56). The human α-DB gene encodes three distinct syntrophin-binding sites, resulting in a greatly enhanced functional repertoire. Therefore, α-DB absence in humans might be significantly more damaging.

REFERENCES


**FOOTNOTES**

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**FIGURE LEGENDS**

**Fig.1.** Leaky BBB in α-dystrobrevin knockout (ADB) brains. (A) Macroscopic views of wild-type, ADB and Mdxβgeo (positive control) brains showing Evans blue extravasation in specific brain areas. (B) Confocal micrographs showing Evans blue extravasations (*yellow fluorescence*) into the parenchyma of ADB and Mdxβgeo brains. (C) Confocal micrographs of blood vessels in the wild-type and ADB brain cortices stained for fibrinogen (top pair), IgG (middle) and albumin (bottom). Immunoreactivity for these blood components (red) is present outside the GS-IB4-labelled (*green*) blood vessels. (D) Quantitative evaluation of fibrinogen and IgG immunoreactivities expressed as number of positive blood vessels per area (mm²) of cortex showed highly statistically significant differences (*p< 0.001*) between wild-type and ADB brains. Error bars represent ±SEM.

**Fig.2.** Structural and functional abnormalities in endothelia - ADB astrocyte co-cultures. (A) Phase-contrast images of morphological developments in BEC-astrocyte co-cultures: (1) Confluent monolayer of wild-type primary astrocytes (▲) is penetrated by BEC added into the culture (2). BEC presence (▲) triggers rearrangement of glia into islands (2, arrows denote astrocytic island margins) interconnected by thick multicellular glial columns. The size of the islands decreases with time in culture (3), while the columns elongate and the complexity of their network increases. (B) Comparison of phase-contrast images of BEC co-cultured with wild type or ADB astrocytes. Control astrocytes (a, b) formed typical arrangements, which were highly reproducible and stable. The ADB glia lattice framework was significantly less complex and fragile (c, d) as shown by statistically significant differences (p<0.01) in morphometric analyses (C) where ADB columns were longer and the surface areas in between lattices larger. (D) Normalised ECIS plot (% relative resistance *versus* time) showing endothelial resistance development in BEC cultured on wild-type or ADB astrocyte-derived ECMs (i.e. no live cell contact). (E) Relative resistance in BEC co-cultured with either wild-type or ADB astrocytes (live cell-cell contact). Note the significant divergence in ECIS values between cultures containing the two astrocyte types but no difference when glia-derived ECM were used.

**Fig.3.** Alterations at glial endfeet in the absence of α-dystrobrevin
Representative confocal immunofluorescence images taken under identical settings through glia in BEC/astrocyte co-cultures. (A,B) In ADB co-cultures, analyses revealed vacuolized spaces (arrowheads) near the cell attachment points on the basal surface. Staining for Kir4.1 (A, green) and AQP4 (B, green) revealed significantly decreased expression levels and disruption of normal co-localisation of these proteins with β-dystroglycan (β-DG, red) of DAP complexes: Note the diffuse distribution and loss of co-localisation (yellow signal, arrows) in ADB samples (A,B). This coincided with disruption of the DAP assembly itself in the absence of α-DB (C) as shown here by the lack of signal co-localisation for β-DG and its extracellular ligand, laminin (D).

**Fig.4.** Malformation of 3D assemblies of BEC co-cultured with ADB glia.
Wild-type astrocytes induced the formation of well delineated tubular endothelial structures clearly evident at Day 4. Confocal microscopy analyses at Day 6 confirmed that GFAP-labelled wild-type astrocytes were in contact with the GS-IB4-labelled endothelial tubes while ADB astrocytes did not support the endothelial tubulogenesis. Here, remnants of cyst-like structures that initiated the early
branching of endothelial tubes were still fairly intact at Day 4 and 6. Although contacts between ADB astrocytes and endothelial tube-like structures were made, the latter appeared unusually thick. Left-side panels. Phase-contrast images of 3D cultures of glia with BEC. WT glia: Note fine, dense connections with intricate vessel-like structures at day 4 in vitro (DIV 4), becoming more pronounced and networked by day 6. ADB glia: fewer, broader and discontinuous structures and cysts (arrows) - a sign of early vessel development are evident. By day 6 some cyst remained and vessel assemblies broadened. Right-hand side panels. Representative confocal images in three-dimensional co-cultures: BEC and wild type astrocytes formed well-defined tubular structures, with astrocytes (positive for GFAP, green) arranged in a continuous sheath along and around the endothelial vessel-like assemblies (stained with GS-IB4, red). In the presence of ADB glia, these orderly arrangements were lost and cells have grown in large, dense clusters (arrows).

Fig.5. Abnormalities in expression and assembly patterns of glial and endothelial proteins in ADB co-cultures. (A) Representative immunoblots of protein extracts are shown. (Solo cultures): BEC cultured alone expressed ZO-1, occludin (OCLN), low levels of β-DB, dystroglycans (DG), syntrophins (SYN) and laminin (LAM). In astrocytes alone dystrobrevins, dystroglycans, syntrophins, dystrophin Dp71 and ZO-1 were found. Molecular mass (kDa) of each protein is indicated. Actin was used as a control for equal protein loading. Immunoblots of protein extracts from co-cultures of BEC with wild-type (WT) or ADB astrocytes over 8 days in culture show dystrophin and dystrophin-associated proteins (DAP), DAP-interacting proteins and BBB markers. There were significant delays in temporal expression patterns and in levels of expression of AQP4, laminin (LAM) and Kir4.1 in ADB co-cultures. Expression of dystrophin Dp71 was lower in ADB and the time course analysis showed gradual decrease of Dp71 levels from day 4 onwards in both control and ADB samples. Expression of BBB marker occludin (OCLN) was detectable from day 2 in wild-type cultures but in ADB samples it was noticeable from day 6 only. (B) BN-PAGE analysis of orthogonal arrays of particles (OAP) formation. AQP4 immunoblots following BN-PAGE of cell lysates of wild-type and ADB astrocytes either pre-treated with increasing concentrations of laminin-1 (20 nM and 40 nM) or co-cultured with BEC. Under both conditions ADB astrocytes failed to form OAP, in clear contrast to the wild-type cells.

Fig.6. Abnormal localisation of Kir4.1 and AQP4 in ADB brains. Representative micrographs of immunolocalisations for Kir4.1 (A) and AQP4 (B) in mouse brains. (A1) In the small magnification view of hippocampus, AQP4 staining (green) delineates capillaries in the wild-type but not in ADB brains. Confocal co-localisations of AQP4 (A2, green) and Kir4.1 (B, green) with GS-IB4-labelled endothelia (A2 and B, red) show AQP4 and Kir4.1 signals located in close proximity to endothelia (yellow, arrows) in WT but not ADB brains. (inset) Larger magnification image confirms that this corresponds with the loss of signals from ADB astrocytic endfeet (arrows). Hoechst (blue/white) was used as a nuclear counterstain, where indicated. (C) Representative immunoblots of brain protein extracts demonstrate the relative levels of AQP4, Kir4.1 syntrophin and β-DB in the absence of α-DB. Actin was used as a control for equal protein loading. (D) Total brain mass and baseline water content analyses showed significant differences (p<0.005 and p<0.0005) between age-matched 3-month old WT and ADB brains.

Fig.7. Absence of α-DB causes spongiform neurodegeneration and progressive brain edema. (A) H&E staining of hippocampal areas of 18 month old wild-type (WT) and ADB mice showing massive spongiosis evident in the parenchyma of the pyramidal cell layer, particularly regions CA2 and CA3 and in the granule cell layer of the dentate gyrus in ADB brains. (B) Electron-microscopic images of brain cortex of WT (a) and ADB (b) mice. (a) Regular capillary vessel (V) with pericytes (P) and endothelium (E) characterized by necrotic features in some cells (en), typically associated with ageing. (b) Capillary vessel (V) in ADB cortex surrounded by vast astrocytic edema (*). Endothelial (E) cell cytoplasm is rich in pinocytotic vesicles. Arrow points to an improper tight junction between endothelial cells.
Figure 1
Figure 2
Figure 3
Figure 4
**A**

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**DAP**

- α-DB-1
- β-DB
- α-DG
- β-DG
- Dp71
- SYN

**DAP-interacting proteins**

- AQP4
- Kir4.1
- LAM

**Tight junction proteins**

- OCLN
- ZO-1
- Actin

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**B**

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Lam-1 (nM) BEC (BEC) Glial cells

**C**

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co-cultured BEC Glial cells

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Figure 5
Figure 7
Absence of glial alpha-dystrobrevin causes abnormalities of the blood-brain barrier and progressive brain edema
Chun-Fu Lien, Sarajo Kumar Mohanta, Malgorzata Frontczak-Baniewicz, Jerome Swinny, Barbara Zablocka and Dariusz C. Gorecki

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