Assessment of outer retinal function in genetically modified zebrafish larvae by electroretinography (ERG)

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Publication Date:
2005

Permanent Link:
https://doi.org/10.3929/ethz-a-005071343

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Assessment of outer retinal function in genetically modified zebrafish larvae by electroretinography (ERG)

A dissertation submitted to the
Swiss Federal Institute of Technology Zurich

For the degree of
Doctor of Sciences

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2005
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Summary

The zebrafish is increasingly used to study the genetics of vision. Although our knowledge of visual system development is increasing rapidly, we know considerable little about the genetics of visual system function.

In the present studies, I have developed an experimental tool to study visual function by recording electroretinograms (ERG). The electroretinogram measures the sum field potential of the retina in response to changes in light stimulation. This experimental approach was then applied to a number of genetically modified (by random mutagenesis or antisense nucleotide mediated gene knockdown) mutant strains to probe the physiological properties of the outer retina.

In an introductory chapter, I first summarize our current knowledge of electrophysiological properties of the vertebrate retina, with an emphasis on the outer retina and properties of the zebrafish retina. The second chapter describes a simple ERG setup for measuring outer retinal activity in intact both larval and adult aquatic vertebrates, followed by a chapter on the hands on use of this instrument. This chapter is aimed to be used as a manual for future researchers to use the ERG set-up.

The following chapters describe the application of this powerful electrophysiological technique on a number of selected zebrafish strains with visual defects.

In chapter 4, the function of protocadherin 15 is studied. A mutation of this gene causes Usher syndrome, congenital hearing and vision loss, in humans. In the zebrafish two paralogous exist, with one being essential in hearing, while the other paralogue is involved in outer retina morphology. Consequently, the functional inactivation of only paralogue leads to an unaltered ERG, while knocking down the other version leads to disrupted outer retina function.

In chapter 5, I describe the function of the cone specific kinase GRK7. Functional inactivation of this enzyme leads to a delayed recovery of b-wave amplitude in a double flash paradigm. This defect is also apparent at the behavioral level, since dark adaptation and temporal contrast sensitivity was affected.

Finally, in chapter 6, I present a brief overview of my work and discuss the relevance of the obtained results.

In summary, I present a useful electrophysiological technique to study visual system function, particularly of cone photoreceptors. The role of pcd15 and GRK7 in cone photoreceptor function is discussed.
Zusammenfassung

Der Zebrafisch wird vermehrt dazu verwendet die Genetik des Sehens zu erforschen. Obwohl unser Wissen über die Entwicklung des Sehsystems schnell voranschreitet, wissen wir doch vergleichsweise wenig über genetische Faktoren, die die Funktion des Sehsystem steuern.


In einem einführenden Kapitel fasse ich den Stand des Wissen über die elektrophysiologischen Eigenschaften der Wirbeltierretina zusammen. Dabei betone ich besonders die Eigenschaften der äusseren Retina und Eigenschaften der Zebrafischretina.

Im zweiten Kapitel beschreibe ich ein einfaches Gerät zur Messung der äusseren Retinafunktion in lebenden Larven und erwachsenen aquatischen Tieren mittels Elektroretinographie. Das folgende Kapitel enthält eine detaillierte Bedienungsanleitung, die es zukünftigen Forschern ermöglichen soll das Gerät selbstständig zu bedienen.

Die beiden folgenden Kapitel beschreiben die Anwendungen dieser Technik auf zwei genetisch manipulierte Stämme mit Sehdefekten.


Im fünften Kapitel beschreibe ich die Funktion der zapfenspezifischen Kinase GRK7. Inaktivierung dieses Enzmys führt zu einer verzögerten Wiederherstellung der b-Wellenamplitude in einem Doppelblitzversuch. Dieser Defekt spiegelt sich auch im Verhalten wieder, da Dunkeladaptation und die zeitliche Kontrastsensitivität betroffen ist.

In Kapitel 6 gebe ich einen kurzen Überblick über meine Arbeit und diskutiere die Bedeutung der erzielten Ergebnisse.
Zusammenfassung

Zusammenfassend stelle ich eine nützliche elektrophysiologische Methode zur Untersuchung der Sehsystemfunktion, vor allem die der Zapfenphotorezeptoren, vor. Die Bedeutung von pcd15 und GRK7 für die Zapfenfunktion wird diskutiert.
Chapter 1: Introduction
1 Introduction

1.1 The vertebrate visual system

The visual system has been particularly attractive to study neural processing. The optic nerve (ON) is the communication channel by which the retina communicates with the rest of the brain. The retina is easy to stimulate with light of defined properties. First single cell recording was done by Hartline in 1938 (Hartline, 1938) by dissecting out individual optic nerve axons. He divided them into three groups of fibers that had different responses: Group I showed responses only during stimulation; Group II showed responses at the cessation of illumination; Group III responds at both onset and cessation of the light. Another electrophysiologist, Ragnar Granit (Granit, 1947) went further by introducing extracellular microelectrode recordings from the optic nerve. He showed the similar responses from ganglion cells in a wide range of animals. Horace Barlow (Barlow, 1953a; Barlow, 1953b) and Stephen Kuffler (Kuffler, 1953) showed that single ganglion axons were capable of sending more significant information to the brain visual centers rather than the simple message that a light was just switched either “on” or “off”. Those studies opened the field of cellular and synaptic organizations – the information that is significant for understanding the underlying circuitry for brain processing.

1.2 The vertebrate retina is an approachable part of the brain

Developmentally, structurally and functionally the retina is a part of the brain. The retina provides an excellent source of information for detailed anatomical and physiological analyses. As a part of the brain, the retina allows to study the neural mechanisms underlying elementary visual information processing by the brain.

The retina lines the back of the eyecup and is accessible (Fig. 1). It has a well-defined and ordered anatomical layered structure. All vertebrate retinae are composed of three layers of nerve cell [photoreceptor cell layer (PCL) consists of photoreceptor cells; inner nuclear layer (INL) consists of horizontal, bipolar and amacrine cells; ganglion cell layer (GCL) consists of ganglion cells and displaced amacrine cells] and two synaptic layers [outer plexiform layer (OPL) consist of synaptic contacts between photoreceptor cells and both bipolar and horizontal cells; inner plexiform layer (IPL) consists of synaptic contact between bipolar
cells, amacrine and ganglion cells]. In the retina network that exists all neurotransmitters are found elsewhere in the Central Nervous System (CNS).

![Diagram of the layers of the developing human retina around 5 months' gestation](image)

Figure 1. Scheme of the layers of the developing human retina around 5 months' gestation (R-rod; C-cone; B-bipolar cell; H-horizontal cell; Am-amacrine cell; M-Müller cell; As-astrocyte) A three-neuron chain-photoreceptor, bipolar cell and ganglion cell-provides direct route for transmitting visual information to the brain. Horizontal cells and amacrine cells mediate lateral interactions in the outer and inner plexiform layers, respectively (Ogden, 1989)

1.3 The development of the eye

The vertebrate eye is derived from three types of embryonic tissue: the neural tube (neuroectoderm), from which the retina arises and its associated pigment cell layer; the mesoderm of the head region, which produces the cornea-scleral and uveal tunics and the
ectoderm, from which the lens is formed (Fig. 2). The eye of all vertebrates develops in a pattern, which produces an "inverted" retina. The initial detection of light takes place at the outermost portion of the eyecup, the photoreceptor layer. The tier of cells at the back of the retina contains photoreceptors cells: rods and cones. Rods are responsible for vision in dim light (scotopic vision) and do not work due to saturation in bright light. Cones do not respond to dim light but are capable to see fine detail and color light during the day (photopic vision). The ratio between both rod and cone densities in the retina varies from species to species, depending on their natural habitat.

![Figure 2](www.colorado.edu/MCDB/MCDB4620/13ppt.pdf)

**Figure 2. Development of the eye.** (A) The optic vesicle evaginates from the diencephalon. (B) The vesicle then invaginates to form the optic cup. (C) The "back" of the optic cup becomes the retinal pigment epithelium (RPE). The "front" of the cup becomes the neural retina. (www.colorado.edu/MCDB/MCDB4620/13ppt.pdf)

### 1.4 The Zebrafish as a model organism to study development and organogenesis

During the last decades, the zebrafish (Danio rerio) has become one of the model organisms to study biological processes in vivo. The pioneer in the Zebrafish field was George Streisinger, who chose the Zebrafish as a model for vertebrate development in 1970s. He first utilized the possibilities of genetical manipulation of the chosen model (Streisinger et al., 1981).

The Zebrafish is a teleost fish of the cyprinid family in the class of ray-finned fishes (Actinopterygii). Nearly all contemporary fish species are teleosts. The Zebrafish genome
comprises 1.7 gigabases. It is distributed over 25 chromosomes (1n). The absolute number of genes in zebrafish is currently unknown.

The combination of excellent embryology and the powers of genetic manipulation make the zebrafish one of the vertebrate model organisms for biological research. Fertilization and subsequent embryonic development are external and occur simultaneously in large clutches. The embryos are relatively large and their development can be observed through the chorion. The embryos are completely transparent, allowing the visualization of developing organs inside the living larva. Embryonic development is rapid. Zebrafish larvae have hatched and are able to swim and search for food as soon as 5 dpf (days post fertilization).

Methods for high-efficiency N-ethyl-N-nitrosourea (ENU) mutagenesis were established and large-scale screens were done resulting in close to 2000 characterized mutants in 1996. A special issue of the Journal of Development was published in 1996 describing the results of these first large scale genetic screens in vertebrates. Numerous zebrafish mutant phenotypes resemble human diseases. Powerful cellular methods, such as cell labeling, transplantation, and microinjection enable a detailed understanding of zebrafish mutant phenotypes. Thus, phenotypic analysis in zebrafish can provide functional information that is difficult to obtain in other species.

1.5 Morpholino “knockdown” of gene activity

There are two types of genetic approaches to study gene function. First, large-scale “forward” genetic screens begin with the isolation of an interesting mutant phenotype and end with the molecular analysis of the genes underlying these phenotypes. Second, “reverse” genetic approaches begin with a gene of interest and end with the analysis of the phenotype which results from their mutation. In the last decades, the breakthrough in the zebrafish reverse genetics came with the recent development of the morpholino synthetic antisense oligonucleotide. Such an antisense nucleotide can be used to efficiently “knock down” gene activity in larval zebrafish at early stages of development (Fig. 3). Morpholinos are chemically modified oligonucleotides. They are able to bind to mRNA and prevent their translation by forming a double helix, thereby preventing protein synthesis. A morpholino consists of monomers that contain any of the four bases (A-adenine, C-cytosine, G-guanine, T-thymine). Unlike nucleic acid, morpholinos are uncharged and non-toxic, even when injected at high concentrations.
The use of morpholinos in zebrafish have shown these compounds to be sequence specific and extremely potent in all cells during the first 50 hours of development. This time period in the zebrafish embryo includes the fundamental vertebrate processes of segmentation and organogenesis. In many cases, this period can be considerably extended up to 7 dpf (e.g. see chapter 5).

Figure 3. Segment a Morpholino-RNA heteroduplex, 8-mer shown (http://en.wikipedia.org/wiki/Morpholino).
1.6 The Zebrafish visual system

The Zebrafish has a functional visual system at 4 dpf (Clark, 1981). Zebrafish photoreceptors develop and mature during larval stages. The zebrafish visual system is tetrachromatic. Their retinae contain four types of cones that absorb light maximally in the red [570 nm; L-cones], green [480 nm; M-cones], blue [415 nm; S-cones] and UV [362 nm; U-cones] regions of the spectrum (Fig. 4) (Nawrocki et al., 1985; Robinson et al., 1993). Zebrafish rods contain rhodopsin with a $\lambda_{\text{max}}$ of 500 nm (Schwanzara, 1967a; Schwanzara, 1967b).

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**Figure 4.** Difference spectra of adult zebrafish photoreceptors. Traces are the difference between absorbance spectra recorded in dark-adapted cells and following a 1 min exposure to light at the $\lambda_{\text{max}}$ of the pigment. (A) SS cone. (B) LD cone (similar difference spectra were obtained in LS cones). (C) SD cone. (D) Rod (Nawrocki et al., 1985).
In adults, cones are arranged in a row mosaic (Fig. 5) (Branchek and Bremiller, 1984a; Robinson et al., 1993).

![Figure 5](image.png)

**Figure 5.** Light micrograph (LM) of mosaic organization of adult retina. Outer segments are indicated as follows: 1-rod; 3-double cone; 5-long single cone; 9-short single cone; 12-rod nuclei. Ellipsoid of long double cone (arrowhead) is positioned scleral to ellipsoid of short double cone (arrow); p-pigment granules of the pigment epithelial cells. Bar=6 μm (Branchek and Bremiller, 1984a).

Red- and green-sensitive cones alternate in sequence and are separated by either a single blue cone (always adjacent to a red-sensitive cone) or a single UV cone (always adjacent to green-sensitive cone) (Robinson et al., 1993). The expression of the red opsin begins at about 52 hpf (hour post fertilization) followed by blue and then UV opsins (Raymond et al., 1995). By about 3 dpf (days post fertilization) the zebrafish has opsin expressing cones distributed throughout the photoreceptor layer of the retina.

The larval eye is quite different structurally from that of the adult. This raises the possibility that image formation might be different at these stages (Easter and Nicola, 1996). The absolute size of the larval eye is smaller. It is about 0.2-0.3 mm in diameter versus several millimeters in the adult. The relative size of the ocular contents are different from adult. The larval lens and retina have approximately the same thickness. The adult lens is 10 times as thick as the retina. The vitreal space is absent in the larva. Therefore, the inner limiting
membrane (ILM) is contiguous with the outer surface of the lens (OSL). The ILM and OSL are separated by the vitreal space in the adult. Sections of 72 hpf retinas show that outer segments were present throughout the sector that included the plexiform layer, but the outer segments were shorter than those in the 96 hpf retinas (Figure 6).

Figure 6. Retinal development between 72 and 96 hpf. (g) shows section from 72 hpf. (h) from 96 hpf. (g,h) Tangential sections through the outer nuclear layer of the nasal retina, showing the transversely cut cone nuclei (Nu) in the central field of both panels and the inner segments (IS) in the surrounding area (Easter and Nicola, 1996).

Anatomical studies of the developing zebrafish retina have shown that rods continue to develop long after hatching. By 12 dpf rod outer segments lengths is only about 25 per cent that of adult rods (Fig. 7) (Branchek and Bremiller, 1984b).
Figure 7. By 8 dpf there were no apparent signs of rod outer segments and by 12 dpf rod outer segment length was only 25% that of adult rods. d2-2 day post fertilization; d2.5-2.5 day post fertilization; d3-3 day post fertilization; d4-4 day post fertilization; d8-8 day post fertilization; d12-12 day post fertilization; d15-15 day post fertilization (Branchek and Bremiller, 1984a)

By 15-20 dpf, rods approach anatomical maturity. Behavioral work has reported that zebrafish rod function begins at about 12 dpf (Clark, 1981). Clark used the optomotor response to measure visual acuity and found that at 7 dpf there were no differences in acuity values obtained under dark- and light-adapted conditions. Hence, there was only cone contribution to the behavioral response at that stage. By 14 dpf, subjects were more sensitive under dark-adapted state than light-adapted one. Clark reasoned that the difference in acuity between the two light conditions reflected the changing contributions of the developing rods with age.

1.7 Behavioral approaches to study visual performance

The visual system of the zebrafish develops quickly. Some behavioral approaches have been implicated to analyze visual performance at both early and adult stages to identify mutant strains with visual defects (Neuhauss, 2003). Some of these behavioral approaches are described briefly below. One of the most robust behavior assays is the OKR (optokinetic response). The OKR is based on the detection of patterned visual cues resulting in stereotypic eye movements (Neuhauss, 2003; Rinner et al., 2005) in larvae (Fig. 8A). The OMR (optomotor response) is based on the detection of moving visual stimuli followed by swimming larvae (Fig. 8B) and adult zebrafish (Fig. 8C) (Neuhauss, 2003). An adult escape response behavioral assay was introduced by the Dowling group (Li and Dowling, 1997). When the zebrafish is exposed to a threatening object, (e.g. a black stripe in a round drum) the fish turn and swim away (Fig. 8D). DLR (dorsal light response) is a reflexive reaction of the fish to tilt along its longitudinal axis (Fig. 8E) in such a way that its dorsal surface faces toward the bright light (Pfeiffer, 1964; Powers, 1978; Silver, 1974; von Holst, 1935).
1.8 Introduction to light and dark adaptation

1.8.1 Dark adaptation

Once bleached, rods and cones recover at different rates. For example, if you go from a very bright light area (a bright sunny day) to a dim one (indoors), it is difficult to see for several minutes after arriving indoors. This phenomenon is called dark adaptation. As seen in figure 9, the difference in the range of illuminance over which rods and cones apparently operate adheres to the duplicity theory of Schultze (1866) (Fig. 9). Max Schultze developed the duplicity theory of vision, based on his study of the retina. He had noticed that in diurnal birds the retina consisted mainly of cones but nocturnal birds possessed a retina with an abundance of rods. This led him to propose that cones must respond to coloured light while rods should be more sensitive to black and white. Dark adaptation forms the basis of the Duplicity Theory:
above a certain luminance level, the cone mechanism is involved in mediating photopic (light) vision. Below this level, the rod mechanism comes into play providing scotopic (night) vision. The point at which the rods become more sensitive is called the rod-cone break.

![Dark adaptation curve](image)

**Figure 9.** Dark adaptation curve. The solid line shows two-stage dark adaptation for a normal subject with a cone branch at the beginning and the rod branch at the end. The dashed line shows the dark adaptation of cones only, which can be achieved by using a small stimulus confined to the fovea. The dotted line shows the rod adaptation curve derived from a rod monochromat observer. The difference in the range of illuminance over which rods and cones apparently operate adheres to the duplicity theory of Schultze (1866). He had noticed that in diurnal birds, the retina consisted mainly of cones but nocturnal birds possessed a retina with an abundance of rods. This led him to propose that cones must respond to coloured light while rods should be more sensitive to black and white.

When light is absorbed by photopigments the prosthetic group isomerizes, turning the opsin into inactive opsin that activated the visual transduction cascade. Our visual system is most sensitive when the photopigments have not absorbed any light in darkness at a certain period. Under these conditions, the photopigments are fully regenerated. When the rod
photopigments are exposed to light, they undergo a process called bleaching. It is called bleaching because the photopigment color becomes almost transparent. In the dark, they regenerate and regain their pigmentation again.

1.8.2 Light adaptation

The cone receptors have outer segments, which contain photopigments (four types of cone opsins in animals such as chicken, ground squirrel, and tree shrew; three types of cone in human being for example). The photopigments in the cones bleach when exposed to light. For example, you go from a dark environment (indoors) to a bright one (bright sunny day). This phenomenon is called light adaptation. Our eyes have to adapt quickly to the background illumination to be able to discriminate objects on the background. One of the major differences between dark adaptation and light adaptation is their time course. Whereas in human dark adaptation takes about 30 minutes to be complete, light adaptation happens very quickly, usually in less than a minute. Another difference between dark and light adaptations is that when you are light adapted and then go into a very dark room for a while you may not see anything at all. As you dark-adapt more and more things become visible. When you go from a darker area to a very bright one you usually are not temporarily blind. It is that our vision temporarily is not very good. To put it another way, our contrast sensitivity is poor until you become light adapted. It means that we will have difficulty in perceiving areas of low contrast. As soon as you quickly light adapt the darker areas become darker and the lighter areas become easier to see. To summarize, light adaptation occurs in two ways: the sensitivity of the retina decreases and retinal neurons undergo rapid adaptation inhibiting rod function.

1.9 Physiological study of the zebrafish retina

The first functional study of rod and cone contribution to the visual performance was done by ERG (electroretinography) in 1984 (Branchek and Bremiller, 1984b). The electroretinographic measurement of flicker fusion frequency (FFF) as a function of light intensity makes it possible to distinguish rod and cone responses. Responses of individual animals gave a biphasic profile characteristic of organisms with duplex retinas. A photoreceptor’s ability to resolve temporal detail can be determined by measuring its FFF electrophysiologically. In photoreceptors functioning in a dim light environment, sensitivity is more important than resolution, which may result in slower photoreceptor transduction and
increased summation time, which would be reflected by a lower FFF. By using the FFF the authors wanted to see whether zebrafish was capable to produce a biphasic FFF response (Fig. 10). In their study, FFF responses have been measured for four series of zebrafish in ten stages of development. The ages were chosen to allow study of the initial appearance of photoreceptors, their growth and their final maturation. Indeed the graph depicted in Fig. 10 showed this biphasic response curve from zebrafish adults. The procedure to apply FFF was as follow. To initiate a recording session, each fish was adapted for 30 minutes before stimulus presentation. A very low intensity stimulus was presented at an initial frequency of 1/second for 100 presentations in 1 log unit increment until the first response was found. Subsequently, the frequency was increased in increments of 2 flashes/second until fusion was obtained. Fusion was defined as the maximum flash frequency for a given intensity at which a response followed the stimulus on a one to one fashion at minimum amplitude of 6 µV. Subsequently, the intensity was increased in 0.5 or 1 log unit increment, in each increasing the frequency of stimulus presentation from 1/second to higher until fusion was obtained for several stimulus intensities.
Figure 10. Regression-fitted plot for pooled data from 25 adults. There was a rod/cone break in the FFF versus intensity function in zebrafish (Branchek and Bremiller, 1984b).

At the age of 4-12 dpf zebrafish larvae showed no response to high intensity stimuli (Fig. 11). Increases in sensitivity and flicker resolution were observed after this age. The authors concluded that functional photoreceptors were present but their functional properties differed from those of the adult.

Figure 11. Regression-fitted plot for pooled data from 50 animals at 4-12 dpf (solid lines); adult profile is shown (dotted line) for comparison. No rod/cone break in the FFF versus intensity function in zebrafish can be recorded at these stages (Branchek and Bremiller, 1984b).

At the stage of 15-24 dpf, the photoreceptor response was close to those of the adults (Fig. 12).
To summarize, prior to age of 15 dpf, there did not appear to be a break in the FFF function (Fig. 11).

In order to determine the nature of the photoreceptor contributions in the developing zebrafish, spectral sensitivity functions must be obtained and compared to the photoreceptor spectra. Recent physiological work has examined adult zebrafish spectral sensitivity under both light-adapted (Hughes et al., 1998) and dark-adapted (Saszik and Bilotta, 1999b) conditions. Under photopic conditions, the spectral sensitivity derived from the b-wave response of the ERG appears to receive contributions from the four known cone types (U-, S-, M- and L-cones). Under dark-adapted conditions, the spectral sensitivity function of the adult zebrafish appears to receive input from rod photoreceptors containing rhodopsin as well as
from U-cones (Saszik and Bilotta, 1999a; Saszik and Bilotta, 1999b). This gives good indication that before 15 dpf the rod system contributes little or nothing at all to vision.

1.10 The electroretinogram (ERG) as a tool to evaluate outer retinal function

1.10.1 Introduction

Electroretinographic (ERG) methods record a sum field potential of the retina in response to light. Holmgren first found in 1865 that a light stimulus could evoke a change in the electrical potential of the amphibian eye. He showed that light illumination caused a slight movement of a galvanometer suggestive a positive electrical change in the cornea relative to the back of the eye. This phenomenon was called electroretinogram (ERG).

Ragnar Granit (Granit, 1933) published a detailed study of the ERG components in cats (Fig. 13). He did ERG from the anesthetized subject and monitored the gradual removal of the ERG components at different levels of anesthesia. Granit termed three different components as P-I, P-II and P-III. The P-I component is a slow cornea-positive wave. P-II is a cornea-positive wave as well. P-III component is a cornea-negative wave. This analysis of the components remains the basis of our ERG understanding up to the present day (Fig. 14).

![Graph of ERG components](image.png)

**Figure 13.** The ERG of a cat in response to a 2-sec light stimulus. The components, P-I, P-II and P-III have been isolated by deeping the state of anesthesia. Granit termed three different components as P-I, P-II and P-III. The P-I component is a slow cornea-positive wave. P-II is a cornea-positive wave as well. P-III component is a cornea-negative wave (Granit, 1933).
Introduction

Figure 14. The ERG of the cat recorded between a chlorided silver wire in the vitreous humor and a chlorided silver reference electrode in the orbit behind the eye. Positivity of the active electrode is recorded as an upward deflection in accordance with convention for ERG work. The retina was stimulated by a light spot delivered in the area of tapetum. The lower record shows when the stimulus begun and terminated. The stimulus duration was 320 sec with 10 sec interstimulus interval (Brown, 1968).

There are two electrical pathways across the vertebrate retina: radial and lateral. The “dark” current of the photoreceptors gives rise to a strong radial extracellular current flowing from the inner part of the retina towards the outer part, the retinal pigment epithelium. These extracellular currents will sum up in case they are directed radially only. Weak lateral currents will obliterate each other since the retinal lateral structure of the retina is symmetrical. Hence, when the retina is exposed to light only radial extracellular currents are formed. These currents will flow through different pathways: (Fig. 15) the local one (A) and the remote one (B). The current in pathway A remains in the retina while the current in pathway B is flowing outside the retina. ERG is measuring the current along pathway B. Figure 16 shows an electrical scheme of the eye as a series of resistances. We need a system of two electrodes to record an ERG. The largest light-induce potential change will be observed in the ERG when an experimenter records an ERG. This is recorded between two points A and B (Fig. 16). Doing ERG from laboratory animals in vivo, the electrodes can not be inserted into the retina. Hence, the alternative is to record the ERG from extraocular sites by placing two electrodes in two points: C and D (Fig. 16). Any changes in one of the resistance (Fig. 16) will induce a
change the properties of the current along the pathway B (Fig. 15). Therefore, it is important to know the different resistance and understand the factors affecting them.

**Figure 15.** A schematic representation of the extracellular currents that are formed following light stimulation. Pathway A represents local currents within the retina, while pathway B shows the currents leaving the retina through the vitreous and the cornea and returning to the retina through the choroid and the pigment epithelium. ERG recording in human is done along the B path. (http://webvision.med.utah.edu/ERG.html#Historical%20view)
Figure 16. An electrical scheme of the resistances through which currents IA and IB (Figure 15) flow when the retina is stimulated with light. The current source I represents the electrical current that is generated in the retina in response to a light stimulus. Pathway A is the local intra-retinal route of current flow and pathway B is the remote route going from the retina and through the vitreous, lens, cornea, extra-ocular tissues and back to the retina through the sclera, choroid and pigment epithelium.

1.10.2 The origin of the major ERG waves

As has been mentioned before, the ERG consists of at least three major components. From those analyses, we know that the negative a-wave is the leading edge of the P-III negative component; the positive b-wave reflects the summation of P-II and P-III while the slow-c-wave is the summation of P-I and P-III components. Two types of approaches, physiological and pharmacological, have been intensively used to gain insights into the origin of ERG.

1.10.3 The a-wave generation

The photoreceptors contribute to ERG by generating the a-wave. It is a corneal negative potential that is the earliest when a light stimulus is applied (Brown, 1968; Brown and Wiesel, 1961). The ERG a-wave is the main part of P-III component. Witkovsky suggested to divide the P-III component into slow- and fast potentials (Fig. 17) (Witkovsky et al., 1975).

The a-wave can be isolated by exogenously applying of L-glutamate (2-amino-phosphonobutyric acid). It has been shown (Slaughter and Miller, 1981) that APB (called L-AP4) is a selective agonist for all Group III mGluRs receptors [mGluR 4, 6, 7 and 8] that block synaptic transmission at the first synaptical level in the retina. It has been shown on separated retina from the pigment epithelium that P-I component can be eliminated and the P-III component can be isolated and studied (Witkovsky et al., 1975).
Figure 17. (Left part of the figure) A series of ERGs recordings from dark-adapted carp retina in response to 30 sec, 500 nm flashes of increasing intensities. Increasing corneal negativity is downward. Note that an early fast deflection (PIII component) is discernible in the response to bright flashes (arrows) followed by a slowly increasing potential (slow PIII component). However, at stimulus intensity, all the fast PIII voltage is generated within 1 sec after response onset. (Right part of the figure) An ERG series in response to 1 sec 500 nm test flashes. Note that no voltage is produced by a 1 sec flash whereas a measurable response is elicited by a 30 sec flash of the same intensity. Both fast and slow PIII components are apparent in the response to a bright 1 sec flash, but only the slow PIII component is elicited by less intense stimuli. The amplitude of the fast and slow PIII components were measured as shown in the schematic drawing above (Witkovsky et al., 1975).

1.10.4 The b-wave generation

The ERG b-wave is the major component of the ERG. Most researchers have suggested that the b-wave generator lies in the inner nuclear layer [INL]. The idea was first suggested by
Granit (Granit, 1947). Granit first concluded that, based on the sensitivity of the b-wave to KCl and the general association of the b-wave with excitation, the b-wave is initiated by membrane depolarization (Granit, 1962). Miller and Dowling (Miller and Dowling, 1970) were first to suggest that glial Müller cells play a role in generating the ERG b-wave. Newman (Newman, 1979; Newman, 1980) proposed his "current-source" analysis of the b-wave (Fig. 18).

Figure 18. The pathways of the extracellular currents that have been suggested to underlie the generation of the ERG b-wave. The two sink (OPL and IPL) reflect the increase in extracellular potassium ions due to light-induced electrical activity. The vitreous serves as a large current source due to the high potassium conductance of the endfeet of the Müller cells (Newman, 1979; Newman, 1980).
The actual b-wave current flow pattern is more complex than depicted in Figure 18. Briefly, elevating of extracellular potassium concentration generates current flows into Müller cells in both proximal and distal retina. These inward currents must be balanced by outward current from other regions of Müller cells. These outward current appear at the place where either smaller increases or actual decreases in extra potassium occur. These outward current exits from the end-foot region (ILM, inner limiting membrane) of Müller cells. The inward currents flow through extracellular spaces at the levels of OPL (distal current sink) and OIL (proximal current sink). These currents establish the b-wave positive voltage.

There are two types of bipolar cells with which photoreceptor make contacts, called ON and OFF bipolar cells (Fig. 19). In response to a light stimulation, the ON BCs depolarize while the OFF BCs hyperpolarize. ON BCs use mGluR6 (Nakajima et al., 1993) that detect the glutamate at synapse. OFF BCs use AMPA/KA receptors (DeVries and Schwartz, 1999; Sasaki and Kaneko, 1996). The ERG b-wave reflects mainly light-induced activity of ON BCs and Müller cells.
Fig. 19. A scheme of ON and OFF pathways in the retina. There are two types of bipolar cells with which photoreceptor make contacts, called ON and OFF bipolar cells. In response to a light stimulation, the ON BCs depolarize while the OFF BCs hyperpolarize. ON BCs use the mGluR6 that detect the glutamate at synapse. OFF BCs use the AMPA/KA receptors. The ERG b-wave reflects mainly light-induced activity of ON BCs and Müller cell (http://webvision.med.utah.edu/cone.html).

It has been reported that third-order retinal neurons (amacrine, ganglion cells) may contribute the b-wave. For example, exogenously applied GABA, glycine, and dopamine affected the b-wave (Gottlob et al., 1988; Naarendorp and Sieving, 1991; Starr, 1975). Other distal retinal neurons appear to modulate the b-wave response under certain conditions. There is evidence that the activity of hyperpolarizing second-order neurons [OFF BCs and amacrine cells] affects both the amplitude and kinetics of the photopic b-wave (Sieving et al., 1994).

Recently, it has been shown which receptor types mediate synaptic transmission from rods and cones to ON and OFF bipolar cells in adult zebrafish using glutamate analogs (Wong et al., 2005a; Wong et al., 2005b; Wong and Dowling, 2005):
1) cone signals onto ON bipolar cells involve mainly EAATs (excitatory amino acid transporters), but also mGluRs (presumably mGluR6) to a lesser extent;
2) rod signal onto ON bipolars by mainly mGluR6;
3) OFF bipolar cells receive signals from both photoreceptor types by AMPA/kainate receptors. These results (Wong and Dowling, 2005) are consistent with the hypothesis that both EAATs and AMPA/kainate receptors are involved in the generation of light-evoked responses in Cabs [cone-driven ON BCs] and that they confer these cells with ON and OFF bipolar cell properties, respectively. Cabs can generate double color-opponent center responses by receiving inputs from certain cones through EAATs and from other cones through AMPA/kainate receptors.

1.10.5 The c-wave generation

The origin of the ERG c-wave [the P-I component of the Granit ERG scheme] was first described by Noell (Noell, 1954). It is believed that the source of the c-wave is in the RPE (Noell, 1954; Pepperberg et al., 1978; Pepperberg and Masland, 1978; Steinberg et al., 1970) (Fig. 20). When the RPE layer was destroyed, the c-wave is abolished but optic tract discharges and both the ERG a- and b-waves were unaffected. There are several possible mechanisms by which the retina could produce the interaction with the RPE. Only one hypothesis was accepted: the hypothesis of potassium version (Oakley and Green, 1976). The hypothesis is that there must be a light-induced decrease in extracellular potassium with time.
course similar to that of the c-wave. Indeed, if the potassium hypothesis of the c-wave is true, then the KRG (potassium retinogram) decrease only needs to have the same time course as the c-wave at the retinal depth where the extracellular potassium decrease would hyperpolarize the RPE (Fig. 21). The conclusion was made as follows: both the c-wave and the light-induced decrease in extracellular potassium concentration should respond to all changes in the stimulus parameters in the same manner (Fig. 21).

Figure 20. A model for the generation of the retinal pigment epithelial hyperpolarization that is reflected as the ERG c-wave. A photon of light (hv) is absorbed by rods (1) causes them to effect a loss of potassium ions from the extracellular fluid (2), resulting in a decrease in the extracellular potassium concentration (3). This ionic change causes the pigment epithelium to hyperpolarize (Oakley and Green, 1976).
The hypothesis is that there must be a light-induced decrease in extracellular potassium with time course similar to that of the c-wave. Indeed, if the potassium hypothesis of the c-wave is true, then the KRG (potassium retinogram) decrease only needs to have the same time course as the c-wave at the retinal depth where the extracellular potassium decrease would hyperpolarize the RPE. Both the c-wave and the light-induced decrease in extracellular potassium concentration should respond to all changes in the stimulus parameters in the same manner (Oakley and Green, 1976).

1.10.6 The d-wave generation

The typical ERG consists of the a-, b-, and d-waves when the prolonged light stimuli (more than 500 ms) were applied (Fig. 22). Bipolar cells in the retina are divided into two types according to their response patterns. “ON” [ERG b-wave] and “OFF” [ERG d-wave] bipolar cells were identified by both electrophysiological and anatomical criteria: the two types of bipolar cells produce postsynaptic currents of opposite polarity and have axon terminals that end in different part of the retina’s inner plexiform layer (Fig. 19). OFF bipolar cells hyperpolarize in response to light. Amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA)/kainate receptors are found postsynaptically in these cells in all species studied so far (Euler et al., 1996; Hensley et al., 1993; Sasaki and Kaneko, 1996). Glutamate activates a nonselective cation channel in these receptors, leading to an inward flow of cations and consequently depolarization of the cell (Ozawa et al., 1998).
Figure 22. ERG from pure cone retina of squirrel. A 1sec light was applied to separate ON and OFF pathways. The typical ERG consists of the a-, b-, and d-waves when the prolonged light stimuli (more than 500 ms) were applied. Bipolar cells in the retina are divided into two types according to their response patterns. “ON” [ERG b-wave] and “OFF” [ERG d-wave] bipolar cells were identified by electrophysiological criteria (Brown, 1968).

Steven and colleagues (DeVries and Schwartz, 1999) showed, by recording from both cone and OFF bipolar cells simultaneously, that transmission is mediated by the ionotropic kainate subtype of glutamate receptors and not by the AMPA subtypes of the same receptor family.
Chapter 2: ERG device for non-invasive electroretinography in small aquatic vertebrates
An inexpensive device for non-invasive electroretinography in small aquatic vertebrates


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Key words: Electroretinogram; Zebrafish; Medaka; Xenopus
2 Authors’ contribution to the project

Oliver Rinner wrote the kernel of the ERG VI (virtual instruments) under LabVIEW (a graphical programming language).

I contributed: all ERG measurements; added some extra features into the ERG VI later on; a strategy using the light source, light paradigms, electrode system; a scheme of entire ERG setup was designed on paper; all other small things were developed, introduced and bought to improve the quality of the system itself.

I wrote the manuscript with the help of Stephan Neuhauss.

Our electrical (Hansjörg Kasper, Marco Tedaldi) and mechanical (Stefan Giger) departments built a system of pre-amplifier-amplifier/filtering and a Faraday cage.

2.1 Abstract

Electroretinographic (ERG) method records a sum field potential of the retina in response to light. It mainly arises in the outer retina and is used as a non-invasive measure in both animal experiments and the clinic. Since it is a comprehensive method to assess outer retinal function, it is becoming increasingly useful in genetic studies of vision. Here we present a simple in-house built setup to measure ERGs of aquatic vertebrates. We have used this setup to efficiently and reliably measure intact larvae of zebrafish (Danio rerio), Medaka fish (Oryzias latipes), and Xenopus laevis tadpoles. By slight modification of the setup, we were also able to measure adult zebrafish and Medaka, demonstrating the general versatility of the setup. We picked these organisms since they are increasingly used to study visual function with genetic means. This setup is easily built and will be particularly useful for laboratories setting up ERG measurements as a complement to their genetic studies.

2.2 Introduction

The electroretinogram (ERG) is a comprehensive and non-invasive method to probe outer retinal function. It measures light-induced changes of electrical activity of the eye in response to light. These sum field recordings are generally measured with an extracellular recording electrode placed on the cornea or in the vitreous chamber of the eye. In the vertebrate retina photoreceptors, bipolar and Müller glia cells are arranged in parallel, so that radially directed extracellular current flows of these cell types sum up.
The vertebrate ERG originates in the outer retina and features three prominent waves, an initial negative a-wave, reflecting photoreceptor activity, a large positive b-wave reflecting mainly ON-bipolar cell activity, and the d-wave reflecting postsynaptic activity involved in the OFF response (Dowling, 1987). The ERG is therefore an elegant method to assess outer retinal responses to variable light stimuli and adaptation conditions. It is used extensively in both basic research and ophthalmic practice.

Recent years have seen a rekindled interest in the use of aquatic vertebrates and their larvae in vision research. This is partly due to an increasing focus on using small aquatic vertebrate larvae in genetic experiments. For instance, forward genetic screens in the zebrafish (Danio rerio) have uncovered a large collection of mutant strains that produce larvae with visual deficits, some of them closely mirroring human diseases. (Bilotta and Saszik, 2001; Goldsmith, 2001; Malicki, 2000; Neuhauss, 2003) This collection of zebrafish strains with visual defects will be an entry point into the genetic dissection of vertebrate visual system development and function.

The ERG is ideally suited to assess outer retinal function in visually impaired animals in order to locate genetic defects in the visual pathway. Lack of an ERG response argues for a defect in photoreceptors, altered traces may be associated with synaptic defects in the inner retina, and normal recordings argue for a defect beyond the first photoreceptor synapse (Brockerhoff et al., 1995; Neuhauss et al., 1999).

Here we describe a simple, reliable, and low-cost ERG setup. We successfully tested our setup on intact aquatic larvae of three species: zebrafish (Danio rerio), Medaka fish (Oryzias latipes), and the African clawed frog (Xenopus laevis). These species were selected for their laboratory use as genetically modifiable model species. With only slight modifications in the animal preparation (Bilotta et al., 2001), ERGs from adult aquatic species can also be obtained, as we show for both adult Medaka and Zebrafish. To our knowledge this is the first report of non-invasive ERG measurements from intact Medaka retinas and one of the few from intact Xenopus retinas.

2.3 Material and methods

2.3.1 Animals

Adult zebrafish (Danio rerio) of the WIK inbred strain were maintained and bred as previously described (Mullins et al., 1994). Larvae were staged by days after fertilization
(dpf) at 28 C in E3 medium (in mM: 5NaCl, 0.17KCl, 0.33CaCl2 and 0.33MgSO4). Larvae were measured at 5 dpf in the afternoon.

Medaka (Oryzias latipes) of the inbred CAB strain were raised and bred as previously described (Yamamoto, 1956). Embryos were raised at 28 C in embryo rearing medium (ERM: in % (w/v); 0.1NaCl, 0.003KCl, 0.004CaCl2, 0.016MgSO4) and measured as young fish at 12 dpf in the afternoon. Stages were determined according to(Iwamatsu, 1994).

Tadpoles (Xenopus laevis) were kept in 0.1 x Marc’s modified ringer solution (MMR: in mM; 10NaCl, 0.2KCl, 0.1MgCl2, 0.2CaCl2, 0.5Hepes, pH 7.5). Tadpoles were tested at stage 42 (98 h post fertilization) in the morning. Larvae were staged according to the normal table of (Nieuwkoop, 1975).

Adult medaka and zebrafish of both sexes ranging in size from 2 to 3 cm in length were used. All experiments were performed at room temperature.

2.3.2 Stimulation

A one channel optical system was used to present visual stimuli. White light was presented over the subject’s eye through the channel, which fitted with a 24 V 250 W projection lamp (Liesegang Diafant 250, Düsseldorf, Germany) as its light source. The light from the beamer was collimated, passed through a neutral density filter wheel and focused onto a mechanical shutter head. A light guide was used to deliver light over the animal’s head. Both ends of the light guide were fitted with diffusers (Fig. 1). The optical system was outside a Faraday cage. The shutter was controlled by software under Windows OS environment (AMPI Master-8, Jerusalem, Israel). Stimulus intensity was controlled using a neutral density filter wheel. Unattenuated light intensity over the subject’s head was 3100 lux (optical density, OD is equal to 0 log unit), as measured by a light meter (Tekronix J17).
2.3.3 Recording setup

Recordings and data acquisition were performed by an in-house built electroretinography setup, with the recording setup inside a grounded Faraday cage (Fig. 1). ERG recordings were performed using an Ag/AgCl micropipette holder with a silver wire (MEH3SW10 WPI, Sarasota, CA, USA) as recording electrode. The chloridized wire was inserted into a glass micropipette with a tip opening of approximately 20 μm. The pipette was filled with E3, ERM or MMR medium, respectively. Reference electrode was an Ag/AgCl pellet assembly (HLA-003, Axon Instruments, Union City, CA, USA) beneath the subject's body, separated by a piece of wet tissue paper.

The electrical signal from both electrodes was differentially amplified 1000 times with a band pass between 3 and 100 Hz. A system of pre-amplifier–amplifier and filters was constructed in-house. Recording signals from the amplifier were sent to a 40 MHz dual channel storage...
oscilloscope HM 408 (Hameg GmbH, Frankfurt am Main, Germany) and to a DAQ Board NI PCI-6035E (National Instruments, Ennetbaden, Switzerland) via NI BNC-2090 accessories.

2.3.4 Experimental procedure

All pre-recording steps were done under red illumination to minimize bleaching of the visual pigment. Preparation and recordings were performed in a tight Faraday cage under visual control using an upright microscope (Zeiss Stemi SV8, Oberkochen, Germany). Larvae and tadpoles were placed on a piece of a moistened sponge in a 35 mm Petri dish. The subjects were dark-adapted for at least 30 min prior to positioning them in the recording chamber. For larval recordings, each larva or tadpole was placed on its side on the surface of the moist sponge. Tadpoles were anesthetized with 0.02% buffered 3-aminobenzoic acid methyl ester (MESAB; Sigma–Aldrich, Buchs, Switzerland) in MMR medium. Zebrafish larvae and young Medaka fish were paralyzed by directly adding a droplet of the muscle relaxant Esmeron (0.8 mg/ml in larval medium; Organon Teknika, Eppelheim, Germany) onto the animals. The electrode was positioned in the approximate center of the cornea. Since the tadpole head is covered by a thin transparent tissue (the pellucida), a small incision had to be made to gain direct access to the cornea.

For adult recordings the setup was slightly modified similar to (Saszik et al., 1999). Briefly, the recording electrode was inserted into the vitreal chamber of the eye through an incision in the cornea. A chlorodized silver wire was fixed on the opposite nostril as a reference electrode. Normal fish water was taken to prepare a medium containing 0.01% buffered 3-aminobenzoic acid methyl ester (MESAB, Sigma–Aldrich, Buchs, Switzerland) and 0.1 mg/ml Esmeron (Organon Teknika, Eppelheim, Germany).

In order to ensure oxygenation of the animal, the medium was flushed by gravity forces over the gills by a plastic tube inserted into the mouth. The flow rate was approximately 1 ml/min. Animals were dark-adapted for at least 30 min before mounting the electrode under dim red light. Before exposure to light animals were adapted in complete darkness for at least 2 min. For photopic measurements, the subject was light-adapted for at least 2 min prior to measurement. Background illuminance was 60 lux. A 1 s stimulus was chosen to separate ON and OFF response with an interstimulus interval of 5 s. Illumination was increased in 1.0 log unit steps over the range from −5 log unit to −1 log unit.
2.4 Results

2.4.1 Electroretinograms

In order to test our setup, we first measured zebrafish larvae at 5 pdf, since a number of studies have published ERG recordings at this stage (Brockerhoff et al., 1998; Saszik et al., 1999; Seeliger et al., 2002). We measured larvae under both dark- and light-adapted conditions with a stimulus of one second duration in order to temporally separate ON and OFF responses (Fig. 2). In the dark-adapted larva, already at the lowest light intensity a positive deflection, the b-wave, appears. At increasing light intensities the b-wave rises in amplitude and the OFF response (d-wave) appears (Fig. 2A). The b-wave usually occludes the a-wave, which is therefore only occasionally visible. Pharmacological blocking of the b-wave can reveal the negatively reflecting a-wave, which also increases in amplitude with increasing light intensity (data not shown). In general the ON response under light-adapted conditions is less sensitive, but similar in waveform (Fig. 2B). The recorded ERGs are typical for vertebrates and conform to published reports of the zebrafish ERG using a similar stimulation paradigm (Seeliger et al., 2002).
Fig. 2. Typical larval zebrafish ERG recording. Intensity series obtained under dark-adapted (A) and light-adapted (B) conditions at 5 dpf. The small a-wave (upward slanted arrow), the prominent b-wave (horizontal arrow), associated with the ON response and the d-wave (downward arrow), associated with the OFF response are apparent. Light intensity is in log units of illuminance.

2.4.2 System performance

A critical feature of any measuring device is the signal to noise ratio. The main source of noise in our system stems from interference from the building's power installation and can be effectively controlled by a tight Faraday cage. Typically noise was below 10 μV, while recorded signals ranged from 20 to 1000 μV. Hence the SNR was lower at lower light intensities, making averaging necessary. Although at high light intensities no averaging was necessary, we generally averaged three to seven traces at all light intensities.

We observed a variation in b-wave amplitude among larvae measured under the same conditions. This variation may be due to biological variation or stem from our setup. Hence we addressed this question by measuring the b-wave amplitude of 10 dark-adapted zebrafish larvae at a given illumination (−3 log). Each individual larva was measured three times after remounting the electrode in between trials. A one-way ANOVA analysis for repeated measurements revealed that most of the variation is due to inter-individual differences (F=411.32; P<0.001) rather than to variation between trials (F=0.70; P>0.5). There was also no significant correlation of b-wave amplitude and order of measurement, indicating that remounting the electrode did not influence subsequent measurements. Taken together, these
data indicate that the described setup is reliable and variation is only marginally influenced by the mounting procedure.

2.4.3 Comparative recordings from aquatic vertebrates

Having successfully tested our ERG device in zebrafish, we next set out to test other aquatic vertebrate larvae and adult fish. An important consideration is the oxygen supply of the retina during measurement. In small larvae, surface exchange should suffice, while in larger animals water has to be flushed over the gills to ensure proper oxygenation.

We measured young Medaka fish at 12 dpf, a stage were all animals have hatched. The resulting ERG is similar to 5-day-old zebrafish larvae (Fig. 3). The ON response kinetics is slightly faster, probably reflecting the more matured state of the Medaka retina. We also recorded ERGs from stage 42 Xenopus tadpoles, a stage where rods and cones are histologically distinguishable. At this stage the pellucida, a transparent tissue covering the eye, has to be penetrated by the electrode to gain access to the cornea.

![ERG waveforms comparison](image)

Fig. 3. Comparison of ERG waveforms of various aquatic larvae and animals. Dark-adapted ERGs were recorded at -3 log unit of unattenuated light intensity: (A) 5 dpf zebrafish larva; (B) 12 dpf Medaka fish; (C) stage 42 Xenopus tadpole; (D) adult zebrafish; (E) adult Medaka.
ERGs from adult zebrafish (Fig. 3D) and Medaka (Fig. 3E) are also easily measurable and are comparable to larval recordings. The measurements tend to be more stable, reflecting the mature state of the visual system.

Having established that the tested aquatic animals display a typical vertebrate ERG, we compared the b-wave amplitude of dark-adapted animals (Fig. 4). The amplitudes differ remarkably little between larvae and adult animals.

Fig. 4. Comparison of dark-adapted b-wave amplitudes over log intensity. Statistical evaluation over n number of animals: (○) larval zebrafish, n=11; (△) young Medaka, n=6; (▼) Xenopus tadpoles, n=8; (○) adult zebrafish, n=6; (□) adult Medaka, n=5. Error bars are mean±S.E.M.

2.5 Discussion

In the present study we report on a simple setup for electroretinographical recordings of vertebrate aquatic animals and their larvae. Such a setup allows a quick and informative survey of outer retinal function, as will be especially useful in assessing visual defects in genetically modified animals.

The experimental setup can be readily and cheaply assembled, giving experimental results comparable to commercial solutions, usually tailored to the use in humans.

Our recordings showed a good signal to noise ratio, so that in most cases averaging was not necessary. As a measure of the reproducibility of ERG recordings, we measured the inter-
2.5 Discussion

experimental variance in a number of zebrafish larvae and showed that the inter-individual variability is much higher than the variance between repeated measurements of the same animal. Hence the variance in ERG measurements is mainly due to biological variation.

Having established that zebrafish ERGs can be reliably recorded in our custom made setup, we set out to apply the technique to other aquatic vertebrate species. Since aquatic vertebrate species are increasingly used for genetic manipulation, we focused on species that are already used as genetic model organisms to study vision. A number of reverse and transgenic approaches have been used to study eye development in Xenopus. Recently these tools have also been employed to study functional aspects of vision, for instance, by transgenic manipulation of photoreceptor properties (Wiechmann et al., 2003). The Medaka is a teleost species with favorable genetic properties (reviewed in Wittbrodt et al., 2002) that will likely play an increasingly important role in studies of vertebrate visual function. Finally, the zebrafish is already extensively used in our and a number of other laboratories in genetic studies of visual function, including electroretinography (reviewed in Neuhauss, 2003).

We were able to record ERGs with our simple setup in all species, including adult zebrafish and Medaka. To our knowledge this is the first report of electroretinography in the Medaka and one of the few using an intact larval preparation in Xenopus. Most ERG studies using aquatic animals measure in eye cup preparations. Although in most cases the individual recorded eye is further used for histological assessment, the option to have the specimen survive the measurement may be of importance. Survival of the specimen allows repeated measurement, as we have used in this study, enabling comparison of retinal responses before and after experimental treatment. Furthermore, electroretinography may serve as decisive criteria which fish to propagate, for instance, after screening adult fish for carriers of dominant founder mutations.

Our setup is easily adjustable to other species and will mainly involve ensuring proper oxygenation of the animal. Any larva below a length of one centimeter will likely be small enough to be sufficiently oxygenated by passive diffusion though the skin. In larger animals oxygenation through the gills is the method of choice with the added advantage that gill flow can be utilized to expose the animal to pharmacological agents. This simple and cheap setup should be suitable for undergraduate teaching and might be utilized by laboratories studying foremost the genetics of vision without a special physiological focus.
Chapter 3: ERG manual
ERG manual

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3 ERG Manual: step by step

3.1 Fish

3.1.1 Larval stage

Between 4 and 8 dpf, larvae do not need a special perfusion system, presumably because oxygen uptake through the skin is sufficient. Larvae rarely survive the ERG, likely due to drying of the gills. To minimize mechanical movements of larval zebrafish during ERG recordings the body of the larva was covered by a piece of damp paper with E3 medium. The larva was fixed properly and it was easy to mount the recording electrode on the surface of the cornea. The larva was itself in a moist environment. The zebrafish larva did not produce any disturbances, which can affect an ERG signal during recordings.

The main question was whether to use both anaesthetic and neuromuscular blocker. It was unclear whether the effects of anesthetic were explored in the case of the zebrafish. It was not clear from the literature why both anesthetic and neuromuscular blocker were required to do ERG from larval zebrafish. Another strategy was applied to fix a larval zebrafish for ERG recordings. A solution of Esmeron was used. The underlying concept was as follows: larval zebrafish could breathe by skin and applying a droplet of Esmeron, it could be possible that a certain amount of Esmeron can penetrate the body leading to an effect of the drug. An extra piece of damp paper was used to mechanically immobilized movement body over the ERG recordings.

3.1.2 Adult and perfusion

The adult stage may vary from 29 dpf until the mature stage of the Zebrafish. The adult zebrafish must be kept alive with oxygenated fresh water during ERG measurements. Therefore, 500 ml of fresh water and Mesab were added to final concentration of 0.01% (see the solutions chapter below). This treatment did not affect the ERG performance. The flow rate of the water is about 1-2 ml per minute sufficient to keep adult zebrafish alive over the course of an experiment (30 minutes at least). The position of the body is upright. The body is held by a plastic holder, which can be formed out of a plastic pipette. A small plastic tube is put into the mouth in such a way that it allows to perfuse the gills. The criterion for that is visible water flow through the gills.

It was an important issue how to immobilize an adult zebrafish to do ERG. It was inconceivable to do an injection of both a neuromuscular blocker and an anesthetic by a
3.2 Solutions

E3 medium (in mM: 5NaCl, 0.17KCl, 0.33CaCl\(_2\) and 0.33MgSO\(_4\)). Take it to fill the glass micropipette up. This micropipette is as a recording electrode. Mesab (3-Aminobenzoic acid ethyl ester methanesulfonate) (Sigma #A-5040) is as a local anesthetic. I prepared a 0.01% of Mesab for adult perfusing. Esmeron is a muscle relaxant. It acts by blocking the passage of impulses between nerves and muscles (also known as neuromuscular blocking agent). I follow the following protocol: 1000 microL=950 microL of E3 + 50 microL of Esmeron (Organon, Inc). Esmeron’s rapid onset is quick, at about 1 minute after administration of the drug. I added a droplet of 1-2 microL on the larval body before doing ERG measurement.

APB (+/-)-2-Amino-4-phosphonobutyric acid (Sigma #A1910) is a metabotropic glutamate receptor agonist blocking the ON pathway in the retina. I used to 1-5 mM of the APB solution.

PDA (Sigma # P8782, cis-2,3-Piperidinedicarboxylic acid) is a NMDA receptor agonist at the glutamate recognition site blocking the OFF pathway in the retina.

3.3 Electrodes and chloridizing

At the electrodes, current must be transformed smoothly from a flow of electrons in the silver wire to a flow of ions in solution [Equation 1]. The most common type of electrodes is a
silver/silver chloride (Ag/AgCl) interface, which is a silver wire coated with silver chloride. If electrons flow from the silver wire to the electrode AgCl pellet, they convert the AgCl to Ag atoms and the Cl\(^-\) ions become hydrated and enter the solution. There are several points to remember about Ag/AgCl electrodes: [1] The Ag/AgCl electrode perform well only in solutions containing chloride ions; [2] Because current must flow in a complete circuit, two electrodes are needed. If the two electrodes face different Cl\(^-\) concentrations (for instance, 2M KCl inside a micropipette and 150 mM NaCl in a bathing solution surrounding the cell), there will be a difference in the half-cell potentials (the potential difference between the solution and the electrode) at the two electrodes, resulting in a large steady potential difference in the two wires attached to the electrodes. To minimize this effect E3 medium was used for both inside a micropipette (the recording electrode) and in bath solution.

The Silver/Silver Chloride Electrode

Electrode reaction:

\[ \text{Ag} + \text{Cl}^- \leftrightarrow \text{AgCl} + \text{electron (e\(^-\))} \quad [\text{Equation 1}] \]

This reaction can also be presented by:

\[ \text{AgCl} \leftrightarrow \text{Ag}^+ + \text{Cl}^- \quad [\text{Equation 2}] \]

The Ag-AgCl system was used to get an ERG signal from the eye.

GC100-10 glass capillaries No. 30-0016 (1 mm O.D x 0.58 mm I.D) was employed to make a recording electrode. The following parameters were adjusted to create glass micropipettes with an opening of 10-30 micron: [HEAT 520; PULL 80; VELOCITY 50; Time 150 and PRESSURE 140] (Model P-87 Sutter Instrument Co.).

As a reference electrode, a Ag-AgCl pellet of Axon Instrument Inc was applied. This company sells only few types of them. All of them can be used. The only critical thing is that is small enough to be placed beneath the larval fish. In the case of adult recordings, a piece of chloridized Ag-wire was used for both recording electrode and reference electrode.

A DC power supply was taken, 1M HCl and micropipette electrode holder for recording electrode and a piece of Ag wire for reference one.

Holders with silver wire should be chloridized before use. This can be done easily by immersing the wire in 1M hydrochloric acid and applying +1.5 V to the holder versus a return electrode, such as a large platinum wire. If, before chloridizing, the wire is tarnished (yellow
3.3 Electrodes and chloridizing

to dark red) it may be cleaned by passing it quickly over a flame, being careful to avoid melting of the acrylic body, or washing in urea solution.

A 20-50 ml beaker was taken and fill in with 1M HCl. Connect the microelectrode holder to plus (+) and the Pt-wire (or Au-wire) to minus (-) using small metallic “crocodiles” clamps. The voltage of the DC supply is about between 1-4 V. Put two wires into the beaker and you will see bubbles. This is an indicator of chloridization. It will take about 20 sec to be completed visible by a patina on the wire. In general, there is an instruction in the box of all commercial microelectrode holders.

Resistor measurement with constant current:

Resistor of the probe: \( R_p = 10^{-10} \) Ohm

Voltage only probe: \( U_p = 9.8 \) Volt \( \rightarrow 9.8 \) MOhm

Voltage with electrode on the eye: \( U_e = 8 \)

Voltage with electrode in bath: \( U_b = 7.5 \)

\[
R_{\text{tot}} = \frac{1}{\left( \frac{1}{R_e} + \frac{1}{R_{\text{prob}}} \right)}
\]

Resistance of two \( R \) in parallel:

The Current is constant! \( \rightarrow \) so the measured voltage is proportional to the resistor

\[
R_{\text{electrode}}(R_{\text{tot}}, R_{\text{prob}}) = \frac{1}{\left( \frac{1}{R_{\text{tot}}} - \frac{1}{R_{\text{prob}}} \right)}
\]

Electrode on the Eye: \( R_{\text{electrode}}(8, 9.8) = 43.556 \) MOhm

Electrode in Bath: \( R_{\text{electrode}}(7.5, 9.8) = 31.957 \) MOhm

\( C = 20 \times 10^{12} \) \( R = 7.5 \times 10^6 \) \( \tau = R \times C \) Timeconstants
3.3 Electrodes and chloridizing

$$U_c(t) = U_e (1 - e^{-\frac{t}{\tau}})$$

Figure I. Time constant of the recording electrode.

Low-pass filter (Bessel Filter 2nd order):

$$C = \begin{pmatrix} 0 \\ 0.4710^9 \\ 1.510^9 \end{pmatrix}$$

$$f_g := 1500 \text{ Hz}$$

A0 := -1

Bessel Factors:

$$a_1 := 1.361$$

$$b_1 := 0.618$$

Proposed C2/C1 Relation:

$$\frac{4b_1(1 - A0)}{(a_1)^2} = 2.666$$

Condition

$$\frac{C_2}{C_1} \geq \frac{4b_1(1 - A0)}{(a_1)^2} = 1$$
3.3 Electrodes and chloridizing

\[ R_2 = \frac{a_1 \cdot C_2 - \sqrt{\left(\frac{a_1}{C_2}\right)^2}}{4 \cdot \pi \cdot f \cdot C_1 \cdot C_2} \cdot 4 \cdot C_1 \cdot C_2 \cdot b_1 (1 - A_0) \]

\[ R_1 = \frac{R_2}{-A_0} \]

\[ R_3 = \frac{b_1}{4 \cdot \pi^2 \cdot f^2 \cdot C_1 \cdot C_2 \cdot R_2} \]

Resistor Value:

\[
\begin{bmatrix}
0 \\
9.135 \times 10^3 \\
9.135 \times 10^3 \\
1.081 \times 10^4
\end{bmatrix}
\]

Figure II. The schematic drawing of the filter system employed for ERG recordings.

I choose:
3.4 Light source and paradigms

3.4.1 Light source

The light source is described in chapter 2 (see above)
3.4.2 Calibration the light

The light intensity was calibrated with a light meter [either Texas Instrument Tektronix J17 or PeakTech 5020 Digital Light meter] using log scale since the intensity of the light is changed over logarithmic scale by the following definition: \( f(x) = \log_{10} X \). For example,

\[
\begin{array}{ccccccccccc}
 x & 3 & 1 & 3^{-1} & 3^{-10} & 3^{-100} & 3^{-1000} & 3^{-10000} & 3^{-100000} \\
 f(x) & 1 & 0 & -1 & -10 & -100 & -1000 & -10000 & -100000 \\
\end{array}
\]

There is a filter wheel marked according to the indicated intensity on the light probe device. I use six levels of light intensity from the lowest (OD5) to the highest one (OD 0). It must be re-calibrated ones every two-three months since the bulb loses intensity with age. I recommend to use the following unit of light: Irradiance (W/m²) [definition: the amount of radian flux received by a unit of surface area] or Luminance (cd/m²) [definition: the amount of light emitted or scattered by a surface], Luminous Intensity (cd) [definition: the luminous flux through a unit of solid angle].

The procedure of the light measurement was as follow: the head of the light meter was positioned at the same place where a larva was. One end of the light guide is positioned close to the head of the larva to create a “Ganzfield” [bowl] illumination. All part of the retina must be illuminated equally. The door of the Faraday cage was closed and the measurement was begun. Try to find an area of filter, which has the maximum attenuation of the light source. It is the minimum of light, which falls on the head of the larva. The filter wheel was marked indicating the value of light intensity. Then, rotating the filter wheel try to find next level of light intensity, which corresponds to the 1 log light intensity more from the initial level of the minimum light. The filter wheel is divided in according with the number of light unit of the monitor of the meter. I use a unit of optical density (OD), but it is not important to have this unit. In this case, there was a scale of 6 level (log step) of light intensity. These levels depend on the range of light attenuation, which depends on the manufactures.

3.4.3 ON-response

To see the ON response (both a- and b-waves): apply 100-200 msec of light stimuli.
3.4.4 ON and OFF responses

To see ON and OFF responses (a-, b- and d-waves): apply 1-2 sec of light stimulus.

3.4.5 Adaptation time

To analyze ERG of larval zebrafish under dark condition: use 30 minutes of time keeping the larvae in full dark before work with them in the Faraday cage. Once, you begin to put a larval zebrafish on the sponge, you work with the deep red ambient light in order to minimize cone photoreceptor bleaching.

To analyze ERG of larval zebrafish under light condition: once you mounted a larval zebrafish on the sponge use about 5 minutes for a certain level of light to adapt fish before the light stimulation onset.

To analyze ERG of adult ERG under dark condition the approach is as follow: it will take longer time to adapt an adult zebrafish to dark, saying, up to 30-40 minutes.

3.4.6 Caution

Before doing any ERG recordings sit and understand the following way:
First - ask the question which must be investigated by ERG?
Second – what kind of either light paradigm or ambient light environment is suitable to answer the question?
Third – if it is necessary, re-build ERG VI (Virtual Instrument) under LabVIEW to control light exposure and recordings simultaneously according to the studied question.

3.5 ERG software and performance

3.5.1 ERG software

The kernel of an ERG VI was built by Oliver Rinner for the project described in our paper published in (Makhankov et al., 2004).

During the ERG measurements, Marco Tedaldi and I were doing corrections and modifications to improve performance of the all ERG VIs.

DESCRIPTION of the ERG VI:
This VI retrieves the specified amount of data from one or more analog input channels each time a digital start trigger, digital stop trigger, or digital start and stop trigger, occur. It shows how to trigger an acquisition multiple times while avoiding the overhead of configuration and buffer allocation each time. This is a timed acquisition, meaning that a hardware clock is used to control the acquisition rate for fast and accurate timing. It is also a buffered acquisition, meaning that the data are stored in an intermediate memory buffer after they are acquired from the DAQ board. If you use only the stop trigger, a software strobe starts your DAQ board and your stop trigger will determine when data are acquired into a buffer to be read by LabVIEW. If you use a start and stop trigger, your start trigger will start the DAQ board, but your stop trigger will determine when data are acquired into a buffer to be read by LabVIEW.

Use this VI to wait for multiple digital triggers, acquiring a relatively small amount of data for each trigger (it must fit in the memory available), where each point does not need to be viewed or processed while it is being acquired.

KEY PARAMETERS:
This VI shows how to use the intermediate analog input DAQ VIs to trigger multiple acquisitions.
AI Config.vi is only called on the first iteration of the while loop. This avoids the overhead of configuration and buffer allocation for each acquisition. However, you must stop and restart the VI if you wish to change input parameters.
The "read/search mode" for AI Read.vi is set to "relative to trigger point", since this is a hardware triggered acquisition.
AI Control.vi (an advanced analog input VI) is called, in the event that AI Read.vi times out, to momentarily stop the analog input clock. This is needed so the input clock is not running when AI Start.vi (which restarts the clock) is called again.

DAQ VIs USED:
AI Config.vi, AI Start.vi, AI Read.vi, AI Control.vi, AI Clear.vi.
3.5.2 The day before ERG measurement

Prepare glass micropipette (see the instruction above);
Prepare solutions;
Prepare yourself.

3.5.3 The day of measurement

Switch all equipment on: shutter controller, oscilloscope, stimulator, PC. Take a Petri dish with larva and put into dark using a small black box, which is next to the Faraday cage. Prepare the environment in the room to be dark: close the door, switch red light on in the Faraday cage.
Recordings of larvae.
A sponge was taken and put it into a 35 mm Petri dish. The Petri dish is fixed on the microscopic stage. An Ag-AgCl pellet was taken and fixed it on the sponge surface. Cover this pellet with a piece of tissue paper. The size of paper is about 2.5 x 2.5 cm. The paper will be between the electrode and the larval body. Change the paper for every larva during experiments. A larva was caught using a plastic pipette, put it on the paper and used a paint brush to gently relocate it in such a way that the one of the eyes are turned towards the end of the light guide. The recording electrode was mounted on the surface and in the middle of the cornea. *Nota Bene!* Do not penetrate the eye.

Recordings of adults.
A 500-ml beaker of fresh fish water was prepared. The beaker was fixed on the back wall of the Faraday cage, inside of the cage. The water flow was calibrated to about 1-2 ml per minute. This is enough to perfuse the gills via the mouth of the adult zebrafish. An adult zebrafish was caught and put into Mesab solution of high concentration, wait for 1-2 minute, take it out and fix it in a vertical position in the plastic box. As soon as you have mounted the body put tip of the plastic tube inside the mouth to perform the perfusion. The gills should still oscillate, but the body is immobilized.

Now you are ready to mount the electrodes. First, a piece of wire was fixed in the opposite side of the recording eye in the nostril. A microelectrode holder was taken with a glass pipette inside and use the micromanipulator to gently penetrate the vitreous chamber of the eye. The criterion of that will be the stable noise line on the oscilloscope. The Faraday cage was covered with the door and experiments began.

### 3.5.4 Measurement of ERG by Virtual Instrument

The design of the Virtual Instruments under LabVIEW was adapted to do the following functions, such as recording, storing and analyses of ERG traces. The main issue was to be able to couple mechanical devices with a computer using LabVIEW software.

I use the following VI (Virtual Instrument) under LabVIEW for conventional experiments (Figure 3.1). To initiate ERG recordings the following parameters must be adjusted in advance: interstimulus interval (ISI) in sec in a range of 5-30 seconds; stimulus duration in a range of 100-2000 mseconds; acquisition time in msec. Sampling rate is in the range of 250-
1000 Hz. To activate the VI the following procedure is as follow: pushing the buttons in sequence “arrow ->” and START. When the experiment is done, a window appears offering you to save the registered ERG signals. An appropriate directory and folder are chosen to save the file.

The ERG VI under LabVIEW consists of the following blocks: the front panel is a panel with the parameters, which must be adjusted for every experiments; the digram panels is a internal connection and nodes.

Figure 3.1 Front panel of a VI for conventional ERG recordings. The following parameters must be tuned prior to the initiation of ERG recording.

Upper part of the VI:
3.5 ERG software and performance

ISI-Time [ms]- a time of interstimulus intervals between light exposures; Stimulus Duration [ms]- the duration of the light exposure either to evoke ON or ON-OFF responses; Acquisition Time [ms]- a time is chosen to record an evoked ERG response (a 1-sec recording time is to record an ON response, a 2-sec recording time is to record both ON and OFF responses); #Trials- how many time do you want to apply light exposure for averaging procedure by the VI; Print to file is a box which must be activated to store ERG raw files on the hard disk.

Lower part of the VI: scan rate [Hz] is a sample rate, this refer to how frequently the analog ERG signal is measured during the sampling process. The more frequently the signal is sampled the better the approximation to the original ERG signal. However, the higher the sample rate the more memory is required to store the samples, so you do not want to sample more than is necessary. I have been applying the following range of the sample rate: 250-1000 Hz.
Figure 3.2 Block 1 of the VI for conventional ERG recordings. A diagram part of the ERG VI.
3.5 ERG software and performance

Figure 3.3 Block 2 of the VI for conventional ERG recordings. A diagram part of the ERG VI.
Figure 3.4 Block 3 of the VI for conventional ERG recordings. A diagram part of the ERG VI.
Figure 3.5 Block 4 of the VI for conventional ERG recordings. A diagram part of the ERG VI.
Figure 3.6 Block 5 of the VI for conventional ERG recordings. A diagram part of the ERG VI.
Figure 3.7 Block 6 of the VI for conventional ERG recordings. A diagram part of the ERG VI.
3.6 Offline analyses after online measurements

First step was collecting the raw data received during ERG recordings. The raw data can be opened, analyzed and stored in Microsoft Excel format. All graph can be made with the help of Microsoft Excel, Microcal Origin. Responses are averaged between three and seven times depending on the signal to noise ratio (SNR). Statistical analysis was performed using SPSS v.11 (SPSS Inc., Chicago, USA) and graphs were generated using Microsoft Excel and Origin v.7.0 (OriginLab, Northampton, USA) and Adobe Illustrator.
Chapter 4: Split function of protocadherin 15 orthologues in zebrafish hearing and vision
Duplicated genes with split function: independent roles of protocadherin 15 orthologues in zebrafish hearing and vision

Published in Development 2005, Feb. 132 (3), 615-623

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Key words: Protocadherin 15, Hair cell, Photoreceptor, Zebrafish, Deafness, Blindness, Outer segment, Stereocilia, Gene duplication, orbiter
4 My contribution to the project

I contributed to all ERG measurements and their interpretation to this paper.

4.1 Summary

In the sensory receptors of both the eye and the ear, specialized apical structures have evolved to detect environmental stimuli such as light and sound. Despite the morphological divergence of these specialized structures and differing transduction mechanisms, the receptors appear to rely in part on a shared group of genes for function. For example, mutations in Usher (USH) genes cause a syndrome of visual and acoustic-vestibular deficits in humans. Several of the affected genes have been identified, including the USH1F gene, which encodes protocadherin 15 (PCDH15). Pcdh15 mutant mice also have both auditory and vestibular defects, although visual defects are not evident. Here we show that zebrafish have two closely related pcdh15 genes that are required for receptor-cell function and morphology in the eye or ear. Mutations in pcdh15a cause deafness and vestibular dysfunction, presumably because hair bundles of inner-ear receptors are splayed. Vision, however, is not affected in pcdh15a mutants. By contrast, reduction of pcdh15b activity using antisense morpholino oligonucleotides causes a visual defect. Optokinetic and electroretinogram responses are reduced in pcdh15b morpholino-injected larvae. In electron micrographs, morphant photoreceptor outer segments are improperly arranged, positioned perpendicular to the retinal pigment epithelium and are clumped together. Our results suggest that both cadherins act within their respective transduction organelles: Pcdh15a is necessary for integrity of the stereociliary bundle, whereas Pcdh15b is required for alignment and interdigitation of photoreceptor outer segments with the pigment epithelium. We conclude that after a duplication of pcdh15, one gene retained an essential function in the ear and the other in the eye.

4.2 Introduction

Sensory receptors in the ear and eye are thought to have evolved from a common ancestral ciliated cell (reviewed by (Popper, 1992). Both receptors have highly specialized apical surfaces. Receptor cells in the ear possess an apical bundle of actin-filled stereocilia that transduce mechanical stimuli (reviewed by (Hudspeth, 1989), whereas the photoreceptors of the eye have an outer segment that contains membrane disks specialized for detection of light. Through genetic studies, several molecules required for both hearing and vision have been
identified. In humans, mutations in these genes cause Usher syndrome (for a review, see (Petit, 2001), one of the most prevalent inherited disorders (Ahmed et al., 2003b). Individuals suffering from Usher 1 syndrome are born deaf and develop visual problems in the second to fourth decade of life due to retinitis pigmentosa. Mutations in most of these genes have been shown to cause deafness in mice as well, but no phenotype comparable to retinitis pigmentosa has been reported (reviewed by (Ball et al., 2003; Libby et al., 2003; Steel and Kros, 2001).

As in mammals, fish sense mechanical stimuli with hair cell receptors in the inner ear and an additional sensory system unique to aquatic animals, the lateral line (Manley and Koppl, 1998; Popper and Fay, 1997). In recent screens for genes necessary for inner ear function in zebrafish larvae, 24 loci linked to an auditory/vestibular-specific phenotype were identified (Nicolson et al., 1998) (T.N., unpublished). Here we show that the zebrafish auditory/vestibular mutant orbiter is affected in the gene pcdh15. Mutations in PCDH15 have been recently shown to be the cause of human Usher syndrome 1F (Ahmed et al., 2001; Alagramam et al., 2001b; Ben-Yosef et al., 2003) and deafness in Ames waltzer mice (Alagramam et al., 2001a). Furthermore, we report the characterization of a second, duplicated pcdh15 gene in zebrafish, which is expressed in the retina. Our results indicate that one gene acquired an essential function in the eye and the other one in the ear. Both genes are required for the structural integrity of the respective receptor cells in these organs.

4.3 Material and methods

4.3.1 Zebrafish strains

All studies were done with larvae in the Tübingen (Tü) background. albino larvae were used for in situ hybridization. Zebrafish stocks were maintained as described (Haffter et al., 1996).

4.3.2 Mapping and cloning

Linkage analysis was performed using WIK wild-type fish crossed with heterozygous orbiterIp263b-Tü fish. The mutation was mapped using SSLP (simple sequence length polymorphisms) and SSCP (single strand conformation polymorphisms) (Cleangels SSCPplus gels were used according to the manufacturer's instruction; ETC, Kirchentellinsfurt, Germany). For fine-mapping, single mutant larvae were tested for recombination events. To initiate a chromosomal walk, we used the marker z26404 to screen PCR-able pools of genomic zebrafish BAC (BioCat, Heidelberg, Germany) or PAC (RZPD, Berlin, Germany)
4.3 Material and methods

libraries. All clones found were sized using pulsefield gel electrophoresis. New SSLP or SSCP markers were created using sequences from the ends of the identified BAC- or PAC-clones. After defining a critical interval, six clones spanning the contig (PAC1, BAC1, PAC10, PAC13, BAC6, BAC7) were sequenced at the Sanger Center (Welcome Trust Sanger Institute, Cambridge, UK). The sequence within the critical interval was analyzed using Genscan (http://genes.mit.edu/GENSCAN.html) and blast searches of the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST).

To clone the 3' and 5' ends of the genes several rounds of RACE with either the Marathon or SMART RACE cDNA amplification kit (Clontech, Palo Alto, CA) were performed according to the manufacturers' instruction. The Goodfellow T51 panel was used to map pcdh15b (Geisler et al., 1999). Mapping prediction on the T51 panel map was done using the 'Instant Mapping' website from The Children's Hospital Zebrafish Genome Project Initiatives (http://zfrhmaps.tch.harvard.edu/ZonRHmapper/instantMapping.html).

4.3.3 GenBank accession numbers and clone names

The official names of the PAC clones from RZPD are the following: PAC1: BUSMP706C1835Q2; PAC5/7: BUSMP706H0192Q2; PAC9: BUSMP706M01204Q2; PAC12: BUSMP706N24105Q2; PAC10: BUSMP706N24105Q2; PAC11: BUSMP706O192Q2; PAC13: BUSMP706J031239. See the RZPD-Database (https://www.rzpd.de) for further information. GenBank accession number of the clones sequenced by the Sanger center: BAC1: AL592289; BAC6: AL592062; BAC7: AL645689; PAC10: AL592202; PAC13: AL592204; PAC1: AL592077. GenBank accession numbers for zebrafish pcdh15a: AY772390; and pcdh15b: AY772391.

4.3.4 Mutation analyses

Total RNA was prepared from pools of 5-10 wild-type sibling or mutant larvae using the NucleoSpin RNAII Kit (MACHEREYNAGEL, Easton, PA). Reverse transcription reactions were performed using the Superscript reverse transcriptase (Invitrogen, Carlsbad, CA) with an oligo (dT) primer.

4.3.5 In situ hybridization

Whole mount in situ hybridization was performed as described (Schulte-Merker et al., 1994). A fragment containing bp 180-1800 from the extracellular domain (Fig. 2) or bp 4516-5908
from the polymorphic intracellular part was used as probes for pcdh15a. Both probes gave the same signal; the signal with the extracellular probe was stronger and appeared more specific. bp 4075-5697 from the intracellular part was used as probe for pcdh15b.

### 4.3.6 Cryosectioning

For cryosectioning, larvae were immersed with increasing concentration of fish gelatin (FG, 8-25%) (Sigma G-7765 or G-7041) and sucrose (SC 8-15%) in Ringers during a 48-hour period at room temperature. They were transferred to forms made of aluminium foil covered with 20% FG, 15% SC and quickly frozen on a metal plate bathing in liquid nitrogen. 14 μm sections were cut at -20°C using a Leica CM 3050 cryomicrotome.

### 4.3.7 Electron microscopy

For electron microscopy, larvae were processed as previously described (Seiler and Nicolson, 1999). Whole larvae were embedded in Epon and oriented such that the eye was sectioned in a transverse dorsal to ventral direction. All sections were done in the plane of the optic nerve. Images were taken from areas adjacent to the optic nerve in the center of the retina.

### 4.3.8 Morpholino injections

Four different morpholinos were used in this study and injected at the one cell stage as described (Nasevicius and Ekker, 2000). One was directed against the translation start (ATG Mo) of pcdh15b with the sequence 5'-CCACGACCTCTGGCGATCTTCATA-3', one against the 3' splice site of coding exon number 4 (GT Mo) with the sequence 5'-AAAAGATAATCACATCTCGGTCCAG-3', and an unrelated control morpholino (a 4 bp mismatched oligo against fkd6; 5'-CGACGAGCTCTGCGATCTGGATA-3'). A second control morpholino was a 4 bp mismatched oligo against pcdh15b (5'-TGCGCGCCAGACAGGGTGATGAC-3'). In the ATG Mo-injected larvae (40 ng), a slightly shorter body axis of the larvae was occasionally observed. Differentiation of other organs like the heart, brain and ear appeared normal, indicating that this effect was not due to a delay in development. To ensure that the 40 ng dose had no effect on the onset of eye differentiation, we stained 14 μm sections of eyes of these larvae with anti-tyrosine hydroxylase antibody and counted the positive cells. No difference in number could be detected between uninjected (1.4 cells/section \(n=13\)) and larvae injected with 40 ng ATG Mo (1.5 cells/section \(n=14\)).
4.3 Material and methods

4.3.9 Fluorescence staining and microscopy

For confocal microscopy, an inverted Leica DM IRBE microscope equipped with a 100x oil lens was used. For antibody staining, the larvae were fixed with 4% formaldehyde in PBS for at least 24 hours prior to sectioning. The sections were incubated with 1% bovine serum albumin. The following antibodies were used in this study: zpr-1 (The zebrafish international resource Center, Eugene, OR, USA), rhod-1 (Ret-P1, Milan, Analytica AG/Switzerland), and Alexa488 goat anti-rabbit Ab (Molecular Probes, Eugene, OR).

4.3.10 Optokinetic stimulation

Single larvae were placed dorsal side up in the center of a petridish (35 mm diameter) containing 3% prewarmed (28°C) methylcellulose. Moving gratings were projected by a Proxima 4200 DLP projector onto a screen within the visual field of the larva, at an apparent distance 4.65 cm from the larva’s right eye. Projection size on the screen was 8 cm x 6 cm, subtending a visual angle of 65.6° horizontally and 53.1° vertically. A custom-made graphics library running under Microsoft DOS, allowing full control of timing and intensity of the projection was used to create the stimuli. Mean intensity levels were adjusted by introducing neutral density filters into the light beam. The contrast C of grating stimuli was calculated from: C = (I_{max} - I_{min})/(I_{max} + I_{min}).

Spatial frequency of the grating stimulus was varied between 0.025 and 0.14 cycles/°. All measurements were made under a mean luminance of 120 cd/m², temporal frequency of 0.4 Hz, pattern contrast of 62.5% and a grating velocity of 7.6°/second.

We measured the CSF in 4-day-old larvae injected with either an ATGMo (40 ng n=7 or 30 ng n=7) or the control Morpholino (40 ng n=7). The experiment has been repeated with similar results in an independently injected clutch.

4.3.11 ERG recordings

Electroretinograms (ERGs) were performed on larvae at 4.5 dpf as previously described (Makhankov et al., 2004). All pre-recording steps were done under red illumination to minimize bleaching of the visual pigment. Preparation and recordings were performed in a tight Faraday cage. All specimens were dark-adapted for 30 minutes prior to positioning them in the recording chamber. Each larva was placed on its side on the surface of a moist sponge.
with E3 medium (mM: 5 NaCl, 0.17 KCl, 0.33 CaCl₂ and 0.33 MgSO₄) and paralyzed by
directly adding a droplet of the muscle relaxant Esmeron (0.8 mg/ml in larval medium;
Organon Teknika, Eppelheim, Germany). The Ag/AgCl electrode system was used to record
the ERG response. The recording electrode was positioned in the center of the cornea. The
reference Ag/AgCl pellet was placed under the body of the larva. A 3-minute period was
chosen to adapt the larvae to both dark- and light-adapted states prior to measurement.
Relative background light intensity was 175 lux for the light-adapted state. The duration of
light stimulus was 100 milliseconds with an interstimulus interval of 5 seconds. Stimulus
illumination was increased in 0.5 log unit steps over the range from -5 log unit to -1 log unit.
Unattenuated light intensity over the subject's head was 20,000 lux (optical density, OD was
equal 0 log unit), as measured by a light meter (Tektronix J17, Texas Instruments, USA). A
virtual instrument (VI) under NI LabVIEW 5.1 was developed to use in all experiments.
Amplified analog signals were sampled by means of NI PCI 6035E DAQ board connected to a
NI BNC-2090 BNC terminal block. Sampling was done in buffered acquisition mode with a
sampling rate of 250 Hz. Recording was triggered by the shutter signal. To analyze the ERG
response with respect to the actual onset of the light stimulus, mechanical shutter delay was
measured by means of a photodiode. Traces were normalized to the baseline by subtracting
the average potential before the stimulus onset. Responses were averaged between three and
five, times depending on the signal-to-noise ratio.

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4.4.1 The orbiter gene encode Protocadherin 15

Mutant orbiter zebrafish larvae have a vestibular defect and cannot sense acoustic-vibrational
stimuli (Granato et al., 1996; Nicolson et al., 1998). Extracellular microphonic potentials of
orbiter⁰²₆₃ᵇ mechanosensory hair cells are absent, suggesting that the primary events in the
mechano-transduction process are affected (Nicolson et al., 1998). To identify the orbiter gene,
we undertook a positional cloning approach. By testing 2951 mutant larvae for recombination
events using polymorphic CA repeat markers, we mapped orbiter to chromosome 13, north of
the marker z26404 (4 recombinations in 2951 larvae) and south of the marker z25253 (99
recombinations in 2780 larvae). Using z26404, we started a chromosomal walk and
constructed a contig comprising several BAC and PAC clones that spanned the critical
interval (Fig. 1A). To identify the affected gene, three PAC and three BAC contig clones were
sequenced. Analysis of these sequences revealed that these clones contain exons present in the
first half of a gene that is homologous to human and mouse protocadherin 15 (PCDH15). The entire contig contained bp 1-3225 of pcdhl5; bp 1352-3225 was within the critical interval. No other genes were present on the clones. We performed RACE to clone the full-length zebrafish pcdh15 gene, which we designated pcdh15a (see below). Sequence analysis revealed that it shares high homology with human and mouse protocadherin 15. The Pcdh15 protein belongs to a class of atypical cadherins: the protein is composed of 11 cadherin repeats, a single transmembrane domain, and an intracellular domain with no similarity to other proteins (Fig. 1B).

The five orbiter alleles can be grouped in two phenotypic classes. One class causes strong defects such as no response to acoustic vibrational stimuli and a vestibular defect, no uptake of the styryl dye FM1-43 into hair cells, and no detectable microphonic potentials [microphonics only measured in th263 (Nicolson et al., 1998)]. This class includes the th263b, t25708, and t288 alleles. The other class is a weak, hypomorphic class with occasional response to acoustic vibrational stimuli but a fully penetrant vestibular defect, weak uptake of FM1-43 (data not shown) (Nicolson et al., 1998; Seiler and Nicolson, 1999), and reduced microphonic potentials [tc256e (Nicolson et al., 1998)]. The weaker alleles include tc256e and t23281.

Sequencing of pcdhl5a cDNA from the mutants revealed that it is the gene affected in orbiter mutants. For the alleles of the strong class, we found that the mutation in t25708 leads to a valine to glutamine exchange (V142Q) in close proximity to the conserved cadherin calcium-binding motif (with the consensus DXNDN) of the first cadherin domain. The mutation in th263b causes a truncation of the protein in the third cadherin repeat (R360X), presumably resulting in a nonfunctional protein. t288 causes a leucine to proline (L386P) exchange, adjacent to the calcium binding domain of the third cadherin domain (here DENNQ instead of DXNDN) (Fig. 1B).

Sequencing of the alleles of the hypomorphic class revealed that tc256e causes an isoleucine to asparagine (I419N) exchange in the linker between the third and fourth cadherin domain. t23281 causes an asparagine to lysine (N1143K) exchange in the tenth cadherin domain, changing the calcium-binding motive from DENDH to DEKDH (Fig. 1B). The weaker phenotype seen in homozygous t23281 animals suggests that this cadherin domain is less critical for Pcdh15 function.
Fig. 1. Zebrafish have two *pcdh15* genes. (A) Cloning of *pcdh15*a. The *orbiter* locus maps to chromosome (LG) 13 between the simple sequence length polymorphic (SSLP)
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markers z26404 and z25253. The critical interval was determined by generating SSLP markers from overlapping BAC and PAC clones and testing for recombination events in larvae identified with the south marker z26404 (indicated by arrowheads pointing to the right) or north marker z25253 (arrowheads to the left). Sequencing of six clones spanning the contig identified exons of the gene pcdhl15a. No other gene was present. (B) Schematic diagram of the Pcdh15a protein. The mutations identified in orbiter are indicated below. (C) Comparison of the predicted extracellular and intracellular domains of zebrafish Pcdh15a and Pcdh15b with the human and mouse PCDH15 proteins. Similarity of the extracellular regions is in the upper right half of the table; the intracellular regions are in the lower left half, shaded in grey. Extracellular domains of human, mouse, and zebrafish Pcdh15 are more conserved than the intracellular domain. (D) Phylogeny of human, mouse, and zebrafish Pcdh15 proteins are shown. The scale indicates phylogenetic distance by substitution events.

4.4.2 A second zebrafish pcdh15 gene

In addition to the pcdh15 gene mutated in orbiter, we identified a second ortholog of pcdh15. We designated the gene mutated in orbiter as pcdh15a, and the other gene pcdh15b. pcdh15b is located on chromosome 12 between the markers dkk1 and zl473 (T51 RH panel). The predicted Pcdh15b protein has the same domain structure and shares high amino acid similarity (70%) with the extracellular domain of zebrafish Pcdh15a, and mouse and human PCDH15 (Fig. 1C). The intracellular domain is less similar to the intracellular domains of Pcdh15a and mouse and human protocadherin 15 (17-33%, Fig. 1C), but the intracellular tail is divergent among all species (Fig. 1C). Nevertheless, all Pcdh15 intracellular domains share the feature of being proline- and serine-rich, and there are short stretches of high similarity. Pcdh15b has a putative PDZ class I binding motif at its C-terminus [consensus X-S/T-X-L (Sheng and Sala, 2001)]. This motif is also conserved in human and mouse PCDH15, but is not present in zebrafish Pcdh15a, which is shorter at the C-terminus. Phylogenetic analysis based on a ClustalW alignment revealed that the two zebrafish proteins are more related to each other than to the mammalian orthologs (Fig. 1D). This suggests that they were duplicated after the split of the mammalian and fish lineage, probably during the suggested genome duplication event in ray-finned fish (Prince and Pickett, 2002; Taylor et al., 2003).

4.4.3 Expression of pcdh15a and pcdh15b in the ear and the eye

To determine the spatial and temporal expression of pcdh15a and pcdh15b, we performed mRNA in situ hybridization. At the four-somite stage (11 hours post fertilization, hpf), pcdh15a is expressed in the anterior neuroectoderm and in the first forming somites (Fig. 2A).
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At 24 hpf, pcdh15a is expressed in the eye (Fig. 2B) and hatching gland (out of focus). Expression is also detectable in the newly developed mechanosensory hair cells (Fig. 2B,C). Expression of pcdh15a in hair cells was visible in all later stages investigated (Fig. 2D-F'). At day 3, additional expression in regions of the brain is present, together with expression at the lateral borders of the epiphysis (Fig. 2D). At day 4, expression in the brain persists and is detectable in the hair cells of neuromasts (Fig. 2E,F'). Within the neuroepithelia of the ear and lateral line, levels of pcdh15a mRNA are highest in the hair cells (Fig. 2F,F').

Fig. 2. Expression pattern of pcdh15a using in situ hybridization. (A-E) pcdh15a expression in embryos and larvae. At the four-somite stage (A), pcdh15a is expressed in the anterior neuroectoderm and in the somites. At 24 hpf (B), there is expression in the eye, the hatching gland (blue shading under the eye, out of focus) and the first hair cells of the ear at the margins of the otic vesicle (arrowhead and panel C). At 3 dpf (D), expression of pcdh15a is not detectable in the eye, whereas expression is present in the ear (asterisk), the brain and the lateral parts of the epiphysis (arrowheads). At 4 dpf (E), expression is present in the hair cells of the ear (arrows indicate the posterior maculae present in this focal plane) and the neuromasts (arrowheads). (F) Higher magnification view of the pcdh15a-positive hair cells of the anterior macula. (F') Higher magnification view of a trunk neuromast. Scale bar in F': 180 μm for A,B; 50 μm for C; 120 μm for D; 150 μm for E; 30 μm for F; 20 μm for F'.

pcdh15b is weakly expressed in the whole embryo at 24 hpf (data not shown). At 48 hpf, strong expression in the epiphysis is visible but not in the eye (data not shown). At day 2.5, the gene is expressed at the ventral margin of the eye (Fig. 3A), where the first photoreceptors
differentiate. Following the wave of differentiation of photoreceptors (Larison and Bremiller, 1990; Raymond et al., 1995), pcdh15b is expressed in the proximal regions of the eye around day 3 and restricted to the lateral margin at day 5 (Fig. 3B,C). In thick sections, expression is evident in photoreceptors (Fig. 3E,F). In addition, pcdh15b is expressed in the brain and center of the epiphysis at day 3 (Fig. 3D) and 5. Weak expression was detected in the ear and neuromasts (data not shown). No signal was detected with the corresponding sense probes.

4.4.4 Orbiter/pcdh15a is required for hair bundle integrity

To analyze the hair bundles in orbiter mutants in more detail, we stained the actin-filled stereocilia of the hair bundles with fluorescently-labeled phalloidin (Fig. 4). We observed a
moderate degree of splaying in the hair bundles in the semicircular canals in the strong allele th263b (Fig. 4B). Not all stereocilia were split apart, and some bundles appeared normal. Hair bundles were less affected in larvae carrying the weak allele tc256e (Fig. 4C). The defect in th263b is much weaker in comparison to other splayed-bundle mutants like mariner (Fig. 4D).

Fig. 4. orbiter mutants have a splayed hair-bundle phenotype. (A-D) The actin of the mechanosensory hair bundles of the lateral cristae were labeled with Alexa488-Phalloidin, and visualized in transverse sections with a confocal microscope. In wild-type larvae (A), the bundles are conical and intact in comparison to bundles in orbiter<sup>th263b</sup> (B). A similar phenotype is visible tc256e bundles (C), but many intact bundles are still present. A null allele of mariner/myosin VIIA (D) causes a more severe phenotype than th263b (B). (E,F) Horizontal optical sections of lateral line hair bundles. The unstained insertion point of the kinocilium is located at the caudal or rostral margin of the hair cells. No differences in planar polarity were observed between wild-type or th263b bundles as indicated with arrows (pointed toward each kinocilium) in (E') and (F'). Scale bar: 1 μm.

In Ames waltzer/Pcdh15 mutant mice, the planar polarity of the hair bundle of cochlear hair cells was sporadically disturbed, with hair cells rotated at 90° (Alagramam et al., 2001a; Raphael et al., 2001). By staining the actin in the bundle and the cuticular plate with fluorescently-labeled phalloidin, we could visualize the polarity of the hair bundles in
zebrafish neuromasts (NM), because the kinocilium and its insertion point are not stained with phalloidin. The kinocilium is located at either the caudal or rostral margin of the hair cell (n=12 NM, three specimens) (Fig. 4E-F). No difference in polarity could be detected in \textit{th263b} (n=10 NM, 5 spec) (Fig. 4E'-F'; arrows indicate orientation each bundle with arrowhead pointed towards the kinocilium; see Fig. S1 in the supplementary material for more examples). The number of hair cells (HC) in neuromasts was reduced (12.5 HC/NM in \textit{tc263b}; 17HC/NM in WT; counting NM above and around the ear) (Fig. 4F). A similar reduction was seen in \textit{mariner} neuromasts (13,25 HC/NM, n=12, 3 spec). It is not clear why the hair bundles in Ames waltzer mice have a polarity defect, whereas mutant \textit{orbiter} hair bundles do not.

![Fig. S1. Orientation of hair bundles in individual trunk neuromasts. Larvae were fixed and labeled with Alexa488-Phalloidin. Left column, neuromasts of wild-type siblings. Right column, neuromasts of mutant \textit{orbiter} \textit{th263b} larvae. Round shadows represent unstained kinocilia. Two orientations of the kinocilia along the anteroposterior axis are visible. The arrangement of these opposing groups of hair bundles is variable between neuromasts. The axis of orientation does not differ between wild-type siblings and mutant \textit{orbiter} larvae. Scale bar: 1 \mu m.](image-url)
4.4.5 Pcdh15b morphants have reduced contrast sensitivity and retinal function

To determine the function of Pcdh15b, we designed two morpholinos: one against the ATG translational start site (ATG Mo), and one against the GT splice site of the fourth coding exon (GT Mo). The ATG Mo is predicted to inhibit the initiation of translation of pcdh15b message, whereas the GT Mo should prevent the correct splicing of exon 4. Because the ATG Mo inhibits protein synthesis of Pcdh15b, its efficiency cannot be evaluated at the mRNA expression level. However, we could test the efficiency of the GT Mo by amplifying cDNA with primers flanking the targeted exon. RT-PCR analysis confirmed that the GT Mo indeed caused aberrant splicing of the targeted exon. Wild-type transcript levels are reduced by approximately 60% on day 3, and reduced by 40% on day 4 (Fig. 5D). The results of the histological analysis and electreretinograms (see below) suggest that the ATG Mo was more effective than the GT Mo. Morpholino-injected animals appeared to develop normally and overall morphology was normal (see Fig. S2 in the supplementary material). No obvious defects in balance or the acoustic startle reflex or hair bundle morphology were observed in pcdh15b morpholino-injected (morphant) larvae (data not shown). In addition, we examined uptake of the vital dye, FM1-43, an indicator of mechanotransduction in zebrafish hair cells (Seiler and Nicolson, 1999). Uptake was unaffected in pcdh15b morphants (see Fig. S3 in the supplementary material).
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Fig. S2. Injection of *pcdh15b* morpholinos (40 ng) does not affect overall morphology of larvae (4 dpf). (A) Uninjected larvae. Larvae injected with a 4 bp mismatch control (B), the *pcdh15b* GT (splice site) (C) or *pcdh15b* ATG (start site) morpholino (D).

![Figures S2A-D](image)

Fig. S3. Injection of *pcdh15b* morpholinos (40 ng) does not affect hair-cell transduction. Live larvae (4 dpf) were labeled with 167 mm FM1-43 dye for 20 seconds in E3 medium. Labeled head neuromasts are shown with the focus on a neuromast near the eye (arrows). Image acquisition settings were the same for all images. No difference in uptake of FM1-43 was observed among uninjected (A), 4 bp mismatch control (B), *pcdh15b* GT (splice site) (C) or *pcdh15b* ATG (start site) morpholino-injected larvae (D). Scale bar: 20 mm.

![Figures S3A-D](image)

To test whether morphant larvae have a visual defect, we measured the optokinetic response (OKR). The OKR is a reflexive movement of the eye when stimulated with a moving pattern. The velocity of the eye movement depends on the density and contrast of the pattern. We measured the gain (eye velocity/stimulus velocity) of 4-day-old larvae injected with 40 ng of an unrelated control morpholino (control Mo) and larvae injected with 30 ng or 40 ng ATG Mo during stimulation with increasing spatial frequency (i.e. decreasing stripe width of the moving grate) (Fig. 5A). Comparison of the *pcdh15b* morphants with control injected larvae (40 ng, n=7) revealed a significant difference in sensitivity (ANOVA with repeated measures, d.f.=2, F=44.1, \( p<0.001 \)). The comparison of responses in larvae injected with 40 ng or 30 ng *pcdh15b* ATG Mo is significant as well (\( p<0.001 \)), indicating an obvious dosage effect. The experiment was repeated, obtaining similar results in an independently injected clutch of eggs.
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(data not shown, n=7, 40 ng ATG Mo, 31% reduction in contrast sensitivity). In contrast, no OKR defect was detectable in orbiter$^{h263b}$ mutant larvae when compared to wild-type larvae (data not shown).

To determine whether injection of pcdh15b morpholinos results in physiological defects that are specific for retinal function, the electroretinogram (ERG) b-wave was measured in 4.5-day-old larvae. Larvae injected with 40 ng of the ATG Mo (n=6) or 16 ng of the GT Mo (n=6) were compared with uninjected larvae (n=5) and larvae injected with the control Mo (n=5). As shown in Fig. 5B and C, the b-wave increases in amplitude with increasing stimulus intensity in all experimental groups. Injection of both morpholinos resulted in a significant reduction in b-wave amplitude. (3-way ANOVA d.f.=3, F=11.7, $a<0.001$). Although both morpholinos are targeted against different sequences, they cause a comparable reduction in b-wave amplitude, indicating that this effect is specific for the knockdown. Comparison of the pcdh15b morphants with control-injected and uninjected larvae is significant for 40 ng ATG MO ($a<0.001$) and 16 ng GT MO ($a<0.001$; Scheffe test). The weaker b-wave amplitude reduction between the GT Mo compared to the ATG Mo injected larvae was not significant, though. Representative traces from individual specimens are shown in Fig. 5E and F. Due to a very low amplitude and differing width, we were not able to evaluate the a-wave data conclusively. Nevertheless, our behavioral and physiological analyses indicate a clear defect in retinal responses in pcdh15b morphants.
Fig. 5. Behavioral analysis and ERG recordings of pcdh15b morphants. (A) Optokinetic responses were measured as a function of spatial frequency of a moving square pattern.
Larvae injected with 40 ng ATG Mo (filled circles) have reduced contrast sensitivity and visual acuity compared with control injected larvae (filled squares). Larvae injected with 30 ng ATG Mo (open circles) show a slightly reduced visual performance. (B) 

*pcedh15b* knock-down results in significantly reduced retinal sensitivity to light under dark- and light-adapting states. The ERG b-wave amplitudes in the dark-adapted (B) and light-adapted state (C) are plotted as a function of relative light intensity. Uninjected larvae (open squares), larvae injected with control MO (closed squares), 40 ng ATG Mo (closed circles) and 16 ng GT Mo (triangles) were analyzed. Averaged data are plotted with error bars showing mean±s.e.m. (D) RT-PCR analysis of aberrant splicing of *pcdh15b* transcript. The upper gel contains products amplified with primers flanking exon 4. The lower band is the predicted product without exon 4. The uppermost band presumably includes an extra intronic sequence. The lower gel shows a control reaction with *elongation factor*-1alpha. Lane 1, GT Mo-injected larvae, day 3; lane 2, uninjected larvae, day 3; lane 3, GT Mo-injected larvae, day 4, lane 4, uninjected larvae, day 5 (pool of 5 larvae per lane). Representative examples of ERG records from four different specimens under dark-adapted (E) and light-adapted states (F). Each trace is an average of 3-7 consecutive responses. Stimulus duration was 100 milliseconds. Attenuated light intensity was presented as OD (optical density unit).

### 4.4.6 Reduction of *pcdh15b* activity affects photoreceptor morphology

To determine if the visual defect is due to a morphological defect in the retina, we examined cryosections of morpholino-injected larvae using immunohistochemistry. In larvae injected with 30 ng (*n*=5) or 40 ng (*n*=7, 2 independent experiments) of the ATG Mo, we observed a dose-dependent phenotype in the retinal photoreceptor cell layer (Fig. 6). The zpr-1 antibody labels double cone photoreceptor cell bodies and outer segments (Larison and Bremiller, 1990). In 4-day old morphants, the general morphology of the photoreceptors labeled with zpr-1 antibody appeared disorganized. Labeled cells appeared wider, shorter, and deformed (Fig. 6A-F). Effects were also evident using an anti-rhodopsin (*rhod-1*) antibody that labels outer segments. Outer segments of the rod photoreceptors appeared shorter and less organized than wild-type outer segments (Fig. 6G-I). The effects on both rods and cones seen with the antibodies were stronger in larvae injected with 40 ng (Fig. 6F, I) than in larvae injected with 30 ng ATG Mo (Fig. 6E, H).
Fig. 6. Knock-down of *pcdh15b* function shows a dose-dependent effect on photoreceptor structure. (A-I) Cryosections of the proximal region of day 4 retinas were stained with Alexa568-Phalloidin (red), and DAPI (blue). All layers are present in the ATG Mo-injected larvae (B,C). The outer segments of rod photoreceptors were stained with zpr-1 antibody (A-F, green) or double cone photoreceptors were stained with rhod-1 antibody (G-I, green). Uninjected larvae (A,D,G), larvae injected with 30 ng ATG Mo (B,E,H), and larvae injected with 40 ng ATG Mo (C,F,I) are shown. Double cone receptors are disorganized and broader in appearance (E,F), and rod outer segments appear shorter and clumped together (H,I). Scale bar in 1, 20 μm for A-C; and 5 μm for D-I. gc, ganglion cells; hc, horizontal cells; inl, inner nuclear layer; is, inner segments; os, outer segments; pr, photoreceptors; rpe, retinal pigment epithelium.

To examine the morphological defect at the ultrastructural level, we performed transmission electron microscopy (TEM) with thin sections of 4-day-old larvae. We compared uninjected (n=2) larvae to larvae injected with either 40 ng ATG Mo (n=3), 16 ng GT Mo (n=2) or 40 ng control (n=2) or 4 bp mismatch (n=3) control Mo. In the ATG Mo-injected larvae, the layer of photoreceptor nuclei appeared more compacted than in uninjected larvae (Fig. 7A,B). The outer segments of the photoreceptors were often tilted sideways and clustered together. In addition, the number of pigment cell granules positioned between the outer segments was greatly reduced (Fig. 7C). This effect was still present in 7-day-old larvae, though the outer segments were more clustered and less tilted (Fig. 7E,F). A similar but weaker defect was visible in the GT Mo-injected larvae (data not shown). A lack of interdigitation of the outer
segments and therefore the absence of the protective ensheathing by the pigmented epithelium is consistent with the poorer performance of the pcdh15b morphants under bright-adapting conditions. Defects were not detectable in the control Mo-injected larvae or in larvae injected with a pcdh15b 4 bp mismatch morpholino (Fig. 1A'). In addition, we also examined orbiter<sup>h263b</sup> retinas using TEM, and did not observe any obvious defects (data not shown).

Fig. 7. Photoreceptor outer segments are tilted and clumped in pcdh15b morphants. Electron micrographs of day 4 (A-C) and day 7 (D-F) retinas of uninjected or 40 ng pcdh15b ATG Mo-injected larvae. The outer segments are slanted sideways and the nuclear layer is more condensed in morphants (B) in comparison to segments in uninjected larvae (A) or control 4 bp mismatch Mo-injected larvae (A'). In pcdh15b morphants, pigments cell melanosomes are rarely present between photoreceptor outer segments (B,D). The phenotype does not recover at day 7 and outer segments are often clustered together (D). (C,F) Higher magnification views of (B) and (E). Scale bar in F: 1 μm for A,B,D,E; 350 nm for C,F.

4.5 Discussion

Here we show that protocadherin 15, responsible for retinitis pigmentosa and congenital deafness in humans (Ahmed et al., 2001; Alagramam et al., 2001b), is duplicated in zebrafish. In larvae, Pcdh15a is specifically required in the ear, whereas Pcdh15b is required in the developing eye. The expression patterns of the two pcdh15 paralogs provides insight into the evolution of these two genes. After gene duplication, one of the paralogous genes is often lost from the genome due to its redundant function (Prince and Pickett, 2002). If duplicated genes acquire non-redundant functions, then both are likely to be retained (Force et al., 1999). In the
case of the pcdh15 zebrafish paralogues, the high divergence of the intracellular domains may account for an adaptation or specialization of function of these two genes in the eye and ear. Although pcdh15a and pcdh15b are required in different sensory organs, both pcdh15 genes retained a function in maintaining the structural integrity of the specialized apical surfaces of the respective sensory receptor cells.

In a recent study, PCDH15 protein was shown to be localized at the stereociliary bundle of hair cells in mammals (Ahmed et al., 2003a). This result is consistent with the bundle phenotype we detected in orbiter mutants, suggesting that orbiter/pcdh15a encodes a component of the stereociliary bundle. The large extracellular domain of Pcdh15a, along with the localization data from mammals, suggests that Pcdh15a may be part of the outer membrane calyx or a component of the extracellular links spanning neighboring stereocilia. Although the bundle phenotype is mild in orbiter mutants, microphonic potentials are not detectable in larvae carrying the strong allele. This phenotype raises the possibility that Pcdh15a plays a functional role in hair bundles.

In the pcdh15b morphants, the tilted outer segment phenotype suggests at least two possible functions of pcdh15b during differentiation. First, Pcdh15b may be necessary for adhesive contact between the outer segment of photoreceptors and the retinal pigment epithelium. Interestingly, when the retinal pigment epithelium is absent in the zebrafish retina, the outer segments are tilted sideways in a similar way (C. Seiler and A. Rojas-Munoz, unpublished). This phenotype suggests that the outer segments are actively intercalated into the pigmented epithelium. This paradigm requires trans interactions of at least two cadherins expressed in photoreceptors and the retinal pigment epithelium, but we could not detect pcdh15b in the pigment epithelium. Another possibility is that Pcdh15b is required for stabilization of photoreceptor outer segments. Such contacts may help to align the outer segments, making interdigitation with the retinal pigment epithelium possible. The localization pattern of PCDH15 in photoreceptor outer segments in the human and monkey retina (Ahmed et al., 2003a) appears to be consistent with these notions. PCDH15 protein was also detected in other layers of the fetal and adult human retina, especially the synaptic layers, and may play a role in synaptogenesis as well (Alagramam et al., 2001b).

To date, the only mouse mutant in an Usher gene reported to have a morphological defect in the eye is the shaker1/Myo7a mutant. In shaker1 mutants, the melanosomes of the retinal pigment epithelium do not migrate between photoreceptors, suggesting that myosin VIIa is needed for transport of these pigmented structures into the apical processes of retinal pigment cells (Liu et al., 1998). Slight reductions in ERG responses have also been detected in myosin
VIIA mutant mice (Libby and Steel, 2001). Moreover, myosin VIIA has been shown to be expressed and required in the retinal pigment epithelium for uptake of outer segment disks (Gibbs et al., 2003). These results suggest that myosin VIIA also participates in photoreceptor and retinal pigment epithelium interactions.

In human Usher syndrome, retinitis pigmentosa develops in the second to third decade of life. This late defect in humans contrasts with the very early onset of photoreceptor malformation in zebrafish pcdh15b morphants. It is worth noting that in zebrafish, the eye develops much faster than in mammals (O'Brien et al., 2003; Sharma et al., 2003). Within 2.5 days, the zebrafish eye develops from a undifferentiated neuroepithelium to a layered and differentiated functional organ (Easter and Malicki, 2002). Furthermore, a fully populated photoreceptor cell layer develops within 30 hours (Raymond et al., 1995).

Thus, the contact and migration of outer segments into the retinal pigment epithelium happens much faster in zebrafish, and proper function of Pcdh15 appears critical at this timepoint. In various mouse models for retinal degeneration, the photoreceptor cell layer is initially disorganized and shows severe degeneration later in life [for example rd8/Crb1 (Mehalow et al., 2003) rd6/Mfrp (Hawes et al., 2000; Kameya et al., 2002)]. Due to the limited efficiency of antisense morpholinos, we were not able to investigate juvenile stages to look for degeneration. However, photoreceptor interactions with the retinal pigment epithelium have been shown to be important for the outer segment disc phagocytosis, visual pigment renewal, and nutrient supply (Boulton and Dayhaw-Barker, 2001; Pacione et al., 2003). Moreover, progressive death of photoreceptors was observed in the vitiligo/Mtf mouse mutant in which retinal pigment epithelium initially failed to interdigitate with photoreceptors (Sidman et al., 1996), and in mutant vestigial outer segments zebrafish, which exhibit a similar phenotype to pcdh15b morphants (Mohideen et al., 2003). Thus, impaired contact between the retina pigment epithelium and the outer segments may cause degeneration at later stages.

The phenotypes observed in either mutant orbiter/pcdh15a zebrafish or pcdh15b morphants indicate a clear requirement for Pcdh15 proteins in hearing and vision in zebrafish. The sensory receptors in either the ear or the eye rely upon these protocadherins for structural integrity. Our study establishes a role for Pcdh15 in maintaining the structural integrity of the photoreceptor outer segment, and highlights the usefulness of an alternative animal model in studying the function of Usher genes.
Chapter 5: Role of GRK7 in larval zebrafish visual system
Knockdown of cone specific kinase GRK7 in larval zebrafish leads to impaired cone-response recovery and delayed dark adaptation

Accepted for publication in Neuron, 2005

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key words: Light adaptation, dark adaptation, GRK7, zebrafish, cone opsin
5 My contribution to the project

I contributed to all ERG measurements and some of the experimental ERG design to this project.

5.1 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 opsin is phosphorylated following light stimulation. 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.

5.2 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 and Bremiller, 1984b; 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 and dark adaptation. In psychophysical experiments, temporal contrast sensitivity was markedly reduced under light adapting conditions.

5.3 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, 1984b). Developing rods are sparse and not functional in the larva (Branchek and Bremiller, 1984a; 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.

5.3.1 Cloning of grk7 and grkl homologues in zebrafish

To explore the role of cone specific kinases in the cone dominant zebrafish retina we cloned orthologs of the rhodopsin kinase grkl, 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 (grkl) gene denoted as grklα and grklβ, 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.

5.3.2 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, grklα, grklβ, and c-arr. 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).
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.

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 is 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.
5.3 Results

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+B 20 μm; C 5 μm.

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
The 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).

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.

5.3.3 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).
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 grka7MO 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.

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) was 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 indicated knockdown below 5% of control levels.

### 5.3.4 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 measure of outer retinal function (Dowling, 1987). In the larval zebrafish retina the a-wave is largely masked by the positive deflection of the b-wave and therefore is not a robust measure of photoreceptor function. As a functional measure we used the ERG b-wave amplitude recorded from live larvae. The b-wave of the ERG originates from retinal interneurones as a result of photoreceptor activity and thus is 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, recovery was below 10% after 1 s (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 insert).
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 1 s. (B) In grk7aMO injected larvae the recovery after 1 s 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 (open triangles) and controlMO (black square). 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.

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, 2005). 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 1 s 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 high flash intensity of 2120 lux. Under these conditions photoresponse recovery is still significantly reduced (p < 5%; Fig. S2).

5.3.5 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.
5.3 Results

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.

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 show optokinetic gain measured with a low temporal frequency pattern (0.034 cycles/deg, 0.7 cd/m²). Under these conditions grk7aMO and controlMO 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 7dpf 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
5.3 Results

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.

As a negative control we also injected a morpholino directed against grkl1a, 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 in rods, which are not functional at this developmental stage, no difference was found between grkl1aMO and controlMO injected larvae. However, as no antibodies are available for zebrafish Grk1a, effectiveness of this morpholino could not be verified (Fig. S3).

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 only. 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 S4).

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.

5.3.6 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.
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.

5.4 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 in a light dependent manner (Liu et al., 2005). 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)).

5.4.1 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. Furthermore, electrophysiological and behavioral defects recover correlated to the reappearance of Grk7a protein at later stages of development in morphant larvae.

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.

5.4.2 Grk7a deficiency leads to prolonged dark adaptation

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 (Yamamoto et al., 1997) attributed this to a delayed recovery of opsin by 11-cis retinal. 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 (Yamamoto et al., 1997) attributed this to a delayed recovery of opsin by 11-cis retinal. Lamb and Pugh (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.
5.4 Discussion

5.4.3 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 (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 of cone opsin (Imai et al., 1997). Kennedy (Kennedy et al., 2004) showed 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 larva 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 (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 it was shown that shutoff kinetics is markedly delayed if putative phosphorylation sites are mutagenized (Kefalov et al., 2003). Our in vivo data indicates that in absence of Grk7a 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.
5.5 Methods

5.5.1 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 CaCl₂, and 0.33 mM MgSO₄) and staged according to development in days postfertilization (dpf).

5.5.2 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.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>grk7a</td>
<td>grk7a_fw</td>
<td>5'-GCATCTTTCTAGTCTGATAGC-3'</td>
</tr>
<tr>
<td>grk7a</td>
<td>grk7a_rev</td>
<td>5'-ACAGCTTTCAATCATGTATGTA-3'</td>
</tr>
<tr>
<td>grk7a</td>
<td>grk7a_fw_rec</td>
<td>5'-GTGCAGCTGTGTGACATGGGGGAC-3'</td>
</tr>
<tr>
<td>grk7b</td>
<td>grk7b_fw</td>
<td>5'-CCCAGAGCGCTCATATA-3'</td>
</tr>
<tr>
<td>grk7b</td>
<td>grk7b_rev</td>
<td>5'-AGTCACAGGAATAAGCTATGAA-3'</td>
</tr>
<tr>
<td>Rka</td>
<td>grk1a_fw</td>
<td>5'-CTCGCTGTGACCCGTACGTC-3'</td>
</tr>
<tr>
<td>Rka</td>
<td>grk1a_rev</td>
<td>5'-CCTTCTGTGATGGGTAGTG-3'</td>
</tr>
<tr>
<td>Rkb</td>
<td>grk1b_fw1</td>
<td>5'-ATGTITAAACCTCATACTCC-3'</td>
</tr>
<tr>
<td>Rkb</td>
<td>grk1b_fw2</td>
<td>5'-AAGAGGTGAAAGAAACGTA-3'</td>
</tr>
<tr>
<td>Rkb</td>
<td>grk1b_rev</td>
<td>5'-TTAATTATTTCCAGCGACAC-3'</td>
</tr>
<tr>
<td>c-arr</td>
<td>c-arr_fw</td>
<td>5'-GCTGCTCTGTGCTCCCGCTAT-3'</td>
</tr>
<tr>
<td>c-arr</td>
<td>c-arr_rev</td>
<td>5'-TAAAGGATGATGAGCATTAGG-3'</td>
</tr>
</tbody>
</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 grk7aJrev. The forward primer contained a Sall restriction site. The PCR product was ligated with a TOPO PCR-II cloning vector. A 1755 bp Sall/Notl 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.

5.5.3 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).

5.5.4 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.

5.5.5 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 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 N2; 25μm thick sections were cut at −20°C, mounted on superfrost slides, and air dried at 37°C for at least 2h. 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 1h. 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 both, biotinylated secondary antibody followed by ABC reagent (Avidin DH and biotinylated horseradish peroxidase; Vectastain ABC-Kit, Vector Laboratories, Burlingame, CA) and visualized using hydrogen peroxidase and diaminobenzidine tetrahydrochloride (DAB), and 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.

5.5.6 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).

5.5.7 Targeted gene knockdown

For targeted knock-down of the grk7a protein an antisense morpholino (MO) oligonucleotide (grk7aMO) (GeneTools, LLC, Philomath, OR) was used covering -1 to +24 of the grk7a mRNA: 5’-ATCGAGTCCCCCCCATGTCACACATT-3’. For control-injections, a standard control morpholino oligonucleotide, controlMO, 5’-CCTCTTACCTCAGTTACATTATA-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 MgSO₄, 0.6 mM Ca(NO₃)₂, 5 mM Hepes, pH 7.6) to obtain a stock concentration of 2 mM. The stock solutions were diluted to 500 μM. 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.

5.5.8 Western blots

To quantify gene knockdown, 10 larvae were homogenized in 50 μl 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.

5.5.9 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, 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 = n1) and control larvae (n = n2) under certain experimental conditions, data was pooled (n = n1 + n1) and two samples of the sizes n1 and n2 were drawn with replacement. OKR amplitudes A1* and A2* were determined and the bootstrap distribution of ΔA* = A1* - A2* 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.

5.5.10 Electroretinography

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 and 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.
6 Summary and outlook

6.1 Dissection of retinal physiology in zebrafish by ERG

The developing zebrafish visual system provides an opportunity to correlate anatomical maturity with function because the different photoreceptors develop at different times. For the last decades, zebrafish has become an important model to study visual processing in the retina. The major knowledge about zebrafish retinal processing has been obtained employing the electroretinogram. At the beginning of my PhD project, I mainly focused on establishing an ERG setup to study the different level of gene expression in mutated zebrafish larvae and adult in vivo.

Little is know about mechanisms of dark and light adaptations, visual cycle and pigment regeneration in zebrafish visual system. All studies has been done in those field of interest employing higher vertebrate organisms such as rat, mouse, monkey and human beings to get insight the problem of visual processing in cone dominant model.

In the present study a simple setup for electroretinographical recordings of vertebrate aquatic animals was developed. The ERG data gave significant value for understanding the problem at the level of retinal processing. This approach was mainly focused on the vertical processing pathway of the outer part of the zebrafish retina. The established ERG was used for studying the role of pcdh15b (chapter 4) and the role of grk7a (chapter 5) in cone-dominant zebrafish retina.

However, there were more advantages to employ our ERG technique to study for example, spectral sensitivity function from the b-wave component, rod/cone contributions to the dark/light adapted spectral sensitivity of the b-wave, direct contact between photoreceptors and retinal pigment epithelium. The ERG was as a final step to assess the functional effects of genetic manipulations, which were done by my colleagues. Without in vivo ERG data, it was difficult to improve the role of gene expression at functional level.

6.2 Dissection of pcdh15 genes in larval zebrafish by ERG

Protocadherins, a subclass of the cadherin superfamily, are expressed in neuronal tissues where they are thought to be involved in cell adhesion. In humans, mutations in protocadherin 15 are known to result in Usher syndrome type 1F (USH1F). Patients with USH1F are born with profound hearing loss and have visual problems. The only one model, called the Ames waltzer mice, was described as a potential model for USH1F so far. Activity of both rod and
cone photoreceptor activities were evaluated by ERG analyses under different ambient illuminations. The conclusion was that protocadherin 15 did not affect ERG response in the Ames waltzer mice. Both ERG a-wave and b-wave were comparable between control and mutated groups. The mutation of pcdh15 did not result in retinal abnormalities in the different group of alleles tested in this study.

In contrast to the unaffected mouse model of USH1F, a phenotype observed in either mutant orbiter/pcdh15a or pcdh15b (Chapter 4) zebrafish morphants indicate a clear requirement for protocadherin proteins in hearing and vision. In collaboration with a group of Teresa Nicolson the ERG approach was chosen to study pcdh15a-deficient larvae as an alternative model to USH1F.

Protocadherin 15 is duplicated in zebrafish. One form, pcdh15a is required in the ear, whereas another form, pcdh15b is required in the eye. The pcdh15a-deficient larvae have a morphological defect between photoreceptor outer segment and retinal pigment epithelium cells. Protocadherin 15b was expressed on the surface of photoreceptor outer segments only. To determine the function of pcdh15, two morpholinos were designed to inactivate protein synthesis. One of the valuable approach to determine whether injection of pcdh15b morpholinos result in physiological defects that are specific for retinal function, are measurement of ERG a-wave or c-wave. The a- and c-wave mainly reflect the activity of photoreceptor and interaction them with retinal pigment epithelium cells. It was not possible to record both of those waves due to technical difficulties in our ERG setup. The ERG b-wave was chose as an indirect functional measure in 4.5 day old larvae. Two light paradigms were developed to determine a defect in ERG responses. Since outer segments of photoreceptors are tilted sideways and clustered together there must be a physiological defect in generation of the b-wave in electroretinogram recordings. Perhaps, the ERG response must be different under dark and light conditions due to the phenomenon called retinomotor (photochemical) movement [RM]. The photoreceptors and retinal pigment epithelium undergo positional changes in response to changes in different ambient light conditions. The RM is found in teleost fishes, like zebrafish, with duplex retinas and other lower vertebrates but not in mammalian. It has been proposed that the RM depends on circadian regulation and absorbing scattered rays refracted from cone ellipsoids protecting the photoreceptors from bleaching and damaging in bright light. At the different ambient light illumination there is different elongation of photoreceptor outer segments and interdigitation them into the RPE. Consequently, there is a decrease in a magnitude of the ERG response that is electronically conducted from outer segment to synaptic terminal. The contact and migration of outer
segments into the RPE take places in zebrafish and proper function of pcdh15b is critical at this point. Thus, impaired contact between the RPE and the outer segments can be investigated to see regeneration at different stage of photoreceptor development under different level of ambient illuminations.

Our ERG physiological analysis demonstrated a clear defect in retinal responses in pcdh15b-deficient larvae. Although both morpholinos were targeted against two different sequences, they cause a significant reduction in the b-wave amplitude under dark and light conditions. The level of difference in the b-wave amplitude was significantly different at higher level light stimuli under both dark and light ambient illumination. An additional hypothesis is that this interaction between RPE and photoreceptor outer segments affected retinal pigment regeneration cascade due to abnormal morphological contact. The retinal pigment regeneration is delayed as a consequence the ERG has diminished b-wave amplitude. ERG proved to be a comprehensive combination with other techniques and methods in examining the role of protocadherin 15 in zebrafish larvae. The ERG study established a role for protocadherin 15 in maintaining the structural integrity in the retina.

6.3 Dissection of cone specific kinase GRK7a in larval zebrafish visual system by ERG

The zebrafish has a cone dominant retina up to 15 dpf as determined by ERG (Chapter 1). Little is known about the cone photoreceptor responses measured by electroretinogram during and after exposure to intense illumination in zebrafish. For the last decades the sensitivity of the overall human visual system during light and dark adaptation has been studied exhaustively over many decades and the electroretinogram (ERG) a-wave analysis has long been used as a method for extracting information about photoreceptor response recovery. In case of zebrafish, it is difficult to evaluate the kinetic of the ERG a-wave as it has been done by many groups in human and mouse models. To test cone response recover in larval zebrafish, we used ERG b-wave as a functional measure. The b-wave amplitude is an indirect measure of cone response in larval zebrafish. For this purpose, a cone-specific kinase, grk7a was cloned and its function was studied by ERG. The expression pattern of the kinase was identified in the cone outer segments. It was postulated that cone opsin phosphorylation played a role in light adaptation similar to that of rhodopsin phosphorylation in rod photoreceptors. To explore the role of GRK7 function in cone desensitization in vivo larval zebrafish was used to test whether deficiency of Grk7a affects cone response recovery. The
similar approach was used to examine role of GRK1 in mammalian cone function with the GRK1 gene inactivated. This study showed that indeed GRK1 was essential for normal deactivation of murine cone phototransduction and provided the first functional evidence for a major role of a specific GRK in the inactivation of vertebrate cone phototransduction. Knockdown of grk7a does not alter the fish morphology and results in a prolonged recovery time in a paired flash ERG assay and reduced gain under light-adapting conditions in the OKR assay. This study established that zebrafish morpholino experiments can be used effectively to study phototransduction.

It is interesting to note that cone opsin phosphorylation by GRK7 does not seem to be needed for vision at low intensities based on the OKR studies whereas it is needed at high intensities. This seems to imply that another inactivation mechanism is sufficient in dim light presumably mediated by cones since the larvae have few rods at this stage. But in bright light an additional inactivation mechanism is required. The next steps would be more appropriate to examine this aspect of light-adaptation, the need for multiple mechanisms for different light levels, could be investigated further to reveal new insights. In addition, it would be interesting to include morpholino analyses of rod and cone arrestins.

Our results presents a novel set of experiments addressing a long-standing question in vertebrate cone vision: the role of cone opsin kinase (GRK7) in photoresponse recovery and adaptation. This is a hot topic in the vision community because several previous studies, including those with human subjects, have suggested that opsin phosphorylation is not necessary for normal cone vision. Here, we presented data that decisively show that GRK7 is essential for normal ERG paired flash responses, a measure of the cone response recovery. The employment of both ERG and behavioral measures demonstrated the efficacy of the knockdown; these measures constituted both internal and external controls, as they (e.g., the use of the control morpholino) demonstrated that the effects were specific for the grk7a morpholino, while at the same time showing the animals were otherwise healthy and seeing well.
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7 Acknowledgment

I thank Prof. Stephan Neuhaus for inviting and supervising my PhD project, the steady support, helpful discussions and the freedom to follow my own ideas and strategy.

I thank Prof. Martin Schwab and Prof. Kevan Mertin for supervision of my thesis.

My thanks to our colleagues from the zebrafish group for their friendship and support. It was a pleasure to work as a team. Special thanks to: Oliver Rinner for our enormous collaboration to study different scientific questions during our study at the same group; Ronja Bahadori for her friendship and help; Oliver Biehlamaier, the senior postdoc and leader and big man, for his advices and help to manage problems not only in my scientific field. I thank Honsjörg Kasper, Stefan Giger and Marco Tedaldi for their technical help to develop the magnificent ERG setup.

My private thank to my wife and relatives outside Switzerland.

The present project has been done between October 2001 and July 2005 at the Brain Research Institute, University of Zurich and Swiss Federal Institute of Technology (ETH) Zurich, Switzerland. Many thanks to all of you who helped me to carry this project out. The thesis was funded by ETH TH grant.
8 Curriculum Vitae

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Scholarships

Since 2001              ETH Internal grant
1994-2000               MSU Internal scholarship

Published
papers


Submitted


In the pipeline


Role of NYX in the development of retina. Ronja Bahadori, Oliver Rinner, Yuri V. Makhankov, Oliver Biehlmaier, and Stephan C. F. Neuhauss.

Altered retinal morphology in laminin chain deficient zebrafish mutants. Oliver Biehlmaier, Yuri V. Makhankov and Stephan C. F. Neuhauss.