AAV-mediated gene therapy in Dystrophin-Dp71 deficient mouse leads to blood-retinal barrier restoration and oedema reabsorption

Ophélie Vacca1,2,* Hugo Charles-Messance1, Brahim El Mathari1, Abdoulaye Sene3, Peggy Barbe1, Stéphane Fouquet1, Jorge Aragón1,4, Marie Darche1, Audrey Giocanti-Aurégan1,5, Michel Paques1,6, José-Alain Sahel1,6, Ramin Tadayoni1,7, Cecilia Montañez4, Deniz Dalkara1 and Alvaro Rendon1

1Sorbonne Universités, UPMC Univ Paris 06, INSERM, CNRS, Institut de la Vision, 75012 Paris, France, 2Neuroscience Paris-Saclay Institute (NeuroPSI)–CNRS UMR 9197–Université Paris-Sud, Cognition & Behavior, 91405 Orsay cedex, France, 3Department of Ophthalmology, Therapeutic, Saint Louis, MO 63103, USA, 4Department of Genetics & Molecular Biology, CINVESTAV: Research Centre for Advanced Studies, IPN, C.P. 07360 México, 5Ophthalmology Department, Avicenne Hospital, 93000 Bobigny, France, 6CHNO des Quinze-Vingts, DHU Sight Restore, INSERM-DHOS CIC, 75012 Paris, France and 7Ophthalmology Dept, Hôpital Lariboisière, AP-HP, Univ Paris Diderot, 75010 Paris, France

*To whom correspondence should be addressed at: Email: ophelie.vacca@gmail.com

Abstract

Dystrophin-Dp71 being a key membrane cytoskeletal protein, expressed mainly in Müller cells that provide a mechanical link at the Müller cell membrane by direct binding to actin and a transmembrane protein complex. Its absence has been related to blood-retinal barrier (BRB) permeability through delocalization and down-regulation of the AQP4 and Kir4.1 channels (1). We have previously shown that the adeno-associated virus (AAV) variant, ShH10, transduces Müller cells in the Dp71-null mouse retina efficiently and specifically (2,3). Here, we use ShH10 to restore Dp71 expression in Müller cells of Dp71 deficient mouse to study molecular and functional effects of this restoration in an adult mouse displaying retinal permeability. We show that strong and specific expression of exogenous Dp71 in Müller cells of Dp71 deficient mouse leads to correct localization of Dp71 protein restoring all protein interactions in order to re-establish a proper functional BRB and retina homeostasis thus preventing retina from oedema. This study is the basis for the development of new therapeutic strategies in dealing with diseases with BRB breakdown and macular oedema such as diabetic retinopathy (DR).
Introduction

Dp71 belongs to the dystrophin superfamily, and is a short product of the DMD gene, located on the X chromosome, involved in Duchenne muscular dystrophy (DMD) (4). Dp71—a membrane-bound cytoskeletal protein (5)—plays an important role in glial and vascular functions in the retina. It forms, with other proteins called dystrophin associated proteins (DAPs), a dystrophin glycoprotein complex (DGC) establishing a molecular link between the cytoskeleton and the surrounding extracellular matrix. It contributes to anchor ion/water channels and receptors in specific membrane fractions. DAPs include dystroglycan, syntrophin, dystrobrevin, and sarcoglycan. These are found in association either with Dp71 or utrophin—dystrophin autosomal homolog—(6) and play both structural and intracellular signal transduction roles. Dp71, absent from skeletal muscle, is predominant in the central nervous system (CNS). In the retina, all products of the DMD gene are expressed through the activation of internal promoters for each dystrophin. Dp71 is mainly localized in Müller cells and more specifically in their inner endfeet, around retinal vessels, closely related to the blood retinal barrier (BRB) (6, 7).

Tight junctions located between endothelial cells of retinal blood vessels, pericytes and Müller glial cell endfeet, form the BRB. Indeed, Müller cells participate in the maintenance and functioning of the BRB (8–10). Previous work on mice has shown that the deletion of Dp71 causes a BRB breakdown suggesting a crucial role of Dp71 in BRB integrity (1). Indeed, genetic inactivation of Dp71 (11) alters β-dystroglycan, potassium and water channels’ (Kir4.1 and AQP4) distribution along Müller glial cells that results in a disorganization of water transport through Müller cells (1). Thus, Dp71 associated with β-dystroglycan is required for proper clustering and precise membrane localization of Kir4.1 and AQP4 channels (12, 13). From now on, we will call Dp71, β-dystroglycan, Kir4.1 and AQP4 of Müller glial cell endfeet, the Dp71-dependent protein complex.

Vascular leakage and impaired fluid absorption from the retinal tissue across the glio-vascular interface are major pathogenic events of BRB breakdown (14). Both pathological mechanisms are stimulated by the absence of Dp71. Nevertheless, the Dp71-null retina is not naturally oedematous since we did not measure any increase in the retinal thickness of this mouse model (2). In the clinic, BRB breakdown of retinal detachment is a major cause of vision loss. Moreover, BRB breakdown in macular oedema is an important complication occurring in uveitis, diabetic retinopathy (DR), and macular telangiectasia (MacTel2) (15). The BRB breakdown is a symptom, which is the core of many important eye pathologies. In previous studies, we have shown in both retinal detachment (1) and Irvine Gass syndrome mouse model that BRB breakdown leads to Dp71 down-regulation (L.Siqueiros Marquez and A.Giocanti-Auregan, manuscript in preparation). Thus, the absence of Dp71 causes BRB breakdown and conversely BRB breakdown induces Dp71 down-regulation making of Dp71-null mouse, a good animal model to study BRB permeability and of the Dp71 protein, an ideal therapeutic target to elaborate novel strategies to improve BRB integrity. We recently tested an AAV capsid variant, ShH10 that has become the most promising ocular gene delivery vehicle over the past 15 years. Its low immunogenicity, ability to infect the majority of retinal cells, and long-term transgene expression make the virus a safe and very efficient gene delivery vector. We recently tested an AAV capsid variant, ShH10 that efficiently and specifically transduces Müller glial cells from the vitreous of the Dp71-null mouse (2, 16). Here, we employed this variant to restore Dp71 in Müller glial cells of Dp71 deficient mice. This virus was intravitreally injected to 8-weeks-old Dp71-null mice. GFP fluorescence was observed by fundus images and retinal thickness was measured by optical coherence tomography (SD-OCT).

We found that Dp71 expression leads to proper localization of this protein in Müller glial cell endfeet and consecutively leads to the up-regulation of several genes coding for transmembrane and ECM proteins related to Dp71. Furthermore, proteins of the Dp71-dependent complex re-localized to their proper compartments after treatment. Importantly, restoration of Dp71 leads to the total reabsorption of oedema caused by an intravitreal saline injection of Dp71-null mice as well as restoration of normal BRB permeability. Our data strongly suggest that Dp71 restoration is able to alleviate all symptoms related to the absence of the Dp71 protein despite the fact that intervention was made after development. These findings open promising avenues in the development of gene based therapies in diseases with permeable BRB where Dp71 is down-regulated.

Results

ShH10 delivers Dp71 to Müller glial cells

To restore Dp71 expression, we cloned the complete murine Dp71 sequence from exon 63 to exon 79 (GenBank: JN900253.1) without splicing and GFP under the control of a ubiquitous CBA promoter. To obtain a bicistronic expression of both genes, we inserted the viral 2A peptide between coding sequences, on GFP C-terminal and Dp71 N-terminal (17). Thus, we produced a AAV with ShH10 capsid and GFP-2A-Dp71 transgene (referred to: ShH10-GFP-2A-Dp71), since ShH10 targets specifically and efficiently Müller glial cells in Dp71-null mice (2, 13).

One microliter stock containing 1.8 × 1010 particles of ShH10-GFP-2A-Dp71 was injected intravitreally to 8-weeks-old Dp71-null mice. Control eyes received vehicle (PBS) or ShH10-GFP injection. Fundus imaging showed GFP expression across transduced retinas (Fig. 1A–D). We obtained a similar transduction pattern and the same GFP intensity using ShH10-GFP-2A-Dp71 or ShH10-GFP (Fig. 1E and F). Both viruses had the same intense transduction pattern only targeting Müller glial cells (Fig. 1E and F).

Quantitative RT-PCR and protein analysis of Dp71-treated eyes

Two months after ShH10-GFP-2A-Dp71 injection, RNA and protein were extracted from treated retinas by Trizol method. In parallel, we performed qRT-PCR and Western Blot analysis to evaluate expression levels of Dp71-dependent complex, utrophin (dystrophin homolog, present in Müller glial cell endfeet) and other ECM genes coding for proteins located in Müller glial cell endfeet binding Dp71-dependent protein complex (Tables 1 and 2).

We observed that Dp71 mRNA and protein were significantly over-expressed in the Dp71-null retinas in respect to wild-type Dp71 levels (Figs 2, 3A and C). Thus, ShH10-GFP-2A-Dp71 restores Dp71 expression in Müller glial cells.
It is known that Dp71 is associated with β-dystroglycan, AQP4 and Kir4.1 clustering in Müller glial cell endfeet. This Dp71-dependent protein complex is linked to the inner limiting membrane by the interaction of laminin and agrin with α-dystroglycan (13,18). Therefore, we analysed the expression levels of all these mRNA, and we found that AQP4, Kir4.1, laminin and agrin mRNA in ShH10-GFP-2A-Dp71-treated retinas were significantly higher compared to both wild-type and Dp71-null retinas (Fig. 2). At the protein level, we observed that β-dystroglycan was also over-expressed in ShH10-GFP-2A-Dp71-treated retinas (Fig. 3B and D). Full-length utrophin messenger, dystrophin autosomal homolog expressed in Müller cell endfeet, was slightly up-regulated. These results suggested that Dp71 over-expression induced the stimulation of a Dp71-dependent transcriptome (Fig. 2).

It is known that Müller glial cells become activated in response to retinal damage or cellular defects. This activation is characterized by the induction of glial fibrillary acidic protein (GFAP). To verify if AAV injection or Dp71 over-expression induce Müller cell gliosis, we quantified GFAP mRNA levels. We found that the amount of GFAP messenger did not increase in treated retinas versus controls (Fig. 2).

Relocalization of Dp71-dependent protein complex

In wild-type Müller glial cells, we have already established that Kir4.1, AQP4 and β-dystroglycan co-clustered in the endfeet of these cells. In the absence of Dp71, these three proteins are distributed along Müller glial cells (13). Here, we investigated the localization of these proteins after Dp71 restoration. We isolated Müller glial cells and immunostained with the following antibodies: anti-glutamine synthetase or anti-GFAP to identify Müller glial cells, H4 (pan-specific antibody) to localize exogenous Dp71, anti-β-dystroglycan, anti-AQP4 and anti-Kir4.1. We observed that the restoration of Dp71 rescued the localization at the Müller glial cell endfeet of Dp71, β-dystroglycan, AQP4 and Kir4.1 (Fig. 4A). We also performed retinal cryosections of Dp71-null-treated mice and we confirmed the relocalization of Dp71, β-dystroglycan, AQP4 and Kir4.1 at the Müller glial cell endfeet and around vessels (19)(Fig. 4B and Supplementary Material, Fig. S2). Thus, Dp71 expression through ShH10 allowed relocalization of the Dp71-dependent protein complex at the inner limiting membrane and at the glio-vascular interface forming the BRB of Dp71-null mouse retina, which was the first step prior to rescue Müller glial cell functions. Moreover, we remarked a morphological difference between Dp71-null and Dp71-restored MGC endfeet. Whereas Dp71-null MGC endfeet looked looser

<table>
<thead>
<tr>
<th>Protein</th>
<th>Species</th>
<th>References</th>
<th>Immunostaining</th>
<th>Western Blot</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamin Synthetase</td>
<td>mouse</td>
<td>MAB302</td>
<td>1:2000</td>
<td>–</td>
<td>Millipore</td>
</tr>
<tr>
<td>GFAP</td>
<td>rabbit</td>
<td>Z0334</td>
<td>1:1000</td>
<td>–</td>
<td>Dako</td>
</tr>
<tr>
<td>Dystrophins (H4)</td>
<td>rabbit</td>
<td>–</td>
<td>1:1000</td>
<td>1:5000</td>
<td>Homemade by D.Mornet</td>
</tr>
<tr>
<td>Dystrophins</td>
<td>mouse</td>
<td>DYS2-CE</td>
<td>1:20</td>
<td>1:10</td>
<td>Novocastra</td>
</tr>
<tr>
<td>AQP4</td>
<td>rabbit</td>
<td>AQP-004</td>
<td>1:500</td>
<td>1:100</td>
<td>Alomone</td>
</tr>
<tr>
<td>Kir4.1</td>
<td>rabbit</td>
<td>APC-035</td>
<td>1:200</td>
<td>1:500</td>
<td>Alomone</td>
</tr>
<tr>
<td>β-dystroglycan</td>
<td>mouse</td>
<td>B-DG-CE</td>
<td>1:200</td>
<td>1:100</td>
<td>Novocastra</td>
</tr>
<tr>
<td>Utrophin (K7)</td>
<td>rabbit</td>
<td>–</td>
<td>–</td>
<td>1:500</td>
<td>Homemade by D.Mornet</td>
</tr>
<tr>
<td>Utrophin (DRP2)</td>
<td>mouse</td>
<td>DRP2CE</td>
<td>–</td>
<td>–</td>
<td>Novocastra</td>
</tr>
<tr>
<td>GFP</td>
<td>chicken</td>
<td>ab13970</td>
<td>1:500</td>
<td>–</td>
<td>Abcam</td>
</tr>
<tr>
<td>β-Actin</td>
<td>mouse</td>
<td>A2228</td>
<td>–</td>
<td>1:7500</td>
<td>Sigma</td>
</tr>
</tbody>
</table>
Figure 2. Dp71 mRNA over-expression leads to over-expression of mRNAs associated with Dp71. Quantitative RT-PCR showed an over-expression of Dp71, dystroglycans, AQP4, Kir4.1, agrin and laminins mRNAs in the treated Dp71-null mouse in comparison with the wild-type. Quantitative RT-PCR showed that the utrophin mRNA is slightly up-regulated while GFAP didn’t show any variation. Bars represent means ± SEM for triplicate data points; n = 7. *P < 0.05, **P < 0.01 significant differences versus wild-type; †P < 0.01 significant differences versus wild-type; ‡P < 0.05, ‡‡P < 0.01 significant differences versus Dp71-null (Mann-Whitney test).

Table 2. Primer sequences for quantitative RT-PCR

<table>
<thead>
<tr>
<th>Name</th>
<th>Sense</th>
<th>Antisense</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dp71</td>
<td>ATGAGGGAACCCCTCAAAGGCCACG</td>
<td>TCTGGAGCTTCTGAGCTTC</td>
</tr>
<tr>
<td>Dystroglycan</td>
<td>CTGGAGGTCATCGACT</td>
<td>GCGCAATGAACTGGTGAAATGC</td>
</tr>
<tr>
<td>AQP4</td>
<td>CTTCCTGAAAGGGCAATCTCAG</td>
<td>GCCACCGAGCAGAAAAACAAAGAT</td>
</tr>
<tr>
<td>Laminin alpha 1</td>
<td>CAGGAACCATGCTACCTGT</td>
<td>GAATCTGAACTGTACGGTCAGCA</td>
</tr>
<tr>
<td>Laminin gamma 3</td>
<td>CGGACCCCTGTCATCAAATA</td>
<td>AAGCAAGGTCTGCTCTCAAAGC</td>
</tr>
<tr>
<td>Kir4.1</td>
<td>CCCTTTTTTATCCAGACG</td>
<td>AGATCCCTGAGGAGAGAA</td>
</tr>
<tr>
<td>Utrophin/exon1</td>
<td>GGATCTGGAAAGGCTTTGGGA</td>
<td>TGGGTTTGCGCTATCACAG</td>
</tr>
<tr>
<td>GFAP</td>
<td>CCACAAACTGTTGATGCTCTAC</td>
<td>TTCTCTGCCAAATCCACAGAG</td>
</tr>
<tr>
<td>Agrin</td>
<td>CACCCGGGGACATGAAATCTT</td>
<td>GACCTACCATGACCAGGCA</td>
</tr>
<tr>
<td>β-Actin</td>
<td>GCTCTTTTCCAGCTTCTT</td>
<td>CTTCTGGAATCCTGAGCA</td>
</tr>
</tbody>
</table>

Downloaded from https://academic.oup.com/hmg/article/25/14/3070/2525784 by guest on 03 May 2024
Receptor segments were unchanged (Supplementary Material, Vascular Endothelial Growth Factor), a vascular permeability factor is also increased (20). We assessed retinal vascular permeability, in wild-type and vehicle-injected Dp71-null mice in comparison to Dp71-null mice treated with ShH10-GFP-2A-Dp71. Vascular permeability was evaluated using the Evans blue dye technique (21). We showed that restoration of Dp71 by viral-mediated expression can rescue normal vascular permeability (Fig. 6 left). We also quantified VEGF-A mRNA expression, and we showed that Dp71 restoration leads to VEGF-A mRNA restoration to the wild-type expression level (Fig. 6 right). This observation is in agreement with the postulated direct correlation existing between Dp71 in Müller glial cells and BRB permeability (1). Thus, we can say that Dp71 plays a pivotal role in retinal homeostasis and in BRB permeability.

Discussion

AAV-mediated Dp71 expression (ShH10-GFP-2A-Dp71) in Müller glial cells shows that it is possible to restore Dp71 expression and thereby, relocate Dp71-dependent protein complex. This in turn leads to normal retinal homeostasis, restoring BRB permeability and reabsorbing retinal oedema.

ShH10-GFP-2A-Dp71 leads to Dp71 expression in Müller glial cells deficient in Dp71. The expression level was 7-fold higher compared to wild-type Dp71 level. A similar result was obtained for Dp71-dependent genes or proteins as α/β-dystroglycan, AQP4, Kir4.1, laminins, agrin and utrophin (Figs 2 and 3). These high levels did not seem to cause any adverse effects.

In the present study, we have restored cDNA of the unspliced Dp71, called the Dp71d (22,23), thus eliminating any transcriptional regulation and therefore the expression of other Dp71 isoforms (24–29). However, Dp71 undergoes several alternative-splicing events. Restoration of others isoforms could allow the regulation of the Dp71-dependent complex expression. Therefore, it would be very interesting to study the functional effects of the restoration of each Dp71 isoform in the Dp71-null retina.

We have demonstrated for the first time that Dp71 restoration in MGC re-establishes a normal BRB permeability proving that MGC recovered their ability to regulate the vascular permeability (Fig. 6) (8,9). This is most likely a result of the relocation of potassium and water channels (Kir4.1 and AQP4) and of the consequent restoration of the water transport regulation through MGC.

We have shown that Dp71-null retina is more sensitive to needle injury than wild-type retina since a single saline intravitreal injection induces an increase of the inner retina thickness that persists at least two months after the injection (Fig. 5). The retinal thickness increase might be due to the deregulation of water transport associated with the K+ current in the Dp71-null MGC. The deregulation of water transport that results in the swelling of MGC when exposed to an osmotic stress (1) could lead in Dp71-null mice to retinal oedema. It is very difficult to develop macular oedema animal models because most experimental animals do not possess macula. Dp71-null retina does not naturally present retinal oedema (2) whereas after a single intravitreal injection of saline solution, Dp71-null mice could be used as macular oedema animal model.

In addition, we have found that Dp71 restoration leads to the relocation of Dp71, β-dystroglycan and potassium/water channels (Kir4.1/AQP4) in MGC endfeet at the inner limiting membrane (Fig. 4). The localization of this macromolecular complex could allow fluid reabsorption by the MGC, hence the decrease in the thickness of the retina corresponding to a reuptake of retinal oedema (Fig. 5). Moreover, we have previously

Reabsorption of induced Oedema in Dp71-null mouse retinas

Although, in a previous work, we have shown that intact retinal layers were thinner in the Dp71-null mice compared to wild-type retinas (2). Here, we observed that two months after an intravitreal injection of PBS, the retinas of Dp71-null mice presented a significant thickness increase in the whole retina with respect to the wild-type mice (Fig. 5B). The thickness was increased in the inner retina (Fig. 5C) whereas the ONL and photoreceptor segments were unchanged (Supplementary Material, Fig. S1). These results suggested that Dp71-null mouse is more sensitive to retinal oedema than wild-type mouse and that oedema cannot be reabsorbed due to deficient Müller glial cell function. However, upon restoration of Dp71 through ShH10-GFP-2A-Dp71 treatment, Dp71-null retinas did not show oedema in response to PBS injection (Fig. 5B and C). This absence of thickness variation in ShH10-GFP-2A-Dp71-treated retinas after PBS injection suggests that Dp71 expression helps the oedema reabsorption by restoring the role of Müller glial cells in retinal homeostasis (Fig. 5B and C).

Restoration of blood-retinal barrier permeability

We have demonstrated in previous studies that the absence of Dp71 in Müller glial cells is accompanied by an increased permeability of the blood-retinal barrier (BRB) (1) and that the VEGF (Vascular Endothelial Growth Factor), a vascular permeability

![Figure 3. Dp71 and β-dystroglycan over-expression two months after intravitreal injection of ShH10-GFP-Dp71 in the Dp71-null mouse retina. Western blot and subsequent band density semi-quantification relative to β-actin of Dp71 (A, C) using H4 antibody and of β-dystroglycan (B, D).](https://academic.oup.com/hmg/article/25/14/3070/2525784)
shown that Dp71 by linking Kir4.1, and AQP4 allows the aggregation of these channels in “lipid raft” membrane domains (13). The aggregation in membrane microdomains of Kir4.1 and AQP4 channels in MGC is crucial for the functional coupling of these channels since these channels co-locate and support K\textsuperscript{+} and water transport in glial cells (30–34).

Based on the present data, we propose the following model for Dp71 restoration in Dp71-null mice. First, the ShH10-GFP-2A-Dp71 intravitreal injection induces Dp71 restoration in Müller glial cells. Second, Dp71 restoration allows Dp71-dependent protein complex up-regulation and relocalization in Müller glial cell endfeet at the ILM and around vessels. Finally, recovering a functional Dp71-dependent protein complex in Müller glial cell endfeet leads to BRB reinstatement and retinal oedema reabsorption. Knowing that Dp71 is down-regulated in pathologies showing BRB breakdown, we highlighted that the successful Dp71 restoration is an important step forwards the development of new treatments for retinal diseases with symptoms of BRB breakdown and macular oedema such as DR.

Materials and Methods

Animals

The Dp71-null mice (11) was a kind gift from Pr David Yaffe and were produced by replacing, via homologous recombination, most of the first and unique exon of Dp71 and of a small part of Dp71 first intron with a sequence encoding a β-gal-neomycin-resistance chimeric protein (β-geo). In this mouse line, Dp71 expression is abolished without interfering with the expression of other products of the DMD gene. C57BL/6J mouse strain (JANVIER, France) was used as controls for this study. All animals used in this study were cared for and handled according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.
Generation and purification of AAV vectors

Recombinant AAVs were produced by the plasmid co-transfection method (35), and the resulting lysates were purified via iodixanol gradient ultracentrifugation as previously described (36). Briefly 40% iodixanol fraction was concentrated and buffer exchanged using Amicon Ultra-15 Centrifugal Filter Units. Vector stocks were then titered for DNase-resistant vector genomes by real time PCR relative to a standard (37). Each vector contained a self-complimentary genome encoding the viral 2A peptide for bicistronic expression (17) of GFP and Dp71 under the control of a ubiquitous CBA promoter. The GFP-2A-Dp71 cDNA was synthesized by GENEWIZ, Inc. (USA) and cloned into an AAV plasmid (pTR-SB-smCBA) containing inverted terminal repeat (ITR) regions for the packaging of the interest sequence into ShH10 capsid. The vector was further modified with a single Y445F tyrosine to phenylalanine mutation for enhanced intracellular and nuclear trafficking (38), which was introduced into the ShH10 capsid plasmid using a site directed mutagenesis kit (QuikChange Lightning, Agilent Technologies).

Injections

Before vector administration, mice were anesthetized with ketamine (50 mg/kg) xylazine (10 mg/kg Rompum). Pupils were dilated by the ocular instillation of neosynephrine 5% and mydriaticum 0.5% (Théa) eye drops. An ultrafine 30-gauge disposable needle was passed through the sclera, at the equator and next to the limbus, into the vitreous cavity. Injection of 1 μl stock containing 1.8 x 10^{10} particles of AAV was made with direct observation of the needle in the centre of the vitreous cavity. Left eyes were injected and right eyes served as control.

Fundus photography

Fundus examinations were performed at one or two months after the intravitreal injection of ShH10 coding GFP or GFP-2A-Dp71 under the ubiquitous CAG promoter. Fundus photographs were obtained with a Micron III fundus camera. Mouse pupils were dilated by the application of neosynephrine (5%) and mydriaticum (0.5%) eye drops prior to imaging.

Optical coherence tomography

OCT was performed using an SD-OCT system (Bioptigen Inc., Durham, NC) (39). Hydration with normal saline was used to preserve corneal clarity. Volume analysis centered on the optic nerve head was performed, using 100 horizontal, raster, and consecutive B-scan lines, each one composed of 1200 A-scans. The volume size was 1.4 x 0.1 mm either side of the optic nerve. A custom software was used to generate the en face fundus image using reflectance information obtained from the OCT sections (volume intensity projection), so that the point-to-point correlation between OCT and fundus position was possible and accurate.

Müller glial cell isolation

The retinas were isolated and incubated in papain (Papain from Carica papaya, 10108014001, Roche) for 30 min at 37 °C, followed by a washing step with saline (PBS) and a 10 min fixation step with 4% paraformaldehyde (PFA). After this step, the tissue pieces were shortly incubated in PBS supplemented with DNase I (160 U/ml; Invitrogen) and triturated by a pipette to obtain a suspension of isolated cells. The cells were stored at 4 °C in serum-free minimum essential medium (Sigma) until use within three hours after isolation.

Immunostaining of isolated cells

Suspension of freshly dissociated Müller cells was distributed on glass slides and air-dried. Adherent cells are...
overnight at 4°C for 1 h at room temperature then incubated membranes were blocked in PBS containing 5% dry milk (BIO- membranes according to the manufacturer’s instructions. PVDF and electrotransferred to polyvinylidene difluoride (PVDF) NuPAGE Tris–Acetate 3–8% gradient gels (Invitrogen, France) Germany). Healthcare, Germany) and documented on film (GE Healthcare, formed using ECL plus Western blotting detection system (GE Immunoresearch laboratories). Chemiluminescence was per-

Western blot analysis

Western blot analysis was performed as previously described (40). In brief, retinal protein extracts were resolved using NuPAGE Tris–Acetate 3–8% gradient gels (Invitrogen, France) and electrotransferred to polyvinylidene difluoride (PVDF) membranes according to the manufacturer’s instructions. PVDF membranes were blocked in PBS containing 5% dry milk (BIO-RAD, Herts, UK) for 1 h at room temperature then incubated overnight at 4°C with the primary antibody in the same blocking buffer. Blots were then washed and incubated with the secondary antibody conjugated to horseradish peroxidase (Jackson Immunoresearch laboratories). Chemiluminescence was performed using ECL plus Western blotting detection system (GE Healthcare, Germany) and documented on film (GE Healthcare, Germany).

Quantitative RT-PCR analysis

Total RNA from retina was extracted using Trizol reagent (Invitrogen, France) according to the manufacturer’s instructions. Reverse transcription was performed on 1μg total RNA using SuperScript III and random hexamers (Invitrogen, France). PCR amplifications of cDNA were performed using Master plus SYBR Green I (Roche Diagnostics, Germany) on a LightCycler instrument (Roche Products, Basel, Switzerland). PCR primers were designed using Primer3 software (41) (Table 2). For relative comparison, the Ct values of real-time PCR results were analysed using the ΔΔCt method according to the manufacturer’s instructions. The amount of cDNA was normalized to the standard internal control obtained using primers for β-Actin.

Quantification of blood-retinal barrier permeability

Vascular permeability was quantified by measuring albumin leakage from blood vessels into the retina using the Evans blue method (21). Briefly, mice were anesthetized and Evans blue (45 mg/kg; Sigma-Aldrich, Germany) was injected through the penile vein (3). Blood samples were taken 3 h after injection of the dye and mice were perfused for 2 min via the left ventricle with a citrate buffer (0.05 M, pH 3.5) pre-warmed to 37°C. After perfusion, both eyes were enucleated and carefully dissected. Retinas were dried in a Speed-Vac for 5 h, weighed and the Evans blue dye was extracted by incubating the retina with 100 μl of formamide for 18 h at 70°C. Retinal supernatant was filtered with Nanosep 30k omega tubes (VWR) at 14 000 g during 15 min. Both supernatants were used to measure absorbance. A background-subtracted absorbance was determined by measuring each sample at both 620 nm (absorbance maximum for Evans blue) and 740 nm (absorbance minimum)(TECAN infinite M1000). Evans blue concentration in the plasma and the retina was calculated from a standard curve of Evans blue in formamide. Blood-retinal barrier (BRB) permeability was expressed in
microliter of Evans blue per gram of dry retina per hour (μl Evans blue x g dry retina-1 x h-1).

Data analysis

Results are expressed as mean ± Standard Error of the Mean (SEM). Confocal stacks of 50 images were taken with the same settings. These images were Z projected and the fluorescence area was quantified with Fiji (Fiji Is Just ImageJ) software. Fluorescence data were then analysed using Mann Whitney U test with Prism 5 (GraphPad Software, San Diego, CA). P values < 0.05 accepted as statistically significant.

Supplementary Material

Supplementary Material is available at HMG online.

Acknowledgements

We acknowledge Mélissa Desrosiers for skillful technical assistance with AAV preparations.

Conflict of Interest statement. Conflict of Interest statement. AFM, CONACyT, ECOS Nord, Labex, AVOPH, ANR DysTher are public or non-commercial organizations. Allergan has offered an unrestricted grant to the laboratory used for this study. Authors also disclose the following Conflict of Interests with Commercial Entities: Ramin Tadayoni is a board member of and consultant for Alcon, Switzerland; Novartis, Switzerland; Allergan, USA; Bausch and Lomb, USA; Alimera, USA; Bayer, Germany; FCI-Zeiss, France; Thomboengage, Belgium; Roche, Switzerland; Genentech, USA; Zeiss, Germany. He has received lecture fees from Alcon, USA; Bausch and Lomb, USA; Novartis, Switzerland; Allergan, USA; Bayer, Germany; Alimera, USA; Zeiss, Germany, and meeting expenses from Novartis, Switzerland; Alcon, Switzerland; Allergan, USA; Bayer, Germany, and Novartis, Switzerland. She has received lecture fees from Novartis, Switzerland; Allergan, USA; Bayer, Germany, and meeting expenses from Novartis, Switzerland; Alcon, Switzerland; Allergan, USA; Bayer, Germany, and Alimera, USA.

Funding

This work was supported by (i) the Association Française contre les Myopathies (AFM) for a research project grant number 14853 to A.R. and (ii) a PhD grant number 14768 to O.V., (iii) Allergan INC., (iv) the Institut National de la Santé et de la Recherche Médicale (INSERM), (v) the French State program “Investissements d’Avenir” managed by the Agence Nationale de la Recherche [LIFESENSES: ANR-10-LABX-65], and (vi) EcosNord program number M11502.

References


