Differential effects of P2Y₁ deletion on glial activation and survival of photoreceptors and amacrine cells in the ischemic mouse retina

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Gliosis of retinal Müller glial cells may have both beneficial and detrimental effects on neurons. To investigate the role of purinergic signaling in ischemia-induced reactive gliosis, transient retinal ischemia was evoked by elevation of the intraocular pressure in wild-type (Wt) mice and in mice deficient in the glia-specific nucleotide receptor P2Y₁ (P2Y₁ receptor-deficient (P2Y₁R-KO)). While control retinae of P2Y₁R-KO mice displayed reduced cell numbers in the ganglion cell and inner nuclear layers, ischemia induced apoptotic death of all retinal layers in both, Wt and P2Y₁R-KO mice, but the damage especially on photoreceptors was more pronounced in retinae of P2Y₁R-KO mice. In contrast, gene expression profiling and histological data suggest an increased survival of amacrine cells in the postischemic retina of P2Y₁R-KO mice. Interestingly, measuring the ischemia-induced downregulation of inwardly rectifying potassium channel (Kir)-mediated K⁺ currents as an indicator, reactive Müller cell gliosis was found to be weaker in P2Y₁R-KO (current amplitude decreased by 18%) than in Wt mice (decrease by 68%). The inner retina harbors those neurons generating action potentials, which strongly rely on an intact ionic homeostasis. This may explain why especially these cells appear to benefit from the preserved Kir4.1 expression in Müller cells, which should allow them to keep up their function in the context of spatial buffering of potassium. Especially under ischemic conditions, maintenance of this Müller cell function may dampen cytotoxic neuronal hypoperfusion and subsequent neuronal cell loss. In sum, we found that purinergic signaling modulates the gliotic activation pattern of Müller glia and lack of P2Y₁ hasJanus-faced effects. In the end, the differential effects of a disrupted P2Y₁ signaling onto neuronal survival in the ischemic retina call the putative therapeutic use of P2Y₁-antagonists into question.

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Gliotic alterations of Müller cells, the dominant macroglia of the vertebrate retina, have been observed in various models of retinal diseases.⁹,¹⁰ A prominent feature of Müller cell gliosis is the downregulation of the inwardly rectifying K⁺ conductance mediated by inwardly rectifying K⁺ (Kir) channels.⁹ It has been demonstrated in astrocytes that downregulation or conditional knockout of Kir4.1 results in an impairment of glial glutamate (Glu) uptake.¹¹,¹² In addition, it has been suggested that autocrine/paracrine purinergic signaling may have a causative role in the development of reactive gliosis in brain and retina.¹³,¹⁴ Müller cells express different subtypes of P2 nucleotide receptors including P2Y₁ and P2Y₂.¹⁵,¹⁶ P2Y₁ receptors have been demonstrated to be functionally expressed by Müller cells and microglial cells, rather than by neurons.¹⁵–¹⁸

Retinal ischemia, a characteristic of various important human blinding diseases including diabetic retinopathy, results in neuronal degeneration and reactive gliosis.¹⁹,²⁰ The reduced K⁺ permeability of Müller cell membranes is associated with an impaired cell volume regulation under hypoxosmotic stress after high intraocular pressure (HIOP)-induced ischemia.²¹ It has been observed that tandem-pore domain K⁺ channels may...
fulfill certain functions under conditions where Kir channels are downregulated or lacking.\textsuperscript{22,23} A malfunctioned Müller cell volume regulation was also found after deletion of P2Y\textsubscript{1} in the mouse retina.\textsuperscript{16} It has been suggested that impaired glial K\textsuperscript{+} buffering and cell volume regulation may contribute to neuronal degeneration in the ischemic retina by inducing neuronal hyperexcitation and Glu-induced cell death.\textsuperscript{14} In order to determine whether endogenous purinergic signaling is implicated in mediating and/or protecting from neuronal degeneration, we investigated the effects of HIOP-induced ischemia in the retinae of P2Y\textsubscript{1}-deficient mice.

Results

Retinal morphology. In order to compare the cell numbers between retinae from Wt and P2Y\textsubscript{1} receptor-deficient (P2Y1R-KO) mice, we counted cell nuclei in the ganglion cell layer (GCL), inner nuclear layer (INL) and outer nuclear layer (ONL) in TO-PRO-3-labeled retinal slices from untreated eyes within a 100-μm-wide area close to the optic nerve head (Figure 1a). As shown in Figure 1b, the retina of P2Y1R-KO mice displayed significantly reduced numbers of cell nuclei in the GCL and INL as compared with the Wt control retina. Moreover, the inner plexiform layer (IPL) of untreated P2Y1R-KO mice was significantly thinner than the IPL of Wt animals (Figure 1c).

Retinal ischemia-reperfusion results in neuronal degeneration.\textsuperscript{19,20} In order to determine ischemic retinal degeneration, we induced transient retinal ischemia in mice for 90 min and quantified the number of cell nuclei in retinal slices 7 days after reperfusion. Transient ischemia caused significant reductions in the numbers of cell nuclei in retinas of Wt and P2Y1R-KO mice compared with untreated controls (Figure 1b). Interestingly, the number of cell (photoreceptor) nuclei in the ONL was more reduced (P<0.05) in P2Y1R-KO mice than in Wt retinae (Figure 1b). Although the thickness of the IPL was significantly reduced in the postischemic retinae of Wt and P2Y1R-KO mice, the decrease in the thickness of the IPL was significantly less pronounced in P2Y1R-KO mice (Figure 1c).

Apoptotic cell death was determined with terminal deoxynucleotidyl transferase-dUTP nick end labeling (TUNEL) labeling of retinal slices obtained 1 day after ischemia, the presumed time point of the peak apoptotic activity in the HIOP-induced rodent retinal ischemia model.\textsuperscript{24} No TUNEL-labeled cell nuclei were found in untreated retinae, neither of Wt nor of P2Y1R-KO mice (Figure 1d). Between 40 and 50% of the cell nuclei in the INL were found to be TUNEL labeled in the postischemic retinae of Wt and P2Y1R-KO mice (Figure 1e). A similar percentage of cells was TUNEL positive in the ONL of Wt mice, whereas this number was significantly increased in the ONL of P2Y1R-KO (P<0.01; Figure 1e).

Differential retinal gene expression in Wt and P2Y1R-KO mice. To assess information about a differential gene expression in control and ischemic retinae as a consequence of P2Y\textsubscript{1} receptor deficiency, which may explain the described differential degeneration pattern in Wt and P2Y1R-KO mice, we performed a RT\textsuperscript{2} Profiler PCR Array cAMP (3′-5′-cyclic adenosine monophosphate)/Ca\textsuperscript{2+} Signalling PathwayFinder. Expression levels of a total of 84 genes (see also Supplementary Table S1) from retinae 7 days after transient ischemia of 90-min duration were investigated. For 21 genes, no major changes in expression levels were observed — neither if expression levels in postischemic retinae were compared with the respective control eyes, nor if expression levels were compared between Wt and P2Y1R-KO retinae (Figure 2a). We found 14 genes with different expression in the untreated retina of P2Y1R-KO mice compared with Wt retinae; all but one were upregulated (Figure 2b). The expression levels of these genes remained unaltered after ischemia in retinae of Wt and P2Y1R-KO mice. At least five of the upregulated genes are known to be expressed in Müller cells, including the transcription factors Junb and Stat3 (see Supplementary Table, for abbreviations of genes).\textsuperscript{25,26} A higher expression level of the enzyme, inducible nitric oxide synthase (iNOS; Nos2), was also observed (Figure 2b). Nos2 is known to be expressed in microglial and Müller cells of the ischemic retina.\textsuperscript{27,28} The higher expression levels of Junb, Nos2, Stat3 and Tacr1 (Figure 2b) may suggest a slight chronic activation of microglia and Müller cells in the P2Y1R-KO retina.

Transient ischemia of the Wt retina induced alterations in the expression of 24 genes of which 21 were downregulated (Figure 3a). Calbindin (Calb) is expressed in amacrine and horizontal cells of the rat retina and was downregulated after retinal ischemia (Figure 3a).\textsuperscript{29} Amacrine cells (ACs) also express neuropeptide Y (Npy), vasoactive intestinal polypeptide (Vip) and tyrosine hydroxylase (Th).\textsuperscript{30–32} In the postischemic retina of P2Y1R-KO mice, only the expression of Ppp2ca, the gene of the x-isoform of the catalytic subunit of the protein phosphatase 2 (PP2A), was markedly decreased (Figure 3a). PP2A is present in rod outer segments where it is involved in vesicle trafficking within photoreceptor synapses.\textsuperscript{33–35}

Finally, we found a group of 16 genes that were differentially expressed in control and ischemic retinae of Wt and P2Y1R-KO mice (Figure 3b). The expression levels of these genes were higher in control retinae of P2Y1R-KO mice than in control retinae of Wt mice (Figure 3b). Among these genes are those for transcription factors (Atf3 and Egr2), enzymes (Eno2, Phrrk1a, Ppp1r15a and Ptgs2), and the neuropeptides glucagon (Gcg) and proopiomelanocortin (Penk) (Figure 3b). Both neuropeptides are expressed in ACs.\textsuperscript{36,37} We found increased expression of the presumable microglial voltage-gated potassium channel Kv1.5 gene (Kcnas)\textsuperscript{38} in control retinae of P2Y1R-KO mice compared with Wt retinae (Figure 3b).

Glial cells are one source of thrombospondin-1, a matricellular glycoprotein with manifold functions. It supports neurite formation, has anti-inflammatory effects and prevents angiogenesis in the retina.\textsuperscript{39–42} Putative downstream signaling mechanisms involve activation of transforming growth factor-β (TGF-β).\textsuperscript{43} Here we found a remarkably higher level of thrombospondin-1 gene (Thbs1) expression together with a slightly upregulated gene expression of TGF-β3 (Tgfβ3) in control P2Y1R-KO retinae if compared with the Wt tissue. In agreement with recent studies,\textsuperscript{44} we found a moderate upregulation of Thbs1 in posts ischemic Wt retinae, but the expression level did not reach the level detected in retinae of P2Y1R-KO animals.

Effects on specific retinal neurons. The gene profiling data suggest that various retinal cell types are differently
Figure 1  Ischemic degeneration of the murine retina after transient retinal ischemia of 90 min. (a) Counting of cell nuclei was performed in 100-μm-wide areas close to the optic nerve head from retinae after 7 days survival — marked as the white square left and shown at larger magnification right. Cell nuclei were labeled with TO-PRO-3 (blue), astrocytes were immunostained with anti-GFAP antibodies (green) and Müller cells with anti-CRALBP antibodies (red). OPL, outer plexiform layer. (b and c) Significant differences compared with the respective control eyes of the same genotype: ***P<0.001, **P<0.01. Significant differences between the indicated strains: K.P<0.05, KK.P<0.01, KKK.P<0.001. Data were obtained from 4 to 6 animals. From each animal, 4–6 central slices were analyzed leading to n=17–33. (d) Apoptotic cell death determined with TUNEL staining 1 day after transient retinal ischemia of 90 min. Staining of all cell nuclei (blue in the left parts of the images) and of TUNEL-labeled nuclei (red in the left parts and white in the right parts of the images) in retinae from Wt and P2Y1R-KO mice. Bars, 50 μm. (e) Mean ± S.E.M. number of TUNEL-labeled cell nuclei in the INL and ONL. **P<0.01. Apoptotic activity was determined in 13–18 central retinal slices derived from 4 to 5 animals.
affected by ischemia and absence of P2Y1R. Retinal slices were immunolabeled for the marker proteins calretinin (ganglion cells, ACs), \( \text{calbindin} \) (amacrine and horizontal cells), protein kinase C\( \alpha \) (PKC\( \alpha \); bipolar cells) and cellular retinaldehyde-binding protein (CRALBP; Müller cells) to quantify the respective cell types. Calretinin immunostaining revealed positive cell bodies in the GCL and INL and three positive bands in the IPL (Figure 4a). Whereas this staining was markedly reduced after ischemia in the Wt retina in both the GCL and INL, the effect was less prominent in the P2Y1R-KO retina (Figure 4a). The total number of calretinin-positive cells was significantly higher in the postischemic P2Y1R-KO retina compared with values found in Wt mice in both layers (Figure 4c). Of note, we also found major differences in the thickness of the IPL in ischemic retinae of Wt and P2Y1R-KO animals than in ischemic retinae of Wt animals.

Calbindin immunoreactivity was observed in ACs (located in the inner part of the INL) and horizontal cells (located in the outer part of the INL) with a tendency of a minor loss of calbindin-labeled cells in the postischemic P2Y1R-KO retina as compared with the retinae of Wt mice (Figures 4b and e). We found no apparent differences in the PKC\( \alpha \) immunolabeling of bipolar cells (Figure 4b) and in the CRALBP immunostaining of Müller cells (Figure 4a) between the retinae of both mouse strains.

Müller cell gliosis. In the normal retina, the intermediate filament protein, glial fibrillary acidic protein (GFAP), is determined the overall fluorescence signal of calretinin immunoreactivity in the IPL. In line with the above-mentioned assumption and with the observed superior survival of calretinin-positive cells per se, the level of calretinin immunoreactivity in the IPL (Figure 4d) was significantly higher in ischemic retinae of P2Y1R-KO animals than in ischemic retinae of Wt animals.

Figure 2 Gene expression profiling in retinae of Wt and P2Y1R-KO mice. The tissues were isolated 7 days after transient retinal ischemia of 90 min and from untreated control eyes from three mice of each genotype. (a) Genes that did not show any differences in expression levels according to the analysis using the RT\(^2\) Profiler PCR Array Data Analysis software. (b) Genes are depicted that are differentially expressed in control retinae of Wt and P2Y1R-KO mice. Genes in red were significantly upregulated, genes in blue were significantly downregulated compared with the respective reference group.
predominantly expressed by retinal astrocytes rather than by Müller cells; upregulation of GFAP is an early marker of Müller cell gliosis. We found that Müller cells were devoid of GFAP immunoreactivity in the untreated retinae of Wt and P2Y1R-KO mice (Figure 5a). In contrast, Müller cells in the postischemic retinae of Wt and
Figure 4  Effects of transient ischemia on retinal neurons 7 days after 90 min of transient ischemia. (a) Retinal slices were immunostained for calretinin (green) and CRALBP (red). Cell nuclei were labeled with TO-PRO-3 (blue). Ischemia resulted in a marked reduction of the calretinin staining of distinct cell bodies and a disappearance of the calretinin-labeled bands in the IPL. These effects were more pronounced in the retina of WT mice than in the P2Y1R-KO retina. (b) Calbindin (red) and PKCa (green) immunoreactivities in retinal slices. Cell nuclei were labeled with TO-PRO-3 (blue). Postischemic retinae displayed reduced levels of calbindin immunoreactivity in the INL compared with untreated control retinae. The level of PKCa immunoreactivity was only slightly different between control and postischemic retinae. (c) Mean±S.E.M. number of calretinin-positive cells counted in the INL and the GCL. (d) Mean±S.E.M. relative intensity of calretinin immunoreactivity in the IPL, reflecting the calretinin content of AC dendrites. The data were obtained in a 460-μm-wide area. (e) Mean±S.E.M. number of calbindin-positive amacrine and horizontal cells. (c–e) Data were obtained by analysis of 10–14 central retinal slices derived from 3 to 4 animals. Significant difference to values from the respective untreated control: *P<0.05, **P<0.01, ***P<0.001; # P<0.05, ## P<0.01, ### P<0.001. Scale bars, 20 μm. OPL, outer plexiform layer.
P2Y1R-KO mice were immunolabeled for GFAP over their entire length. In line with findings from Hirrlinger et al.,47 we found that Müller cells of Wt animals, isolated 7 days after transient retinal ischemia for 60 min, displayed a reduction of the Kir currents by approximately 25% as compared with cells from untreated retina (Table 1). Prolongation of ischemia up to 90 min aggravated this effect resulting in a decrease of the Kir currents in Wt Müller cells by almost 70%. Ischemia also induced an increase in the membrane capacitance of Wt Müller cells (Table 1), suggesting a hypertrophy of the cells.

As P2Y1R signaling was implicated to be involved in gliosis induction,48 we investigated the dependence of the ischemic induced an increase in the membrane capacitance of Wt Müller cells by almost 70%. Ischemia also 90 min aggravated this effect resulting in a decrease of the Kir currents in Wt Müller cells of Wt animals, isolated 7 days after transient retinal ischemia for 60 min, displayed a reduction of the Kir currents of Wt Müller cells of Wt animals as compared with cells from Wt animals suggesting an increase in the size of Müller cells of P2Y1R-KO animals (Table 1). The membrane capacitance was explicitly increased only in some but not all cases of ischemia, whereas ischemia always induced a decrease of inward current densities (Table 1).

The predominant Kir channel subtype expressed by Müller cells is Kir4.1.49,50 It is concentrated in cell membranes contacting inner limiting membrane and in those surrounding blood vessels. Kir4.1 immunolabeling was similar in retinal slices from untreated Wt and P2Y1R-KO eyes (Figure 5c). Kir4.1 immunoreactivity was more evenly distributed and partially downregulated in the postischemic Wt and P2Y1R-KO retina.

**Microglia activation.** In addition to Müller cells, microglial cells were shown to express P2Y1. 51,52 To assure that observed effects of P2Y1R deficiency were largely due to an altered gliotic activation of Müller glia rather than being mediated by a changed activation pattern of microglia, we characterized the latter on the basis of cell numbers and morphological parameters (Supplementary Figure S1) in retinal tissues isolated 1 day after HIOP-induced retinal ischemia for 90 min. We only found minor differences in the characteristics of microglia in the control and postischemic retina of Wt and P2Y1R-KO mice (see Supplementary Information), which do not explain the finding of an altered degeneration pattern in postischemic Wt and P2Y1R-KO retina.

**Discussion**

**Effects of P2Y1 deficiency in the control retina.** Analysis of the untreated retina revealed significantly less cells in the GCL and INL of P2Y1R-KO compared with Wt mice (Figure 1b). As P2Y1 stimulates the proliferation of retinal progenitors, 53, 54 our data may indicate that P2Y1-mediated signaling has a role in the development of the murine retina. Alternatively, and although we did not find TUNEL-positive cells in the control P2Y1R-KO retina (Figure 1d), slow degenerative processes in the adult P2Y1R-KO retina leading to the reduced cell numbers cannot be excluded. Interestingly, cellular hypertrophy and decreased Kir current amplitudes (Table 1), but no elevation in GFAP expression, suggest a role of P2Y1R signaling has a role in the development of the murine retina.
activation in P2Y1R-KO animals.\textsuperscript{97} It remains to be determined whether the low-level glial activation in the P2Y1R-KO retina reflects degenerative processes or alterations of glial function without significant effects on retinal integrity.

**Ischemic retinal degeneration.** Significantly less photoreceptors survived in P2Y1R-KO mice than in Wt retinae (Figure 1b). The TUNEL assay, revealing more apoptotic cells in the ONL of P2Y1R-KO than in Wt mice, confirmed this finding (Figure 1e). Interestingly, the IPL in postischemic P2Y1R-KO retinae was thicker than in Wt mice, despite only 15% of putative ACs survived in postischemic Wt but stably expressed in the postischemic P2Y1R-KO mice. Gene expression profiling points to ACs as receptors in ischemia. Müller cells support photoreceptors by delivering of lactate and neurotrophic factors.\textsuperscript{61,62} Selective

### Table 1 Electrophysiological properties of murine Müller cells

|               | Untreated | HIOP 60 min | HIOP 90 min | P2Y1R-KO | Untreated | HIOP 60 min | HIOP 90 min |
|---------------|-----------|-------------|-------------|----------|-----------|-------------|-------------|
| Inward current amplitude (pA) | 3082 ± 606 | 2237 ± 1069*** | 989 ± 680*** | 2753 ± 761○ | 2238 ± 803** | 2358 ± 975○○○ |
| Relative inward currents (%) | 100 ± 19 | 75 ± 35*** | 32 ± 22*** | 100 ± 20 | 90 ± 48 | 82 ± 37*** |
| Membrane potential (mV) | –86 ± 4 | –85 ± 3 | –78 ± 17*** | –85 ± 4 | –85 ± 5 | –86 ± 5 ○○○ |
| Membrane capacitance (pF) | 46 ± 14 | 67 ± 9*** | 53 ± 15 | 56 ± 13○ | 70 ± 16*** | 64 ± 10** ○○○ |
| Current density (pA/pF) | 74 ± 25 | 34 ± 15*** | 29 ± 12*** | 53 ± 21○○○ | 36 ± 17*** | 37 ± 15*** |

The cells were isolated 7 days after HIOP-induced retinal ischemia. For the calculation of the relative inward currents, the mean current amplitude recorded from cells of the untreated control eye was set to 100% for each mouse, and the relative value for each cell from the contralateral postischemic eye was calculated. *P < 0.05, **P < 0.01, ***P < 0.001 compared with the respective value of the untreated eye from animals of the same strain. ○P < 0.05, ○○P < 0.01, ○○○P < 0.001 compared with the respective value from Wt mice. Data are given as mean ± S.D.
abolition of Müller cells results in photoreceptor loss.\textsuperscript{63,64} It remains to be clarified in future studies, which of these putatively involved mechanisms are affected in the postischemic P2Y1R-KO retina. Further mechanisms, for example, the regulation of the extracellular space volume,\textsuperscript{65} may contribute to the glial support of photoreceptor survival (Figure 6). One major alternation in Müller cell physiology in P2Y1R-KO mice is the impaired capability to maintain cellular volume control under hypoosmotic conditions.\textsuperscript{16} The latter involves a signaling cascade induced by an activation of metabotropic Glu and P2Y\textsubscript{1} receptors, and glial release of adenosine 5\textsuperscript{-}triphosphate and adenosine (AD).\textsuperscript{14} In the outer plexiform layer, this signaling cascade may be continuously activated by the constant release of Glu from photoreceptor terminals.\textsuperscript{16} Interruption of this feedback loop in P2Y1R-KO retinae may contribute to photoreceptor degeneration because of the impaired Glu-induced release of the neuroprotectant AD from Müller cells (Figure 6).\textsuperscript{67}

Anti-punniceral agents were suggested to serve as therapeutics for the treatment of ischemic disorders in neural tissues. The present results of differential effects of P2Y\textsubscript{2} deficiency on the survival of neuronal subtypes in the ischemic retina call the use of such agents into question. This is consistent with conflicting results regarding a potential protective effect of P2Y signaling in ischemia published by others. P2Y\textsubscript{2} antagonists applied in a rat model of cerebral ischemia reduced the infarct volume and improved motor function recovery.\textsuperscript{68} In contrast, neuroprotective effects of P2Y\textsubscript{1}-mediated signaling in brain astrocytes under various pathological conditions have been reported.\textsuperscript{69–71} Further research is required to determine — and to separate — the beneficial and detrimental effects of P2Y\textsubscript{1}-mediated signaling in the ischemic brain and retina.

**Materials and Methods**

**Materials.** Papain was from Roche (Mannheim, Germany). All other substances used were from Sigma-Aldrich (Taufkirchen, Germany), unless stated otherwise. The following primary antibodies were used: rabbit anti-Kir4.1 (1:200, Sigma-Aldrich), mouse anti-GFAP (1:200, G-A-5 clone, Sigma-Aldrich), rabbit anti-ionized calcium binding adaptor molecule 1 (1:1; 1:500, Wako, Neuss, Germany), goat anti-calretinin (1:500, Swant, Marly, Switzerland), mouse anti-calbindin (1:400, Swant), rabbit anti-PKCa (1:300, Santa Cruz Biotechnology, Heidelberg, Germany), rabbit anti-CRALBP (1:300, Santa Cruz Biotechnology) and mouse anti-glutamine synthetase (1:1000, Merck Millipore, Darmstadt, Germany). The following secondary antibodies were used: Cy3-conjugated donkey anti-goat, Cy2-conjugated donkey anti-rabbit, Cy2-conjugated donkey anti-mouse, Cy3-conjugated goat anti-rabbit and Cy2-conjugated goat anti-mouse. All secondary antibodies were applied in a 1:200 dilution and were obtained from Dianova (Düsseldorf, Germany). The apoptosis rate was detected using the in situ cell death detection kit, tetramethylrhodamine red (Roche).

**Animals.** All experiments were done in accordance with the European Communities Council Directive 86/609/EEC, and were approved by the local authorities. Animals were maintained with free access to water and food in an air-conditioned room on a 12-h light–dark cycle. Adult (2–6 months old) mice deficient in the nucleotide receptor P2Y\textsubscript{1} (P2Y1R-KO; 129Sv background) were conditioned as described.\textsuperscript{72} Briefly, for genotyping of the P2Y1R-KO mice and wild-type (Wt) littermate controls, the genomic region of the P2Y\textsubscript{1} receptor was characterized. PCR analysis was made with the following primers: knockout, common, 5'-GCAGTGGTTGCGTGCAAT-3', neo, 5'-GGGAGAATTCTCGAGTTG-3'; Wt, common, 5'-GCAGTGGTTGCGTGCAAT-3', Wt, 5'-AACTACCG CTGAGGCTCT-3'. Age- and weight-matched littermate Wt controls were used.

**Retinal ischemia.** Transient retinal ischemia was induced in one eye by the HIOP method. The other eye remained untreated as internal control. Anesthesia was induced with ketamine (100 mg/kg body weight, intraperitoneal (i.p.); Ratiopharm, Ulm, Germany), xylazine (5 mg/kg, i.p.; Bayer Vital, Leverkusen, Germany) and atropine sulfate (100 mg/kg, i.p.; Braun, Melsungen; Germany). The anterior chamber of the test eye of anesthetized mice was cannulated from the pars plana with a 30-gauge infusion needle, connected to a saline bottle. The intrascleral pressure was increased to 160 mm Hg for 60 or 90 min by elevating the bottle. After removing the needle, the animals survived for 1 or 7 days and, subsequently, were killed with carbon dioxide.

**Preparation of isolated Müller cells.** Isolated retinae were incubated in papain (0.2 mg/ml)-containing Ca\textsuperscript{2+/-}Mg\textsuperscript{2+/-}-free phosphate-buffered saline, pH 7.4, for 30 min at 37 °C, followed by several washing steps with saline. After short incubation in saline supplemented with deoxyribocnuclease I (200 U/ml), the tissue pieces were triturated by a 1-ml pipette tip, to obtain isolated retinal cells. The cells were stored at 4 °C in serum-free minimum essential medium until use within 4 h after cell isolation. Müller cells were identified in the cell suspensions according to their characteristic morphology.

**Whole-cell patch-clamp records of isolated Müller cells.** The whole-cell currents of freshly isolated Müller cells were recorded at room temperature using the Axopatch 200A amplifier (Axon Instruments, Foster City, CA, USA) and the ISO-2 computer program (MFK, Niedernhausen, Germany). The signals were low-pass filtered at 1, 2 or 6 kHz (eight-pole Bessel filter) and digitized at 5, 10 or 30 kHz, respectively, using a 12-bit AD converter. Patch pipettes were pulled from borosilicate glass (Science Products, Hofheim, Germany) and had resistances between 4 and 6 MΩ when filled with a solution containing (mM) 10 NaCl, 130 KCl, 1 CaCl\textsubscript{2}, 2 MgCl\textsubscript{2}, 10 ethylene glycol tetraacetic acid and 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), adjusted to pH 7.1 with tris(hydroxymethyl)aminomethane (Tris). The recording chamber was continuously perfused with extracellular solution that

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**Figure 6** Artist’s view on the putative janus-faced effect of P2Y\textsubscript{1} deficiency in the postischemic retina. Left: schematic drawing of a Müller cell (light green) and its associated neurons (dark blue). Right: down-regulation of Kir4.1 in Wt mice prevents Müller cells from fulfilling their important function to maintain the retinal potassium homeostasis by transcellular clearance of the latter from the extracellular space into bigger fluid filled compartments such as the blood system. This may lead to cytotoxic neuronal hyperexcitation especially of cells generating action potentials such as retinal ganglion cells and subtypes of ACs (indicated by red membrane) in the inner retina. In contrast, photoreceptors in the outer retina appear to benefit from functional P2Y\textsubscript{1}R, probably due to P2Y\textsubscript{1}-mediated release of neuroprotective substances like AD or other trophic factors. In P2Y1R-KO mice the stable Kir4.1 expression appears to allow a better survival of neurons in the inner retina, while the lack of P2Y\textsubscript{1}R considerably disrupts the essential support of photoreceptors by Müller glia. BV, blood vessel; mGluR, metabotropic Glu receptor; NT, nucleoside transporter; OPL, outer plexiform layer; PRC, photoreceptor cell; RCC, retinal ganglion cell cell death and Disease.
Statistical analysis was made using Prism (Graphpad Software, San Diego, CA, USA). Changes in gene expression levels were calculated according to the ΔΔCt method using the web based RT2 Profiler PCR Array Data Analysis software (http://pcralignanalysis.sabiosciences.com/prc2/arrayanalysis.php).

Statistics. Data are expressed as mean ± S.E.M. or S.D. (patch-clamp data). Statistical analysis was made using Prism (Graphpad Software, San Diego, CA, USA); significance was determined by the non-parametric Mann–Whitney U-test.

Conflict of Interest
The authors declare no conflict of interest.

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