Dopamine D1 receptor-mediated upregulation of BKCa currents modifies Müller cell gliosis in a rat chronic ocular hypertension model

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Abstract
Müller cell gliosis is a common response in many retinal pathological conditions. We previously demonstrated that downregulation of Kir channels contributes to Müller cell gliosis in a rat chronic ocular hypertension (COH) model. Here, the possible involvement of outward K+ currents in Müller cell gliosis was investigated. Outward K+ current densities in Müller cells isolated from COH rats, as compared with those in normal rats, showed a significant increase, which was mainly contributed by large-conductance Ca2+-activated K+ (BKCa) channels. The involvement of BKCa channels in Müller cell gliosis is suggested by the fact that glial fibrillary acidic protein (GFAP) levels were augmented in COH retinas when these channels were suppressed by intravitreal injections of iberiotoxin. In COH retinas an increase in dopamine (DA) D1 receptor (D1R) expression in Müller cells was revealed by both immunohistochemistry and Western blotting. Moreover, protein levels of tyrosine hydroxylase were also increased, and consistent to this, retinal DA contents were elevated. SKF81297, a selective D1R agonist, enhanced BKCa currents of normal Müller cells through intracellular cAMP-PKA signaling pathway. Furthermore, GFAP levels were increased by the D1R antagonist SCH23390 injected intravitreally through eliminating the BKCa current upregulation in COH retinas, but partially reduced by SKF81297. All these results strongly suggest that the DA-D1R system may be activated to a stronger extent in COH rat retinas, thus increasing BKCa currents of Müller cells. The upregulation of BKCa channels may antagonize the Kir channel inhibition-induced depolarization of Müller cells, thereby attenuating the gliosis of these cells.

KEYWORDS
Dopamine D1 receptor, glaucoma, gliosis, large-conductance Ca2+-activated K+ channel, Müller cells

1 INTRODUCTION
Müller cells, a major type of glial cells in the retina, play a crucial role in regulating and maintaining retinal neuronal functions (Bay & Butt, 2012; Bringmann et al., 2006). Under many pathological conditions Müller cells undergo reactivation (gliosis; Bringmann et al., 2006, 2009), which is characterized by upregulated expression of glial cytoskeletal proteins, glial fibrillary acidic protein (GFAP) and vimentin (Bringmann et al., 2000a; Francke et al., 1997, 2001; Pannicke et al., 2006). Abundant evidence has suggested that Müller cell gliosis may be involved in retinal neurodegeneration (Bringmann et al., 2009; Goureau, Régnier-Ricard, & Courtois, 1999; Kashiwagi, Lizuka, Araie, Suzuki, & Tsukahara, 2001; Tezel, Li, Patil, & Wax, 2001; Tezel & Wax, 2003).

Müller cells express a variety of inward and outward K+ channels, such as inward rectifying K+ (Kir), transient outward K+ (Kto), delayed rectifying K+ (Kdr) and large-conductance Ca2+-activated K+ (BKCa)
channels (Bringmann, Faude, & Reichenbach, 1997; Chao et al., 1994; Felmy, Pannicke, Richt, Reichenbach, & Guenther, 2001; landiev et al., 2006b; Kofufi et al., 2000; Pannicke, Faude, Reichenbach, & Reichelt, 2000, 2005; Skatchkov et al., 2006). These channels play important roles in maintaining physiological functions of Müller cells by affecting the membrane potential and/or the excitability of the cells (Bringmann et al., 2009), and they may be related to the pathogenesis of some retinal diseases by modifying Müller cell gliosis (Bringmann et al., 2006b; landiev et al., 2006a,b,c; Ji et al., 2012). A previous work in this lab, for instance, demonstrated that the suppression of Kir channels induced by the activation of group I metabotropic glutamate receptors (mGlur I) contributes to Müller cell gliosis in a rat chronic ocular hypertension (COH) model (Ji et al., 2012). Recent studies have also revealed that outward K\textsuperscript+ currents are altered under some pathological conditions (Bolz et al., 2008; Bringmann et al., 2007; Pannicke et al., 2005). In particular, a remarkable reduction of outward K\textsuperscript+ currents was observed in Müller cells isolated from rat retinas with retinal ischemia-reperfusion or diabetic retinopathy (Pannicke et al., 2005, 2006). These studies raise a possibility that outward K\textsuperscript+ currents may also contribute to the pathogenesis of glaucoma. In the present study we explore how outward K\textsuperscript+ currents of Müller cells are altered in a rat COH model and whether they may be involved in Müller cell gliosis. We show that outward K\textsuperscript+ currents, mainly BKCa currents, are significantly upregulated in Müller cells in COH rats and these upregulated currents attenuate Müller cells gliosis. We further provide evidence showing that the upregulation of BKCa currents in Müller cells is mediated by the dopamine (DA)/D1 receptor (D1R) system.

2 | MATERIALS AND METHODS

2.1 | Animals and rat COH model

Male Sprague-Dawley rats weighing 100–150 g were obtained from SLAC Laboratory Animal Co. Ltd (Shanghai, China) and maintained under a 12/12 hr light/dark circle, with standard food and water provided ad libitum. All experimental procedures described here were in accordance with the National Institutes of Health (NIH) guidelines for the Care and Use of Laboratory Animals and the guidelines of Fudan University on the ethical use of animals. During this study, all efforts were made to minimize the number of sacrificed animals and their sufferings.

Rat COH model was reproduced following a procedure described previously in detail (Chen, Miao, Wang, & Wang, 2011; Dong et al., 2015; Ji et al., 2012). Briefly, three episcleral veins of the right eyes of anesthetized rats were ligated or cauterized under an OPMI VISU 140 microscope (Carl Zeiss, Germany). Sham-operated treatment, following a similar procedure (except for not occluding the veins), was conventionally done on the eyes of other rats. Intraocular pressure (IOP) was measured using a handheld digital tonometer (Tonolab, Icare, Helsinki, Finland), and the average value of five consecutive acceptable measurements with a deviation <5% was recorded. All measurements were performed in the morning to avoid possible circadian fluctuations. The IOPs of both eyes were measured before surgery as a baseline, and then measured immediately after surgery (day 0, G0d), the first, third days and 1 week after surgery (G1d, G3d, and G1w), and weekly thereafter.

2.2 | Preparation of isolated Müller cells

Müller cells were acutely dissociated by enzymatic and mechanical methods, as previously described (Ji et al., 2012; Yang et al., 2016) with minor modifications. Briefly, isolated retinas were incubated in oxygenated Hank’s solution containing the following (in mM): NaCl 137, KCl 3, CaCl\textsubscript{2} 2, MgSO\textsubscript{4} 1, NaHCO\textsubscript{3} 0.5, NaH\textsubscript{2}PO\textsubscript{4} 1, sodium pyruvate 1, HEPS 20 and glucose 16, adjusted to pH 7.4 with NaOH. And they were then digested with 1.6 U/ml papain (Worthington Biochemical, Freehold, NJ) in Hank’s solution supplemented with 0.75 mg/ml l-cysteine for 34 min at 34°C–35°C. After several rinses in Hank’s solution, the retinas were mechanically dissociated with fire-polished Pasteur pipettes, and the cell suspension was plated onto a culture dish mounted on an inverted microscope (IX 70; Olympus Optical, Tokyo, Japan). Typical Müller cells were chosen for whole-cell patch-clamp recording within 2–3 hr after dissociation. These cells were characterized by long bipolar processes, thick proximal stalks and large endfoot (Ji et al., 2012; Yang et al., 2016), which are typical of Müller cells, but quite different in morphology from isolated bipolar cells and amacrine cells shown immunohistochemically. The rod-ON-type bipolar cell was characterized by short dendrites emerging at one end of the soma and a long axon with an enlarged terminal bulb, whereas the OFF-type bipolar cell had two long processes, thin proximal stalks and a large terminal bulb (Yang et al., 2011). In addition, the amacrine cell had a larger soma, but without dendritic terminal bulbs.

2.3 | Whole-cell patch-clamp recording

Whole-cell membrane currents of dissociated Müller cells were conventionally recorded by patch-clamp techniques (Ji et al., 2012; Yang et al., 2016; Zhang et al., 2013). The cells were continuously perfused with an external solution containing (in mM): NaCl 135, KCl 3, CaCl\textsubscript{2} 2, MgCl\textsubscript{2} 1, BaCl\textsubscript{2} 1, HEPS 10 and glucose 11, adjusted to pH 7.4 with NaOH and to 290–300 mOsm/L. Patch pipettes were made by pulling BF150-86-10 glass (Sutter Instrument Co., Novato, CA) on a P-97 Flaming/Brown micropipette puller (Sutter Instrument Co., Novato, CA) and fire polished (Model MF-830; Narishige, Tokyo, Japan) before being used. The pipette resistance was typically 4–5 M\textOmega while filling with an internal solution containing (in mM): K-glucuronate 130, NaCl 20, CaCl\textsubscript{2} 1, MgCl\textsubscript{2} 2, EGTA 1, HEPES 10, GTP-Na 0.1, ATP-Na\textsubscript{2} 2, adjusted to pH 7.2 with NaOH and to 290–300 mOsm/L. The currents were recorded by a patch amplifier (Axopatch 700B; Molecular Devices, Foster City, CA) with Digidata 1440A data acquisition board and pClamp 10.0 software. Analog signals were sampled at 5 kHz, filtered at 1 kHz, and stored for further analysis. Fast capacitance was fully cancelled and cell capacitance was partially cancelled as much as possible by the amplifier circuits. Seventy percent of the series resistance of the recording electrode was compensated. During the recordings, the access resistance was monitored. Once the change of the resistance was greater than ±20%, the cell was discarded. Outward K\textsuperscript+ currents were recorded under current-clamp mode with the holding potential set at 0 mV.
and BKCa currents of Müller cells were evoked by a series of voltage pulses with duration of 400 ms from a holding potential of −60 to +60 mV at increments of 10 mV. All recordings were made at room temperature (22°–24°C).

2.4 Intravitreal injection

The procedure for intravitreal injection was described in previous studies (Dong et al., 2015; Ji et al., 2012). Briefly, the pupils of anesthetized rats were dilated with tropicamide drops, 4 μM ibotenic acid (IBTX), 10 μM SCH23390, 10 μM SKF81297, or 10 μM (S)-3,5-dihydroxyphenylglycine (DHPG), dispersed in 0.9% saline (2 μl), were injected into the vitreous space through a postlimbus spot using Hamilton microinjector (Hamilton, Reno, NV) under a stereoscopic microscope (Carl Zeiss, Jena, Germany). A 30-gauge needle was inserted 1–2 mm behind the temporal limbus and directed toward the optic nerve. Eyes that received normal saline injections in the same manner served as a control.

2.5 Immunohistochemistry

Immunofluorescence staining was performed following the procedure described previously in detail (Chen et al., 2011; Dong et al., 2015; Ji et al., 2012; Yang et al., 2011). Eyeballs, removed quickly from anesthetized rats, were fixed with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB, pH 7.4) for 2 hr at 4°C, followed by dehydration with graded sucrose solutions at 4°C (2 h each in 15%, 20%, and overnight in 30%). The eyecups were then embedded in OCT compound (Tissue Tek, Torrance, CA). The retinas were vertically sectioned at a thickness of 14 μm on a freezing microtome (Leica, Nussloch, Germany), and the sections were collected and mounted on chromalum-gelatin-coated slides (Fisher Scientific, Pittsburgh, PA). After washing with 0.01 M PBS (pH 7.4), the sections were blocked in 6% bovine serum albumin (Sigma, St. Louis, MO) in PBS plus 0.1% Triton X-100 at room temperature for 2 hr, which was followed by incubating with primary antibodies at 4°C for 48 hr: rabbit polyclonal anti-D1R (1:100 dilution; Alomone labs, Jerusalem, Israel), rabbit polyclonal anti-BKCa1.1 (1:100 dilution; Alomone labs, Jerusalem, Israel), mouse monoclonal anti-GFAP (1:1000 dilution; Sigma), mouse polyclonal anti-TH (1:800 dilution, Millipore, Burlington, MA) or mouse monoclonal anti-β-actin (1:1000, Sigma) overnight at 4°C. The blots were washed with Tris-buffered saline-Tween (TBST) and incubated with horseradish-peroxidase (HRP)-conjugated donkey anti-mouse (1:10000, Jackson Immuno-Research Laboratories) or anti-rabbit IgG (1:10000, Jackson Immuno-Research Laboratories) for 2 hr at room temperature. The blots were visualized by incubating with enhanced chemifluorescent reagent ECL (Thermo Scientific, Rockford, IL) and exposed to X-ray film in the dark. These experiments were performed in triplicate, and the protein bands were quantitatively analyzed with NIH Image J or AlphaView analysis software.

2.7 High-performance liquid chromatography (HPLC) analysis

Levels of DA were measured by an HPLC system with electrochemical detection (HPLC-ECD; UltiMate 3000 system, Thermo Fisher Scientific, Waltham, MA), as described previously, with some modifications (Wu et al., 2015). Briefly, retinas and vitreous humor of rats were collected and homogenized with ice-cold 0.1 M perchloric acid containing 10 μM ascorbic acid, 0.1 mM EDTA disodium salt. The samples were centrifuged at 20,800g for 10 min and the supernatants were injected onto an Acclaim C18 column (2.2 μm, 2.1 × 100 mm; Thermo Fisher Scientific) at 38°C. Separations were performed at a flow rate of 0.2 ml/min using a mobile phase of phosphate buffer, containing (in mM) 0.05 EDTA, 1.7 orthosilicic acid (OSA), 90.0 Na2HPO4, 50.0 citric acid. The ECD was fitted with an ANTEC DECRAK SDC detection cell set at +700 mV, and with the guard cell set at +750 mV. The data were collected and analyzed by using Chromeleon chromatography workstation (Thermo Fisher Scientific). Peaks and relative concentrations were identified by comparison to known external standards.

2.8 Reagents and drug application

BaCl2, 4-AP, tetraethylammonium (TEA), Rp-cAMP, H-89, IBTX and forskolin were obtained from Sigma-Aldrich. All the other chemicals were from Tocris Bioscience. Forskolin was dissolved in dimethyl sulfoxide (DMSO) and then added to the extracellular or internal solution, with the final concentration of DMSO being less than 0.1%. The other
chemicals were freshly dissolved in the extracellular or internal solution. Drugs were delivered by a rapid drug application system (RSC-160, Bio-Logic, France), which has nine reservoirs, each with its own control valve to feed fluid through tubing manifolds (500 μm inner diameter). The open/close switch of each valve was manually controlled. Once the valve was open, the solution in the corresponding reservoir was fluxed by gravity.

2.9 | Data analysis

Data analysis was performed by using Clampfit 10.0 (Molecular Devices, Foster City, CA) and Sigmaplot 10.0 (Jandel Scientific, Erkrath, Germany). In the present work, the “n” represents the cell number in electrophysiological recordings, but the animal number in Western blot and immunohistochemistry experiments. For electrophysiological recordings, only one or two cells in a single rat were recorded and tested. Steady-state K<sup>+</sup> current amplitudes were measured at 350 ms of the voltage pulses. Data are presented as means ± SEM. A one-way analysis of variance (ANOVA) with Bonferroni’s post-hoc test (multiple comparisons), Mann-Whitney test (comparisons between two groups) and t test (paired data) were appropriately used. A value of p < .05 was considered significant.

3 | RESULTS

3.1 | Upregulation of BK<sub>Ca</sub> currents of Müller cells in COH rats

In COH rats the IOPs obtained from G1d to G4w (ranging from 24.4 ± 0.1 mm Hg to 29.8 ± 0.2 mm Hg, n = 16–198) of the operated eyes were significantly higher than those of unoperated eyes (20.0 ± 0.1 mm Hg, n = 16–198) and of sham-operated eyes (19.4 ± 0.1 mm Hg, n = 62; p all < .001).

Outward K<sup>+</sup> currents were recorded from Müller cells isolated from COH retinas at two different post-operative times (G1w and G2w; Figure 1a). When these data were compared with the results obtained in Müller cells isolated from sham-operated retinas (control), it was immediately evident that the current amplitudes in operated eyes were overall significantly larger. On average, the current density obtained at membrane potential of −150 mV at G1w was increased from 6.2 ± 1.4 pA/pF (control, Ctr; n = 14) to 9.6 ± 2.6 pA/pF (n = 13, p < .001). No further increase was seen at G2w (9.1 ± 2.4 pA/pF; n = 9, p = .001; Figure 1b). The increases obtained at both G1w and G2w were voltage-dependent, as demonstrated by the current-voltage relationships (Figure 1c).

Outward K<sup>+</sup> currents contain several components, which are respectively induced by activation of BK<sub>Ca</sub> channels, K<sub>CR</sub> channels and ATP-sensitive K<sup>+</sup> (K<sub>ATP</sub>) channels (Clark, Kurth-Nelson, & Newman, 2009; Ettaiche et al., 2001; Fohlmeister, Cohen, & Newman, 2010; Koeberle, Wang, & Schlichter, 2010; Lipton & Tauck, 1987). To determine the identity of the upregulated current components, outward K<sup>+</sup> currents were first recorded in the presence of 1 mM BaCl<sub>2</sub> and 5 mM 4-AP (Figure 1d). When the currents reached a stable level, representative current traces recorded in the presence of BaCl<sub>2</sub> and BaCl<sub>2</sub> + 4-AP + TEA (e) were subtracted from those recorded in the presence of BaCl<sub>2</sub> and BaCl<sub>2</sub> + 4-AP (d) to reveal the TEA-sensitive current components (f).

FIGURE 1 Upregulation of BK<sub>Ca</sub> currents of Müller cells in COH rat retinas. (a) Representative current traces recorded in Müller cells isolated from control and COH rats at G1w and G2w. (b) Summarized data showing that the average outward K<sup>+</sup> current densities under different conditions. (c) I–V relationships, showing the voltage-dependent increase of outward K<sup>+</sup> current amplitudes in Müller cells of COH rats at G1w and G2w. (d and e) Representative current traces recorded in a Müller cell in the presence of BaCl<sub>2</sub> + 4-AP (d) and BaCl<sub>2</sub> + 4-AP + TEA (e). (f) Current amplitude differences between the data shown in (d) and (e), representing TEA-sensitive current components. (g) Representative current traces, showing the upregulated TEA-sensitive K<sup>+</sup> current recorded from a Müller cell at G1w in COH rat, as compared with the current of a normal Müller cell (control, Ctr). (h) Summarized data showing the average BK<sub>Ca</sub> current densities at different post-operative times. *p < .05, **p < .01 and ***p < .001 versus control (Ctr) [Color figure can be viewed at wileyonlinelibrary.com]
or without addition of TEA should be TEA-sensitive BKCa currents (Figure 1e). The difference between these two currents recorded with was added in the bath resulting in a reduction in current amplitudes (Figure 1f). Overall, the BKCa currents recorded from Müller cells at G1w in COH retinas were larger than those from sham-operated retinas. This is illustrated by comparing the two representative currents (Figure 1g). Figure 1h shows how the average current density at +50 mV was changed as a function of post-operational time. It was noteworthy that the density was significantly increased as early as at G3d and fluctuated around the elevated level during the next 3 weeks, without further increase.

Since TEA is a non-specific blocker of BKCa channels, we further used IBTX, a selective BKCa blocker (Tao et al., 2011) to confirm how the IBTX-sensitive component of the whole cell currents in Müller cells was changed in COH retina. Figure 2a,b show the results obtained in two representative Müller cells isolated from normal (control) and COH retinas respectively. The current differences recorded from the cell in the COH retina (G1w) in the presence of 100 nM IBTX (IBTX-insensitive components) were comparable to those obtained in the control retina. In contrast, the current differences obtained before and after the addition of IBTX (IBTX-sensitive components) in the COH retina were larger in size than those in the control retina. On average, the IBTX-sensitive current density at +50 mV was increased to 5.2 ± 0.4 pA/pF (n = 7, p = .002) from the control value of 2.6 ± 0.5 pA/pF (n = 8), but the IBTX-insensitive current density (14.5 ± 0.9 pA/pF, n = 7, p = .268) was not different from the control values (17.5 ± 2.6 pA/pF, n = 8; Figure 2c). It should be noted that the role either TEA or IBTX plays is similar as far as the change in BKCa currents is coincident. That is, either TEA or IBTX-sensitive current components were up-regulated in COH retinas, but TEA- or IBTX-insensitive components were unaffected. These results, together with above ones, suggest that it was BKCa current, but not other outward K+ currents, that was upregulated in Müller cells of COH retinas.

Expression of BKCa channels in Müller cells was examined in retinal sections by double immunostaining (Bringmann et al., 2007). Figure 3a shows that BKCa1.1 was extensively expressed in the control retina (a1), including the ganglion cell layer (GCL), the inner plexiform layer (IPL), the inner nuclear layer (INL) and the outer plexiform layer (OPL). Specifically, BKCa1.1 positive fluorescence signals were co-localized with glutamine synthase (GS), a Müller cell marker (a3). Furthermore, in COH retinas obtained at different post-operative times (G1w–G4w), BKCa1.1 positive signals were remarkably stronger from G2w to G4w (c1–c4) as compared with control (a1). When the antibody of BKCa1.1 was replaced by PBS (negative control), no fluorescence signal was seen in the section (f), demonstrating the specificity of the antibody. These results suggest that IOP elevation increased the expression of BKCa channel in Müller cells.

Whether the upregulation of BKCa currents may be involved in Müller cell gliosis in COH rats was further examined. GFAP upregulation, characteristic of Müller cell gliosis has been reported in COH retinas (Bringmann et al., 2006; Francke et al., 1997; Ji et al., 2012). We studied whether and how upregulated GFAP levels in Müller cells could be changed when BKCa channels were suppressed by IBTX administration in COH rats. Figure 4a shows that the retinal GFAP levels, assessed by Western blotting, were significantly increased to 182.9% ± 9.1% of control (n = 6, p < .001) at G1w (Figure 4b), which is consistent with the results reported previously (Dong et al., 2015; Ji et al., 2012). Intravitreal injections of IBTX (4 μM, 2 µl) prior to the operation further increased the GFAP levels to 222.3% ± 13.4% of control (n = 6, p < .001 vs. control and p = .026 vs. G1w). This result demonstrated that Müller cell gliosis became more severe in COH.
retinas when BKCa channels were suppressed by IBTX. It implies that activation of BKCa channels could make Müller cell gliosis less severe in COH retinas.

3.2 Enhanced activity of DA-D1R system in COH rat retinas

The cause for increased activity of BKCa channels in Müller cells in COH rats was then explored. Müller cells express D1Rs (Kubrusly et al., 2008), and DA concentrations are increased in the aqueous humor of glaucoma patients (Cooper, Constable, & Davidson, 1984). Moreover, activation of PKA is known to increase the activity of single BKCa channels (Bringmann et al., 1997). It seemed to be reasonable to speculate, as a working hypothesis, that the elevated BKCa currents in Müller cells of COH retinas may be resulted from an enhanced activation of retinal DA-D1R system. To test this hypothesis, changes in D1R expression in Müller cells in COH rats were first examined by double immunostaining. Figure 5a1 shows that D1R-positive signals (green) were seen mainly in the GCL and the IPL in a normal retinal section (control). D1R expression in Müller cells exhibited a remarkable increase in retinal sections obtained at different post-operative times (G1w–G4w) from COH rats (Figure 5b–e). In the merged images D1Rs were clearly co-localized with GS (red; Figure 5b3–e3). No positive fluorescence signal was seen when the antibody of D1R was replaced by PBS (negative control; Figure 5f). Consistently, total D1R proteins extracted from COH retinas, assessed by Western blotting, were significantly increased (see the representative result shown in Figure 5g). The average density of D1R proteins was significantly increased to 147.8% ± 5.5% of control at G1w (n = 6, p = .027), and further to 156.0% ± 8.3% (n = 6, p = .001), 190.4% ± 9.4% (n = 6, p = .001), and 190.8% ± 11.9% of control (n = 6, p < .001) at G2w, G3w and G4w, respectively (Figure 5h).
3.3 Activation of D1Rs enhances BK\textsubscript{Ca} currents in normal Müller cells

How activation of D1Rs may modulate BK\textsubscript{Ca} currents was further analyzed in normal Müller cells. In these experiments, BK\textsubscript{Ca} currents were evoked by a depolarizing voltage pulse from −60 to +50 mV. As shown in Figure 8a, extracellular application of SKF81297 (10 \( \mu \)M), a selective D1R agonist, increased the current amplitude (left panel), with the average being 123.9% ± 11.7% of control (\( n = 7 \), \( p = .008 \); right panel). The SKF81297-induced increase in BK\textsubscript{Ca} current amplitude was reversed by co-application of SCH23390 (10 \( \mu \)M), a specific D1R antagonist. The average current amplitude, obtained in the presence of SKF81297 and SCH23390, was 100.2% ± 10.5% of control (\( n = 7 \), \( p = .754 \)). In contrast, bath application of quinpirole (10 \( \mu \)M), a selective DA D2 receptors (D2Rs) agonist, hardly changed the current amplitude, with the average being 100.8% ± 12.6% of control (\( n = 8 \), \( p = .965 \); Figure 8b).

Activation of D1Rs is positively coupled to adenylate cyclase (AC), thus upregulating the cAMP-PKA signaling pathway (Schmidt, Pilgrim, & Beyer, 1998; Stramiello & Wagner, 2008; Zhao et al., 2013). To test if this signaling pathway may be involved, the cells were pre-incubated with Rp-cAMP (10 \( \mu \)M), a membrane permeable cAMP inhibitor for 10 min, bath perfusion of SKF81297 (10 \( \mu \)M) hardly changed the current amplitude (100.5% ± 7.5% of control; \( n = 9 \), \( p = .956 \); Figure 8c). Similarly, the SKF81297-induced increase of BK\textsubscript{Ca} currents in Müller cells was also blocked by H-89 (10 \( \mu \)M), a membrane permeable PKA inhibitor (102.3% ± 9.0% of control, \( n = 4 \), \( p = .852 \); Figure 8d). Furthermore, the SKF81297 effect was mimicked by extracellular application of forskolin, a membrane permeable AC activator. That is, forskolin (10 \( \mu \)M) significantly increased BK\textsubscript{Ca} current amplitudes to 121.3% ± 3.7% of control (\( n = 8 \), \( p < .001 \)), and addition of 10 \( \mu \)M SKF81297 for 2 min did not further change the current amplitude (125.9% ± 6.6% of control, \( n = 8 \), \( p = .398 \) vs. forskolin alone; Figure 8e). These results suggest that cAMP-PKA signaling pathway is likely involved in D1R activation-induced upregulation of BK\textsubscript{Ca} currents in Müller cells.

3.4 Activation of D1Rs reduces GFAP expression in Müller cells in COH retinas by upregulating BK\textsubscript{Ca} currents

How activation of D1Rs was related to the upregulation of BK\textsubscript{Ca} currents in Müller cells in COH retinas was then studied. SCH23390 (10 \( \mu \)M, 2 \( \mu \)l) was intravitreally injected three days before the COH operation. BK\textsubscript{Ca} current densities were determined in Müller cells isolated at G1w and G2w. The density obtained at G1w was 3.1 ± 0.3 pA/pF (\( n = 14 \)), being comparable to the value obtained in sham-operated animals (3.9 ± 0.4 pA/pF, \( n = 11 \)). The density obtained at G2w tended to increase slightly (3.7 ± 0.7 pA/pF, \( n = 10 \)), but was still lower than that in COH retinas without SCH23390 injection and comparable to that in sham-operated animals (Figure 9a,b). These results suggest that upregulation of BK\textsubscript{Ca} currents of Müller cells in COH retinas may be mediated by stronger activation of D1Rs.
Association of D1R activation-mediated upregulation of BKCa currents with Müller cell gliosis in COH retinas was further examined. As reported previously (Ji et al., 2012), retinal GFAP levels were significantly increased in COH retinas at G1w, as revealed by Western blotting (Figure 10a), with the average being 189.2% ± 3.7% of control (n = 6, p < .001; Figure 10b). With intravitreal injection of SKF81297...
two days prior to the operation, GFAP levels were remarkably reduced to 133.7% ± 1.3% of control (n = 6, p < .001 vs. COH alone), even though they were still higher than control (p < .001; Figure 10a,b). This result suggests that D1R-mediated upregulation of BKCa current may attenuate Müller cell gliosis in COH retinas.

We previously showed that suppression of Kir channels induced by the activation of mGluR I contributes to Müller cell gliosis in COH retinas (Ji et al., 2012). Whether activation of D1Rs may affect mGluR I-mediated Müller cell gliosis was also studied. Intravitreal injection of DHPG (10 μM, 2 μl) induced an increase in GFAP expression in the

(10 μM, 2 μl) two days prior to the operation, GFAP levels were remarkably reduced to 133.7% ± 1.3% of control (n = 6, p < .001 vs. COH alone), even though they were still higher than control (p < .001; Figure 10a,b). This result suggests that D1R-mediated upregulation of BKCa current may attenuate Müller cell gliosis in COH retinas.

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(a) Ctr G1w G2w G3w G4w
(b) TH β-actin

FIGURE 6 Changes in TH protein levels in COH rat retinas. (a) Representative immunoblots showing the changes in TH protein levels in control (Ctr) and COH rats at different post-operative times (G1w, G2w, G3w, and G4w). (b) Bar charts summarizing the average densitometric quantification of immunoreactive bands of TH expression at different post-operative times. All data are normalized to control. n = 6 for each group. *p < .05 and **p < .01, versus control.

FIGURE 7 Changes in retinal and vitreal DA contents in COH rats. (a and b) Bar charts showing the changes in retinal (a) and vitreal DA contents (b) at different post-operative times (G1w, G2w, G3w, and G4w). n = 6–8. *p < .05, **p < .01, and ***p < .001, versus control (Ctr).

(a) SKF81297 (SKF) increased BKCa currents, which was reversed by adding SCH23390 (SCH). (b) Quinpirole, a selective D2R agonist, did not alter the current amplitude. (c and d) Pretreatment with Rp-cAMP (c) or H-89 (d) blocked the SKF-induced effect on BKCa currents. (e) Forskolin enhanced BKCa currents and SKF81297 failed to further increase the current. n = 4–9. **p < .01 and ***p < .001 versus control (Ctr). [Color figure can be viewed at wileyonlinelibrary.com]
A retinal slice obtained two weeks after the injection (Figure 10c, c2), as compared with control (Ctr; Figure 10c,c1). But GFAP labeling was clearly reduced by intravitreal injection of SKF81297 (10 μM, 2 μl) made one day after the DHPG injection (Figure 10c,c3). Similarly, Western blotting revealed that total GFAP protein levels, extracted from DHPG-injected retinas, were increased to 149.2% ± 8.2% of control (n = 9, p < .001), but reduced to 110.3% ± 6.3% of control by coinjection of SKF81297 (n = 9, p = .004) (Figure 10d,e).

4 | DISCUSSION

4.1 BKCa channels are negatively involved in Müller cell gliosis in COH retinas

Müller cell gliosis is considered to contribute to the pathogenesis of several major retinal diseases (Bringmann et al., 2009; Goureau et al., 1999; Kashwagi et al., 2001; Tezel et al., 2001; Tezel & Wax, 2003). Since K+ channels play important roles for maintaining the homeostasis of [K+]i in Müller cells, thus being essential for normal retinal functions, it might be expected that these channels could be involved in Müller cell gliosis. It was previously revealed that suppression of Kir channels in the rat COH model could be in part responsible for Müller cell gliosis (Ji et al., 2012). In the present work we demonstrated that both BKCa current densities and BKCa channel protein expression in Müller cells were increased in COH retinas, which was in parallel with the elevation of GFAP expression levels. As shown in Figure 4, GFAP expression levels of Müller cells were further enhanced in COH retinas when BKCa currents were suppressed by IBTX. This strongly suggests that BKCa channels are involved in Müller cell gliosis. Moreover, attenuation of Müller cell gliosis by D1R-mediated upregulation of BKCa currents provided an indirect evidence for the involvement of BKCa channels in Müller cell gliosis. This finding implies that Müller cell gliosis might have been more severe in COH retinas if no BKCa channels existed.

FIGURE 9 Inhibition of D1R blocks the upregulation of BKCa currents in Müller cells of COH rats. (a) Representative current traces showing that intravitreal injection of SCH23390 blocks the increase of BKCa currents in Müller cells of COH rats at G1w and G2w. SCH23390 (2 μl, 10 μM) was intravitreally injected 3 days before the operation. (b) Bar charts showing the average BKCa current densities in Müller cells at G1w and G2w, as compared with that of control [Color figure can be viewed at wileyonlinelibrary.com]

FIGURE 10 Activation of D1Rs attenuates GFAP expression in both COH retinas and DHPG-treated retinas. DHPG (10 μM), SKF81297 (SKF, 10 μM) or vehicle (0.9% saline; control, Ctr) were injected intravitreally in a volume of 2 μl. In COH retinas, SKF was injected 2 days before the COH operation, but 1 day after DHPG injection in normal retinas. (a) Representative immunoblots showing the changes in GFAP protein levels in control (Ctr) and COH rats at G1w with or without SKF injection. (b) Bar charts summarizing the average densitometric quantification of immunoreactive bands of GFAP expression under different conditions. ***p < .001 versus control, ###p < .001 versus G1w. (c) Immunofluorescence images showing GFAP protein expression profiles (green) in retinal slices obtained from Ctr-(a1), DHPG-(a2), and DHPG + SKF-(a3) injected rats. (d) Representative immunoblots showing the GFAP protein levels in Ctr-, DHPG-, and DHPG + SKF-injected eyes. (e) Bar chart shows summarized data. ***p < .001 versus control, ##p < .01 versus DHPG. All data are normalized to control. n = 9 for each group.
is of particular interest that Kir channels and BK$_{Ca}$ channels work in opposite directions in experimental glaucoma. While Kir channels are downregulated, thus causing Müller cell depolarization (cell gliosis; Ji et al., 2012), BK$_{Ca}$ channels are upregulated, which could push the depolarized membrane potential toward the normal level. It seems likely that BK$_{Ca}$ as far as Müller cell gliosis is concerned, works as an intrinsic mechanism protecting retinal neurons from detrimental effects of gliosis in some pathological conditions.

While increased expression of BK$_{Ca}$ channels in Müller cells was found in porcine detached retinas (Bringmann et al., 2007), no evidence was provided for BK$_{Ca}$ channels being causative of Müller cell gliosis modification. In experimental diabetes outward K$^+$ currents of Müller cells are downregulated (Pannicke et al., 2006), which could be explained by a decrease of weakly rectifying Kir4.1 channels. However, it seems not the case in the present work since outward K$^+$ currents were recorded with Kir channels being blocked by Ba$^{2+}$ (1 mM). Fast inactivating K$_a$ currents, which were recorded in Müller cells isolated from post-ischemic retinas and those with diabetic retinopathy (Pannicke et al., 2005, 2006), exhibited significant upregulation following Müller cell gliosis (Felmy et al., 2001, Pannicke et al., 2005). However, these currents were not detectable in Müller cells in the present work (Figure 1).

4.2 Upregulation of BK$_{Ca}$ channels is mediated by enhanced activity of DA-D1R system

The present study demonstrated that both D1R expression in Müller cells (Figure 5) and TH expression were increased in COH retinas (Figure 6). The increase in D1R expression appeared to precede the increase in TH expression by around one week. While the density of D1R proteins was sharply increased at G1w, a significant change in TH expression did not occur until G2w. Consistent to the increase in TH expression, retinal and vitreal DA levels were increased in parallel and they jumped to a higher level at G2w. Although the increase in BK$_{Ca}$ currents in COH retinas occurred as early as at around G3d, the change profiles of BK$_{Ca}$ current amplitudes and the DA-D1R system were overall similar in COH retinas. Moreover, in normal Müller cells the cAMP-PKA signaling pathway mediates the modulation of BK$_{Ca}$ channels of Müller cells through stronger activation of the DA-D1R system. Since the increase of BK$_{Ca}$ currents in Müller cells isolated from COH retinas could be eliminated by intravitreal pre-injection of SCH23390, we could come to a conclusion that stronger activation of DA-D1R system is causative of upregulated BK$_{Ca}$ channels.

Intravitreal injection of SKF81297 attenuated GFAP expression in COH retinas and reduced DHPG-induced upregulation of GFAP expression in normal retinas, and both the effects were mediated by modulating BK$_{Ca}$ channels. These results raise an intriguing possibility that enhancing the activity of the DA-D1R system may serve as a new strategy for reducing the damage of neural retina caused by Müller cell gliosis in glaucoma by elevating the activity of BK$_{Ca}$ channels. Needless to say, one must be cautious to develop this strategy for curing glaucoma, given the fact that the DA-D1R system may be involved in modulation of multiple retinal functions (Aung et al., 2014; Feldkaemper & Schaeffel, 2013; Nishimura & Kuriyama, 1985; Reis, Ventura, Kubrusly, de Mello, & de Mello, 2007; Witkovsky, 2004).

CONFLICT OF INTEREST

The authors declare no competing financial interests.

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