Knockdown of cone specific kinase GRK7 in larval zebrafish leads to impaired cone-response recovery and delayed dark adaptation

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Summary
Phosphorylation of rhodopsin by rhodopsin kinase GRK1 is an important desensitization mechanism in scotopic vision. For cone vision GRK1 is not essential. However, cone opsins is phosphorylated following light stimulation by an unknown kinase. In cone dominant animals as well as in humans - but not in rodents – GRK7, a cone specific homolog of GRK1 has been identified in cone outer segments. To investigate the function of GRK7 in vivo we cloned two orthologs of grk7 in zebrafish and knocked down gene expression of grk7a in zebrafish larvae by morpholino antisense nucleotides. Photoresponse recovery in Grk7a deficient larvae was delayed in electroretinographic measurements and temporal contrast sensitivity was reduced particularly under bright light conditions. These results show that function of a cone specific kinase is essential for cone vision in the zebrafish retina and it argues that pigment bleaching and spontaneous decay alone are not sufficient for light adaptation and rapid cone response inactivation.
Introduction

Light levels of the visual world change approximately by 10 orders of magnitude over the course of the day (Normann and Werblin, 1974). On photoreceptor level most components of the phototransduction cascade are involved in adaptation to ambient light levels (for a review see (Pugh et al., 1999) and (Burns and Baylor, 2001). In rod photoreceptors rhodopsin kinase (RK) operates on the first stage of phototransduction. It phosphorylates Rh*, the active form of rhodopsin (Hurley et al., 1998; Kuhn and Dreyer, 1972; Ohguro et al., 1995). Phosphorylated Rh* is quenched by arrestin (Kuhn, 1978), which disconnects Rh* from transducin. Deficiency of RK or arrestin leads to a severe impairment in scotopic vision in humans (Yamamoto et al., 1997), causing Oguchi disease. RK recognizes and phosphorylates also cone opsins in vitro (Fukada et al., 1990). However, mutations in RK leading to night blindness affect only scotopic vision whereas cone mediated photopic vision is only slightly affected (Dryja, 2000; Yamamoto et al., 1997). This implies either that another kinase, distinct from RK, is functional in cones or that cone opsin phosphorylation is not essential for cone vision. The latter possibility has been proposed by some authors (Burkhardt, 1994; Cideciyan et al., 1998; Rushton, 1977) arguing that photopigment bleaching (bleaching adaptation) is sufficient for desensitization under bright background conditions. However there is evidence that cone opsin phosphorylation plays a role in light adaptation similarly to rhodopsin phosphorylation. (Lyubarsky et al., 2000) measured cone isolated ERGs in RK knockout mice and found – in contrast to humans - a markedly prolonged regeneration time after a bright probe flash and (Zhu et al., 2003) showed that cone opsins become phosphorylated in the mouse retina and bind cone arrestin in the
phosphorylated state. In isolated carp photoreceptors cone opsin is phosphorylated by an unidentified kinase in a light dependent manner with a time course faster than in rhodopsin (Tachibanaki et al., 2001).

Evidence for a cone specific kinase came with the identification of GRK7, a member of the GRK family (Hisatomi et al., 1998), which is expressed in teleost cone outer segments. This kinase was found to be expressed in the cone dominated retinas of ground squirrel and eastern chipmunk (Weiss et al., 1998) as well as in the human and monkey retina. Remarkably, GRK7 is not present in rat and mouse cone outer segments (Weiss et al., 2001).

To explore the role of GRK7 function in cone desensitization in vivo we used larval zebrafish. The zebrafish has sophisticated cone vision (Bilotta et al., 2001). The visual system develops rapidly (Easter and Nicola, 1996; Easter and Nicola, 1997) and can be assayed by visual psychophysics (Rinner et al., 2005) and electroretinography (ERG) (Branchek, 1984; Makhankov et al., 2004). Like in other species zebrafish cone opsin is phosphorylated following light stimulation at the c-terminal end (Kennedy et al., 2004).

We found two paralogs of GRK7 in zebrafish. Our experiments show that deficiency of Grk7a, which is expressed in cone outer segments, results in delayed cone response recovery. In psychophysical experiments, temporal contrast sensitivity was markedly reduced under light adapting conditions.
**Results**

The zebrafish retina contains four types of cones. Double cones, which are composed of a principle and accessory member, express red- and green-sensitive opsins. Single cones can be categorized on morphological grounds in long single cones (blue sensitive) and UV-sensitive short single cones (Branchek and Bremiller, 1984). Developing rods are sparse and not functional in the larva (Branchek, 1984; Clark, 1981). Rods only begin to function at later stages (older than 15 dpf) as determined by ERG (Bilotta et al., 2001). Consequently, physiological and behavioral visual responses measured in larval zebrafish can be regarded as derived from cones only.

**Cloning of grk7 and grk1 homologs in zebrafish**

To explore the role of cone specific kinases in the cone dominant zebrafish retina we cloned orthologs of the rhodopsin kinase *grk1*, and the cone specific kinase *grk7* from zebrafish cDNA. In zebrafish many genes are found to be duplicated as a result of a genome duplication, which occurred most likely after the divergence of ray-finned and lobe-finned fishes before the teleost radiation (Postlethwait et al., 2000). Accordingly, two paralog genes for *grk7*, denoted as *grk7a* and *grk7b*, were identified by database searches. Specific primers were designed and the full coding sequences for both genes were amplified from zebrafish cDNA. Both genes code for proteins that are highly homologous to human GRK7, showing 59% (Grk7a) and 56% (Grk7b) sequence identity on protein level. We cloned two orthologs of the rhodopsin kinase (*grk1*) gene denoted as *grk1a* and *grk1b*, which are also duplicated in the zebrafish genome. The protein
sequence is 66% and 64% conserved compared to human GRK1. Furthermore, a partial
coding sequence of the homolog of cone-arrestin c-arr (59% homology to human c-
arrestin) was cloned.

**Grk7a and grk7b are expressed in cone photoreceptors and the pineal gland**

In order to determine expression patterns of genes putatively involved in opsin
phosphorylation, we prepared riboprobes specific for grk7a, grk7b, grk1a, grk1b, and c-
arrest. As revealed by in situ hybridization, all these genes are expressed in light-sensitive
structures. Their transcripts are detectable in the eye and the pineal gland, a light
sensitive endocrine organ in lower vertebrates (Allwardt and Dowling, 2001) (Fig1).
Expression of grk7a is found in the outer nuclear layer where cones are located. The
paralog grk7b is expressed in the pineal gland and was hardly detectable in the eyes.
Transcripts of c-arr, the cone specific homolog of rod arrestin (Zhu et al., 2003), were
also identified in cones. The rhodopsin kinase homologue grk1a shows a characteristic
sparse rod staining (Raymond et al., 1995). The paralog grk1b is expressed throughout
the outer nuclear layer, indicating expression also in cones. This indicates a divergence
in expression pattern of these paralogs, after the genome duplication.

Expression of grk7a in the outer nuclear layer is developmentally regulated. At 3 dpf
grk7a probes label photoreceptors in the whole outer nuclear layer. In later stages (4 dpf
and 5 dpf) only receptors close to the marginal zone where new photoreceptors are born
strongly express grk7a mRNA. This indicates that grk7a mRNA expression level is high
in newborn cells and reduced in older cells (Fig. 1g-i). In situ hybridization with
riboprobes against grk7a was consistent with a cone specific expression of Grk7a. It was
also stronger expressed in the cones compared to the paralog grk7b. We therefore decided to target grk7a for functional characterization.

To verify that Grk7a protein is expressed in cones and to see if Grk7a was expressed specifically in cone photoreceptors, a polyclonal antibody (anti-Grk7a) was raised against two peptides of zebrafish Grk7a. In immunohistochemical stainings, this antibody specifically and strongly stained cone outer segments (Fig. 2) whereas no labeling could be observed in the negative control stainings with pre-immun serum (Fig. S1). High magnification pictures (Fig. 2B,C) of anti Grk7a labeled sections clearly showed that the strongest labeling was restricted to cone outer segments whereas cone inner segments, processes, and pedicles were only faintly labeled (Fig. 2C). The region of the cone somata was always devoid of any Grk7a staining.

The gradient of grk7a mRNA levels between central and lateral retina seen in in situ experiments (Fig.1) is not reflected on the protein level. Anti-Grk7a stains cone outer segments throughout the retina.

Double labeling with anti-Grk7a and anti-rhodopsin showed that Grk7a is expressed in cones and not in nascent rods as there was no colocalization identifiable (Fig. 3). This expression pattern is in agreement with the expression of the homologs in humans, and ground squirrels where GRK7 is expressed cones but not in rods (Weiss et al., 2001).

**Morpholino mediated knockdown of grk7a**

In order to address the function of Grk7a in cone vision, we performed a loss-of-function study using antisense morpholino oligonucleotide (MO) mediated gene knock-down. A
morpholino antisense nucleotide was designed to specifically block translation of grk7a mRNA. Morpholino knockdown experiments have mostly been performed in early developmental stages (Nasevicius and Ekker, 2000). Recently it has been shown that MO mediated gene knockdown is feasible at 4 and 5 dpf (Seiler et al., 2005; Sollner et al., 2004), depending on the targeted gene.

Larvae were injected with a morpholino directed against the start codon of grk7a (grk7aMO) or a control morpholino (controlMO). Only larvae which hatched normally and which had no visible abnormalities were selected for functional characterization. No obvious behavioral differences including touch response and background adaptation were apparent in these larvae. Histological sections through the eyes did neither reveal any shortening of photoreceptor outer segments nor other retinal defects (Fig. 4a).

To quantify knockdown of Grk7a in grk7aMO injected larvae, Western Blots were made with protein extracts from these larvae and compared to samples from controlMO treated larvae. Anti-Grk7a detects a single band in Western Blots from zebrafish whole protein corresponding to the predicted molecular weight of Grk7a (62 kDa). To test cross reaction with recombinant Grk7a we expressed the full coding sequence as a GST-tagged fusion protein in bacteria. In a Western Blot a single band on the predicted height (82 kDa) is recognized only if bacteria were transformed with a plasmid containing the grk7a coding sequence. Transformation with the empty plasmid did not result in a signal (Fig. 4c).

In three independent injection experiments Grk7a was barely detectable in Western Blots at 4 dpf. Only after autoradiographic overexposure a faint Grk7a band became visible in
grk7aMO injected larvae (Fig 4c). Semiquantitative evaluation of the Grk7a protein amount indicates knockdown below 5% of control levels.

**ERG response recovery is delayed in Grk7a deficient larvae**

To test if deficiency of Grk7a affects cone response recovery, we measured ERG responses to paired light flashes in 4 dpf larvae. The ERG is the sum field response of the retina to light and is a direct assessment of outer retinal function (Dowling, 1987).

The a-wave amplitude is not a robust measure of photoreceptor function in the larval zebrafish retina and recordings from single cones have not been reported so far. Therefore we used the ERG b-wave amplitude recorded from live larvae as an indirect measure of cone photoreceptor function. The b-wave reflects mostly bipolar cells activity and is therefore an indirect measure of cone responses in larval zebrafish.

To measure cone response recovery after light stimulation a probe-flash paradigm was used. A conditioning flash (212 lux, 500 ms) was followed by a probe flash of the same intensity with varying interstimulus intervals (ISI). Recovery of b-wave amplitude was tested relative to the preceding conditioning flash. Recovery of ERG b-wave was 55% complete after 1s in control morphants (Fig. 5a). In grk7aMO injected larvae however, after 1s recovery was below 10% (Fig. 5b) and recovery was not complete before 10 s (Fig. 5d). Half-life of response recovery was estimated by a logarithmic fit to the non-saturated part of the recovery curve as 0.9 s for control larvae and 2.4 s for grk7aMO injected larvae (Fig. 5d inset).

To exclude the possibility that this delay in grk7aMO treated larvae reflects unspecific morphological changes or a defect in visual pigment regeneration, we measured as negative control the fading vision (fdv) mutant at 5 dpf with the same paradigm. This
mutant has significantly reduced cone outer segments, and the recycling of visual pigment is impaired in *fdv* mutant larvae due to a defect in the retinal pigment epithelium. Biochemical analysis shows that the intermediate product retinyl ester accumulates at the expense of the regenerated visual chromophore 11-*cis* retinal (Schonthaler et al. *in revision*). Cone response recovery in larvae homozygous for *fdv* was 58% complete after 1 s (n = 7; Fig. 5c). No significant difference in recovery kinetics was found compared to sibling larvae (n = 5; data not shown).

As an additional control we performed a similar experiment with a lower dose of *grk7a* morpholino injected to promote recovery of protein expression. Response recovery was measured with an ISI of 1s at 4, 5, 6, and 7 dpf. Protein expression recovers to 33% under these conditions as measured on Western Blots. From 6 dpf on response recovery is indistinguishable when tested with a conditioning and probe flash of 212 lux. On 7dpf response recovery was tested in addition with a flash intensity of 2120 lux. Under these conditions photoresponse recovery is still significantly reduced (p < 5%; Fig. S2).

**Temporal contrast sensitivity is reduced under light adapting conditions in Grk7a deficient larvae**

Next we tested the effect of delayed photoresponse recovery on behavioral visual performance in *grk7a* morphants. For this purpose, we applied a behavioral test based on the optokinetic response (OKR) which consists of stereotyped eye movements elicited by moving stimuli in the field of vision. Visual performance was quantified by measuring the velocity of eye movements upon projecting various computer-generated motion stimuli into the visual path of the larva. The optokinetic gain (eye velocity/stimulus velocity) is a function of stimulus contrast and indicative for psychophysical contrast sensitivity (Rinner et al., 2005).
From studies in mammals it is known that temporal contrast sensitivity is improved under light adapting conditions compared to the dark adapted state. Except for very high temporal frequencies this relationship reflects Weber’s law for light adaptation (Kelly, 1961).

We tested if cone vision was slowed down under steady state light adapting conditions in Grk7a deficient larvae. Control and grk7a knockdown larvae were stimulated under dark (0.7 cd/m²) and bright (363 cd/m²) background conditions with moving sine gratings of constant spatial frequency and varying temporal frequency. Larvae from three clutches that had been pooled before injection were tested at 4, 5 and 7 dpf.

For controlMO injected larvae the temporal contrast sensitivity measured as optokinetic gain was higher under light adapting conditions compared to the dark conditions. This effect was most pronounced at high temporal frequencies, consistent with psychophysical results in humans (Kelly, 1961). In grk7a knockdown larvae the effect of light adaptation on temporal contrast sensitivity is reversed; sensitivity was markedly reduced under light adapting condition compared to control larvae (bootstrap resampling test of pooled data as described in the methods part, p < 0.05) and also compared to the dark adapting conditions at 4 and 5 dpf (p < 0.05). This reversal persists up to 7 dpf. Control larvae do not show higher temporal contrast sensitivity under dark adapting conditions at any stage of development tested. We can therefore exclude that this effect is due to an unspecific developmental delay in grk7aMO treated larvae.

A control measurement with low spatial (0.05 cycles/deg) and temporal frequency (0.23 cycles/s) pattern was done at the beginning of the experiment with a low intensity grating (0.7 cd/m²). Larvae injected with controlMO could not be discriminated from
grk7aMO treated larvae at 4, 5 and 7 dpf (Fig. 6a) under these conditions. This shows that the observed reduction in visual sensitivity is specific for gratings with high temporal frequency, particularly under light adapting conditions.

As a negative control we injected a morpholino directed against grk1a, the ortholog of GRK1 in rods. We tested temporal contrast sensitivity under light adapting conditions that showed the strongest effect in grk7a morphants. As expected from the expression in rods, which are not functional at this developmental stage, no difference was found between grk1aMO and controlMO injected larvae. However, as no antibodies are available for zebrafish Grk1a, effectivity of this morpholino could not be verified (Fig. S4).

Grk7a protein content at 4, 5, and 7dpf was determined with Western Blots. The amount of Grk7a is strongly reduced in grk7aMO injected larvae from 4 – 7 dpf. There is however a slight recovery of expression at 5 and 7dpf. Accordingly, under dark adapting conditions the difference between control and grk7aMO treated larvae becomes smaller with increased age of the larvae. At 7 dpf the two groups can not be discriminated under dark adapting conditions. We performed a similar experiment with a lower dose of morpholino injected to promote a faster protein recovery. Temporal contrast sensitivity was measured under light adapting conditions. Sensitivity recovers correlated to the reappearance of Grk7a signal in Western Blots. At 9 dpf, when protein expression is recovered, visual performance is indistinguishable between control and grk7a morphants (Figure S3).

In order to establish a dose-response relationship of temporal contrast sensitivity, larvae from a single clutch were injected with different amounts of grk7aMO (5 ng - 30 ng).
Temporal contrast sensitivity was measured at 4 dpf as above but with a mean intensity of 41 cd/m². Sensitivity is reduced at temporal frequencies > 1 cycles/s for all grk7aMO injected groups compared to the control group (p < 0.05; Fig. 6c). Reduction of sensitivity is correlated with the amount of grk7aMO. Increasing amount of injected morpholino leads to reduced temporal contrast sensitivity (all group differences are significant, p < 0.05). This dose response relationship is in agreement with a concentration dependent enzymatic activity of Grk7a.

**Cone dark adaptation is delayed in Grk7a deficient larvae**

In human Oguchi disease mutations either in GRK1 or arrestin interferes with rhodopsin regeneration. Unphosphorylated photoactivated rhodopsin species continuously activate transducin. This leads to prolonged rod dark adaptation after a bleaching light stimulus independent from pigment regeneration.

To examine if dark adaptation after a bleaching stimulus is similarly affected in cones we measured recovery of b-wave responses to a dim test stimulus (21 lux, 100 ms) at different time points after 10 s bleaching with 2120 lux. The exact ratio of bleached pigment under these conditions was not determined because parameters such as pigment regeneration constant and half-bleaching intensities are not known for zebrafish. By comparison with recovery kinetics from humans (Mahroo and Lamb, 2004), we estimate that less than half the pigment is temporarily bleached under these conditions. Larvae injected with the controlMO (n = 7) recovered with a half-life of about 5 s, estimated by a fit to the logarithmic linear part of the recovery data (Fig. 7). Deficiency of Grk7a leads to a delayed dark adaptation; the b-wave in grk7aMO treated siblings (n = 6) recovers with a half-life of about 35 s. This shows that Grk7a deficient cones regain their
sensitivity slower after strong light stimulation, resembling delayed bleaching adaptation of rods in Oguchi disease.
Discussion

Light adaptation involves mechanisms in various retinal pathways. Range extension and desensitization according to Weber’s Law are hallmarks of light adaptation. In rods, a reduction in the time constant of cGMP hydrolysis and a reduction in lifetime of activated rhodopsin are thought to contribute most to light adaptation (Koutalos et al., 1995; Nikonov et al., 2000). The lifetime of activated rhodopsin depends on rhodopsin phosphorylation by rhodopsin kinase (RK), which is regulated by a calcium recoverin feedback loop (Makino et al., 2004).

If rhodopsin phosphorylation is compromised by a deficiency in RK response recovery in ERG double flash paradigms is delayed in humans. Furthermore rod dark adaptation after pigment bleaching is also slowed (Cideciyan et al., 1998; Yamamoto et al., 1997). Cone responses in contrast are almost normal under these conditions, suggesting that cones do not rely on opsin phosphorylation (Burkhardt, 1994; Cideciyan et al., 1998; Rushton, 1977). However, two lines of evidence argue against this conclusion. The first indication comes from biochemical studies which show that cone opsin like rhodopsin is phosphorylated in vitro (Tachibanaki et al., 2001) and in vivo (Kennedy et al., 2004; Zhu et al., 2003) in a light dependent manner. Evidence for an in vivo function of cone opsin kinases comes from results in RK deficient mice, which in contrast to Oguchi disease patients show severe impairment in cone response recovery (Lyubarsky et al., 2000). There is, however, a notable difference between mice and men with respect to the set of kinases expressed in cones. Humans and cone dominated animals such as pigs and dogs express the rhodopsin kinase homolog GRK7 besides GRK1 in cones. Rod dominant rodents in contrast do not express this kinase in cone outer segments (Weiss et al., 2001).
Biochemical experiments (Chen et al., 2001) showed that GRK7 can phosphorylate opsins in cultured retinas. It has not been shown however that this kinase is essential for cone desensitization or light adaptation in vivo.

In order to perform a loss of function study in larval zebrafish, we cloned the zebrafish kinases that are possibly involved in opsin desensitization. Due to the teleost genome duplication (Postlethwait et al., 2000) many genes including GRK7 and GRK1 are duplicated in zebrafish. Duplication does not necessarily imply functional redundancy of these paralog genes. Often, spatiotemporal expression pattern or function has diverged after the duplication event (Postlethwait et al., 1998). This is also the case for the opsin kinases. Immunohistochemistry confirmed that Grk7a is the paralog that resembles the cone specific expression of GRK7 in cone dominant mammals (Weiss et al., 2001). For the ortholog of cone arrestin which in mice, frogs, and humans binds to phosphorylated opsin (Craft and Whitmore, 1995; Sakuma et al., 1998) we identified only one gene in the zebrafish genome, suggesting that the presumed paralog was lost in the ancestry of zebrafish. The functional relevance of these duplications is not clear. If, as for grk1a and grk1b, the paralogs are expressed in different tissues it is conceivable that they have also diverged in function (for an example see Lister et al. (2001)).

**Larval zebrafish as genetic model for cone vision**

To study the function of GRK7 in an in vivo model we used larval zebrafish. At larval stages only few nascent rods are formed that do not contribute significantly to visual function before 15 dpf (Bilotta et al., 2001).
Consequently the larval retina can be regarded as functionally cone only. This opens the door for a genetic analysis of vertebrate cone vision. For the study of Grk7a function we made use of the precocious development of cone photoreceptors, allowing us to study cones in larval stages, where morpholino mediated knockdown is feasible. Morphant larvae can be a powerful complement to genetically modified mice, if carefully controlled. It has been shown before that morpholino activity can be long lasting (Seiler et al., 2005; Sollner et al., 2004) while in many cases gene expression recovers after 2 or 3 dpf. For morpholino experiments at later larval stages it is therefore indispensable to monitor efficacy of the knockdown on the protein level. For this purpose we raised polyclonal antibodies directed against zebrafish Grk7a. Knockdown of Grk7a protein was more than 95% in 4 dpf lava as judged by western blots, and Grk7a expression recovered only slightly up to 7 dpf. A ready explanation for this efficiency comes from our expression study. We found that after 3 dpf grk7a mRNA levels are only high in newborn photoreceptors close to the marginal zone, while grk7a is down-regulated in older cones. Blocking of translation at 3 dpf is therefore predicted to result in a low Grk7a expression persistent at later stages. Besides efficacy, the specificity of morpholino action has to be shown. We established a dose response relationship to correlate morpholino dose with defect in temporal contrast sensitivity. The most convincing indication for a specific effect of the Grk7a knockdown is the specificity of the phenotype. Visual performance is not affected under dark adapting conditions with low spatial and temporal frequency stimuli, but strongly reduced under light adapting conditions. Therefore we can, in agreement with our morphological data, rule out a general nonspecific effect on the retina.
Grk7a deficiency leads to prolonged dark adaptation
To study functional consequences of Grk7a deficiency on outer retinal function we examined cone responses with a double flash paradigm. Grk7a deficient zebrafish larvae showed recovery that was delayed by a factor of about 3. This delay resembles defects found in Oguchi disease in rods (Cideciyan et al., 1998). Besides the delayed response recovery in these patients also a delayed rod dark adaptation is observed. The reason for this defect is still unclear. (Yamamoto et al., 1997) attributed this to a delayed recovery of opsin by 11-cis retinal. Lamb and Pugh (2004) argue that slower decay of an active metarhodopsin species which is not bound to arrestin is the cause for delayed bleaching adaptation in RK and arrestin deficient patients. Both hypotheses agree though that active opsin species have to be removed in order to regain rod sensitivity after light stimulation, and rhodopsin phosphorylation accelerates this process. Our results suggest that inactivation of photoactivated cone opsin plays a similar role in cone bleaching adaptation as has been shown for rhodopsin phosphorylation.

Grk7a knockdown leads to reduced temporal contrast sensitivity specifically under light adapting conditions
Besides electrophysiological defects also visual performance measured by psychophysical tests based on the OKR was impaired in Grk7a deficient larvae. Under light adapting conditions temporal contrast sensitivity was lower compared to the dark adapted state, which is the reverse of the normal situation where light adaptation improves the sensitivity to high temporal frequency patterns (Kelly, 1961). The acceleration of the temporal transfer function that is seen in wild-type larvae under light adapting conditions likely results from the reduced lifetime of activated opsin in the presence of steady light. A prolonged lifetime of activated opsin in Grk7a knockdown
larvae is in turn expected to result in a slower temporal transfer function and decreased temporal contrast sensitivity.

However, the defects we found in Grk7a deficient larvae are not in agreement with the conclusions from a correlative study with enhanced S cone syndrome (ESCS) patients (Cideciyan et al., 2003). This disease leads to a photoreceptor mosaic dominated by cones expressing short-wavelength-sensitive opsin. For unknown reason, in postmortem analysis of a single ESCS donor, L/M cones did not show GRK7 immunoreactivity, S cones even lacked immunoreactivity to GRK1 and GRK7. In ERG measurements done with ESCS patients, deactivation of L/M cones was normal. From the eye donor however no ERGs could be measured. It remains to be seen if absence of GRK7 in L/M cone is a general phenotype of ESCS.

In the light of our results the most parsimonious explanation for the role of cone specific opsin kinases is that in humans GRK1 is involved in rod desensitization but only little in cone desensitization. For humans and other species with sophisticated cone vision another kinase, namely GRK7, is involved in cone desensitization. Mice in contrast use rhodopsin kinase for rods and cones and do not express GRK7. Such a model has been proposed before by (Chen et al., 2001) on the base of the expression differences of GRK1 and GRK7 between rodents and cone dominant animals.

The function of a cone specific kinase could be phosphorylation mediated desensitization with an enzymatic rate constant that is specific for the requirements of daylight vision. It has been shown in vitro that the kinetics of cone opsin phosphorylation is different to rhodopsin phosphorylation (Tachibanaki et al., 2001). It is however unclear, if this difference is due to higher reaction rate of the cone opsin kinase or to the shorter lifetime

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of cone opsin (Imai et al., 1997). Kennedy et al. (2004) reported that cone opsin phosphorylation is calcium dependent in larval zebrafish. Since cytoplasmic calcium regulates light adaptation in cones, this finding is consistent with a phosphorylation dependent light adaptation mechanism. It remains to be shown that calcium can affect cone opsin kinase activity.

Although cone response recovery and behavioral light adaptation were reduced in our experiments, even under bright light stimulation contrast sensitivity was not completely lost in Grk7a deficient larvae. This could be due to the small amount of remaining Grk7a which we estimate to be smaller than 5%, but it is unlikely that this contributes considerably under bright light conditions. Enzymatic rate constants are proportional to enzyme concentration under substrate saturating conditions. Consequently, a small remaining amount of active Grk7a can have only proportionally little opsin kinase activity. Alternatively other kinases could be involved in phosphorylation of bleached opsin. Possible candidates for the larval zebrafish are Grk1b and Grk7b. Transcripts of these kinases we found in cones by in situ hybridization; grk7b however was mainly transcribed in the pineal gland and only little in photoreceptors.

Besides the involvement of other kinases, spontaneous decay probably also contributes to inactivation of cone responses. Photoactivated cone opsin decay considerably faster compared to rod opsin (Imai et al., 1997). In principle this process could contribute to the faster cone response recovery. (Kennedy et al., 2004) reported from studies in larval zebrafish cones that after a bright flash that saturates 20% of cone opsin not more than 25% of bleached pigment is phosphorylated. The remaining part is predicted to decay spontaneously. However, in isolated Xenopus rods that transgenically express cone opsin

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it was shown that shutoff kinetics is markedly delayed if putative phosphorylation sites are mutagenized (Kefalov et al., 2003). Our *in vivo* data together with results of (Lyubarsky et al., 2000) in GRK1 knockout mice also indicate that in absence of kinase activity cone response recovery is delayed. In conclusion, we showed that the zebrafish paralog of the cone specific opsin kinase GRK7 does function in vivo and is essential for normal cone response recovery, similar like rhodopsin kinase in rods, suggesting that mechanisms such as spontaneous decay or pigment bleaching alone are not sufficient for cone light adaptation.
Methods

Fish care
Experiments were carried out in accordance with the European Communities Council Directive for animal use in science (86/609/EEC). Wild-type fish from the inbred WIK strain were bred and crossed as previously described (Mullins et al., 1994). Embryos were raised at 28°C in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl$_2$, and 0.33 mM MgSO$_4$) and staged according to development in days postfertilization (dpf).

Cloning
About 50 6 day old zebrafish larvae were homogenized and total RNA was prepared using the Qiagen RNeasy kit (Qiagen, Hombrechtikon, Switzerland) according to the manufacturer's protocols. Total RNA was reverse-transcribed with reverse transcriptase using oligo dT primer (First Strand Kit, Stratagene, La Jolla, CA). Polymerase chain reaction (PCR) was performed with Taq polymerase (Taq Gold, Applied Biosystems Biosystems, Switzerland) using nondegenerate oligonucleotide primers. Primer sequences were derived from transcripts predicted from ESTs and genomic sequence.

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<td>grk1b_rev</td>
<td>5’-TAAATTATTTCCACGCACAC-3’</td>
</tr>
<tr>
<td>c-arr</td>
<td>c-arr_fw</td>
<td>5’-GCTGGCCCTGTGCTCTCCGCTA-3’</td>
</tr>
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</table>
Amplified fragments were ligated to TOPO PCR 2.1 or TOPO PCR-II (Invitrogen, Basel, Switzerland) and sequenced. To verify the accuracy of the cDNA sequences, a minimum of 2 clones from different PCR reactions were analyzed for each region. Additionally searches against the Ensembl trace database (http://trace.ensembl.org/) and EST sequences were performed. The final sequence was derived from multiple alignments of sequenced and retrieved traces. Homology of cloned genes to their respective paralogs was derived with the bl2seq program using the BLOSUM62 scoring matrix (Tatusova and Madden, 1999). The full coding nucleotide sequences (cds) for zebrafish grk7a and grk7b have been deposited in the GenBank database under the GenBank Accession Number (AY900004) and (AY900005) respectively. The nucleotide sequences for zebrafish grk1a and grk1b have been deposited under the accession numbers (AY900002) and (AY900003). The nucleotide sequence for the partial cds of zebrafish c-arrestin got the accession number (AY900006).

A recombinant GST-Grk7a fusion protein was constructed by amplifying a 1722 bp product from the grk7a cds with primers grk7a_rec_fw and grk7a_rev. The forward primer contained a SalI restriction site. The PCR product was ligated with a TOPO PCR-II cloning vector. A 1755 bp SalI/NotI fragment was then ligated into the expression vector pGEX-2T that had been cut with the same enzymes. Sequencing verified that the plasmid contained the GST-tag in frame with the full grk7a cds.

**Whole-mount in situ hybridization**

Full-length grk7a was amplified using the forward primer grk7a-fw and reverse primer grk7a_rev. The 1789 bp fragment was cloned and used as a template to synthesize a
digoxigenin labeled in situ RNA probe. For grk7b the forward primer grk7b_fw and reverse primer grk7b_rev was used to amplify a 1798 bp fragment containing the full cds. To make probes from the c-arrestin cds the forward primer c-arr_fw and reverse primer c-arr_rev was used to amplify a 1249 bp fragment containing partial cds. The full rka cds was amplified with the forward primer grk1a_fw and the reverse primer grk1a_rev resulting in a fragment of 1819 bp. Probes from a 1069 fragment of grk1b were made with the forward primer grk1b_fw2 and the reverse primer grk1b_rev.

All PCR-products were cloned into TOPO PCR-II or TOPO PCR 2.1 vectors (both Invitrogen) and sequenced. In vitro transcription of DIG labeled probes was done with the Roche RNA Labeling Kit (Roche Diagnostics, Rotkreuz, Switzerland). RNA-probes were hydrolyzed to yield fragments of an approximate size of 300 bp. Whole-mount in situ hybridization with hydrolyzed probes was performed in PTU treated larvae as described in (Thisse et al., 1993). For transverse cross sections stained unpigmented embryos homozygous for sdy, which encodes tyrosinase, (Page-McCaw et al., 2004) were embedded in Technovit 7100 (Kulzer Histotechnik, Germany).

**Antibodies**

Rabbit antisera against two peptides of Grk7a (peptide1: 385 - 398 CFKGPDAKKEKVEKE and peptide2: 521 – 535; CLFDELSDPNRKESSG) were made by Eurogentec (Seraing, Belgium) with a N-terminal C added. Affinity purified antibodies against peptide2 are referred to as anti-Grk7a in this paper.

**Immunostaining and histology**

For immunohistochemistry, fish larvae were anesthetized on ice, and then immediately fixed in 4% paraformaldehyde in 0.2 M phosphate buffer, pH 7.4 for 1 h (4°C). Fixed

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larvae were cryoprotected in 30% sucrose for at least 4 h. Whole larvae were embedded in Cryomatrix (Tissue Freezing Medium; Jung-Leica, Germany), and rapidly frozen in liquid N$_2$; 25µm thick sections were cut at −20°C, mounted on superfrost slides, and air dried at 37°C for at least 2 h. Slides were stored at −20 °C. Before further use, slides were thawed and washed three times in phosphate-buffered saline (PBS; 50 mM), pH 7.4, and treated with 20% normal goat serum (NGS), 2% bovine serum albumin (BSA) in PBS containing 0.3% Triton X-100 (PBST) for 1 h. Sections were then incubated overnight in primary antibodies in PBST at 4°C.

For Grk7 immunostaining, rabbit antisera against Grk7a 1:1000, and the respective rabbit preimmuniserum (negative control) were used as primary antibodies. The immunoreaction was visualized by using Alexa Fluor 488 anti-rabbit IgG (Molecular Probes, Leiden, Netherlands) 1:1000 as a secondary antibody.

For the Grk7a-rhodopsin double staining, rabbit anti-Grk7a 1:1000 and mouse anti-rhodopsin (Rho; Biodesign, Saco, ME) 1:250 were used in a cocktail. The immunoreaction was visualized by using a mixture of Alexa Fluor 488 anti-rabbit 1:1000 and Alexa Fluor 568 anti-mouse IgG (Molecular Probes, Leiden, Netherlands) 1:1000 as secondary antibodies. For all immunocytochemical experiments, negative controls were carried out in the same way but without using the first antibody.

For Richardson stainings sections (7 µm) were stained with 1% methylene blue, 1% borax in deionized water for 1 min and coverslipped.
**Photography**

Stained whole mount embryos were photographed in 100% glycerol under a dissecting microscope (Leica MZ FLIII with a Leica DC300F). Technovit cross-sections and immunolabeling on slides was either viewed with a Zeiss Axioskop 2 MOT light microscope (Carl Zeiss, Jena, Germany) or a Zeiss LSM 410 confocal microscope (Carl Zeiss, Jena, Germany). The obtained images were processed using Adobe Photoshop 8.0 (Adobe Systems, San Jose, CA).

**Targeted gene knockdown**

For targeted knock-down of the Grk7a protein an antisense morpholino (MO) oligonucleotide (\textit{grk7aMO}) (GeneTools, LLC, Philomath, OR) was used covering -1 to +24 of the \textit{grk7a} mRNA: 5’-ATCGAGTCCCCCCATGTCACACATT-3’. For control-injections, a standard control morpholino oligonucleotide, \textit{controlMO}, 5’-CCTCTTACCTCAGTTACAATT TATA-3’ was used. The MOs were dissolved in 0.3x Danieau’s solution (1x Danieau: 58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO$_4$, 0.6 mM Ca(NO$_3$)$_2$, 5 mM Hapes, pH 7.6) to obtain a stock concentration of 2 mM. The stock solutions were diluted to 500 $\mu$M. Morpholino solutions were injected into the yolk of the fertilized egg between 1 and 4 cell stage. If not otherwise indicated, the injected amount was approximately 30 ng morpholino oligonucleotide per zygote.

**Western blots**

To quantify gene knockdown, 10 larvae were homogenized in 50 $\mu$l Tricine buffer (50 mM Tricine, 100 mM NaCl, pH 7.5). Insoluble material was removed by ultracentrifugation. Prior to loading, samples were adjusted to equal protein amount by quantification of total protein with DC Protein Assay (Bio-Rad, Hercules, Ca).
Additionally, protein amount loaded was controlled by staining for Beta-actin (accession no. Q7ZVI7) with a monoclonal antibody (A 5441, Sigma, Saint Louis, MO) against mouse Beta-actin (accession no. NP_031419), which shares 98% identity to zebrafish Beta-actin. For this purpose membranes were cut at 50 kDa. The upper part was incubated with anti-Grk7a antibodies. The lower part was incubated with anti-Beta-actin. Semiquantitative analysis of the GRK7a knockdown ratio was done by evaluating 3 independent injection experiments with the gel analysis tool in ImageJ (NIH, Bethesda, MD). Autoradiographic films were imaged with a scanner. The relationship between autoradiography values and gray values was linearized by blotting a dilution series which was used to calibrate scanned blots.

**Psychophysics**

Behavioral temporal contrast sensitivity under dark and light conditions was measured as described (Rinner et al., 2005). Briefly, single larvae were placed dorsal up in the center of a petri dish (35 mm diameter) containing 3% methylcellulose. Moving sine-wave gratings were projected by a Proxima 4200 DLP projector onto a cylindrical screen within the visual field of the larva, at an apparent distance 4.65 cm from the larva’s right eye. The image was wrapped on the screen spanning a visual angle of 118 deg horizontally and 52 deg vertically. Mean intensity levels were adjusted by introducing neutral density filters into the light beam. Eye angle and velocity under visual stimulation were recorded by means of an infrared sensitive CCD camera. Custom-developed software on the basis of LabView IMAQ (National Instruments, version 5.1) was used to control stimulation and camera and to analyze the resulting images. Eye velocity was determined by Levenberg-Marquardt fitting of sine functions to the eye
velocity recordings. Amplitudes of the fitted curves indicated the eye velocity for a
certain stimulus configuration. Measures of dispersion for the fitted amplitude $A$ were
derived from bootstrap sampling. Random samples with the size of the original sample
were drawn for each experimental condition, sine curves were fitted for each resampled
data set ($n = 1000$) and amplitudes $A^*$ where determined. The standard deviation of the
bootstrap distribution of $A^*$ constitutes the confidence intervals in this statistical method
(Efron and Tibshirani, 1993). Inference statistics was done according to (Simon, 1985).
Significance of group differences of eye velocity was determined by comparison with
the bootstrap sampling distribution of differences between randomly drawn samples of a
pooled data set. For example, to get significance limits for the difference in OKR gain
between morphants ($n = n_1$) and control larvae ($n = n_2$) under certain experimental
conditions, data was pooled ($n = n_1 + n_1$) and two samples of the sizes $n_1$ and $n_2$ were
drawn with replacement. OKR amplitudes $A_1^*$ and $A_2^*$ were determined and the
bootstrap distribution of $\Delta A^* = A_1^* - A_2^*$ was derived from 10,000 resampling
iterations. Percentiles according to the chosen $p$ level indicate the significance limit for
the measured differences between morphant and control larvae. Significance level
chosen was 5% for all experiments, for multiple comparisons it was adjusted by
Bonferroni correction for $n$ comparisons by $\alpha/n$.

Contrast sensitivity functions for grk7a morphants and their controlMO treated siblings
were measured by the gain (eye velocity/grating velocity) as a function of temporal
frequency and intensity of a moving grating. Movement direction of the grating was
varied by a temporal square function with a frequency of 0.17 Hz.
To determine temporal contrast sensitivity under bright light and dark conditions, the pattern velocity of a sine grating was varied keeping all other parameters constant. Stimulus intensity was adjusted by neutral density filters in the light beam. Initially larvae were adapted for 2 minutes to a background of 0.7 cd/m². Then a low temporal frequency pattern (0.033 cycles/deg) with a velocity of 4.5 deg/s was presented and optokinetic gain was measured for 2 periods. Spatial frequency was increased to 0.084 cycles/deg and gain was determined for 4.5, 8.9, 17.8, 26.6, and 35.4 deg/s, then neutral density filters were changed to provide an averaged intensity of 363 cd/m². Larvae adapted to this condition for 30 s and measurements were repeated with a grating of 0.084 cycles/deg as above.

**Electroretinography (ERG)**

ERGs were performed on larvae at 4 dpf as described previously (Makhankov et al., 2004). All pre-recording steps were done under red illumination. Preparation and recordings were performed in a tight Faraday cage. The subjects were dark-adapted for at least 30 min prior to positioning them in the recording chamber.

To test cone response recovery a 500-msec conditioning flash (212 lux) was followed by a probe flash of the same light intensity duration with different interstimulus intervals (1, 2, 3, 5, 10 sec). Recovery was measured as ratio of b-wave peak responses to conditioning and probe flash.

To examine whether cone dark adaptation is affected after bleaching stimulus, ERG responses to a dim test stimulus (2.1 lux, 100 msec) after a bleaching stimulus were recorded. Initially larvae were dark adapted for 30 min. Then a 10 s bleaching light pulse
(2120 lux) was applied. Responses to the test flash were measured at different time points after pulse.

ERG b-wave amplitudes were measured as range of minimum potential between 20 – 80 ms and maximum 100 – 200 ms after onset of light stimulus. Measures of dispersion for the measured amplitudes $A$ were derived from bootstrap sampling. Random samples with the size of the original sample were drawn for each experimental condition, b-wave amplitude $A^*$ was determined for each resampled data set. The standard deviation of the bootstrap distribution of $A^*$ from 1000 resampling iterations is an estimation of the standard error of the mean b-wave amplitude.

Acknowledgments

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Figure legends

Figure 1. In situ hybridization with riboprobes against cone and rod specific kinases and arrestin. (A-F) Whole mount in situ hybridization with PTU treated 4 dpf larvae. (A) grk7a is expressed in cones photoreceptors and the pineal gland. (B) grk7b is expressed in the pineal gland and in low amounts in the photoreceptors. (C) c-arr is expressed in cone photoreceptors and the pineal gland. (D) grk1a is only expressed in rods whereas grk1b (E) stains cone photoreceptors. (F) control staining with rhodopsin showing staining of nascent rods. (G-I): Sagittal sections of whole mount stainings in sandy larvae with grk7a antisense riboprobes. Expression in 3 (G), 4 (H), and 5 dpf (I) larvae shows that grk7a mRNA levels are strongest in newborn cells at the marginal zone. Expression of grk7a is reduced toward the medial retina where older cells are located.

Figure 2. Confocal images of immunostaining for Grk7a in transverse sections of 5dpf zebrafish larvae. (A) Grk7a-positive cones are labeled ubiquitously throughout the entire retinal section.
(B) Higher magnification shows the strong Grk7a labeling, specifically in cone outer segments (asterisks). (C) Grk7a labeling is most pronounced in cone outer segments (asterisk) and weaker in cone inner segments (open triangle), cone processes (arrowhead) and cone pedicles (arrow) in the outer plexiform layer. Scale bars: A and B 20 μm; C 5 μm.

Figure 3. anti-Grk7a and anti-rhodopsin double labeling in transverse sections of 5 dpf larvae. (A, B) Immunostaining of Grk7a (green) is specific for cones. Colocalization of Grk7a and rhodopsin was not observed; nascent rods are exclusively labeled by the anti-rhodopsin antibody (red). Scale bars: 20 μm.

Figure 4. Morpholino knockdown of GRK7a. (A) Eye morphology of 4 dpf grk7aMO injected larvae as compared to siblings injected with a control Morpholino. Transversal sections through the eyes show no morphologically defect in the retina of grk7aMo treated larvae. (B) Western blot analysis indicates nearly complete knockdown of Grk7a in 3 and 4 dpf grk7a morphants. Total protein of larvae raised from zygotes injected with 30 ng grk7aMO or controlMO was incubated with anti-Grk7a antibody resulting in a single band of approximately 62 kDa which is almost completely down-regulated in grk7aMO treated larvae. (B') The same blot overexposed shows a faint signal of Grk7a in 4 dpf grk7aMO treated larvae. (C) The anti-Grk7a antibody cross-reacts with recombinant Grk7a expressed in E. Coli as GST-tagged protein with a molecular weight of 82 kDa. Transformation with the empty vector does not result in a signal.

Figure 5. Cone photoresponse recovery is impaired in grk7a knockdown larvae. (A-C) ERG recordings with 500 ms conditioning (dark line) and probe flash (light gray line) with an ISI of 1 s. (A) Averaged ERG recordings of conditioning and probe flash. Recovery of the ERG b-wave is about 55% complete in control morphants after 1s. (B) In grk7aMO injected larvae the recovery after 1s is below 10%. (C) The fading vision mutant, which has a delayed visual cycle and shortened cone outer segments, shows normal cone response recovery. (D) Time course of the b-wave recovery in grk7aMO (black triangles) and controlMO (open triangles). The inset shows the regression to the logarithmic linear part of the recovery kinetics. Error bars indicate standard error of the mean derived from the bootstrap sampling distribution.

Figure 6. Temporal contrast sensitivity is reduced in larvae deficient for Grk7a. (A) Optokinetic gain as function of temporal frequency of a sinewave grating (0.084 cycles/deg) at 4, 5, and 7 dpf. OKR was measured under dark (0.7cd/m², solid symbols) and light adapting (363 cd/m²) conditions (open symbols). Offset symbols shows optokinetic gain measured with a low temporal frequency pattern (0.034 cycles/deg, 0.7 cd/m²). Under these conditions grk7aMO and control MO injected larvae respond with similar optokinetic gain. Light adaptation improves temporal sensitivity in control morphants. In grk7a morphants sensitivity is reduced under light adapting condition compared to the dark adapting condition. Sensitivity in the dark condition is slightly reduced in grk7a morphants at 4 and 5 dpf but recovers at 7 dpf. (B) In a Western Blot made with larvae from the same clutch as in (A) Grk7a is strongly reduced in grk7a morphants between 4dpf and 7 dpf and increases only slightly after 4 dpf. (C) Dose response relationship in grk7aMO injected larva. Temporal contrast sensitivity for a sinewave grating (0.084 cycles/deg; 41 cd/m²) measured with larvae injected with varying doses of grk7aMO (c1: 5 ng, c2: 10 ng, c3: 30 ng). Sensitivity decreases with increasing amount of injected morpholino. Error bars indicate standard error of the mean derived from the bootstrap sampling distribution.

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Figure 7. Dark adaptation is delayed in grk7aMO injected larvae. Recovery of the ERG b-wave in response to a dim test light (21 lux, 100 ms) at different time points after bleaching with a 10 sec light pulse of 2120 lux indicates delayed cone dark adaptation in grk7a morphants. (A) ERG recordings of a 500 ms stimulation (2.1 lux, 100 msec) after 30 min. dark adaptation (control) and at different times after 10 s bleaching with 2120 lux (B) b-wave recovery relative to the pre-bleaching recording with regression to the logarithmic linear part of the kinetics. Error bars indicate standard error of the mean derived from the bootstrap sampling distribution.


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Figure 1.
Figure 2. Function of GRK7 in zebrafish.
Figure 3.

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Figure 5. Function of GRK7 in zebrafish.
Figure 6.

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