

## New Developments

# Protection of Glial Müller Cells by Dexamethasone in a Mouse Model of Surgically Induced Blood–Retinal Barrier Breakdown

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**PURPOSE.** Breakdown of the inner blood–retinal barrier (iBRB) occurs in many retinal disorders and may cause retinal edema often responsible for vision loss. Dexamethasone is used in clinical practice to restore iBRB. The aim of this study was to characterize the impact of a surgically induced iBRB breakdown on retinal homeostatic changes due to dystrophin Dp71, aquaporin-4 (AQP4), and Kir4.1 alterations in Müller glial cells (MGC) in a mouse model. The protective effect of dexamethasone was assessed in this model. Moreover, retinal explants were used to control MGC exposure to a hypoosmotic solution containing barium.

**METHODS.** Partial lens surgery was performed in C57BL6/J mice. Dystrophin Dp71, AQP4, and Kir4.1 expression was analyzed by quantitative RT-PCR, Western blot, and immunohistochemistry. Twenty-four hours after surgery, mice received a single intravitreal injection of dexamethasone or of vehicle.

**RESULTS.** After partial lens surgery, iBRB permeability increased while Dp71 and AQP4 were downregulated and Kir4.1 was delocalized. These effects were partially prevented by dexamethasone injection. In the retinal explant model, MGC were swollen and Dp71, AQP4, and Kir4.1 were downregulated after exposure to a hypoosmotic solution containing barium, but not in the presence of dexamethasone. Heat shock factor protein 1 (HSF1) was overexpressed in dexamethasone-treated retinas.

**CONCLUSIONS.** Partial lens surgery induces iBRB breakdown and molecular changes in MGC, including a downregulation of Dp71 and AQP4 and the delocalization of Kir4.1. Dexamethasone seems to protect retina from these molecular changes by upregulating HSF1.

**Keywords:** blood-retinal barrier, retinal edema, Irvine Gass syndrome, dystrophin Dp71 mouse model, ocular surgery, macular edema, dexamethasone

Müller glial cells (MGC) and astrocytes participate in the formation and maintenance of the inner blood–retinal barrier (iBRB), which is required for a normal vision. Transport processes and tight junctions between the vascular endothelial cells composing the iBRB regulate the flow of ions, nutrients, water, and toxic molecules between the retina and vessels to preserve the proper balance necessary for neuronal function.<sup>1</sup> The loss of iBRB alters the balance of fluids between retinal tissues and retinal vessels. It may cause retinal edema with intraretinal fluid accumulation that may be partially compensated by the continuous fluid reabsorption by MGC<sup>2</sup> and the retinal pigment epithelium.<sup>3</sup>

Müller glial cells are the predominant type of glial cells in the vertebrate retina, and they regulate retinal extracellular

ionic homeostasis<sup>4,5</sup> through cooperation between the rectifying potassium channel Kir4.1<sup>6,7</sup> and the selective water transport protein, aquaporin-4 (AQP4). In normal mouse retina, AQP4 and Kir4.1 are strongly expressed in MGC and mainly localized at MGC endfeet and in processes surrounding the blood vessels. In many retinal diseases, including diabetic retinopathy, retinal vein occlusion, and retinal detachment with iBRB breakdown, the expression and distribution of these channels are altered in MGC.<sup>8–11</sup> Another important function of MGC is the uptake of neurotransmitters supporting the synaptic activity. A MGC is a neurotransmitter recycler whose neuroprotective effect prevents retinal neurons from the long-lasting toxicity of neurotransmitters.<sup>12,13</sup> These phenomena are



particularly important to maintain the retinal homeostatic balance in case of retinal disorder.

Dystrophin Dp71, a membrane-associated cytoskeletal protein that forms the core of the dystrophin-associated protein complex, is predominantly localized in retinal MGC, mainly at their endfeet and around the retinal vessels,<sup>14,15</sup> and is responsible for the clustering and anchoring of AQP4 and Kir4.1.<sup>8,14,16</sup> We have previously shown in mice that a retinal detachment induces reactive MGC gliosis and changes in AQP4, Kir4.1, and Dp71 expression or localization.<sup>15</sup> Moreover, the genetic inactivation of Dp71 (in Dp71-null mice)<sup>17</sup> is associated with iBRB breakdown.<sup>14</sup> The osmotic swelling capacity of MGC exposed to a hypoosmotic solution is exceeded in the absence of Dp71,<sup>14</sup> increasing the vulnerability of retinal nerve cells to transient ischemia in Dp71-null mice.<sup>8,14</sup> Moreover, in the absence of Dp71, AQP4 is downregulated, Kir4.1 distribution in MGC is modified, the retina is in a proinflammatory state,<sup>18</sup> and the morphology of retinal astrocytes is altered.<sup>19</sup>

Dexamethasone is currently injected intravitreally in clinical practice to treat retinal edema because of its anti-inflammatory and antiedematous properties on the retina. Furthermore, in the rat retina, dexamethasone induces alterations in the expression of the potassium channel Kir4.1 in MGC.<sup>20</sup> Intravitreal injections of dexamethasone in a rat model of streptozotocin-induced diabetes inhibit significantly the iBRB breakdown.<sup>21,22</sup> Moreover, as suggested by Zhao et al.,<sup>20</sup> a lower dose of dexamethasone than the doses used in clinical practice may have therapeutic effects.

The aim of this study was to determine the pathway leading to AQP4, Kir4.1, and Dp71 impairments over the postoperative course of a surgically induced iBRB breakdown in a mouse model and to explore the effects of a low dose of dexamethasone on altered AQP4, Kir4.1, Dp71, and iBRB in this model.

## MATERIALS AND METHODS

### Animal Care and Surgical Procedure

For this study, 8-week-old C57BL/6J mice (Janvier, St Bethervin, France) were used in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Surgical procedure was performed using isoflurane; pupils were dilated with topical phenylephrine (2.5%) and tropicamide (1%). Under the operating microscope, a 30-gauge needle was inserted into the posterior part of the lens to remove it (Fig. 1A), allowing a direct contact between remaining lens fragments and the vitreous to create vitreous inflammation and iBRB breakdown in the context of an Irvine Gass syndrome model.

### Quantification of Blood–Retinal Barrier Permeability

Vascular permeability was quantified using the Evans blue (EB) method.<sup>14,23</sup> Evans blue (Sigma-Aldrich Corp., St. Louis, MO, USA) was injected (6 mg/mL) through the penile vein under isoflurane anesthesia. Blood samples were taken 3 hours after dye injection. Mice were perfused for 2 minutes with citrate buffer (0.05 M, pH 3.5) warmed at 37°C. Eyes were enucleated. Retinas were dried in a SpeedVac (Thermo Fisher Scientific, Inc., Waltham, MA, USA) overnight. Evans blue was extracted with formamide (100  $\mu$ L for 18 hours, at 70°C). Retina filtrates were centrifuged at 14,000g (2 hours) and blood samples at 12,000g (15 minutes). Supernatant absorbance was measured at 620 and 740 nm. Plasma and retina concentrations of EB were calculated using a standard curve of EB in formamide. Inner BRB permeability was expressed as microliters of EB per

gram of dry retina per hour ( $\mu$ L EB/g dry retina/h) and quantified 24, 48, and 72 hours post surgery ( $n = 8$ ) based on the previously established formula.<sup>23</sup>

### Intravitreal Dexamethasone Injection and Experimental Procedure

Under isoflurane, a single dose (1  $\mu$ L) of 10 mg/mL dexamethasone (Sigma-Aldrich, Saint-Quentin, France) diluted in balanced salt solution (BSS) (Alcon Laboratories, Inc., Rueil-Malmaison, France) was injected intravitreally with a 33-gauge microinjector (Hamilton, Bonaduz, Switzerland). A control group received an equal volume of vehicle (BSS). Lens surgery was performed at day 0. Twenty-four hours post surgery, mice received a dexamethasone or vehicle injection ( $n = 22$ ). Inner BRB permeability was measured 24 hours ( $n = 5$  per group) and 48 hours ( $n = 5$  per group) after injection, and at 48 hours, the total proteins were extracted ( $n = 12$  per group). A timeline illustrating experimentation chronology is presented in Supplementary Figure S1.

### Quantitative RT-PCR Analysis of Retinal RNA

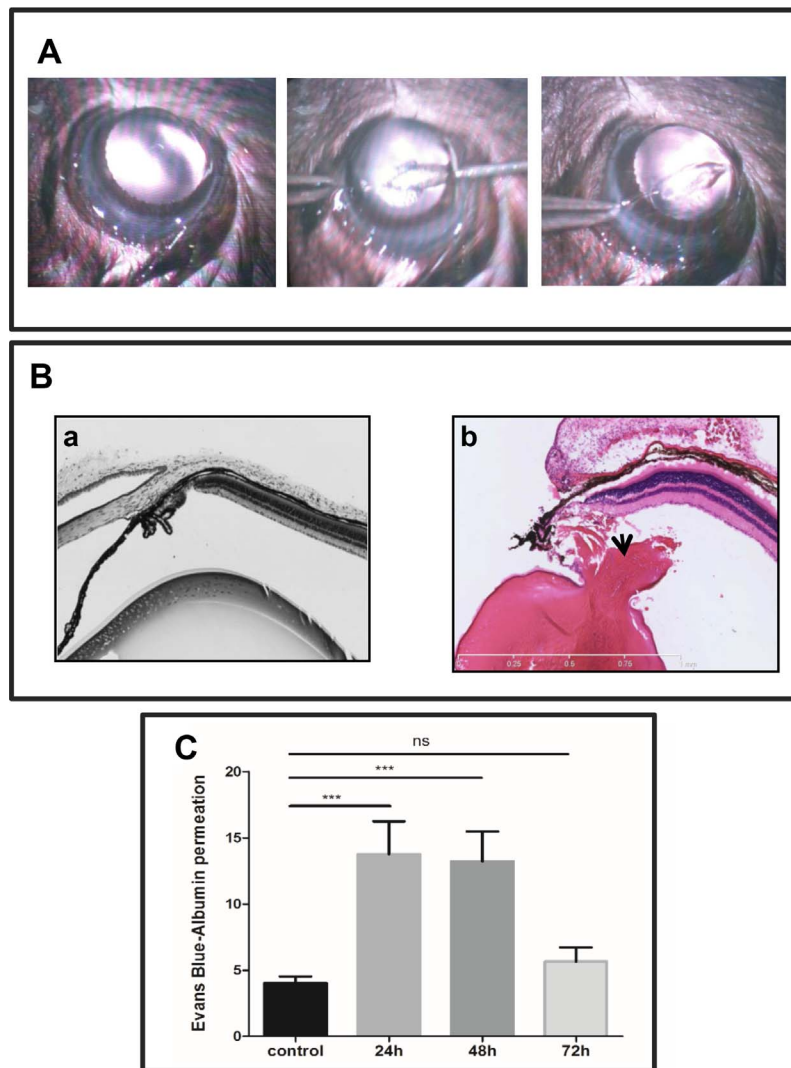
Total RNA was extracted using Trizol reagent (Invitrogen, Waltham, MA, USA):  $n = 4$  at 24, 48, and 72 hours after lens surgery. Reverse transcription was performed on 5  $\mu$ g total RNA using SuperScript III (Thermo Fisher Scientific) and random hexamers (Invitrogen). Complementary DNA were amplified using power SYBR Green (Applied Biosystems, Waltham, MA, USA) on a LightCycler instrument (Applied Biosystems) following the manufacturer's instructions. Polymerase chain reaction primers were designed using Primer3 software<sup>24</sup> and are listed in the Table. The Ct values of real-time PCR products were compared using the dCt method. The amount of Dp71 cDNA was normalized to  $\beta$ -actin used as the standard internal control.

### Western Blot Analysis

Western blot analysis<sup>25</sup> was performed on total proteins extracted 24, 48, and 72 hours post surgery. Retinal proteins were separated on NuPAGE Tris-Acetate 4–12% gradient gels (Invitrogen) and electrotransferred onto polyvinylidene difluoride (PVDF; Millipore, Billerica, MA, USA) membranes. Polyvinylidene difluoride membranes were blocked in PBS containing 0.1% Tween 20, 5% dry milk (Bio-Rad, Marnes-la-Coquette, France) for 1 hour at room temperature, then incubated with the primary antibody at 4°C for the night in the same blocking buffer. Polyclonal antibodies directed against dystrophins (H4) were previously characterized,<sup>26</sup> whereas antibodies directed against Kir4.1 and AQP4 were from Alomone Labs (Souffelweyersheim, France), a rabbit polyclonal anti-Kir4.1 (APC-035) at the dilution of 1:100, and a rabbit polyclonal anti-AQP4 (AQP-004) at the dilution of 1:100. A rabbit polyclonal anti-HSF1 (HSF1-4356) from Cell Signaling Technology (Danvers, MA, USA) at the dilution of 1:1000 was also used. Blots were washed and incubated with the secondary anti-rabbit antibody (Interchim, Montluçon, France) conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). A chemiluminescence substrate (ECL plus Western blotting detection system, using X-ray) was added and chemiluminescence was detected using X-ray films (GE Healthcare, Little Chalfont, UK).

### Immunohistochemistry

Dissected eyes were fixed in 4% paraformaldehyde for 1 hour, cryoprotected in 30% sucrose, frozen, embedded in Cryomatrix



**FIGURE 1.** Partial lens surgery induces iBRB breakdown for 48 hours. **(A)** Surgical technique: partial lens aspiration with a needle. **(B)** Hematoxylin-eosin staining of a control eye without surgery **(a)** and an eye after surgery **(b)**: posterior capsular lens discontinuation (*arrow*). **(C)** Quantification of iBRB permeability post surgery at different time points (expressed in microliters of EB per gram of dry retina per hour). Vascular permeability increased significantly 24 and 48 hours after surgery compared to control retinas and returned to normal after 72 hours. *Scale bar*: 1 mm. Data are expressed as means  $\pm$  SEM,  $n = 8$ .  $***P < 0.001$  was considered significant versus controls.

(Thermo Scientific, Strasbourg, France), cut into 10- $\mu$ m cryostat sections, and mounted on SuperFrost/Plus slides (Microm, Francheville, France). Sections were prepared for hematoxylin and eosin (HE) staining or immunohistology. Sections were permeabilized for 10 minutes with 0.1% PBS (phosphate-buffered saline)-Triton X-100 and blocked for 1 hour in 1%

bovine serum albumin, 0.1% Tween 20, and PBS, then incubated with the primary antibody at 4°C overnight. Primary antibodies used were a mouse monoclonal anti-glutamine synthetase (MAB302; Millipore), dilution 1:500, a rabbit polyclonal anti-Kir4.1 (APC-035 from Alomone Labs) at the dilution of 1:500, a rabbit polyclonal anti-AQP4 (AQP-004,

**TABLE.** List of PCR Primers

Primer Name		Sequence	Target
$\beta$ -Act	Sense	5'-GCTCTTTTCCAGCCTTCCTT-3'	$\beta$ -Actin
	Antisense	5'-CTTCTGCATCCTGTCAGCAA-3'	
Dp71	Sense	5'-ACAACCATGAGGGAAACACCT-3'	Dystrophin Dp71
	Antisense	5'-CTGGAGCCTTCTGAGCTTC-3'	
AQP4	Sense	5'-CTTTCTGGAAGGCAGTCTCAG-3'	Aquaporin-4
	Antisense	5'-CCACACCGAGCAAAAACAAAGAT-3'	
Kir4.1	Sense	5'-CCGCGATTTATCAGAGC-3'	Inwardly rectifying K channel Kir4.1
	Antisense	5'-AGATCCTTGAGGTAGAGGAA-3'	
HSF1	Sense	5'-AACGTCCC GGCTTCCTAA-3'	Heat shock factor protein 1
	Antisense	5'-AGATGAGCGCGTCTGTGTC-3'	



Alomone Labs) at the dilution of 1:500, and a mouse monoclonal anti-GFAP (glial fibrillary acidic protein) (Sigma-Aldrich) at 1:500. Secondary antibodies (Interchim) coupled to Alexa Fluor (Invitrogen) were used diluted 1:800 in 1% bovine serum albumin, 0.1% Tween 20, and PBS for 1 hour. Sections were mounted with Fluorsave reagent (Millipore) and observed by confocal microscopy. ImageJ software (<http://imagej.nih.gov/ij/>; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA) was used for pixel quantification. For Kir4.1 immunohistochemistry we used six retinas and 10 sections per retina. For AQP4 immunohistochemistry, we used six retinas, 44 sections for vessel quantification, and an average of 20 sections at the inner limiting membrane (ILM). For H4 staining, we used six retinas, six sections per retina. For each section, two images were taken.

### Semiquantification of Dystrophins, AQP4, Kir4.1

Retinal section preparations were captured using a Zeiss LSM 700 Meta Axioplan 2 laser scanning confocal microscope (Carl Zeiss, Jena, Germany) on the same area, using the same objective and intensity. Glial fibrillary acidic protein, dystrophins, AQP4, and Kir4.1 were then immunolocalized on retinal sections. Staining was quantified using a semiquantitative measurement with ImageJ software. Density and mean gray value were integrated after subtraction of the unspecific signal. The corrected total cell fluorescence (CTCF) was obtained using the following formula:  $CTCF = \text{Integrated density} - (\text{Area of selected cells} \times \text{Mean fluorescence of background readings})$ .

### Retinal Explants

In a sterile six-well plate containing 500  $\mu\text{L}$ /well of isotonic solution (136 mM NaCl, 3 mM KCl, 2 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$ , 10 mM HEPES, and 11 mM glucose, adjusted to pH 7.4 with Tris) or hypoosmotic solution (60% of isotonic solution) containing 1 mM of ions  $\text{Ba}^{2+}$ , which are potassium channel blockers, three Millicell insert (Millipore) membranes were placed into each well with the hydrophilic side facing the well bottom in contact with the culture medium.<sup>27</sup> After enucleation, eyes were maintained in  $\text{CO}_2$ -independent medium (Gibco, Waltham, MA, USA). Retinas were placed with the photoreceptors facing the membrane, and 1 mL solution was added to each well. Dexamethasone (10 mg/mL) was added on the retina top. The plates were incubated at 35°C in a humidified incubator with 5%  $\text{CO}_2$  for 8 hours. After retinal explant incubation, the retinas were processed to obtain RNA as previously described or were treated with papain (0.3 mg/mL) and immunostained using anti-GS antibody and DAPI (Sigma-Aldrich) to identify MGC soma. Images were captured using the confocal microscope (Carl Zeiss). The quantification of soma size was performed selecting the soma area of individual cells and using the area measure tool of ImageJ software ( $n = 30$  cells); the values are expressed in percentages.

### Statistical Analysis

All data were analyzed using the nonparametric Mann-Whitney test and expressed as means  $\pm$  SEM. GraphPad Prism 6 software (La Jolla, CA, USA) was used for all analyses, and statistical significance was set at  $P < 0.05$ .

## RESULTS

### Partial Lens Surgery Induces iBRB Permeability

Posterior capsular lens discontinuation was confirmed post surgery on cryosections stained with HE (Fig. 1Bb, black

arrow). In C57BL/6/J control mice, the EB extravasation rate was of  $4.03 \pm 0.5 \mu\text{L EB/g dry retina/h}$ , and it was increased to  $13.78 \mu\text{L EB/g dry retina/h}$  ( $\pm 2.5$ ) and to  $13.23 \mu\text{L EB/g dry retina/h}$  ( $\pm 2.5$ ) at 24 hours ( $P < 0.001$ ) and 48 hours ( $P < 0.001$ ) post surgery, respectively. After 72 hours, iBRB impermeability was restored with an extravasation rate of  $5.65 \mu\text{L EB/g dry retina/h}$  ( $\pm 1.1$ ) (Fig. 1C).

### Partial Lens Surgery Causes Dystrophin Dp71, AQP4, and Kir4.1 Alterations

Dp71 is mainly localized at MGC endfeet in the retina.<sup>15,17</sup> Dp71 mRNA was quantified by real-time PCR 24 and 48 hours post surgery and was downregulated by 30% and 45%, respectively, compared to control retinas (Fig. 2A). After 72 hours, Dp71 mRNA level returned close to normal, as it was downregulated by only 15% compared to control retinas. Western blot analysis showed a downregulation of Dp71 72 hours post surgery (Fig. 2B). Immunostaining on control retinal sections with H4, a pan-specific dystrophin antibody, showed the previously reported dystrophin expression pattern<sup>8,15</sup> mainly around the blood vessels (arrow, Fig. 2Cb) and at MGC endfeet at the ILM (arrowhead, Fig. 2Cb). In these areas, a dramatic decrease (50%) in H4 staining was observed 72 hours post surgery, assessed by measuring pixel intensity both at the ILM and around the blood vessels (Figs. 2Cc, 2D).

In control retinas, as previously described<sup>6,7</sup> (Fig. 3Ab), Kir4.1 was localized at MGC endfeet at the ILM and around the blood vessels. Kir4.1 was distributed all along MGC membranes 72 hours post surgery (Fig. 3Ac), mainly at the inner plexiform layer (IPL) (Fig. 3B) without change in overall protein expression as shown by Western blot (Fig. 3D).

In control retinas, AQP4 was localized at MGC endfeet (Fig. 3Ae). However, 72 hours post surgery, an overall downregulation of AQP4 was observed (Fig. 3Af) by 30% at the ILM and by 15% around the vessels (Fig. 3C). Despite this finding, AQP4 was still mainly localized around the vessels and at the ILM (Fig. 3Af). A downregulation of AQP4 was confirmed by Western blot (Fig. 3E). Thus, Dp71 mRNA and protein levels were strongly reduced post surgery, possibly leading to the mislocalization of Kir4.1 and the downregulation of AQP4.

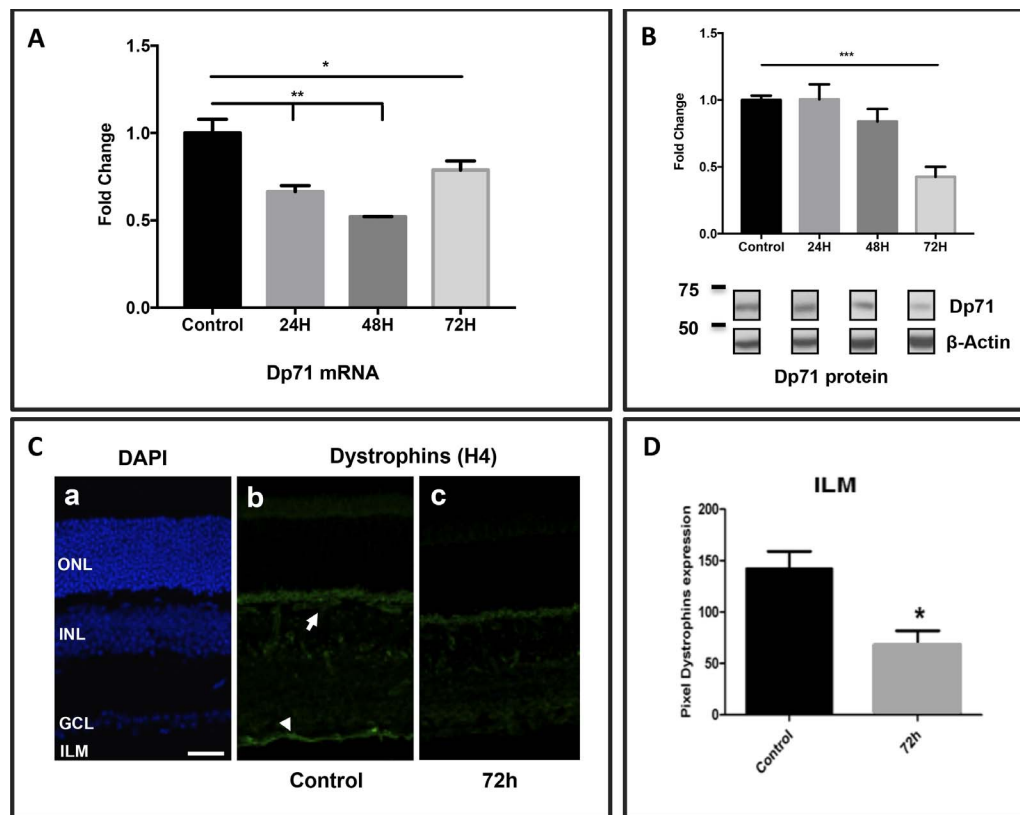
Moreover, as previously described by our team in a model of retinal detachment,<sup>14</sup> MGC gliosis may occur in cases of retinal injury. Here, in control retinas, GFAP was expressed only in astrocytes (Fig. 3Aa, arrow), but 72 hours post surgery, GFAP was unequivocally upregulated in MGC (Fig. 3Ad, arrowhead).

### Partial Lens Surgery Does Not Alter Astrocyte Morphology and Causes No Retinal Inflammation

As shown previously, the constitutive lack of Dp71 induces a change in astrocyte morphology<sup>19</sup> and retinal inflammation.<sup>18</sup> Here, we explored if similar phenomena could be observed after downregulation of Dp71 induced by partial lens surgery and associated with a transient iBRB breakdown. No change in astrocyte morphology (Supplementary Fig. S2) and no significant change in the expression of retinal inflammation biomarkers were observed post surgery with the exception of the *TNF $\alpha$*  gene 48 hours after surgery (Supplementary Table S1).

### Dexamethasone Injections In Vivo Prevent Dp71, AQP4, and Kir4.1 Alterations Without Preventing iBRB Breakdown

A low dose of dexamethasone (23 nM) was injected intravitreally 24 hours post surgery and the iBRB permeability was assessed 48 and 72 hours post surgery: iBRB permeability was



**FIGURE 2.** Downregulation of dystrophin Dp71 after partial lens surgery. (A) The mRNA level of Dp71 was quantified by real-time PCR in control retinas (prior to surgery) and in retinas 24, 48, and 72 hours after lens surgery, and was normalized to the level of  $\beta$ -actin. Dp71 mRNA level decreased significantly until 48 hours post surgery. (B) Western blot analysis of Dp71 and  $\beta$ -actin in control retinas (prior to surgery) and in retinas 24, 48, and 72 hours after lens surgery. Values were normalized to controls. Approximate molecular weights (at the left side of the boxes) given in kilodaltons. (C) DAPI (a) on control retina section. ILM, inner limiting membrane; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; IPL, inner plexiform layer; OLM, outer limiting layer. Immunostaining on retinal sections of control retinas (b) and retinas 72 hours post surgery (c) with H4, a pan-specific dystrophin antibody (green). Dystrophin Dp71 staining is localized at the ILM (arrowhead) and around the blood vessels (arrow). (D) Pixel quantification of dystrophins at the ILM of retinal sections with ImageJ software. Dystrophin expression decreased at the ILM 72 hours after lens surgery. Scale bars: 30  $\mu$ m. Data were expressed as means  $\pm$  SEM,  $n = 6$ . \* $P < 0.05$ ; \*\*\* $P < 0.001$  considered significant versus control retinas.

respectively 5- and 6-fold higher than in control retinas (Fig. 4A) and was not reduced compared to vehicle injection. This showed that, at this concentration, dexamethasone was unable to restore the increased iBRB permeability observed post surgery in mice.

However, using the same experimental conditions, Western blot analysis showed that dexamethasone significantly prevented the downregulation of Dp71 and AQP4 observed 72 hours post surgery (Figs. 4B, 4D, respectively) but had no effect on Kir4.1 expression (Fig. 4C). Moreover, immunofluorescence of dystrophins and AQP4 showed that the intravitreal injection of dexamethasone not only prevented their downregulation but also unequivocally preserved Dp71 immunoreactivity (Figs. 5Ab, 5D) and AQP4 localization mainly at MGC endfeet and around the blood vessels (Figs. 5Ch, 5F). Likewise, the distribution of Kir4.1 around the blood vessels and at the ILM was also preserved (Figs. 5Be, 5E) compared to retinas injected only with the vehicle (Fig. 5Bf).

#### Ex Vivo Dexamethasone Prevents MGC Swelling and the Downregulation of Dp71, Kir4.1, and AQP4

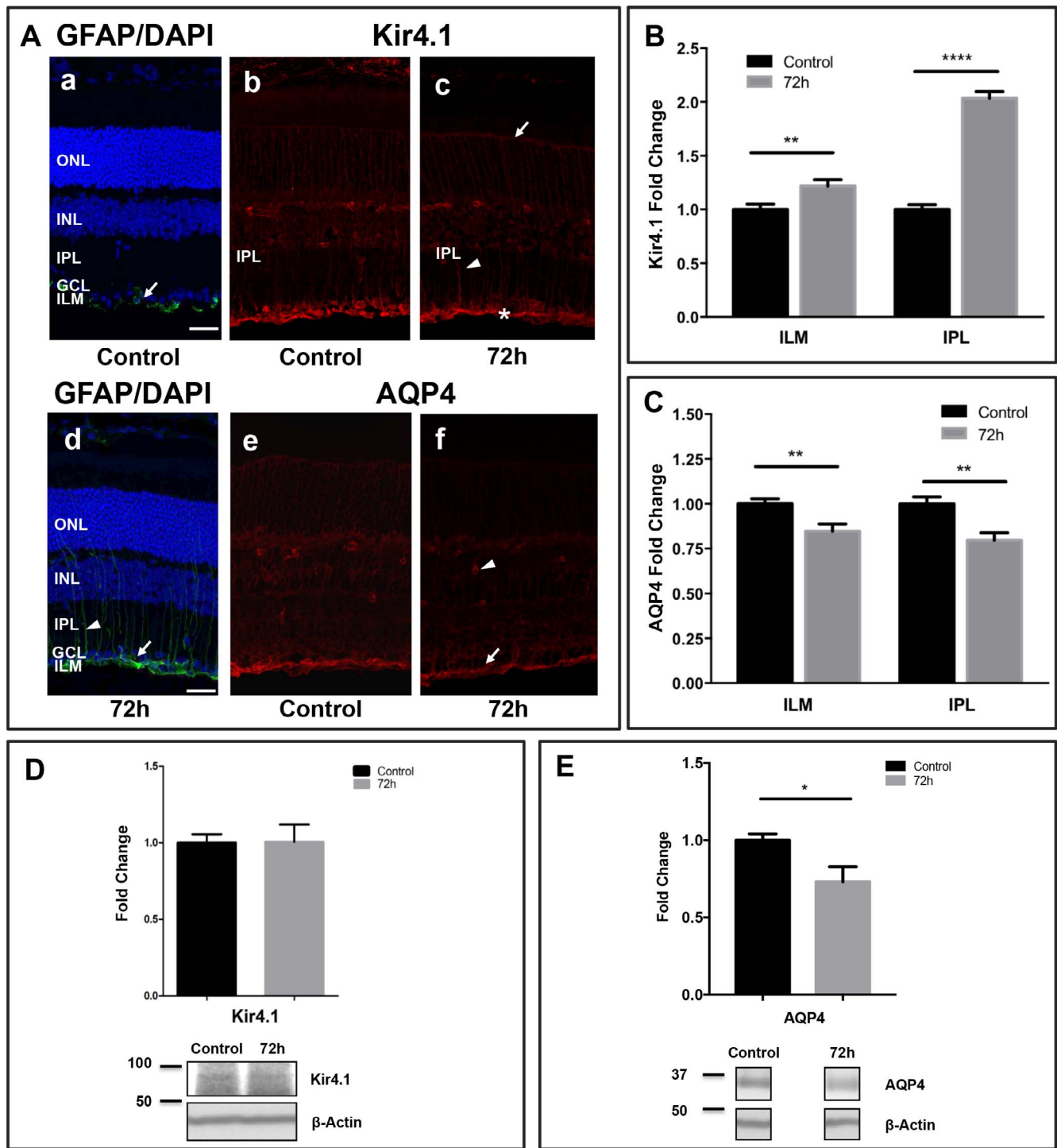
To explore the toxicity of intraretinal fluid resulting from iBRB breakdown on MGC and the effect of dexamethasone, retinal

explants<sup>27</sup> embedded in a controllable fluid environment capable of inducing MGC swelling<sup>28</sup> were used. As previously described,<sup>28</sup> we used a fluid composed of a hypoosmotic solution containing  $Ba^{2+}$  in the presence or absence of a low dose of dexamethasone.

In retinal explants incubated in the hypoosmotic solution containing  $Ba^{2+}$ , a significant increase in MGC soma area size was observed (Fig. 6A). Under these conditions, the hypoosmotic treatment led to a significant downregulation of Dp71, Kir4.1, and AQP4 mRNA (Figs. 6B–D). Moreover, dexamethasone administration also prevented the downregulation of Dp71, Kir4.1, and AQP4 mRNA (Figs. 6B–D).

#### Dexamethasone Regulates HSF1 Expression

Heat shock factor 1 (HSF1) has recently been identified as a regulator of Dp71 expression,<sup>29</sup> acting as a transcription factor that can bind the heat shock-responsive element (HSE) on Dp71 promoter. To further investigate the role of dexamethasone in the prevention of the downregulation of Dp71, Kir4.1, and AQP4 associated with MGC swelling, the level of HSF1 mRNA was quantified. In the retinal explants incubated with the hypoosmotic solution containing  $Ba^{2+}$ , the expression level of HSF1 was similar to that found in control explants. However, in retinas incubated with dexamethasone, HSF1 expression level was significantly increased (Fig. 6E), while Dp71 was not

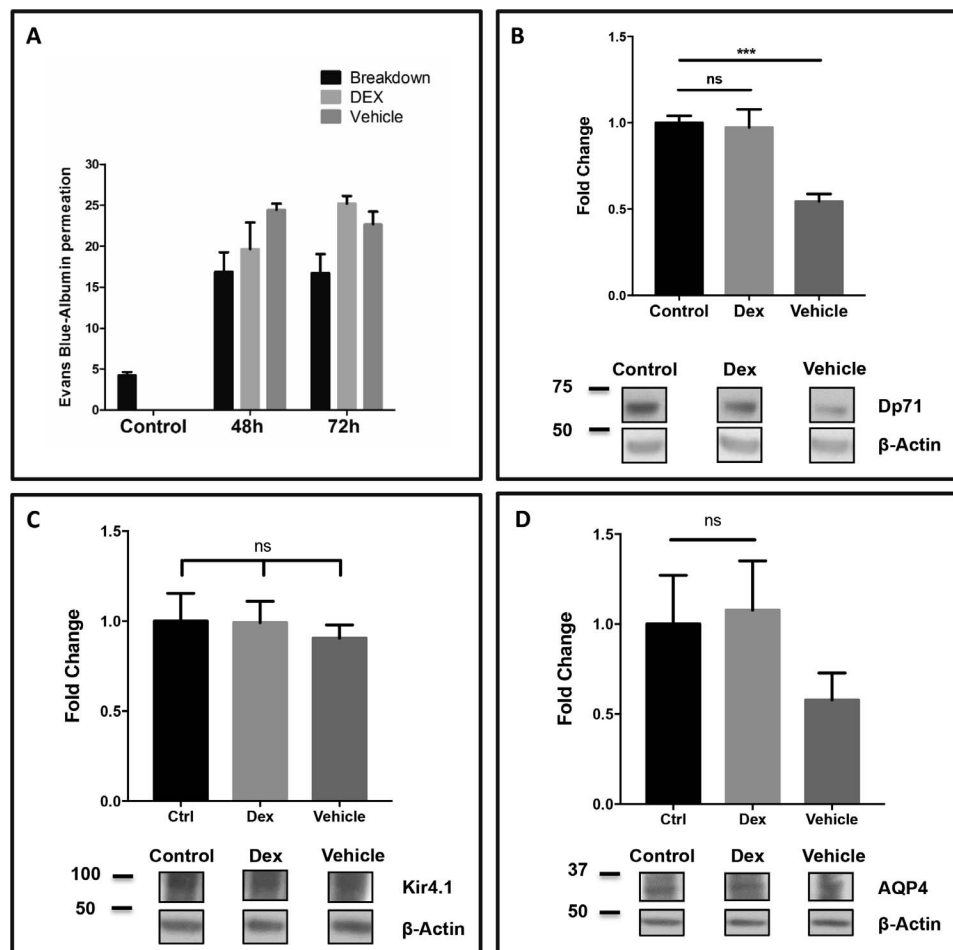


**FIGURE 3.** Delocalization of Kir4.1 and downregulation of AQP4 channels in the retina post surgery. (A) Immunostaining of GFAP (green), DAPI (blue) on retinal sections in control retinas (a), and in retinas after surgery (d). Lens surgery induced an upregulation of GFAP along Müller cell membranes (arrowhead) and in astrocytes from inner retinas (arrow). iBRB breakdown induced a mislocation of Kir4.1 along Müller cell membranes (arrowhead) and at the ONL (arrow) while the staining at the ILM (star) remained unchanged (b, c). AQP4 staining at the ILM (arrow) and around the blood vessels (arrowhead) decreased post surgery (e, f). (B) Pixel quantification of Kir4.1 at the ILM and IPL of retinal sections using ImageJ software. Kir4.1 expression remained stable at the ILM but increased along Müller cell membranes (IPL area). (C) Pixel quantification of AQP4 at the ILM and around the blood vessels: A downregulation was observed for both localizations. (D, E) Western blots of Kir4.1 and AQP4 versus β-actin in control retinas and in retinas 72 hours post surgery. Only AQP4 was downregulated post surgery. Molecular weights given in kilodaltons. Scale bars: 30 μm. Data expressed as means + SEM, n = 4. \*P < 0.05; \*\*\*\*P < 0.001 considered significant versus control retinas.

downregulated and no MGC swelling was induced after exposure to the hypoosmotic solution containing Ba<sup>2+</sup>.

In order to explore the effect of dexamethasone on HSF1 expression, in vivo, we quantified HSF1 retinal protein

expression by Western blot analysis 72 hours after lens surgery, in eyes treated or not by intravitreal injection of dexamethasone. We showed a downregulation of HSF1 72 hours post surgery (Fig. 6F) while dexamethasone intravitreal injection



**FIGURE 4.** Dexamethasone prevented Dp71 downregulation, but did not restore permeability after partial lens surgery. **(A)** iBRB permeability post surgery  $\pm$  dexamethasone/vehicle injection. At 48 or 72 hours post surgery, dexamethasone did not restore iBRB permeability compared to control retinas. **(B)** Western blot of Dp71 and  $\beta$ -actin in control retinas, retinas injected with vehicle or dexamethasone 72 hours after surgery. Dexamethasone prevented the downregulation of Dp71, but not the vehicle. **(C)** Western blot of Kir4.1 72 hours post surgery. Injecting dexamethasone or the vehicle did not change the overall expression of Kir4.1 after surgery. **(D)** Analysis of the Western blot of AQP4. There was a trend toward a downregulation of AQP4 after intravitreal injection of the vehicle, while dexamethasone prevented this downregulation. Data are expressed as means  $\pm$  SEM,  $n = 10$ .  $**P < 0.01$ ;  $***P < 0.001$  were considered significant versus control retinas. **(B, C, D)** Values were normalized to controls.

after surgery significantly prevented the downregulation of HSF1.

## DISCUSSION

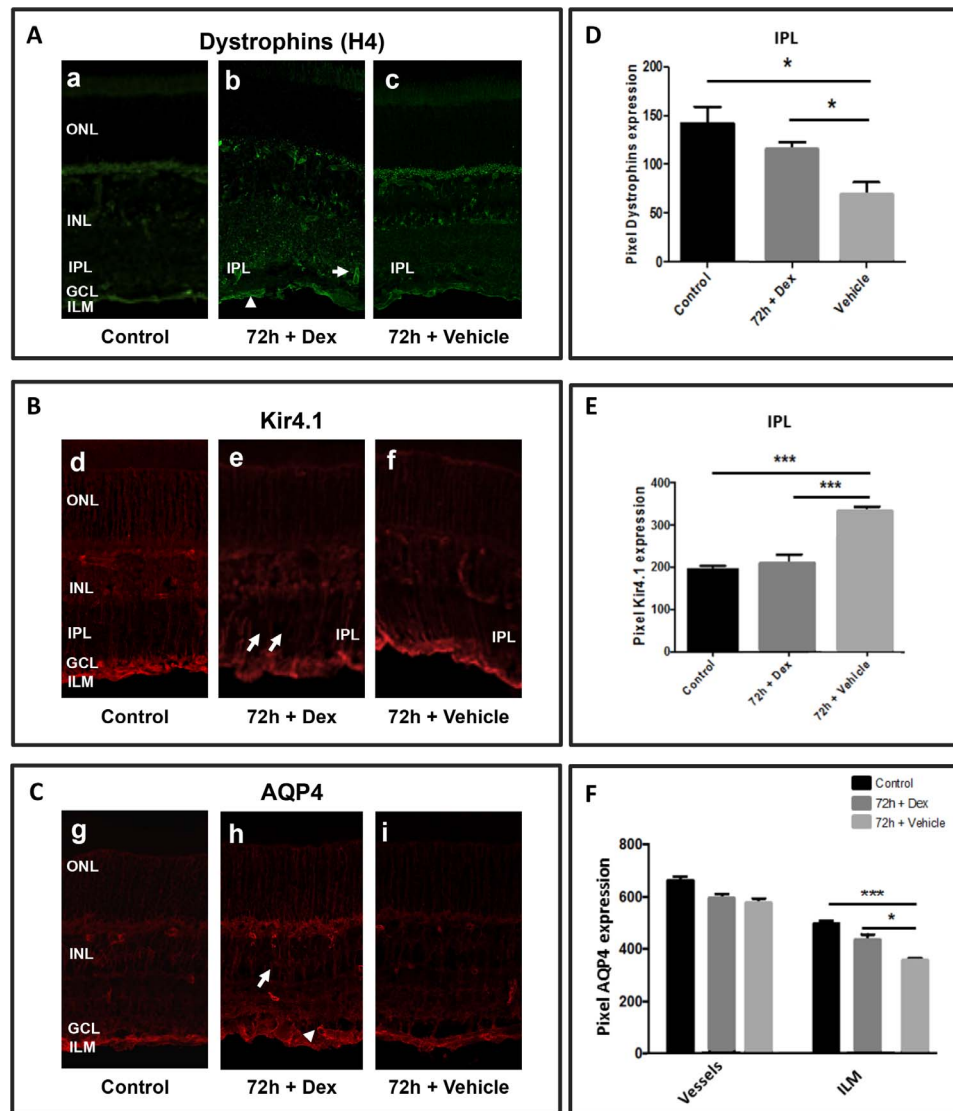
Using an experimental mouse model of partial lens surgery, we (1) confirmed the subsequent occurrence of a transient iBRB breakdown; (2) showed that reactive MGC were strongly altered with a downregulation of Dp71 and AQP4 and a delocalization of Kir4.1; (3) reversed the impairments of Dp71, AQP4, and Kir4.1 using low-dose dexamethasone both in vivo and ex vivo while no effect was observed on iBRB; and, finally, (4) observed that using dexamethasone led to the overexpression of HSF1, one of the transcription factors of Dp71.

Inner BRB breakdown is one of the factors leading to the formation of macular edema, one of the leading causes of vision loss worldwide in diabetic patients and patients with other retinal diseases. In clinical practice, the loss of visual acuity usually poorly correlates with the retinal thickness<sup>30</sup> and iBRB breakdown. Our results suggest that retinal homeostatic alterations could be independent of iBRB breakdown since the

injection of a low dose of dexamethasone prevented retinal homeostatic alterations without preventing iBRB breakdown.

An adapted model of iBRB breakdown induced by partial lens surgery and confirmed using the EB method was used in this study.<sup>23</sup> Inner BRB breakdown due to partial lens surgery appeared similar to the Irvine Gass syndrome observed in humans, which is a rare situation in which an iBRB breakdown and a retinal edema appear after cataract surgery. The localization and proper clustering of AQP4/Kir4.1 seem to be crucial to control the osmotic balance of the retina. In healthy retina, the proteins Kir4.1 and AQP4 are strongly expressed in MGC and mainly localized at MGC endfeet or in the processes surrounding the retinal blood vessels. After partial lens surgery, AQP4 was downregulated and Kir4.1 had lost its polarization. We also showed that dystrophin Dp71, a protein that plays a crucial role in MGC functions such as retinal water and potassium homeostasis and in iBRB maintenance that is the core of a complex responsible for anchoring and proper clustering of the channels Kir4.1 and AQP4, was also downregulated. Chronologically after lens surgery, the iBRB was restored while the downregulation of Dp71 and AQP4 and delocalization of Kir4.1 were not, suggesting that iBRB





**FIGURE 5.** Immunofluorescence of dystrophins, Kir4.1, and AQP4 on retinal sections. Dexamethasone prevented Dp71, Kir4.1, and AQP4 alterations. (A) (a) Dystrophin immunofluorescence in a normal retina (b); there was no downregulation of dystrophin Dp71 at the ILM and around the vessels (arrow and arrowhead) after dexamethasone injection compared to eyes injected with the vehicle after surgery (c). (B) Kir4.1 immunofluorescence in a normal retina (d); dexamethasone prevented delocalization of Kir4.1 at the IPL (star) and around the vessels (arrow) after surgery (e) while the vehicle did not (f). (C) AQP4 immunofluorescence in a normal retina (g); dexamethasone prevented downregulation of AQP4 (h) at the ILM (arrowhead) and around the blood vessels (arrow) after surgery compared to vehicle injection (i). (D) Decreased expression of Dp71 prevented by dexamethasone at the ILM (pixel quantification of retinal sections). (E) Increased expression of Kir4.1 at the IPL (= delocalization) prevented by dexamethasone. (F) Decreased expression of AQP4 at the ILM partially prevented by dexamethasone. Data are expressed as means  $\pm$  SEM,  $n = 3$ . \* $P < 0.05$ ; \*\*\* $P < 0.001$  considered significant versus control retinas.

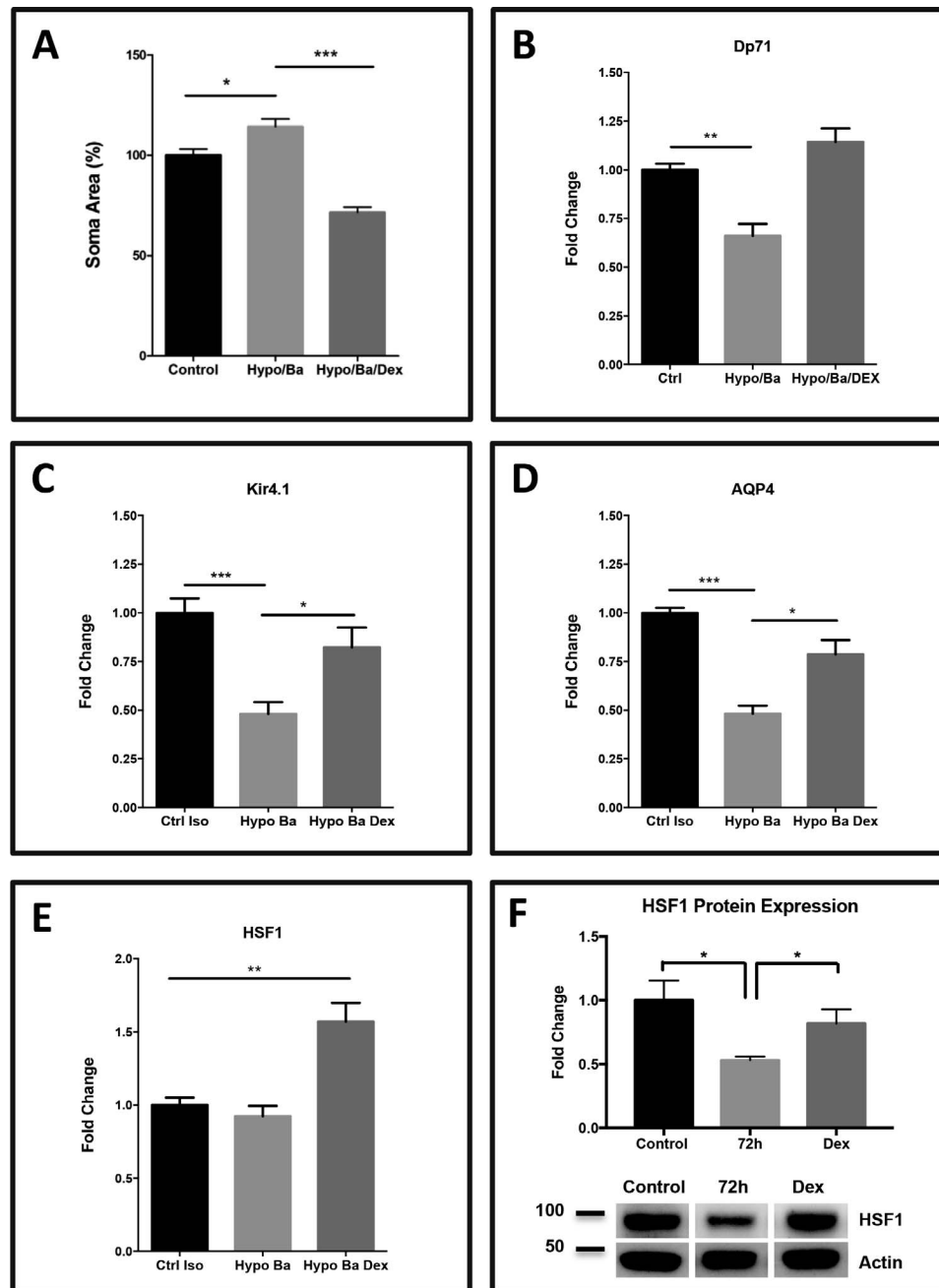
breakdown could be an important factor altering Dp71, AQP4, and Kir4.1.

In previous work from our laboratory, it was suggested that the constitutional lack of Dp71 in Dp71-null mice could be at the origin of the downregulation of AQP4, changes in Kir4.1 localization, and increase in iBRB permeability. However, conversely here, we showed that the transient iBRB breakdown model induced by partial lens surgery preceded the downregulation of Dp71, which could be responsible for the changes observed in AQP4 and Kir4.1 expression in MGC. We could assume that one of the mechanisms responsible for this phenomenon could be the effect of retinal fluid toxicity on MGC and the resulting MGC gliosis (Fig. 3), as previously suggested in a model of retinal detachment,<sup>14</sup> or we can also

suggest that after breakdown of the iBRB, blood-derived factors may induce alterations in Müller cells.

We chose to inject dexamethasone in this model because its efficacy as a treatment for retinal edema has previously been shown in humans, and its anti-inflammatory effect is higher than that of other corticosteroids. Previous works have demonstrated that intravitreal dexamethasone restored an effective iBRB in diabetic rats<sup>31</sup> and inhibited VEGF-induced vascular leakage in rabbits.<sup>32</sup> At a high dose (1  $\mu$ M), in vivo, dexamethasone increased Kir4.1 level in a normal rat retina while a low dose (100 nM) had no effect.<sup>20</sup> However in a pathological model of endotoxin-induced uveitis (EIU), at low dose (100 nM), dexamethasone was able to regulate the expression of Kir4.1.





**FIGURE 6.** Protective effect of dexamethasone tested on retinal explants. The Müller glial cell soma size (A), measured by ImageJ software and expressed as a percentage (considering that control retinas, i.e., explants exposed to an isotonic solution, were 100%), was considerably reduced after dexamethasone exposure while a swelling was observed after exposure to a hypoosmotic solution containing  $Ba^{2+}$ . The decreased expression of Dp71 (B), Kir4.1 (C), and AQP4 (D) was prevented by dexamethasone exposure compared to control. An increase in HSF1 relative expression (E) was observed after dexamethasone exposure compared to control. (F) Western blot of HSF1 72 hours post surgery. Injecting dexamethasone prevented the downregulation of HSF1 72 hours post surgery. Data are expressed as means  $\pm$  SEM,  $n = 4$  for the Western blot. Values were normalized to controls. \* $P < 0.05$  \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  considered significant versus control retinas.

To the best of our knowledge, we were the first to use dexamethasone injections in mice to restore iBRB permeability or altered AQP4/Kir4.1 in MGC during iBRB breakdown. We adapted the intravitreal dose previously used in vivo in rats<sup>31</sup> (50  $\mu$ g or 115 nM) to the weight of the mice, so 10  $\mu$ g was injected. Using this low dose of dexamethasone (23 nM), we observed no effect on iBRB permeability, although Dp71 and AQP4 downregulation and Kir4.1 delocalization were prevented. These results are coherent with those reported by Zhao et al.,<sup>20</sup> who have demonstrated that dexamethasone could

regulate the expression of AQP4/Kir4.1 via its effect on the glucocorticoid receptor. Dexamethasone acts also on Dp71, independently of its effect on the iBRB, suggesting that dexamethasone could have a differential effect depending on the dose used. Based on these observations, we hypothesized that at high dose, dexamethasone could help restore iBRB while at a lower dose, it could act on MGC without restoring iBRB; and as a consequence iBRB permeability, in this model, seemed to be independent of Dp71, Kir4.1, and AQP4 expression. We hypothesized that the paradoxical increase of

the iBRB permeability in comparison to control observed when surgery was followed by intravitreal injection, was due to the effect of intravitreal injection per se in a small eye even without new lens traumatism.

Noteworthy in the explant model, we also observed that MGC swelling, Dp71, AQP4, and Kir4.1 downregulation after exposure to a hypoosmotic solution containing Ba<sup>2+</sup> could be prevented using a low dose of dexamethasone. The regulation of Dp71 remains unclear. However, HSF1 has recently been identified as a regulator of Dp71 expression,<sup>29</sup> acting as a transcription factor. Previous reports have shown that dexamethasone induces and activates HSF1 in mice.<sup>33,34</sup> Here, we observed the upregulation of HSF1 in retinas incubated with dexamethasone. It is conceivable to propose that dexamethasone could prevent the downregulation of Dp71, AQP4, and Kir4.1 and MGC swelling via the upregulation of HSF1. Zhao et al.<sup>20</sup> have previously shown that dexamethasone could regulate the expression of AQP4 and Kir4.1 in MGC. Based on our observations, we could assume that dexamethasone could act on these proteins through the regulation of Dp71 after overexpression of HSF1.

The iBRB-independent regulation of such MGC functions could explain, at least partially, the dissociation between the visual acuity and the edema severity<sup>30</sup> observed in clinical practice. These mechanisms need to be understood. Indeed, in clinical practice, the way to eliminate fluid accumulated in the retina is known most of the time; however, how to protect the retina during iBRB breakdown and macular edema remains unknown, and our findings suggest that the use of corticoids but at a very low dose could be useful. In this study, we showed that dexamethasone was able to prevent MGC swelling ex vivo, suggesting a possible protective role of dexamethasone in MGC. Moreover, since MGC produce trophic substances, remove metabolic waste, recycle neurotransmitters, and modulate the synaptic transmission releasing neurotransmitter precursors,<sup>7,13</sup> dexamethasone's effect on MGC could be useful in a neuroprotective therapeutic approach.

It could help to eliminate potentially toxic neurotransmitters and prevent alterations in water and ionic channels occurring during iBRB breakdown and thus to maintain retinal osmotic balance.

In conclusion, in this study we observed alterations of dystrophin Dp71, AQP4, and Kir4.1 in a mouse model of surgically induced iBRB breakdown. A single injection of low-dose dexamethasone prevented the occurrence of molecular changes without effect on the iBRB. Based on the results of the ex vivo model, we suggest that dexamethasone could upregulate HSF1, one of the transcription factors of Dp71 and a protein stabilizing AQP4/Kir4.1 in MGC. Considering these effects that are independent of its action on the iBRB, low-dose dexamethasone could be a neuroprotective agent with potential utility in clinical practice, probably associated with less complication than when it is used at higher doses.

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