Cone arrestin confers cone vision of high temporal resolution in zebrafish larvae

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Abstract
Vision of high temporal resolution depends on careful regulation of photoresponse kinetics, beginning with the lifetime of activated photopigment. The activity of rhodopsin is quenched by high-affinity binding of arrestin to photoexcited phosphorylated photopigment, which effectively terminates the visual transduction cascade. This regulation mechanism is well established for rod photoreceptors, yet its role for cone vision is still controversial. In this study we therefore analyzed arrestin function in the cone-dominated vision of larval zebrafish. For both rod (arr5) and cone (arr3) arrestin we isolated two paralogs, each expressed in the respective subset of photoreceptors. Labeling with paralog-specific antibodies revealed subfunctionalized expression of Arr3a in M- and L-cones, and Arr3b in S- and UV-cones. The inactivation of arr3a by morpholino knockdown technology resulted in a severe delay in photoresponse recovery which, under bright light conditions, was rate-limiting. Comparison to opsin phosphorylation-deficient animals confirmed the role of cone arrestin in late cone response recovery. Arr3a activity partially overlapped with the function of the cone-specific kinase Grk7a involved in initial response recovery. Behavioral measurements further revealed Arr3a deficiency to be sufficient to reduce temporal contrast sensitivity, providing evidence for the importance of arrestin in cone vision of high temporal resolution.

Introduction
The visual system of vertebrates is effective over a wide range of light intensities as it exploits different types of photoreceptor cells. Rod photoreceptors are exceptionally light sensitive, with their transduction cascade tuned for maximal quantum yield. Cone photoreceptors are less sensitive but allow vision of high temporal and spatial resolution, which necessitates fast response kinetics (reviewed in Rodieck, 1998 and Kawamura & Tachibana, 2008).

Signal amplification and temporal aspects of the photoresponse are precisely regulated by controlling the lifetime of activated photopigment. In the rod phototransduction cascade, phosphorylation of photoexcited rhodopsin reduces the efficacy of transducin activation. Subsequent high-affinity binding of the arrestin protein (Arr) to activated and phosphorylated rhodopsin completely quenches signal transduction by competing with the G-protein transducin for rhodopsin binding (Krupnick et al., 1997). Deficiency in this quenching mechanism in rods causes congenital stationary night blindness in humans but leaves photopic vision unaffected (Fuchs et al., 1995; Yamamoto et al., 1997).

The identification of a cone-specific G-protein-coupled receptor kinase, GRK7 (Hisatomi et al., 1998), and a cone arrestin protein (Craft et al., 1994) strongly supports the hypothesis that, similarly to rods, normal cone response kinetics depend on regulated pigment inactivation.

Impaired cone photopigment phosphorylation has been shown to cause a prolonged photoresponse (Lyubarsky et al., 2000; Nikonov et al., 2005, 2008; Shi et al., 2007) and a delay in photoresponse recovery (Rinner et al., 2005). Furthermore, studies in carp retina have revealed that fast cone response kinetics depend on distinct molecular characteristics of GRK7. Compared to GRK1 found in rods and (in some species) cones, higher expression levels and phosphorylation activity of GRK7 account for fast inactivation of photoexcited opsin and low transducin activation in cones (Tachibana et al., 2005, 2007).

The function of cone arrestin in the final shutoff of cone pigment and its impact on cone response kinetics or visual function is still elusive. In functional studies of transgenic mice expressing S-opsin in rhodopsin-deficient rods the inactivation of S-pigment was slowed in the absence of rod arrestin (Arr1 in the mouse). The prolonged photoresponse of these transgenic rods could not be rescued by expression of cone arrestin (Arr4 in the mouse), indicating that cone arrestin is not sufficient to regulate photoexcited S-opsin (Shi et al., 2007).

Recently, co-expression of mouse Arr1 and Arr4 in the outer segments of cones with higher protein levels of rod arrestin has been demonstrated (Nikonov et al., 2008). Flash responses of cones lacking either cone or rod arrestin did not show any severe impairment of pigment recovery. However, cone response inactivation in arrestin double knockouts was slowed down, which indicates that arrestin is involved in quenching photoexcited cone pigment.

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To directly address the function of cone arrestin in photopigment inactivation and the impact of arrestin on photopic vision we utilized the cone-dominated visual system of zebrafish (Danio rerio) larvae (Bilotta & Saszik, 2001; Fadool & Dowling, 2008). We found that functional loss of cone arrestin causes a prolonged photoresponse recovery and reduced temporal contrast sensitivity of zebrafish larval vision.

Materials and methods

Fish husbandry
Zebrafish of the Tü strain were bred and maintained under standard conditions at 28 °C and a 14 h light and 10 h dark cycle (Westerfield, 1994). Larvae were raised in E3 medium containing (in mM): NaCl, 5; KCl, 0.17; CaCl₂, 0.33; MgSO₄, 0.33; and methylene blue, and were staged in days post-fertilization (dpf) according to Kimmel et al. (1995).

Annotation of arrestin cDNAs
As many genes predicted within GenBank are produced by automated processes and have been shown to contain numerous errors, arrestin cDNA sequences used in this study were manually annotated. Sequences were identified and annotated using combined information from expressed sequence tags and genome databases (GeneBank, http://www.ncbi.nlm.nih.gov; Ensembl, http://www.ensembl.org/index.html; version 50/51, 2008). Human and mouse sequences were used as initial query (for more details on sequence annotation see Gesemann et al., 2010).

Phylogenetic tree analysis
Coding sequences of arrestin genes were translated into proteins using the EditSeq software (Lasergene; DNASTAR, Madison, WI, USA) and obtained protein sequences were used to generate a combined sequence file in FASTA. Sequence alignment and phylogenetic analysis was performed on the Phylogeny.fr platform (Dereeper et al., 2008; http://www.phylogeny.fr/version2.cgi/phylogeny.cgi). Sequences were aligned using MUSCLE (v3.7; Edgar, 2004) configured for highest accuracy (MUSCLE with default settings). After alignment, ambiguous regions (i.e. containing gaps and/or being poorly aligned) were removed with GBLOCKS (v0.91b; Castresana, 2000). The phylogenetic tree was reconstructed by the maximum likelihood method (Guindon & Gascuel, 2003) using the WAG amino acid replacement matrix (Whelan & Goldman, 2001) implemented in the PhyML program (v3.0). The approximate likelihood ratio test (aLRT; Anisimova & Gascuel, 2006) was used to judge branch reliability. Graphical representation and editing of the phylogenetic tree was done using TREEDyn (v198.3) and the obtained svg files were colored using the CORELDRAW program.

Cloning of arrestin genes
Total RNA was isolated from larvae 5 dpf using the RNAeasy kit (Qiagen, Hombrechtikon, Switzerland) and cDNA was synthesized using the SuperScript II Reverse Transcriptase kit (Invitrogen, Basel, Switzerland) according to manufacturer’s instructions.

For identification arrestin genes, the coding sequences were amplified from cDNA using the following primer pairs: arr3a sense ATGGCC-TGACAAAGGTATCAGAAAG, antisense GCCCTGTGGAATCTGGATATG; arr3b sense CATGACAAAGGTATCAGAAAG, antisense TGCTCTCCTAGGCTGGTTAG; arrSa sense CAATGAGTCCAAAGATGTCG, antisense TAAACAGGAATGTCCTTTTCT; arrSb sense ATGAGTCCCGACACATCT, antisense CAGCAG-CTCAAAACAGG. Products were cloned into the pCRII-TOPO vector (Invitrogen) and sequenced using T7 and SP6 primers.

In situ hybridization (ISH)
Arrestin sense and antisense RNA probes were transcribed in the presence of digoxigenin-labeled nucleotides (DIG RNA Labeling Mix; Roche, Rotkreuz, Switzerland) using SP6 and T7 polymerases (Roche), respectively. For the rhodopsin antisense RNA probe T7 polymerases (Roche) were used. Transcripts were hydrolyzed to yield fragments of approximately 300–500 nt in length. For ISH, zebrafish larvae were fixed in 4% paraformaldehyde (PFA), dehydrated and stored in methanol until usage. To reduce pigmentation, larvae were bleached with 3% H₂O₂ and 1% KOH directly before ISH. For mRNA detection in adult eyes, retinas from albino zebrafish were cryosectioned to 14 μm thickness. ISH was carried out in an automated ISH apparatus (Hölle&Hütten, Tübingen, Germany) according to Thisse & Thisse (2008). RNA probes were hybridized overnight at 58 °C and detected using an alkaline phosphatase-based color reaction.

Generation of antibodies
A highly immunogenic hydrophilic peptide specific for either Arr3a or Arr3b but not conserved in ArrS (Supporting Information Fig. S1) has been chosen for immunization. Rabbits were immunized with the Arr3a peptide AKSADDPEKVDKDKTC (147–163) whereas chicken were immunized with the Arr3b peptide ANE-EDNIDEKVEKKDTC (146–162). All antibodies were affinity-purified against the respective peptide by Eurogentec (Seraing, Belgium).

Immunohistochemistry
Immunohistological staining was performed as previously described (Fleisch et al., 2008). For detection of Arr3b, tissue was fixed in 2% trichloroacetic acid (Sigma-Aldrich, Switzerland) for 30 min at room temperature, equilibrated in 30% sucrose and embedded in Tissue Tek (Sakura Finetek Europe, Netherlands). Cryosections of 18 μm thickness were cut. Rabbit anti-Arr3a antibody was used in a 1: 400 dilution and chicken anti-Arr3b antibody in a 1: 250 dilution. The following antibodies were used for co-labeling: Zpr1 antibody (1: 400; Zebrafish International Resource Center, Eugene, OR, USA), rabbit anti-blue opsin and rabbit anti-ultraviolet (UV) opsin (1: 500; kindly provided by David R. Hyde, University of Notre Dame, Notre Dame, IN, USA). For visualization, the secondary antibodies Alexa Fluor 488 goat anti-chicken or goat anti-mouse (1: 1000; Invitrogen) and Alexa Fluor 568 goat anti-mouse or goat anti-rabbit (1: 500; Invitrogen) were applied.

Histology
Larvae were fixed at room temperature for 50 min in 4% PFA in 0.2 M phosphate buffer (pH 7.4). Fixed larvae were dehydrated in a standard ethanol series, infiltrated and embedded in Technovit 7100 (Kulzer Histotechnik, Wehrheim, Germany). Sections (3 μm) were cut and mounted on slides. For contrast, tissue was stained with Richardson

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Microscopy

Histological sections were imaged under a BX61 microscope (Olympus) equipped with a ColorView IIIu digital camera and edited using Adobe Photoshop CS2 (Adobe Systems). Z-stacks of immunostainings were recorded using a CLSM SP2 confocal laser scanning microscope (Leica) and processed in IMARIS 7.0 (Bitplane).

Western blot

Eyes of 35 zebrafish larvae, 5 dpf, were isolated and sonicated in 40 μL Ringer solution (in mM: NaCl, 116; KCl, 2.9; CaCl2, 1.8; and HEPES, 5; pH 7.4) containing serine and cysteine protease inhibitors (complete, Mini, EDTA-free; Roche). Insoluble material was removed by ultracentrifugation. The total protein concentrations were measured using the DC Protein Assay (Bio-Rad, Reinach, Switzerland) according to manufacture’s instruction before samples were mixed with 3X Urea buffer [Tris, 65 mM, pH 6.75; urea, 8 M; glycine, 20%; β-mercaptoethanol, 5%; SDS, 5%; and bromphenol blue]. As loading control, acetylated tubulin of approximately 50 kDa was detected simultaneously with Arr3a or Arr3b by mouse anti-acetylated tubulin antibody (Sigma-Aldrich). Immunoblots were developed using the ECL system (Supra Signal West; Thermo Scientific, Applied Biosystems, Switzerland) and chemiluminescence was detected with the Fujifilm LAS-3000 imaging system. For the detection of Arr3 and acetylated tubulin different exposure times were applied. The arrestin knockdown was semi-quantitatively evaluated in IMAGEJ (NIH).

Targeted gene knockdown

Antisense morpholino oligonucleotides (MOs) covering the translational start site were obtained from Gene Tools (Philomath, Oregon, USA): arr3a-MO (5’-ATAATCCGACCCCCGTCTGGTAG-3’), arr3b-MO (5’-CAAGTTCTGAGCTTTTCAAGTTCTG-3’) and grk7a-MO (5’-ATCGAGTCCCCCCATGTCACACATT-3’). For control, a standard control MO (5’-CCTTCTACCTAGTTACAATTATA-3’) was used.

All MOs were dissolved in ddH2O to a stock concentration of 2 mM. Prior to injection, MOs were diluted in 1× Danieau’s solution [in mM: NaCl, 58; KCl, 0.7; MgSO4, 0.4; Ca(NO3)2, 0.6; HEPES, 5; and phenol red, 0.2%] to the desired concentration. If not indicated differently, embryos were injected in the one- and two-cell stage with 5 ng control-MO, 4.2 ng arr3a-MO, 27 ng arr3b-MO or 10.8 ng grk7a-MO. For double-knockdown experiments we injected 2.8 ng arr3a-MO and 13 ng arr3b-MO.

Electroretinography

Electroretinograms (ERGs) were recorded from unanesthetized zebrafish larvae as described previously (Makhankov et al., 2004). Larvae were first dark-adapted for at least 40 min. The following pre-recording steps were carried out under dim red illumination.

For paired flash recordings, a 500 ms conditioning flash (7000 or 70 lux) followed by a probing flash of the same light intensity and duration was presented. The interstimulus interval between conditioning and probing flash was varied (1, 2, 3, 5, 10 or 20 s). Cone response recovery was calculated as the ratio of b-wave peak amplitudes to conditioning and probe flash (Rinner et al., 2005). The b-wave amplitudes were measured as the range between the minimum and maximum potential within 200 ms of light onset.

Psychophysics

The optokinetic response of zebrafish larvae was measured using the experimental setup described in Mueller & Neuhaus (2010). For immobilization, larvae were placed dorsal up in a 35 mm Petri dish containing 3% methylcellulose.

An LCD Projector (VPL-CX1; Sony Corporation) was used to present a vertical sinusoidal grating pattern on a white paper drum (r = 4.5 cm) with the grating rotating around the larvae. Eye angle and velocity were recorded by means of an infrared-sensitive CCD camera (Guppy F-038B NIR; Allied Vision Technologies). Custom-developed software based on LabVIEW 7.1 and NI-IAQ 3.7 (National Instruments) was used to control stimulation and to record eye movements (Mueller & Neuhaus, 2010).

To determine temporal sensitivity at different illumination levels a sine-wave grating of varying pattern velocity was presented. Stimulus intensity was adjusted via the color values defining the stimulus. Prior to recording, larvae were adapted for 60 s to background light levels of 20 or 120 cd/m2 and then pre-stimulated for 9 s with a low temporal frequency pattern (0.033 cycles/deg) at a angular velocity of 3.5 deg/s. Eye velocity was measured from larvae stimulated with a grating pattern of 0.083 cycles/deg and varying angular velocities of 3.5, 5.25, 7, 14, 21 and 28 deg/s.

Statistical analysis

For electrophysiological experiments, statistical significance was calculated in GraphPad PRISM 4 (GraphPad Software). The b-wave peak amplitude recovery of morphants was compared using two-way ANOVA with Bonferroni’s post-test. For behavioral measurements the eye velocity of the two eyes, the left and the right, were averaged and data were analyzed in PASW Statistics 17.0 (SPSS Inc.) using repeated-measures ANOVA with angular velocity as within-subject effect and genotype as between-subjects effect. Graphs were generated in GraphPad PRISM 4.

Results

The zebrafish genome harbors four visual arrestin genes

To investigate the role of arrestin function in cone vision, we first used a combined bioinformatic and cDNA cloning approach and identified paralogs of the mammalian cone arrestin (arrestin 3, arrestin 4 or X-arrestin). We denoted the two identified zebrafish paralogs as arr3a and arr3b. Similarly, we isolated two rod arrestin paralogs of the mammalian ortholog (arrestin 1, S-arrestin or retinal S-antigen), denoted by arr5a and arr5b. For the two broadly expressed mammalian β-arrestin genes we isolated three orthologs, namely the paralogs β-arr2a, β-arr2b and β-arr1a. The additional arrestin genes found in the zebrafish genome probably originated from the teleost-specific whole-genome duplication event (Postlethwait et al., 2000), as radiation hybrid mapping determined all four visual arrestins to locate on different chromosomes (Supporting Information Table S1). Pairwise amino acid sequence alignment of the visual arrestins revealed that protein identity between two zebrafish paralogs did not exceed 70% (Supporting Information Table S2).
To confirm that the identified zebrafish paralogs are true orthologs of the respective mammalian genes, we performed a phylogenetic analysis, which supported our assumption (Supporting Information Fig. S2).

**Visual arrestins are expressed in a complementary manner in zebrafish photoreceptor subtypes**

In order to determine the cellular distribution of visual arrestin transcripts in the zebrafish retina, we performed whole-mount in situ hybridization experiments with sequence-specific riboprobes at different developmental stages.

At 3 dpf the two cone arrestin paralogs showed distinct expression patterns: arr3a was evenly expressed throughout the photoreceptor cell layer (PRCL) whereas arr3b expression was confined to the ventral half of the retina (Fig. 1A and B). Both arrS transcripts were, in contrast, detected in a ventrotemporal patch (Fig. 1C and D), consistent with expression of rod photoreceptor markers (Raymond et al., 1995; Hamaoka et al., 2002). At 5 dpf arrS transcripts appeared sporadically over the PRCL with prominence at the ventral side whereas mRNAs for both arr3 paralogs were detected evenly throughout the PRCL of the retina (Fig. 1E–H). These expression patterns are consistent with the differentiation of cone (arr3) and rod (arrS) photoreceptors in the developing zebrafish retina (Raymond et al., 1995).

No mRNA transcripts of β-arr genes were found in photoreceptor cells of the zebrafish retina at 3 and 5 dpf (data not shown).

To elucidate if this cell type-specific visual arrestin expression is preserved throughout development we next analyzed adult retina sections. arrS mRNAs could be found in the inner most part of the outer nuclear layer where the nuclei of rods are located (Fig. 1K and L). Comparison of arrS expression with rhodopsin expression confirmed the specificity of both arrS paralogs for rod photoreceptors (Supporting Information Fig. S3).

The expression pattern of cone arrestin transcripts was complementary to the expression of rod arrestins in the adult retina. While arr3a mRNA was detected in the inner segment of most cone photoreceptors, arr3b expression was restricted to a small subset of cone cells (Fig. 1I and J).

To further investigate this distinct expression of arr3a and arr3b, we raised peptide antibodies against the two arr3 paralogs. Immunohistochemical analysis revealed a complementary expression of Arr3a and Arr3b in different photoreceptor cell types (Fig. 2A and D). Interestingly, both arrestin proteins were distributed throughout the photoreceptor cells with high protein levels in the inner segment and the cone pedicles as observed for mouse cone arrestin (Zhu et al., 2002).

In co-immunostainings we ascertained the cell type specificity of Arr3a and Arr3b. Double staining with the Arr3a antibody and the green/red double cone specific Zpr1 antibody (Larison & Bremiller, 1990) revealed zebrafish Arr3a to be specifically expressed in mid- and long-wavelength-sensitive cone (M-cone and L-cone, respectively) photoreceptors (Fig. 2B and E). In contrast, we found Arr3b to be expressed in short- and UV-wavelength-sensitive cones (S-cones and UV-cones, respectively) as revealed by co-labeling of Arr3b and blue- and UV-opsin (Fig. 2C and F).

**Generation of Arr3-deficient zebrafish larvae**

Expression analysis has shown that arr3 is exclusively expressed in zebrafish cone photoreceptors. In order to explore the functional role of arrestin in cone vision, we thus generated Arr3-deficient zebrafish larvae. Protein expression was knocked down using MOs targeted against the translational start sites of arr3a or arr3b. Injection of these MOs did not cause any apparent malformations and the development of injected larvae was indistinguishable from that of control-injected larvae. Similarly, we found no changes to retinal integrity on standard histological sections. Neither arr3 single morphants (arr3aMO and arr3bMO) nor arr3 double morphants (arr3a/3bMO) displayed any morphological abnormality of the retina (Fig. 3A).

The specificity of the knockdown was evaluated in Western blot and immunohistochemical analysis. At 5 dpf, the Arr3a protein level was specifically reduced in arr3a-MO-treated larvae but not in larvae injected with arr3b-MO or control-MO (Fig. 3B and Supporting Information Fig. S4). *Vice versa*, the Arr3b protein level was only reduced in arr3b morphants but not in arr3a or control morphants (Fig. 3C and Supporting Information Fig. S4). Thus, using MOs targeting the translational start site we could specifically knock down either arr3a or arr3b.

To quantify the knockdown efficiency in larvae 5 dpf, acetylated tubulin was additionally detected in Western blot analysis for standardization. Semi-quantitative evaluation determined an estimated knockdown efficiency of 95% for Arr3a and 88% for Arr3b. Hence, MO-mediated knockdown is highly efficient at 5 dpf.

**Cone response recovery is delayed in Arr3a-deficient zebrafish larvae**

The larval zebrafish retina is strongly dominated by cone function. Rod photoreceptors are sparse and exhibit short outer segments (Branchek & Bremiller, 1984). Their function can not be assessed in electroretinographic recordings before 15 dpf (Bilotta et al., 2001).

Taking advantage of this cone dominance we studied the functional impact of cone arrestin on normal photopigment inactivation and cone response recovery using ERG recordings. The ERG is a sum field response of the retina to light (Dowling, 1987). In zebrafish larvae, the ERG is dominated by a prominent b-wave which mainly reflects the response of ON-bipolar cells. As the b-wave largely masks the response of the photoreceptors, represented by the a-wave, we used the b-wave as an indirect measure of photoreceptor function.

Arr3 function was assessed in ERG paired-flash recordings. Two white light flashes, a conditioning and a probing flash (Fig. 4A, black and gray lines respectively) were separated by varying interstimulus intervals. The recovery of the b-wave amplitude between conditioning and probing flash was calculated. In 4 and 5 dpf zebrafish larvae, loss of Arr3b did not cause any alteration in the time course of b-wave recovery when compared to control-injected larvae (Fig. 4B, two way ANOVA, $P = 0.862$; and Supporting Information Fig. S5A, $P = 0.069$). However, knockdown of arr3a caused a significant delay of b-wave amplitude recovery (Fig. 4B; two way ANOVA, $P < 0.0001$). Five-day-old control morphants recovered 75% of their b-wave amplitude within 1.8 s. arr3a morphants, in contrast, required approximately 4 s to recover the b-wave to the same extent. This delay in recovery was enhanced in 4-day-old Arr3a-deficient larvae (Supporting Information Fig. S5A; $P < 0.0001$). As MO efficacy decreases during larval development the reversibility of this delay in response recovery argues against nonspecific effects induced by arrestin knockdown.

To further confirm the specificity of the arr3a morphant phenotype, we analyzed an Arr3a dosage-dependent recovery of the ERG b-wave. Zebrafish larvae treated with increasing concentrations of arr3a-MO revealed a dose–response relationship with MO concentration dependent lengthening of the b-wave recovery time (Supporting
Fig. 1. Spatiotemporal expression analysis of visual arrestin genes in zebrafish. (A–H) Lateral view of zebrafish larvae with dorsal side up and anterior to the left. (I–L) Sections of adult albino retina. (A, E and I) mRNA expression analysis revealed arr3a to be evenly expressed throughout the PRCL at 3 and 5 dpf (A and E) and restrictively in the outer nuclear layer of adult retina (I). (B, F and J) arr3b expression was detected only in the ventral half of the retina in larvae 3 dpf (B) but throughout the PRCL at 5 dpf (F). In the adult retina arr3b expression was restricted to a subset of photoreceptor cells (J). (C and D) arrSa and arrSb were detected in a ventral patch of the retina at 3 dpf. (G and H) At 5 dpf arrSa and arrSb expression had expanded at the ventral site with sparse expression dorsally. (K and L) On adult retina sections, both arrS paralogs were detected in a restricted part of the outer nuclear layer. OS, outer segments; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bars, 150 μm (in A for A–H), 50 μm (in I for I–L).

Fig. 2. Photoreceptor cell type-specific expression of the arr3 paralog genes in zebrafish larvae. Z-projections of confocal image stacks from immunostainings on transverse sections of 5-day-old albino larvae. (A and D) Double immunostaining of Arr3a (red) and Arr3b (green). (B and E) Co-staining with antibody against Arr3a (red) and double cone specific Zpr1 (green). (C and F) Co-labeling of Arr3b (green) and short-wavelength-sensitive blue- and UV-opsin (S-opn, red). Scale bars, 50 μm (A–C), 10 μm (D–F).
Increasing MO concentration and thus decreasing levels of Arr3a resulted in prolonged ERG b-wave recovery times. For arr3b morphant larvae we found no phenotype although Arr3b protein level was efficiently knocked down. Considering the characteristics of the recorded ERG (a small a-wave and dominant b-wave; Fig. 4A), the lack of phenotype in arr3b morphants might be explained by an insufficient stimulation of S- and UV-cones. The larval zebrafish ERG has been shown to possess a large voltage-negative response and a late onset of the b-wave to UV- and short-wavelength stimuli (Bilotta et al., 2005). We therefore tested whether Arr3b function can be assessed in larvae with prolonged M- and L-cone kinetics. For this we generated morphant larvae deficient in both Arr3a and Arr3b. These double-knockdown larvae (arr3a/arr3bMO) displayed a similar response recovery delay as single-injected arr3a morphant larvae (Fig. 4C; two-way ANOVA, \( P = 0.716 \)). Test recordings with chromatic stimuli of wavelength <417 nm revealed that our ERGs were highly dominated by M- and L-cone responses. S- and UV-cone function could not be assessed efficiently as the light source used for ERG measurements exhibits only weak emission at shorter wavelengths. Arr3b function is thus concealed by the fast and dominant photoresponse kinetics of M- and L-cones in our ERG recordings.

For M- and L-cones we have shown that under high bleaching conditions normal ERG b-wave recovery depends on Arr3a inactivating photoexcited pigment (Fig. 4B). To test whether the recovery from low bleaching stimuli similarly requires Arr3a function we next studied the ERG b-wave recovery from dim light flashes. We analyzed paired flash recordings from 70 lux light flashes and found a normal recovery of the ERG b-wave in Arr3a-deficient zebrafish larvae compared to control animals (Fig. 4D; two-way ANOVA, \( P = 0.565 \)). Both control and arr3a morphants recovered 75% of their b-wave amplitude within 1.2 s, indicating that under low bleaching conditions Arr3a function is not limiting for the normal cone response recovery in zebrafish larvae.

**Temporal resolution is reduced in Arr3a-deficient larvae**

To investigate whether the delay in ERG b-wave amplitude recovery observed in arr3a morphants causes visual behavior deficits, we analyzed the optokinetic response of arr3a-MO-injected larvae. The zebrafish optokinetic response is a stereotyped eye movement elicited by moving stimuli in the surround (Clark, 1981; Easter & Nicola, 1996). Eye movement velocity under varying temporal frequencies was recorded at two different light conditions. Under low-contrast conditions (20 cd/m² background illumination) the temporal resolution of Arr3a-deficient zebrafish larvae was only reduced at high temporal frequencies compared to control larvae (Fig. 5A). However, under high-contrast conditions and ambient illumination of 120 cd/m², temporal resolution of arr3a morphants was significantly reduced even at low temporal frequencies (Fig. 5B). Particularly, at high temporal frequencies visual performance of arr3a morphants was strongly impaired compared to arr3b and control morphants (repeated-measures ANOVA, \( P < 0.001 \)). Arr3b-deficient larvae (Fig. 5, gray) performed similarly to control morphants under both contrast conditions (repeated-measures ANOVA, \( P = 0.860 \) and \( P = 0.713 \) respectively). As zebrafish motion vision is mediated by M- and L-cone function (Krauss & Neumeyer, 2003; Orger & Baier, 2005), an altered performance of arr3b morphants was not expected. Hence, our data indicate that cone arrestin function is required for high temporal resolution of motion vision mediated by M- and L-cones.

Fig. 3. Morpholino-mediated arr3 knockdown. (A) Histological sections of 5 dpf control morphants (controlMO), arr3 single (arr3aMO and arr3bMO) and arr3 double (arr3a/arr3bMO) morphants stained with Richardson’s solution. Scale bar, 50 μm. (B and C) Western blot performed on protein extracts from isolated eyes of 5 dpf larvae injected with control-MO (controlMO), arr3a-MO (arr3aMO) or arr3b-MO (arr3bMO). (B) Detection of Arr3a protein levels and acetylated tubulin (Tub) for loading control. (C) Detection of Arr3b protein levels and Tub.
Cone arrestin affects later aspects of photoresponse recovery than cone opsin kinase

Photoexcited visual pigment of rod photoreceptors is inactivated by phosphorylation followed by arrestin binding (Palczewski & Saari, 1997). To establish whether this holds true for the inactivation of the visual pigment in cone photoreceptors, we compared response recovery in phosphorylation-deficient and Arr3a-deficient zebrafish larvae.

GRK7a has recently been identified as the cone-specific rhodopsin kinase important for cone pigment phosphorylation and normal cone response recovery in zebrafish (Rinner et al., 2005). Confirming these results, we found a severe delay in initial response recovery in GRK7a-deficient larvae (Fig. 6; two-way ANOVA, \( P < 0.0001 \)). Control morphants showed a half-maximal recovery of their ERG b-wave amplitude within <1 s. The grk7a morphants, in contrast, exhibited a half-maximal recovery time of 1.7 s. Similarly, half-maximal recovery in Arr3a-deficient larvae was not reached before 1.8 s.

In the late phase of response recovery, arr3a morphants showed a pronounced delay compared to grk7a morphants. GRK7a-deficient larvae recovered 75% of their b-wave amplitude within 3.1 s but arr3a morphants required 4.3 s to recover to the same extent.

Fig. 4. Cone response recovery is prolonged in Arr3-deficient zebrafish larvae at 5 dpf. (A) ERG recordings from a control (controlMO) and an arr3a (arr3aMO) morphant larvae stimulated with 500 ms conditioning (black line) and probing (gray line) flashes separated by an interstimulus interval of 3 s. (B) Time course of the ERG b-wave recovery in control (black, \( n \geq 30 \)), arr3a (red, \( n \geq 19 \)) and arr3b (gray, \( n \geq 10 \)) morphant larvae. For stimulation, 500 ms flashes of 7000 lux were separated by 1, 2, 3, 5, 10 or 20 s interstimulus interval (ISI). (C) Time course of the b-wave recovery in arr3 double morphants (arr3a/3bMO, red, \( n = 14 \)) compared to arr3a (black, \( n \geq 10 \)) and arr3b (gray, \( n \geq 10 \)) single morphants. Larvae were injected with 2.8 ng arr3a-MO and/or 13 ng arr3b-MO. (D) ERG b-wave recovery of cone responses from paired dim light flashes (500 ms, 70 lux) in control-MO-treated (black, \( n \geq 10 \)) and arr3a-MO-treated (red, \( n \geq 10 \)) zebrafish larvae. All data points represent the means ± SEM.

Fig. 5. Temporal contrast sensitivity was reduced in Arr3a-deficient zebrafish larvae. The optokinetic response was recorded from 5 dpf larvae injected with control-MO (black), arr3a-MO (red) or arr3b-MO (gray). Animals were adapted to background illumination for 60 s and eye velocity was measured at the temporal frequencies of 3.5, 5.25, 7, 14, 21 and 28 deg/s. Background illuminations of (A) 20 cd/m² and (B) 120 cd/m². Plotted are mean values ± SEM (\( n \geq 14 \)).

Fig. 6. Cone response recovery differed between GRK7a- and Arr3a-deficient larvae. Time course of the ERG b-wave recovery of arr3a morphants (red, \( n \geq 19 \)) compared to grk7a morphants (black, \( n \geq 10 \)) or control morphants (gray, \( n \geq 30 \)) in response to paired light flashes (500 ms, 7000 lux) separated by an interstimulus interval (ISI) of 1, 2, 3, 5 or 10 s. Data points represent the means ± SEM.
Thus, we found that loss of photopigment phosphorylation in *grk7a* morphants had a severe effect on the initial phase of ERG b-wave recovery. Loss of Arr3a function, in contrast, strongly prolonged late b-wave recovery, indicating sequential actions of GRK7a and Arr3a.

### Discussion

Low light sensitivity and fast response kinetics make cone photoreceptors well suited for vision of high spatial and temporal resolution under bright daylight conditions. Efficient mechanisms to ensure precise signal amplification are indispensable for high resolution cone vision. Regulation of the lifetime of activated photopigment has been elucidated in considerable detail as the first stage of control for signal amplification in the rod visual transduction cascade. Phosphorylation of photoexcited rhodopsin and subsequent quenching by arrestin binding are required for normal recovery of the rod photoreceptor (Xu et al., 1997; Chen et al., 1999).

A similar mechanism for the inactivation of cone visual transduction has remained controversial as thermal decay of activated visual pigment is more rapid in cones than in rods (Shichida et al., 1994; Imai et al., 1997). However, pigment phosphorylation is also faster in cone photoreceptors in the presence of the cone-specific kinase Grk7 (Tachibana et al., 2001, 2005). Recently, the importance of pigment phosphorylation for cone vision has been demonstrated, suggesting a similar quenching mechanism for cone responses (Rinner et al., 2005).

In line with this we now showed that cone arrestin is essential for photopic vision, particularly under high bleaching conditions. In contrast to rats, in which cone co-express rod and cone arrestin (Lyubarsky et al., 1999; Nikonov et al., 2005), zebrafish cone photoreceptors express cone arrestin (*arr3*) exclusively, while rod arrestin (*arr5*) is confined to rod photoreceptors (Figs 1 and 2). This cell-type specificity of zebrafish *arr3* and *arr5* resembles the cone- and rod-specific expression of ortholog genes in the human and salamander retina (McKechnie et al., 1986; Sakuma et al., 1996; Smith et al., 2000). Interestingly, the two *arr3* paralogs of zebrafish exhibited complementary expression in cone photoreceptor subtypes. As these paralogs are located on different chromosomes in the zebrafish genome, they probably arose in a teleost-specific additional whole-genome duplication event (Postlethwait et al., 2000; Taylor et al., 2003). The retention of initially redundant genes in the genome can be explained by subfunctionalization, where complementary degenerative mutations in regulatory elements lead to the preservation of both gene copies (Force et al., 1999). For cone arrestin, we found *arr3a* being exclusively expressed in M- and L-cones whereas *arr3b* was complementary expressed in S- and UV-cone photoreceptors (Fig. 2).

The absence of *arr5* expression in cones and the lack of efficient rod function in larval zebrafish allowed us to specifically probe the contribution of cone arrestin to visual function. Reduced levels of zebrafish Arr3a protein caused a severe delay of b-wave amplitude recovery in the two-flash electroretinogram paradigm (Fig. 4B and Supporting Information Fig. S5A). As the ERG b-wave, generated by ON-bipolar cell responses, depends on cone signaling this delay reflects a prolonged cone response recovery and is consistent with an incomplete inactivation of M- and L-opsin in Arr3a-deficient larvae. This recovery delay is analogous to the prolonged rod photoresponses after rod arrestin depletion in mice (Xu et al., 1997) as well as cone responses in double cone and rod arrestin knockouts (Nikonov et al., 2005).

Hence, our results support a mechanism for photopigment inactivation in zebrafish double cones in which photoactivated cone pigment is shut off through specific phosphorylation and subsequent arrestin binding, similar to the inactivation of rhodopsin in rod photoreceptors (Gross & Burns, 2010). The temporal difference in ERG response recovery under Arr3a-deficient and GRK7a-deficient conditions further affirmed this quenching mechanism (Fig. 6). Comparison of *grk7a* and *arr3a* knockdown revealed a pronounced delay of late response recovery in Arr3a-deficient larvae. This delay exceeded the effect of GRK7a deficiency, thus substantiating the notion that Arr3 binds to light-activated and phosphorylated photopigment to regulate efficient pigment inactivation. Consistent with our data, inactivation of mouse M-cone pigment has been shown to depend on phosphorylation (Nikonov et al., 2005) and arrestin function (Nikonov et al., 2008).

Interestingly, we found normal ERG response recovery to dim light flashes in *arr3a* knockdown larvae (Fig. 4D). Mice lacking both rod and cone arrestin similarly showed a light intensity dependent prolongation of the photoresponse of S-dominant cones (Nikonov et al., 2008). We suggest that the large-scale recovery of bleached cone photopigment strongly relies on arrestin function to assure fast cone response kinetics and normal visual function. For the recovery of low amounts of bleached photopigment cone arrestin appears, however, to be not rate-limiting in zebrafish. We presume that, under low bleaching conditions, the recovery of 11-cis retinal or cGMP (Takekoto et al., 2009) or the lifetime of downstream signaling components dominates response kinetics in cones. In line with this, analysis of rod arrestin function in mouse rods necessitated the acceleration of downstream deactivation kinetics (Gross & Burns, 2010). Alternatively, a different mechanism of pigment inactivation might compensate for arrestin absence. One such mechanism is the thermal decay of light-activated photopigment (Kennedy et al., 2004).

Overall, multiple factors may converge to regulate cone response kinetics. Our data indicate that, at a certain threshold of bleached photopigment, arrestin function becomes limiting for ERG response recovery in larval zebrafish. Differences between the normal dim flash response recovery observed in zebrafish *arr3a* morphant ERGs and the slowed tail-phase recovery from dim light flashes found for mouse cones lacking rod and cone arrestin might on the one hand result from different sensitivities of the measurements. On the other hand, mouse cones express GRK1, not the highly effective GRK7 (Weiss et al., 2001), that is likely to result in different inactivation kinetics. Moreover, bioinformatical analysis revealed a shorter C-terminus of zebrafish cone arrestin compared to its mouse ortholog. C-terminal truncations of arrestin molecules may vary activation kinetics and receptor specificity (Gurevich & Gurevich, 2004).

In physiological analysis Arr3a deficiency was limiting to normal photoresponses depending on the level of bleached photopigment. Its functional relevance for cone-mediated visual behavior was assessed by exploiting the zebrafish opsin-kinetic response, a stereotypic ocular movement probably mediated by modulation of M- and L-cone input (Schaerer & Neumeyer, 1996; Orger & Baier, 2005). Consistent with our electrophysiological data, we found an impaired visual performance of Arr3a-deficient zebrafish larvae (Fig. 5). The opsin-kinetic behavior of the *arr3a* morphant larvae under low-contrast (dark-adapted) conditions was only affected at high temporal frequency patterns, whereas the general temporal sensitivity was highly reduced under high-contrast (light-adapted) conditions. *arr3a* morphants thus showed a deceleration of the temporal transfer function under light-adapted conditions, similarly to *grk7a-morphant* larvae (Rinner et al., 2005). This deceleration contrasts with an improved temporal sensitivity in control larvae under light-adapted conditions (Fig. 5 and Kelly, 1961) and presumably results from differences in the lifetime of photoactivated cone pigment. Hence, Arr3a function in pigment inactivation is essential for high temporal resolution of...
zebrafish cone vision, particularly when exceeding a critical level of bleached photopigment. In accordance with our electrophysiologival results, we assume that under conditions of low pigment bleaching cone response kinetics are dominated by other factors than the lifetime of cone pigment.

Our analysis did not show electrophysiological or behavioral alterations in Arr3b-deficient larvae. Although it was suggested that S-opsin has additional access to an efficient phosphorylation-independent pathway (Nikonov et al., 2005), there is ample evidence that S-opsin inactivation in human, mouse and salamaner can be regulated by phosphorylation and arrestin binding (Lyubarsky et al., 2000; Kefalov et al., 2003). Recent studies in mouse cones have shown that the S-cone response is prolonged in the absence of arrestin (Nikonov et al., 2008), and transgenic expression of S-opsin in mouse rods revealed that Arr1 is effective in S-pigment inactivation (Shi et al., 2007).

Consistently, arrestin is found to localize in the outer segments of mammalian short-wavelength-sensitive photoreceptors, where photopigment inactivation takes place (Sakuma et al., 1996; Nikonov et al., 2008). Similarly, zebrafish Arr3b protein localized in the outer segments of S- and UV-light sensitive photoreceptors (Fig. 2), implying that Arr3b is functional in pigment inactivation. The lack of sensitive physiological and behavioral measurements testing S- and UV-cone function impeded the direct proof of Arr3b function in this study. However, zebrafish S-opsin has been shown to be phosphorylated in a light-dependent manner (Kennedy et al., 2004), indicating that S-pigment may be inactivated by phosphorylation and subsequent arrestin binding.

In conclusion, zebrafish cones complementarily express two arrestin paralogs. Arr3a is expressed in M- and L-cones, and Arr3b is found in S- and UV-cones. In loss-of-function studies we found Arr3a to act in a sequential order to GRK7a in cone response recovery. Behavioral testing of arr3a morphants demonstrated for the first time the crucial role of arrestin in cone vision of high temporal contrast sensitivity.

Supporting Information
Additional supporting information may be found in the online version of this article:
Fig. S1. Sequence alignment of zebrafish arrestin3 and arrestinS paralogs.
Fig. S2. Visual arrestins are duplicated in teleost fish.
Fig. S3. Specificity of arrS transcript expression in zebrafish rod photoreceptors.
Fig. S4. Knockdown of arr3 in cone photoreceptors of the zebrafish retina.
Fig. S5. Arr3 dosage-dependent recovery of the photoreponse.
Table S1. Genome localization of visual arrestin genes
Table S2. Conservation of visual arrestins between zebrafish and human

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Abbreviations
Arr, arrestin; Arr1, rod arrestin in mice; Arr3, cone arrestin in zebrafish; Arr4, cone arrestin in mice; arr5, rod arrestin in zebrafish; dph, days post-fertilization; ERG, electroretinogram; GRK, G-protein-coupled receptor kinase; L-cone, long-wavelength-sensitive cone; M-cone, mid-wavelength-sensitive cone; MO, morpholino oligonucleotide; PRCL, photoreceptor cell layer; S-cone, short-wavelength-sensitive cone; UV, ultraviolet; UV-cone, UV-wavelength-sensitive cone.

References

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