Deletion of Protein Phosphatase 2A Accelerates Retinal Degeneration in GRK1- and Arr1-Deficient Mice

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Purpose. Light detection in retinal rod photoreceptors is initiated by activation of the visual pigment rhodopsin. A critical, yet often-overlooked, step enabling efficient perception of light is rhodopsin dephosphorylation mediated by protein phosphatase 2A (PP2A). PP2A deficiency has been reported to impair rhodopsin regeneration after phosphorylation by G protein receptor kinase 1 (GRK1) and binding of arrestin (Arr1), thereby delaying rod dark adaptation. However, its effects on the viability of photoreceptors in the absence of GRK1 and Arr1 remain unclear. Here, we investigated the effects of PP2A deficiency in the absence of GRK1 or Arr1, both of which have been implicated in Oguchi disease, a form of night blindness.

Methods. Rod-specific mice lacking the predominant catalytic Cα-subunit of PP2A were crossed with the *Grk1*$^{-/-}$ or *Arr1*$^{-/-}$ strains to obtain double knockout lines. Rod photoreceptor viability was analyzed in histological cross-sections of the retina stained with hematoxylin and eosin, and rod function was evaluated by ex vivo electroretinography.

Results. PP2A deficiency alone did not impair photoreceptor viability up to 12 months of age. Retinal degeneration was more pronounced in rods lacking GRK1 compared to rods lacking Arr1, and degeneration was accelerated in both *Grk1*$^{-/-}$ or *Arr1*$^{-/-}$ strains where PP2A was also deleted. In *Arr1*$^{-/-}$ mice, rod maximal photoresponse amplitudes were reduced by 80% at 3 months, and this diminution was enhanced further with concomitant PP2A deficiency.

Conclusions. These results suggest that although PP2A is not required for the survival of rods, its deletion accelerates the degeneration induced by the absence of either GRK1 or Arr1.

Keywords: rhodopsin, rhodopsin kinase, arrestin, protein phosphatase 2A (PP2A), retinal degeneration

Effective detection of light requires the timely shutoff of the photoactivated visual pigment and the rapid inactivation of the phototransduction cascade.[1] As a typical G protein-coupled receptor, the inactivation of the visual pigment in photoreceptors is initiated by phosphorylation, which partially quenches its activity.[2,3] Subsequent binding of arrestin fully inactivates the pigment and terminates the light response.[4,5] It is now well established that visual pigment phosphorylation is carried out by specific G-protein receptor kinases (GRKs). In rods, pigment phosphorylation is catalyzed by GRK1, which phosphorylates several serine/threonine (Ser/Thr) sites on the C-terminus of rhodopsin.[6] Deletion of GRK1,[3] truncation of the opsin C-terminus containing the target Ser/Thr residues,[7] or mutation of these residues themselves,[8] all prevent pigment phosphorylation and result in dramatically slower termination of the light response. This delayed inactivation of the visual pigment in the absence of pigment phosphorylation is believed to cause persistent phototransduction activity and light-induced retinal degeneration in both mice[3] and humans, a disorder known as Oguchi disease.[9,10] Another model of this disorder, the arrestin1 (Arr1) knockout mouse, also undergoes progressive light-induced degeneration.[11] Notably, arrestin has been shown to suppress rhodopsin dephosphorylation in vitro,[12] whereas more recent work indicates that in vivo arrestin could facilitate rhodopsin dephosphorylation, possibly by helping to position the phosphatase at the phosphorylated C-terminus of rhodopsin.[13] These results suggest that the mechanisms of degeneration in *Arr1*$^{-/-}$ mice could be linked to abnormal rhodopsin dephosphorylation. Together, these findings document that the state of phosphorylation of the visual

pigment is tightly regulated and plays a critical role in modulating the phototransduction cascade and the susceptibility of photoreceptors to light-induced damage and degeneration.

Continuous function of photoreceptors in steady light requires regeneration of the photoactivated visual pigment back to its ground state. This process begins with the release from photoreceptors of the spent chromophore, all-trans-retinal, followed by its recycling back to the 11-*cis* form via either the retinal pigmented epithelium (RPE visual cycle, for both rods and cones) or the Müller cells (retina visual cycle, for cones only).[14–16] The recycled *cis*-chromophore is returned to photoreceptors, where it combines with free opsin to regenerate the visual pigment molecule. Notably, phosphorylated rhodopsin activates the phototransduction cascade with reduced efficiency.[17–19] Thus, a necessary but often overlooked step in dark adaptation is the dephosphorylation of the visual pigment.[20] We recently showed that this reaction in mouse rods and cones is carried out by protein phosphatase 2A (PP2A). The PP2A holoenzyme is composed of a catalytic subunit, C, a structural (scaffolding) subunit, A, and a regulatory subunit, B.[21] Deletion of the major Cα catalytic subunit of PP2A in mouse rods results in suppressed rhodopsin dephosphorylation and delayed dark adaptation of the rods.[22] Taken together, these results suggest an important regulatory role of PP2A in the dephosphorylation of rhodopsin required for normal pigment regeneration and photoreceptor dark adaptation. However, despite decades of research on the role of pigment phosphorylation in photoreceptor function and survival, the role of PP2A in retinal degeneration and disease has not been examined. Here, we addressed this question by evaluating the extent of retinal degeneration in mice lacking GRK1, Arr1, or PP2A alone compared to mice deficient in both PP2A and either GRK1 or Arr1.

Methods

Mice

Wild-type C57Bl6/J mice were purchased from Jackson Laboratories (Bar Harbor, MA, USA). *Grk1*$^{-/-}$[3] and rhodopsin-Cre (*iCre75*$^{+}$)[23] mice were a gift from Dr. Ching-Kang Chen (Baylor College of Medicine); *Arr1*$^{-/-}$[4] mice were a gift from Dr. Jeannie Chen (University of Southern California). The generation of the rod-specific mouse line lacking the predominant catalytic Cα-subunit of PP2A (*Ppp2ca*$^{f/f}$ *iCre75*$^{+}$) was described earlier.[22] This line was crossed with *Grk1*$^{-/-}$ or *Arr1*$^{-/-}$ strains to obtain corresponding double knockout lines. All of these lines were homozygous for the Met-450 isoform of RPE65,[24] and were free of the Crb1/rd8 mutation.[25] The rod transducin α-subunit knockout (*Gnat1*$^{-/-}$) mouse line lacking rod phototransduction and carrying the Leu-450 RPE65 isoform was described previously.[26] All mice were maintained under a 12 hour light (10–20 Lux)/12 hour dark cycle and dark-adapted overnight prior to physiological recordings. All experimental protocols were in accordance with the Guide for the Care and Use of Laboratory Animals, were approved by the Institutional Animal Care and Use Committee at the University of California Irvine, and were conducted in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Visual Research.

Histology

Mice were euthanized in a CO_2 chamber prior to enucleation. Eyes were fixed in Hartman's Fixative Solution (Sigma-Aldrich) overnight at room temperature (RT) and embedded in paraffin. Paraffin sections were stained with hematoxylin and eosin (H&E). Spider plots were generated by manual counting of photoreceptor nuclei in the outer nuclear layer (ONL) at several fixed locations from the optic nerve head, visualized through H&E staining and expressed as the number of nuclei per column.

Rod ERG Recordings From Isolated Mouse Retinas

Mice were dark-adapted overnight and sacrificed by CO_2 asphyxiation. The whole retina was removed from each mouse eyecup under infrared illumination and stored in oxygenated aqueous L15 (13.6 mg/mL, pH 7.4) solution (Sigma-Aldrich) containing 0.1% BSA, at RT. The retina was mounted on filter paper with the photoreceptor side up and placed in a perfusion chamber[27] between two electrodes connected to a differential amplifier. The tissue was perfused with bicarbonate-buffered Locke's solution supplemented with 1.5 mM L-glutamate and 40 µM DL-2-amino-4-phosphonobutyric acid to block postsynaptic components of the photoresponse[28]; and with 70 µM $BaCl_2$ to suppress the slow glial PIII component.[29] The perfusion solution was continuously bubbled with a 95% O_2/5% CO_2 mixture and heated to 36 to 37°C.

The photoreceptors in the retina were stimulated with 20-ms test flashes of calibrated 505 nm LED light. Recordings from Arr1-deficient retinas required several times longer periods of dark recovery between test flashes compared to those from controls, to allow their photovoltage to return to its pre-flash level. The light intensity was controlled by a computer in 0.5 log unit steps. Intensity-response relationships were fitted with the following Naka-Rushton hyperbolic function:

$$R = \frac{R_{max} \cdot I^n}{I^n + I_{1/2}^n},$$

where R is the transient-peak amplitude of the rod or cone response, R_{max} is the maximal response amplitude, I is the flash intensity, n is the Hill coefficient (exponent), and $I_{1/2}$ is the half-saturating light intensity. Photoresponses were amplified by a differential amplifier (DP-311; Warner Instruments), low-pass filtered at 300 Hz (8-pole Bessel), and digitized at 1 kHz. Data were analyzed with Clampfit 10.7 and Origin 2021b software.

Single-Cell RNA Sequencing

A wild-type murine retina scRNA-seq dataset was sourced from Hoang et al.[30] and analyzed for native expression of PP2A catalytic and regulatory subunits across all retinal cell types. Average transcriptional expression was quantified in normalized unique molecular identifier (nUMI) counts.

Statistical Analysis

Statistical analysis was carried out using GraphPad Prism version 9.0 or SPSS software. For all experiments, data were

expressed as mean ± SEM and analyzed with 2-way ANOVA or 2-way repeated measures ANOVA (with genotype as main factor and time as repeated measures factor). The level of statistical significance was set at a minimum of $P < 0.05$. Pairwise comparisons were performed using the Bonferroni post hoc test, and $P < 0.05$ was considered significant.

Results

PP2A Deletion Accelerates Retinal Degeneration in GRK1-Deficient Mice

We began our study by investigating how suppressing rhodopsin dephosphorylation through deletion of PP2A affects the progression of retinal degeneration in mice lacking GRK1. Previous morphometric analysis from PP2A-deficient mice (*Ppp2ca*$^{f/f}$ *iCre75*$^{+}$), where the major catalytic Cα-subunit of PP2A was knocked out selectively in rod photoreceptors, has shown that in the absence of PP2A the photoreceptors develop normally and retinas show no sign of degeneration up to 12 months of age.[22] To account for possible influence of rhodopsin-Cre on retinal morphology, we used *iCre75*$^{+}$ mouse retinas as controls for our experiments. As expected, the expression of Cre by itself had no effect on rod survival and caused no discernable retinal degeneration in 3-month-old mice (Fig. 1A, left panels). In contrast, in age-matched *Grk1*$^{-/-}$ mice, we could already observe evidence for thinning in both the dorsal and ventral parts of the outer retina (see Fig. 1A, center panels). Notably, in mice lacking both PP2A and GRK1, retinal degeneration was even more pronounced (see Fig. 1A, right panels).

We next examined how the deletion of GRK1 and PP2A affected retinal morphology in 6-month-old mice. As expected, there was still no evidence for photoreceptor loss in control *iCre75*$^{+}$ mice at that age (see Fig. 1B, left panels). The rod degeneration in 6-month-old GRK1-KO mice appeared more prominent than at 3 months of age (see Fig. 1B, center panels). Consistent with our observations in younger mice, the degeneration in 6-month-old mice lacking both GRK1 and PP2A was more severe than in the age-matched GRK1-deficient mice (see Fig. 1B, right panels).

We quantified the number of photoreceptor nuclei per column across the entire retina in each group by spider plot analysis. This analysis confirmed the retinal

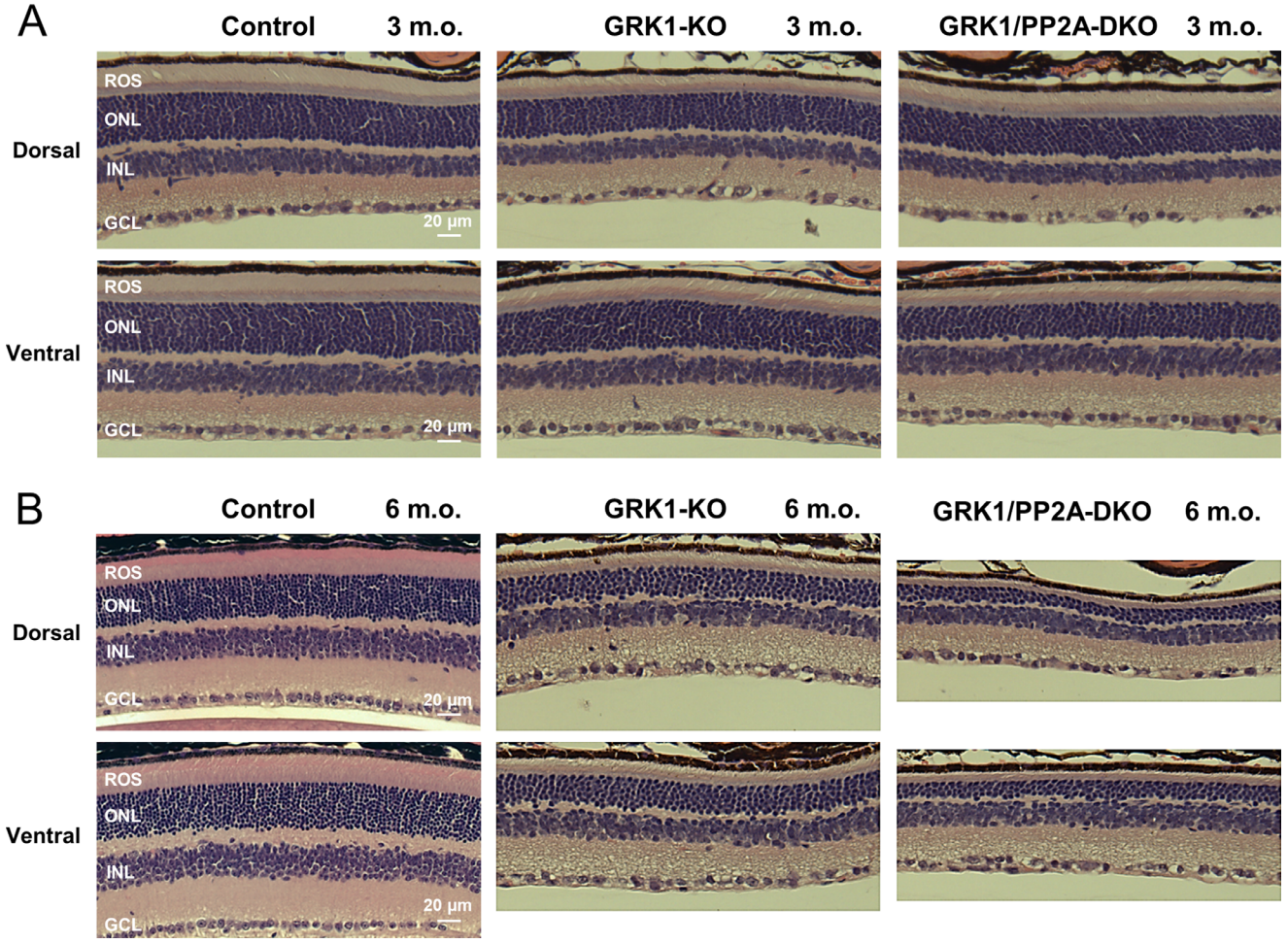

Figure 1. Morphological characterization of the retinas of mice lacking GRK1 alone or GRK1 and the PP2A-Cα subunit in their rods. (**A**) Comparison of retinal morphology in H&E-stained sections of dorsal (*top*) and ventral (*bottom*) parts of the retina from 3-month-old *iCre75*$^{+}$ control (*left*), GRK1-KO (*middle*), and GRK1/PP2A-DKO (*right*) mice. (**B**) Comparison of retinal morphology in H&E-stained sections of dorsal (*top*) and ventral (*bottom*) parts of the retina from 6-month-old *iCre75*$^{+}$ control (*left*), GRK1-KO (*middle*), and GRK1/PP2A-DKO (*right*) mice. In (**A**) and (**B**): ROS, rod outer segments; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bars, 20 µm.

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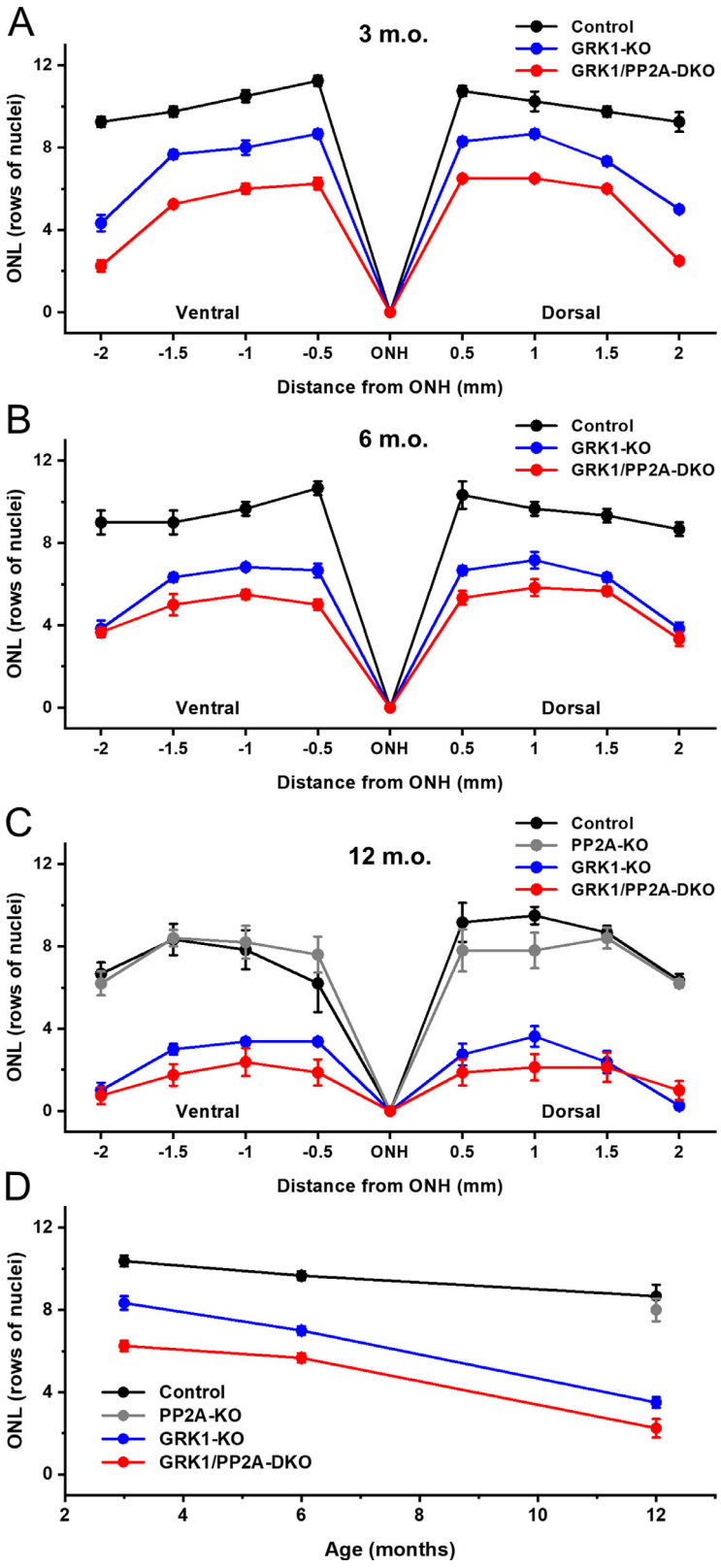

Figure 2. Quantification of age-dependent morphological changes in the retinas lacking GRK1 alone or GRK1 and the PP2A-Cα subunit in their rods. (**A**) The width of the ONL was measured in retinal sections at 500 µm intervals from the optic nerve head (ONH) in 3-month-old *iCre75*$^{+}$ control mice ($n = 4$), GRK1-KO mice ($n = 6$), and GRK1/PP2A-DKO mice ($n = 8$). (**B**) The ONL width was quantified in the same way in the retinas of 6-month-old *iCre75*$^{+}$ control mice ($n = 6$), GRK1-KO mice ($n = 6$), and GRK1/PP2A-DKO mice ($n = 6$). (**C**) The width of the ONL was measured across retinal sections in 12-month-old *iCre75*$^{+}$ control mice ($n = 6$), PP2A-KO mice ($n = 5$), GRK1-KO mice ($n = 8$), and GRK1/PP2A-DKO mice ($n = 8$). (**D**) Combined changes in the ONL thickness in the ventral and dorsal retina (at 1 mm from the ONH) over the span of 3 to 12 months of age in the four mouse lines indicated above (for PP2A-KO mice, ONL thickness was measured only at 12 months). In all panels: error bars represent the SEM.

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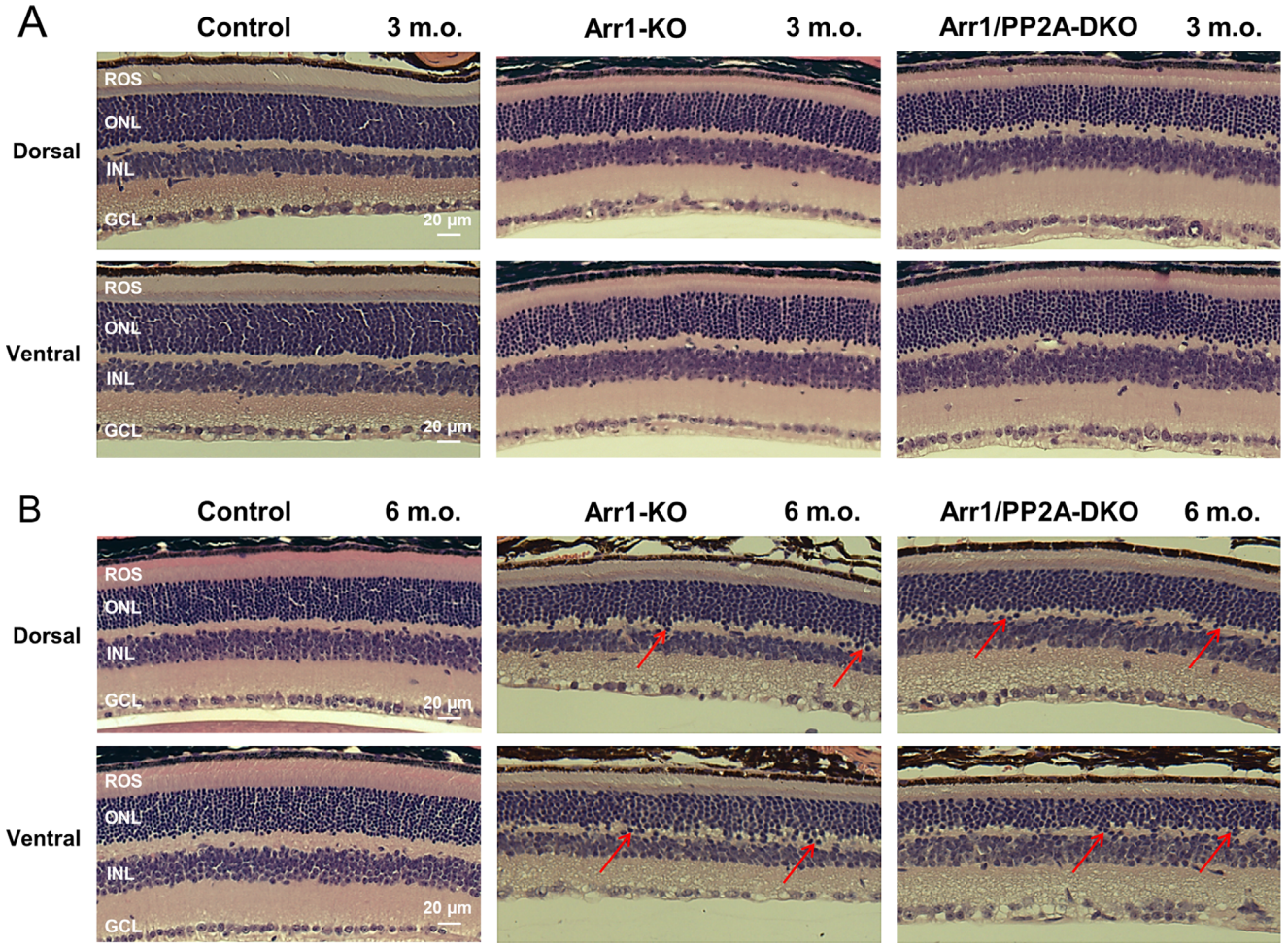

Figure 3. Morphological characterization of the retinas of mice lacking Arr1 alone or Arr1 and the PP2A-Cα subunit in their rods. (**A**) Comparison of retinal morphology in H&E-stained sections of dorsal (*top*) and ventral (*bottom*) parts of the retina from 3-month-old *iCre75*$^{+}$ control mice (*left*), Arr1-KO mice (*middle*), and Arr1/PP2A-DKO mice (*right*). (**B**) Comparison of retinal morphology in H&E-stained sections of dorsal (*top*) and ventral (*bottom*) parts of the retina from 6-month-old *iCre75*$^{+}$ control mice (*left*), Arr1-KO mice (*middle*), and Arr1/PP2A-DKO mice (*right*). *Red arrows* show mislocalized rod nuclei in the retinal plexiform layer. In (**A**) and (**B**): ROS, rod outer segments; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bars, 20 µm.

degeneration in 3-month-old GRK1-KO mice as compared to controls ($F_{(1,72)} = 391$, $P < 0.0001$) and its further advancement in age-matched GRK1/PP2A-DKO animals (Fig. 2A, blue and red symbols, respectively; $F_{(1,108)} = 344$, $P < 0.0001$). Thus, mice lacking GRK1 underwent mild degeneration at 3 months of age that was exacerbated by the deletion of PP2A. The advanced retinal degeneration in 6-month-old GRK1-deficient mice was also confirmed by their spider plot analysis ($F_{(1,90)} = 89$, $P < 0.0001$, compared to controls), with the GRK1/PP2A-DKO line demonstrating a more severe loss in the photoreceptor layer, as shown in Figure 2B ($F_{(1,90)} = 45$, $P < 0.0001$).

For our final time point, we examined retinal morphology in mice aged to 12 months. As reported previously, the deletion of PP2A-Cα did not induce retinal degeneration even at this advanced age. However, both control (*iCre75*$^{+}$) mice and PP2A-KO animals showed some age-dependent loss of photoreceptors and ONL thinning (see Fig. 2C, black and grey symbols, respectively) compared to the younger 3- and 6-month-old mice (see Figs. 2A, 2B, respectively). Retinal degeneration in GRK1-deficient mice progressed further at 12 months than at the younger ages ($F_{(1,108)} = 386$, $P < 0.0001$), with less than 4 nuclei per column remaining in both the dorsal and ventral areas of the retina (see Fig. 2C, blue symbols). Last, we examined the death of rods in mice lacking both GRK1 and PP2A and found that their degeneration also advanced beyond the earlier time points in this strain ($F_{(1,108)} = 143$, $P < 0.0001$), with only approximately 2 nuclei per column still remaining (see Fig. 2C, red symbols). The data followed the trend from earlier age points, with a slightly thinner ONL layer in the double knockout mice compared to GRK1-deficient animals ($F_{(1,126)} = 8.7$, $P = 0.0038$). Together, these data indicate that the degeneration in mice lacking GRK1 is exacerbated by PP2A deficiency across the whole retina except for its very peripheral regions, and this effect initially observed in 3-month-old mice persisted as the mice aged up to 12 months.

The overall time courses of degeneration in central areas of the retinas of GRK1- and GRK1/PP2A-Cα-deficient mice are shown in Figure 2D. Summarizing the results from all age groups, our data demonstrate that GRK1-deficient mice undergo more progressive photoreceptor degeneration with age as compared to that in controls ($F_{(1,26)} = 95$, $P < 0.0001$). The respective degeneration in mice lacking both GRK1 and

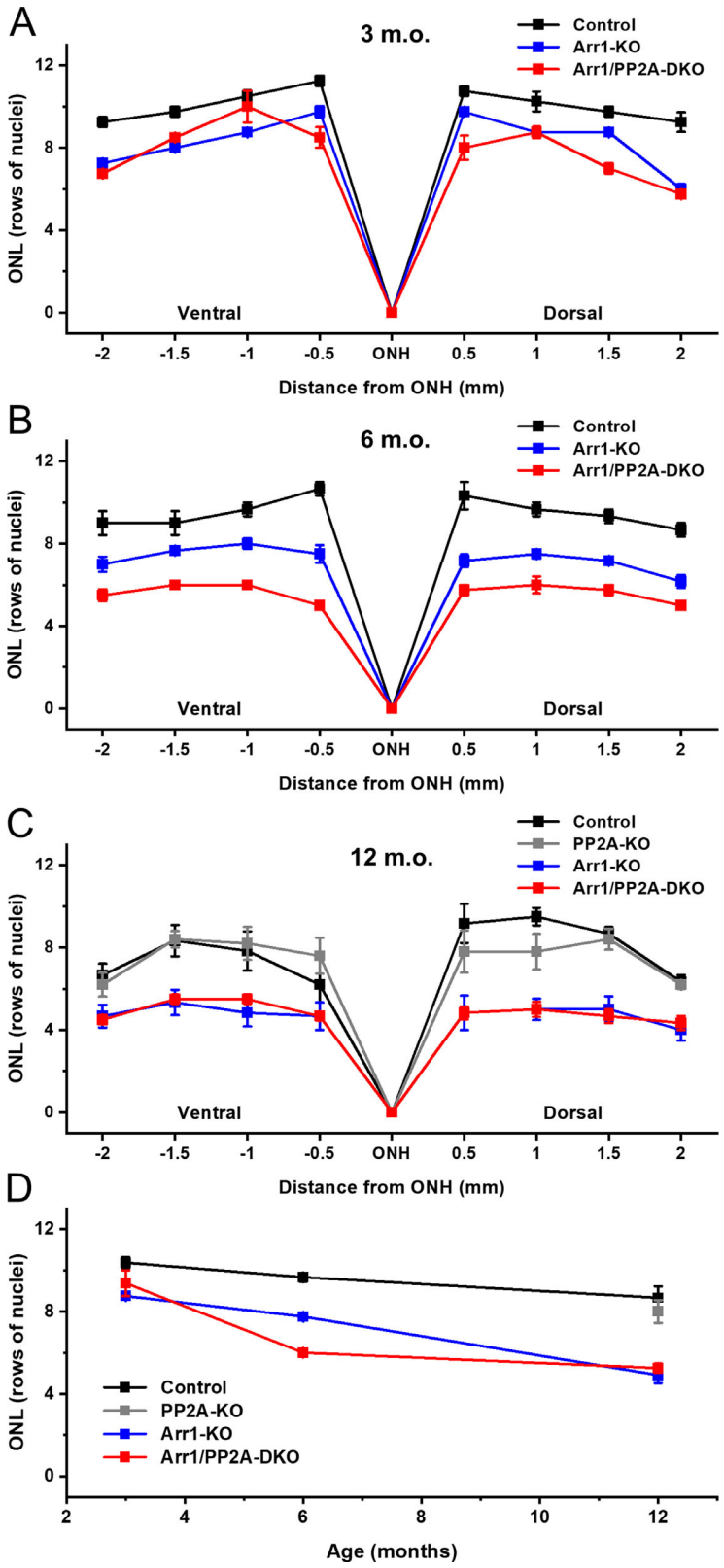

Figure 4. Quantification of age-dependent morphological changes in the retinas lacking Arr1 alone or Arr1 and the PP2A-Cα subunit in their rods. (**A**) The width of the ONL was measured in retinal sections at 500 µm intervals from the optic nerve head (ONH) in 3-month-old *iCre75*$^{+}$ control mice ($n = 4$), Arr1-KO mice ($n = 8$), and Arr1/PP2A-DKO mice ($n = 8$). (**B**) The ONL width was quantified in the same way in the retinas of 6-month-old *iCre75*$^{+}$ control mice ($n = 6$), Arr1-KO mice ($n = 6$), and Arr1/PP2A-DKO mice ($n = 4$). (**C**) The width of the ONL was measured across retinal sections in 12-month-old *iCre75*$^{+}$ control mice ($n = 6$), PP2A-KO mice ($n = 5$), Arr1-KO mice ($n = 6$), and Arr1/PP2A-DKO mice ($n = 6$). (**D**) Combined changes in the ONL thickness in the ventral and dorsal retina (at 1 mm from the ONH) over the span of 3 to 12 months of age in the four mouse lines indicated above (for PP2A-KO mice, ONL thickness was measured only at 12 months). In all panels: error bars represent the SEM.

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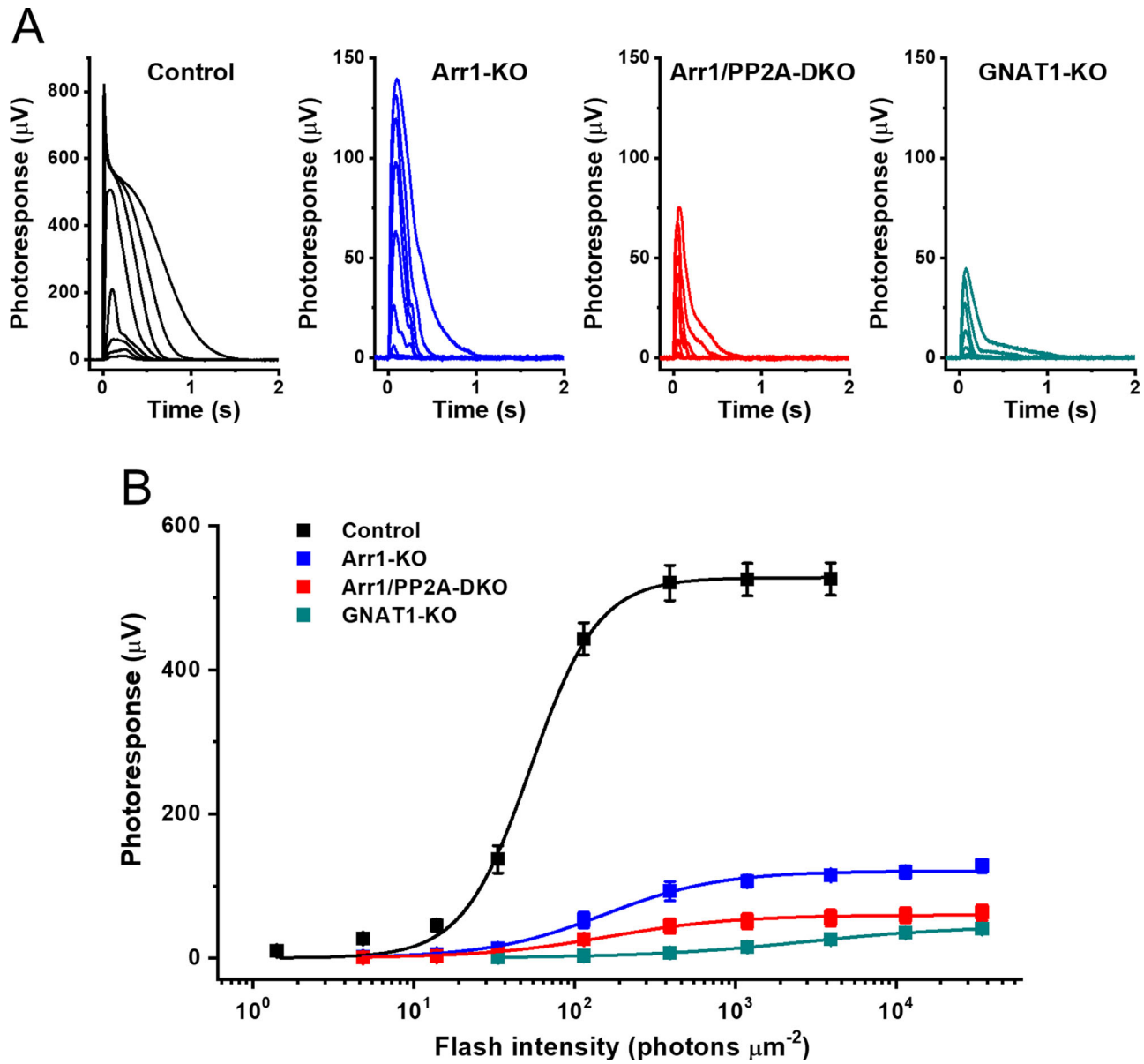

Figure 5. Ex vivo rod ERG responses of 3-month-old *iCre75*$^{+}$ control, Arr1-, and Arr1/PP2A-Cα-deficient mice. Averaged rod intensity-response functions (mean ± SEM) in the retinas from *iCre75*$^{+}$ control mice (*black*; $n = 6$), Arr1-KO mice (*blue*; $n = 4$), and Arr1/PP2A-DKO mice (*red*; $n = 4$). Also shown for comparison are data for retinas from GNAT1-KO mice (*green*; $n = 5$) in which only cones are functional. Points were fitted with hyperbolic Naka-Rushton functions (see Methods). The fits yielded half-saturating light intensities ($I_{1/2}$) of 53 photons μm^{-2} (control), 153 photons μm^{-2} (Arr1-KO retinas), 163 photons μm^{-2} (Arr1/PP2A-DKO retinas), and 2.7 × 10^{3} photons μm^{-2} (GNAT1-KO retinas).

PP2A-Cα is even more severe than that in GRK1-deficient mice ($F_{(1,64)} = 29, P < 0.0001$), suggesting that the absence of functional PP2A enzyme accelerates the retinal degeneration in mice lacking GRK1.

PP2A Deletion Accelerates Retinal Degeneration in Arr1-Deficient Mice

We next investigated how the absence of PP2A-Cα affects photoreceptor degeneration in mice lacking visual arrestin 1 (*Arr1*$^{-/-}$ mice). Analysis of 3-month-old animals revealed very mild rod degeneration in Arr1-deficient retinas compared to control (*iCre75*$^{+}$) retinas (Fig. 3A, center versus left panels). Notably, at this early age, retinas from mice lacking both PP2A and Arr1 were visibly indistinguishable from retinas of Arr1-deficient mice (see Fig. 3A, right panels), yet the 2-way ANOVA test revealed more ONL thinning in the double knockouts (see below). Thus, as in GRK1-KO mice, in mice lacking Arr1, retinal thickness at the young age was affected by the additional absence of PP2A, although the effect of PP2A deficiency was not as prominent here as in the GRK1-KO mice.

We next examined how the deletion of Arr1 and PP2A affected retinal morphology in 6-month-old mice. At this age, we could see clear retinal degeneration in mice lacking Arr1 compared to *iCre75*$^{+}$ controls (see Fig. 3B, center versus left panels). At this more advanced age, retinal

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degeneration was more extensive in mice lacking both Arr1 and PP2A compared to Arr1-deficient mice (see Fig. 3B, right versus center panels). Thus, the deletion of PP2A exacerbated retinal degeneration at 6 months in mice lacking Arr1. In addition, we observed that both Arr1-deficient lines had a greater number of mislocalized rod nuclei in their outer plexiform retinal layer (see Fig. 3B, red arrows) compared to age-matched control and GRK1-KO lines, which could be important for future research.

As in the case of GRK1-deficient mice, we again quantified and compared the level of retinal degeneration in Arr1-deficient mice with spider plots. This analysis confirmed the mild degeneration in Arr1-KO mice at 3 months as compared to controls ($F_{(1,90)} = 204$, $P < 0.0001$). Notably, degeneration was slightly more advanced in Arr1/PP2A-DKO retinas (Fig. 4A, compare blue and red symbols; $F_{(1,126)} = 8.4$, $P = 0.0045$). At 6 months, degeneration became more pronounced in the Arr1-KO line as compared to controls ($F_{(1,63)} = 150$, $P < 0.0001$), and clearly exacerbated in Arr1/PP2A-DKO compared to Arr1-KO retinas (see Fig. 4B; $F_{(1,72)} = 131$, $P < 0.0001$).

Last, we quantified how retinal degeneration is affected by Arr1 and PP2A deficiency in 12-month-old mice. At this advanced age, mild degeneration was observed in both control (*iCre75*$^{+}$) and PP2A-KO retinas, as compared to their respective earlier ages (see Fig. 4C, black and gray symbols, correspondingly). As expected, retinal degeneration was more severe in mice lacking Arr1 (see Fig. 4C, blue symbols; $F_{(1,89)} = 77$, $P < 0.0001$). The additional removal of PP2A did not appear to further drive retinal degeneration at this age, as the thickness of the ONL in Arr1/PP2A-deficient mice was comparable to that in Arr1-deficient mice (see Fig. 4C, red versus blue symbols; $F_{(1,90)} = 0.12$, $P = 0.73$).

The analysis of retinal degeneration in central retinal areas in animals lacking Arr1 or both Arr1 and PP2A-Cα over a substantial part of their lifespan is presented in Figure 4D. It is evident that whereas the absence of PP2A-Cα accelerated the retinal degeneration at the intermediate age of 6 months in mice lacking Arr1 (Bonferroni post hoc analysis demonstrated statistically significant difference between the two strains at this time, $P < 0.0001$), it did not affect either early or final stages of photoreceptor loss at 3 and 12 months of age, respectively ($P > 0.05$ for both ages, as revealed by the same Bonferroni test). Therefore, 2-way repeated measures ANOVA did not reveal an overall significant effect of genotype in this case ($F_{(1,22)} = 0.92$, $P = 0.35$).

PP2A Deletion Exacerbates the Loss of Visual Function in Arr1-Deficient Mice

In contrast to the case of GRK1-deficient mice where rod response recovery is exceedingly slow due to prolonged saturation of the phototransduction cascade, we were able to obtain light responses from Arr1-deficient rods using transretinal ERG recordings (Fig. 5A). This enabled us to investigate independently and quantitatively the function of rods in 3-month-old Arr1-deficient mice and Arr1/PP2A-DKO mice. The responses in both cases were severely reduced relative to wild-type controls (see Fig. 5B, blue versus black symbols, $F_{(1,96)} = 1206$, $P < 0.0001$; and red versus black symbols, $F_{(1,96)} = 1511$, $P < 0.0001$, respectively). To confirm that the responses obtained in the Arr1-deficient retinas were indeed generated by rods and not by cones, we also performed control cone recordings. We collected light responses from isolated retinas where the rods are unable to respond to light due to the absence of the α-subunit of the rod G protein transducin, encoded by the *Gnat1* gene (*Gnat1*$^{-/-}$ mice).[26] We observed robust responses in the retinas of *Gnat1*$^{-/-}$ mice (see Fig. 5B, cyan symbols). However, these cone-driven responses were smaller in amplitude and at least an order of magnitude less sensitive than those from Arr1-deficient ($F_{(1,84)} = 546$, $P < 0.0001$) and Arr1/PP2A-deficient ($F_{(1,84)} = 65$, $P < 0.0001$) retinas. We therefore conclude that the responses from retinas lacking Arr1, although small, are indeed largely generated by rods. Comparison of the responses in Arr1-deficient and Arr1/PP2A-deficient mice revealed that rod function in animals lacking both Arr1 and PP2A was further suppressed compared to the function of rods in Arr1-KO mice (see Fig. 5B, red and blue symbols, respectively, $F_{(1,72)} = 91$, $P < 0.0001$). Thus, consistent with the slightly more advanced retinal degeneration in Arr1/PP2A-DKO mice compared to Arr1-deficient mice at 3 months (see Figs. 3, 4A), the function of rods in mice lacking both Arr1 and PP2A at that age was affected more severely than in animals lacking Arr1 alone.

RNA-Sequencing Analysis of PP2A Subunit Expression in Mouse Retina

The results above demonstrate that deletion of the catalytic subunit of PP2A exacerbates the degeneration and functional decline in the retinas of both GRK1- and Arr1-deficient mice. Although the putative role of PP2A-Cα is to dephosphorylate photoactivated rhodopsin,[22] the observed enhanced degeneration in our GRK1/PP2A-DKO mice is unlikely linked to rhodopsin dephosphorylation, as this

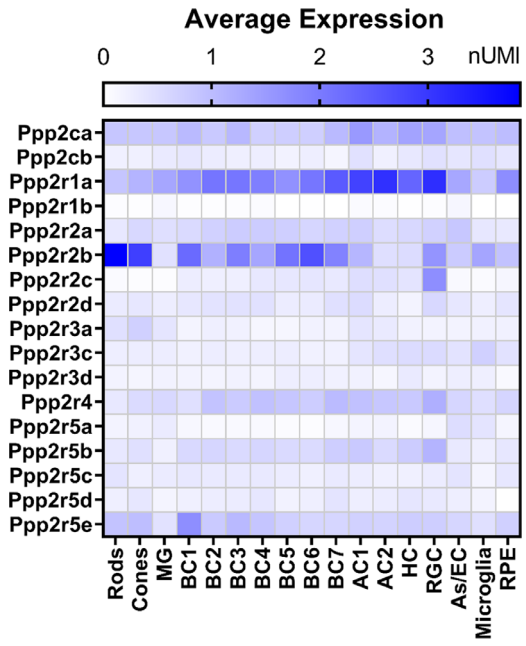

Figure 6. Average gene expression of different protein phosphatase subunits in various cell types of the wild-type mouse retina and RPE, as determined by single-cell RNA-sequencing (scRNA-Seq) analysis and measured in normalized counts of unique molecular identifiers (nUMI).[30] MG, Müller glia; BC1–7, bipolar cells type 1–7; AC1–2, amacrine cells type 1–2; HC, horizontal cell; RGC, retinal ganglion cell; As/EC, astrocytes/endothelial cells; RPE, retinal pigmented epithelium.

process is disrupted in the absence of GRK1. Thus, we hypothesize that PP2A is also involved in the regulation of other homeostatic processes in photoreceptors, as well as the rest of the retina. Indeed, PP2A has previously been implicated in the regulation of Müller glial activity and retinal neuroprotective pathways.[31,32] Our single-cell RNA-sequencing analysis of wild-type murine retina confirmed that the catalytic Cα-subunit of PP2A (gene symbol *Ppp2ca*) is indeed natively expressed in both rod and cone photoreceptors, as well as in every other retinal cell type (Fig. 6). This analysis also demonstrated that genes of regulatory PP2A subunits *Ppp2r1a*, *Ppp2r2a*, *Ppp2r2b*, *Ppp2r4*, and *Ppp2r5e* are also abundantly expressed across all retinal cells. Taken together, these data suggest an important role of PP2A not only in the dephosphorylation of rod and cone opsins for pigment regeneration and photoreceptor dark adaptation, but also in maintaining homeostasis across the many distinct cell types found throughout the retina.

Discussion

In this study, we examined the role of protein dephosphorylation by PP2A in modulating the degeneration of mammalian rods. Our results demonstrate that the rod-specific deletion of the major catalytic Cα-subunit of PP2A in *Ppp2ca*$^{f/f}$ *iCre75*$^{+}$ mice accelerates the retinal degeneration observed in both *Grk1*$^{-/-}$ and *Arr1*$^{-/-}$ mice. As rods lacking PP2A-Cα alone do not undergo degeneration, these results indicate that the deletion of PP2A modulates the retinal degeneration induced by the absence of GRK1 or Arr1 in rods.

It is well established that blockade of rhodopsin phosphorylation by knocking out GRK1 in mice causes severe retinal degeneration.[3] In patients, mutations in GRK1 cause Oguchi disease, a form of night blindness recently shown to be progressive.[9] The prevailing view is that degeneration in the absence of GRK1 is due to persistent phototransduction activation by unphosphorylated metarhodopsins.[33] However, light-independent and phototransduction-independent degeneration in GRK1-deficient mice has also been documented,[34] pointing to a more complex mechanism that may involve multiple degenerative pathways mediated by the absence of rhodopsin kinase.

Indeed, in addition to reduced or absent phosphorylation due to the loss of functional GRK1, abnormally high phosphorylation of rhodopsin has also been linked to retinal degeneration. For instance, the suppression of rhodopsin dephosphorylation by rdgC in *Drosophila* causes extensive light-dependent retinal degeneration.[35] Similarly, enhanced rhodopsin phosphorylation caused by overexpression of GRK1 in transgenic mice also increases the susceptibility of rods to degeneration.[36] A possible link between rhodopsin hyperphosphorylation and retinal degeneration also has been suggested in RCS rats[37] and rhodopsin dephosphorylation is dramatically delayed in P23H rats, with phosphorylated P23H-rhodopsin persisting days after exposure to light.[38] Our results presented here clearly show that the deletion of PP2A accelerates rod degeneration in *Grk1*$^{-/-}$ mice. However, the mechanism of this modulation is not likely to involve rhodopsin, as this protein is not phosphorylated in this line,[3] thus eliminating the need for dephosphorylation. Instead, the blockade of catalytic activity of PP2A is likely to exert its effect on exacerbating rod degeneration by suppressing the dephosphorylation of other, yet unidentified, proteins in the rods. One such potential target is phosducin, an abundant rod phosphoprotein that is likely dephosphorylated by PP2A in a light-dependent manner.[20,39] Normally, phosducin is phosphorylated in darkness and dephosphorylated in light. However, in *rd1* mice, a model of retinitis pigmentosa, phosducin is persistently phosphorylated regardless of light conditions.[40] Interestingly, the increased phosducin phosphorylation in *rd1* mice coincided with increased activation of calcium/calmodulin-activated kinase II, possibly representing an early step in photoreceptor degeneration. As phosducin has been suggested to be dephosphorylated by PP2A, it would be expected that deletion of PP2A would also result in increased phosducin phosphorylation. This, combined with the cellular stress caused by the persistent transduction activation in *Grk1*$^{-/-}$ and *Arr1*$^{-/-}$ mice might explain the accelerated retinal degeneration in these mice caused by the deletion of PP2A. Although the role of phosducin remains to be demonstrated, the increased degeneration in GRK1-deficient rods induced by the deletion of PP2A-Cα indicates a novel rhodopsin phosphorylation-independent mechanism for modulating retinal degeneration, which could be better elucidated by follow-up studies utilizing advanced phosphoproteomic methods and analyses.

Another model of Oguchi disease, the *Arr1*$^{-/-}$ mouse, also undergoes progressive retinal degeneration.[11] Arrestin has been shown to suppress rhodopsin dephosphorylation in vitro[12] but stimulates it in vivo,[13] suggesting that the mechanisms of degeneration in *Arr1*$^{-/-}$ mice could be linked to abnormal rhodopsin dephosphorylation. Whereas rhodopsin dephosphorylation would be expected to be reduced in *Arr1*$^{-/-}$ mice in vivo, the simultaneous deletion of PP2A-Cα would render the dephosphorylation of rhodopsin even less efficient. It is possible that the lack of arrestin allows the other PP2A isoform expressed in rods, PP2A-Cβ, to compensate functionally for the lack of PP2A-Cα. In addition, the gene of one of the PP2A regulatory subunits, *Ppp2r2b*, was also expressed at high levels in both rod and cone photoreceptors, prompting future studies to delineate its role in the function and survival of these cells. Notably, however, we found no evidence for compensatory upregulation in the expression of PP2A-Cβ gene in the absence of PP2A-Cα.[22] Regardless of the exact mechanism, our previous work has demonstrated that, while delayed, rhodopsin dephosphorylation in PP2A-Cα-deficient mouse rods still occurs within a few hours of dark-adaptation.[22] This persistent PP2A-Cα-independent rhodopsin dephosphorylation could possibly prevent excessive accumulation of phosphorylated rhodopsin and the resulting retinal degeneration. Indeed, our results demonstrate that the effect of PP2A-Cα deletion on retinal degeneration in *Arr1*$^{-/-}$ mice was relatively small and transient.

Interestingly, the deletion of PP2A-Cα also resulted in further reduction in the already small photoresponses from Arr1-deficient rods at 3 months of age. As stated above, even in the absence of PP2A, rhodopsin dephosphorylation (which is a much slower process than phosphorylation) is finally achieved after overnight dark-adaptation and the rod responses are normal in PP2A-deficient rods after several hours in the dark.[22] Thus, we do not believe that the reduced responses in dark-adapted Arr1/PP2A-DKO mice are the result of aberrant activation of transducin or delayed rhodopsin regeneration. Instead, the most likely explanation is that the electrophysiological measurements provide

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a more sensitive and accurate measure than histology of the level of degeneration and general health of rods in the retinas of Arr1/PP2A-DKO versus Arr1-KO mice. In this case, the reduced rod responses in 3-month-old DKO mice could be attributed to their slightly more severe degeneration compared to Arr1-KO mice. This subtle histological change could not be visualized easily from examining their retinal sections and could only be established by the ANOVA test. In contrast, the decline in the responses from rods in Arr1/PP2A-DKO mice compared to those of Arr1-deficient mice was readily observable in our transretinal ERG recordings.

In addition to their well-established role in the timely inactivation of rhodopsin and the termination of the light response, GRK1 and Arr1 also control the subsequent thermal decay of photoactivated rhodopsin into all-trans-retinal and free opsin. Indeed, the decay of long-living intermediate metarhodopsin III accelerates with increasing Arr1 expression and in the absence of GRK1.[41] Notably, this modulation of rhodopsin decay also results in altered dark adaptation of rods in vivo. Our current results extend our understanding of the ways in which GRK1 and Arr1 modulate the physiology of rod photoreceptors and demonstrate that the retinal degeneration induced by their absence is exacerbated with suppression of protein dephosphorylation by PP2A.

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