Investigations of Photoreceptor Synaptic Transmission and Light Adaptation in the Zebrafish Visual Mutant nrc

Heather A. Van Epps,1 Chong M. Yim,1 James B. Hurley,2 and Susan E. Brockerhoff1

PURPOSE. To characterize the retinal physiology of the zebrafish visual mutant no optokinetic response c (nrc) and to identify the genetic map position of the nrc mutation.

METHODS. Electroretinograms were recorded from wild-type and nrc zebrafish larvae between 5 to 6 days postfertilization. Responses to flash stimuli, and responses to prolonged light stimuli, and responses to flash stimuli with constant background illumination were characterized. The glutamate agonist, 2-amino-4-phosphonobutyric acid (APB) was used to examine the photoreceptor specific a-wave component of the electroretinogram. Amplified fragment length polymorphism methodology was used to place the nrc mutation on the zebrafish genomic map.

RESULTS. nrc and wild-type zebrafish larvae 5 to 6 days postfertilization have similar threshold responses to light, but the b-wave of the nrc electroretinogram is significantly delayed and reduced in amplitude. On and Off responses of nrc larvae to prolonged light have multiple oscillations that do not occur in normal zebrafish larvae after 5 days postfertilization. Analysis of the b-wave demonstrated a light adaptation defect in nrc that causes saturation at background light levels approximately 1 order of magnitude less than those with wild-type larvae. Application of the glutamate analog, APB, uncovered the photoreceptor component of the electroretinogram and revealed a light adaptation defect in nrc photoreceptors. The nrc mutation was placed approximately 0.2 cM from sequence length polymorphism marker Z7504 on linkage group 10.

CONCLUSIONS. The zebrafish mutant nrc is a possible model for human retinal disease. nrc has defects in photoreceptor synaptic transmission and light adaptation. The nrc mutant phenotype shows striking similarities with phenotypes of dystrophin glycoprotein complex mutants, including patients with Duchenne/Becker muscular dystrophy. Localization of the nrc mutation now makes it possible to evaluate candidate genes and clone the nrc gene. (Invest Ophthalmol Vis Sci. 2001;42:868–874)

A thorough understanding of vision is essential for treatment and prevention of retinal disease. Recently, zebrafish have emerged as a model system for genetic studies of the vertebrate retina. Precedence for genetic analysis of vision lies in Drosophila, where use of classical and molecular genetics has identified novel components of invertebrate vision.1–3 However, invertebrate vision and vertebrate vision are fundamentally different at the molecular level.4 Zebrafish are amenable to genetic screening for visual defects, because they can be bred and maintained with minimal resources and because their sophisticated visual system develops rapidly. Zebrafish have color vision mediated by red, green, blue, and UV-sensitive cone photoreceptors.5 They also have scotopic vision mediated by rod photoreceptors.6 Photoreceptor outer segments appear at 55 hours postfertilization7 and by 3 days postfertilization (dpf) zebrafish have rudimentary visual function.8 By 5 dpf photoreceptors have developed sufficiently to screen for visual behavior,9 although rod function is not distinguishable from cone function until 2 weeks postfertilization.10,11

A genetic screen of mutant zebrafish was initiated to identify fish with abnormal visual responses but no obvious gross morphologic defects.7 Zebrafish larvae display an optokinetic response (OKR), characterized by smooth pursuit and saccade eye movements in response to illuminated rotating stripes. The OKR can be used as a highly effective behavioral assay to isolate zebrafish larvae with visual defects.10 To select for visual mutants with outer retinal defects, electroretinograms (ERGs) are recorded from larvae with abnormal OKRs. Several recessive zebrafish retinal mutants have been isolated by this method.12

Here we describe an ERG analysis of a zebrafish visual mutant, no optokinetic response c (nrc), which has no OKR in white light.5,11 Ultrastructural analyses have revealed specific defects in nrc cone photoreceptor pedicles and the nrc retinal pigment epithelium (RPE).13 We show that nrc has an abnormal b-wave and abnormal On and Off responses, suggestive of a synaptic transmission defect. We also show that nrc has a photoreceptor light adaptation defect. As a first step toward determining the primary molecular defect, we identify the genetic locus of the nrc mutation.

METHODS

Generation and Maintenance of Zebrafish Strains

Zebrafish were maintained as described previously13 on a 10-hour dark:14-hour light cycle. nrc-14 was isolated as described previously.11 nrc larvae were produced for these studies by crossing nrc heterozygous adults. Therefore, OKR responder siblings, referred to here as wild-type (WT) siblings, are genotypically either heterozygous or homozygous WT fish. We did not detect greater than normal variability in the behavioral phenotype or ERGs in this group of larvae. nrc larvae do not eat, and they die at 10 to 15 dpf. Therefore, for control animals we used unfed OKR responder siblings at 5 to 6 dpf. The lack of food does not affect retinal morphology up to 8 dpf.15

OKR Screening

OKR screening for visual mutants was done as described previously.9 nrc mutants show no OKR response in white light. However, their eyes occasionally move spontaneously, indicating that the mutation does not interfere with eye movement.

Electroretinograms

Larvae were positioned on a feather on a sponge in a Petri dish containing Hank’s embryo media14 in dim room light. Larvae were
immobilized on their side between the ridges of a feather. To record ERGs, a glass micropipette (10- to 30-μm diameter tip) containing a silver wire and filled with Hank’s embryo media was positioned on the cornea, and a ground wire was submerged in the Petri dish. The ERG setup was then placed in darkness. WT larvae, dark adapted for at least 2 hours and positioned under dim red light or infrared light, showed a sensitivity threshold of −4.7 ± 0.4 (mean ± SD) log intensity units (n = 11) using a white light flash that has an unattenuated output at the cornea of 7.82 μJ/cm². WT larvae positioned under dim room light showed a similar threshold of −4.5 ± 0.5 log intensity units (n = 5). There is no statistically significant difference between the light- and dark-adapted threshold (P = 0.15). This is consistent with previous reports indicating that rods do not contribute significantly to visual sensitivity at this stage of development. Visual threshold was chosen to be a 20 μV b-wave response measured from the base line. Light was delivered through fiber optics positioned approximately 10 mm above the eye. Brief flash stimuli (<1 msec) were produced by a Canon 540EZ Speedlite Flash at one-sixteenth power. Prolonged light stimuli or background light were produced with an Orion Fiber Optic Illuminator (Model 77501). Interstimulus time was ≥5 seconds. Stimuli were attenuated with neutral density or interference filters. Data were filtered with a high-frequency cutoff of 100 Hz and a low-frequency cutoff of 1 Hz. Data were collected in Igor Pro (Wavemetrics, Lake Oswego, OR) with an Instrutech ITC-16 analog to digital interface filtered with a high-frequency cutoff of 100 Hz and a low-frequency cutoff of 1 Hz. Data were collected in Igor Pro (Wavemetrics, Lake Oswego, OR) with an Instrutech ITC-16 analog to digital interface.

For the analysis of these traces.

Results

ERG Waveform and Sensitivity

To determine whether the nrc mutant has a functional defect in the outer retina we compared ERG responses of nrc and OKR responder siblings to a light flash of less than 1-msec duration. Light stimulates a transient negative potential on the cornea, the a-wave. The rising phase of the a-wave is derived from field currents produced by stimulation of photoreceptors. The a-wave is followed by a corneal positive b-wave. The b-wave is derived from secondary neurons postsynaptic to photoreceptors. In 5 to 6 dpf WT larvae the a-wave is occluded by the b-wave (Fig. 1).

The b-wave of the nrc mutant is reduced in amplitude and delayed compared with WT (Fig. 1). In general in nrc the maximum b-wave amplitude was decreased approximately fourfold, and the b-wave implicit time (flash onset to peak) was increased approximately fourfold (Table 1).

Oscillations in Response to Prolonged Light Stimulus

We used steps of illumination to examine On and Off response signaling pathways. On and Off pathways arise through independent retinal bipolar cells and they are mediated by different glutamate receptors. Off bipolar cells are depolarized, and On bipolar cells are hyperpolarized by glutamate released from photoreceptors in the dark. There is growing evidence that the d-wave, a positive change in potential on the cornea when lights are turned off, is generated by off-bipolar cells. Both the On and the Off responses to prolonged light of nrc mutants are abnormal and oscillatory.

Figure 2a shows averaged responses from one WT larva and three mutant larvae to 1.6-second light stimuli spanning three orders of magnitude of intensity. The WT On response is the corneal positive b-wave, and the Off response is the corneal positive d-wave. In contrast to WT, the nrc On and Off responses to prolonged light are abnormal and are followed by multiple oscillations. Three nrc larvae examples show the variability between fish. The traces shown in Figure 2a are...

Figure 1. The nrc mutant’s response to a flash of 7.8 μJ/cm² white light is abnormal in comparison to WT. In nrc the b-wave is delayed and decreased. Each trace is an average of more than five flash responses.
averages of 5 to 10 individual responses. Often the oscillations are not in phase with one another from one response to the next and are therefore not detected when the data are averaged. Figure 2b shows an example of oscillations in single traces. The number of oscillations in averaged responses to prolonged light stimuli under various conditions ranged from 1 to 5. There was no correlation between the number of oscillations and illumination intensity, illumination duration, or temperature. These studies are summarized in Table 2. All nrc larvae had oscillations in either the On or the Off response or both. The few On responses that lacked oscillations were similar in waveform to the nrc flash response.

**Light Adaptation**

To characterize light adaptation, we measured responses of nrc larvae to flashes of light in the presence of background illumination. These studies are summarized in Table 2. All nrc larvae had oscillations in either the On or the Off response or both. The few On responses that lacked oscillations were similar in waveform to the nrc flash response. The few Off responses that lacked oscillations were similar in waveform to the WT nrc flash response.

**TABLE 1.** ERG a- and b-Wave Implicit Times and Amplitudes in Response to a 7.8 μJ/cm² Flash

<table>
<thead>
<tr>
<th>Flash Onset to Peak</th>
<th>15 msec After a-Wave Onset</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>a-Wave Implicit Time (msec)*</td>
</tr>
<tr>
<td></td>
<td>b-Wave Implicit Time (msec)*</td>
</tr>
<tr>
<td>WT</td>
<td>66 ± 32 (8)</td>
</tr>
<tr>
<td>nrc</td>
<td>37 ± 10 (4)</td>
</tr>
<tr>
<td>P</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>7.9 × 10⁻⁶</td>
</tr>
</tbody>
</table>

The implicit time and maximum amplitude differences between nrc and WT b-waves are statistically significant.

* Values are means ± SD; numbers in parentheses are the sample numbers.
† Values are means ± SEM.

**TABLE 2.** Number of Oscillations in the nrc Larval Response Does Not Correlate with Illumination Duration or Temperature

<table>
<thead>
<tr>
<th>Condition</th>
<th>No. of Larvae Showing Oscillations at Light Onset</th>
<th>No. of Larvae Showing Oscillations at Light Off</th>
</tr>
</thead>
<tbody>
<tr>
<td>28°C, light on 1.6 sec</td>
<td>WT 0/6</td>
<td>nrc 5/5</td>
</tr>
<tr>
<td>22°C, light on 1.6 sec</td>
<td>WT 0/2</td>
<td>nrc 6/9</td>
</tr>
<tr>
<td>22°C, light on 20 sec</td>
<td>WT 0/2</td>
<td>nrc 4/4</td>
</tr>
</tbody>
</table>

Values are presented as number/total number.
Background illumination attenuates the nrc a-wave more than that of the WT a-wave. The a-wave waveform of individual nrc and WT larvae is shown in Figure 4b. Averaged a-wave amplitudes 15 msec after a-wave onset are reported in Figure 4c (n = 4). With no background light, there is no statistically significant difference between the WT and nrc responses to the flash series (all in the flash series are $P \geq 0.18$). However, in response to a flash of 7.8 $\mu$J/cm$^2$ in the presence of 0.012 mW/cm$^2$ background, nrc a-waves are reduced approximately 46%, whereas WT are reduced approximately 25% ($nrc = 49.7 \pm 10.6$ (mean ± SEM), WT $= 61.0 \pm 17.7$). In the presence of 0.11 mW/cm$^2$ background light, nrc are reduced approximately 73%, whereas WT are reduced approximately 41% ($nrc = 24.7 \pm 9.6$, WT $= 47.8 \pm 18.8$). Finally, in 0.96

**FIGURE 3.** (A) Examination of the b-wave reveals an impairment of nrc larvae’s ability to adapt to background illumination. WT and nrc mutant flash responses in no background illumination and constant background illumination are compared. Flash intensities: (A) 7.8 $\mu$J/cm$^2$, (B) 1.0 $\mu$J/cm$^2$, (C) 0.14 $\mu$J/cm$^2$, (D) 0.011 $\mu$J/cm$^2$, and (E) 0.0019 $\mu$J/cm$^2$. Arrow: the initiation of the flash. (B) The normalized adaptation data of seven WT and eight nrc larvae. Error bars, SEM. The data are normalized as explained in the text to clearly show the light adaptation defect.

**FIGURE 4.** (A) To examine the zebrafish a-wave the chemical agonist APB was applied to larvae as described in Methods. Flash responses of untreated WT larvae and APB-treated WT larvae. (B) Comparison of a-waves of WT larvae and nrc larvae flash responses in constant background illumination. All larvae are treated with APB as described in Methods. Flash intensities: (A) 7.8 $\mu$J/cm$^2$, (B) 1.0 $\mu$J/cm$^2$, (C) 0.14 $\mu$J/cm$^2$, and (D) 0.011 $\mu$J/cm$^2$. Arrow: the initiation of the flash. (C) The a-wave amplitudes 15 msec after a-wave response onset of four nrc larvae and four WT larvae are compared in the presence and absence of background light. Error bars, SEM.
mW/cm² background light, nrc a-waves are reduced approximately 92%, whereas WT are reduced approximately 49% (nrc = 4.6 ± 4.0, WT = 33.6 ± 6.2). The adaptation defect appears to increase with increasing background light intensity. In the brightest background light, 0.96 mW/cm², the light adaptation defect is statistically significant (P = 0.01; Fig. 4c, n = 4).

Because nrc has defects in photoreceptor morphology,¹³ we considered that altered morphology of the photoreceptor layer might generally cause defects in light adaptation. As a control, using the same experimental conditions, we examined light adaptation of another visual mutant, partial optokinetic response b (pob). This mutant has a disrupted photoreceptor layer, because of a selective loss of red cones by 5 dpf.¹¹ However, we found unlike nrc, pob adapts normally to background light (data not shown).

nrc Map Position

As a first step in determining the primary molecular defect caused by the nrc mutation we determined its physical map position. We used AFLP technology to identify markers that are closely linked to the mutant gene.¹⁰ Using 256 primer pair combinations, 21 AFLP markers that are linked to the nrc locus were isolated from duplicate DNA samples, each containing 24 nrc mutant or WT sibling larvae. We next examined recombination frequencies in 60 individual larvae for each of the 21 markers to approximate the genetic distance between the nrc locus and the AFLP marker. Five AFLP markers were within 1 cM of the nrc locus. One of these was subcloned, sequenced, and mapped using the LN54 zebrafish radiation hybrid panel¹⁷ and the T51 zebrafish radiation hybrid panel.¹⁶ On the Twoingen map of the zebrafish genome (http://www.map.tuebingen.mpg.de/) this AFLP marker (named unp1417) is between 54.8 and 57.3 cM from the top of linkage group 10 with a LOD score of 11.8. This approximate map position was confirmed by analyzing SSLP markers¹⁶ present at 56.4 cM (http://zebrafish.mgh.harvard.edu/mapping/ssr_map_index.html). Five recombinants out of 2648 meioses were identified using marker Z7504. This places Z7504 approximately 0.2 cM from nrc. Twenty-five recombinants of 2724 meioses, which were different from Z7504 recombinants, were identified using Z9574. This places Z9574 0.9 cM from the nrc locus on the opposite side from Z7504.

DISCUSSION

In this study we showed that (1) the b-wave in response to a flash is reduced in amplitude and delayed in the nrc mutant, (2) both the On and Off responses to prolonged stimuli show abnormal oscillations in nrc, and (3) nrc has a light adaptation defect. The physiological defect in the outer retina is likely to be responsible for the mutant's inability to follow rotating stripes in the optokinetic assay. This study is significant because zebrafish mutants such as nrc are potential models for human retinal disease. Our initial characterization of this mutant provides fundamental functional information about its retinal phenotype.

Morphologic studies have uncovered structural defects in cone photoreceptor synaptic termini and in the RPE of nrc.¹⁵ Proper organization of the photoreceptor terminal is important for visual function. The photoreceptor has a specialized synapse that normally dampen the response are defective. The initiation or termination of prolonged illumination evokes oscillations in nrc. Photoreceptor synaptic terminals tonically release glutamate in the dark. Light stimulates hyperpolarization of the photoreceptor, which ultimately slows this glutamate release. The onset of darkness returns the photoreceptor to a state of tonic neurotransmitter release and renewed sensitivity to light. Release of neurotransmitter must be carefully controlled so that photoreceptors can properly respond to changes in light stimuli.

The oscillatory response to a prolonged light stimulus in nrc suggests a defect in modulation of glutamate release. Perhaps glutamate is released in bursts rather than tonically as is normally supported by the ribbon structure. Activity in the stimulated secondary neurons would then generate corneal positive oscillations. This hypothesis is likely given the morphologic defects in the nrc photoreceptor terminals. Alternatively, it is possible that (1) secondary neurons do not properly respond to the change in glutamate release or (2) feedback mechanisms between photoreceptors and secondary neurons that normally dampen the response are defective.

In our study a flash less than 1 msec consistently showed only a single response. Only 1 in 15 healthy nrc larvae showed an oscillation and only in response to a 7.8 μJ/cm² flash intensity. The 10-msec flash duration may be the minimum time necessary to induce oscillations. It should be noted that constant background light did not cause continual oscillations.

Oscillations in response to a light stimulus have been described in normal ERGs of some vertebrates³⁰ but not in normal zebrafish³⁰ after 5 dpf. The normal oscillatory potentials seen in some vertebrates appear as small waves superimposed on the b-wave after a short light flash. These oscillations are different from the nrc oscillations in phase, onset, and amplitude. Oscillations similar to the ones in nrc have been observed in three to four dpf zebrafish larvae.³¹ This similarity suggests nrc has an incompletely developed photoreceptor synapse at 5 to 6 dpf.

Light Adaptation Defect

Comparison of the b-waves of nrc and WT larvae in the presence of background illumination shows that light adaptation is impaired in nrc. We unmasked the photoreceptor specific a-wave by exposing larvae to APB, an agonist for ON-bipolar cell glutamate receptors, and found that light adaptation is defective in nrc photoreceptors. The initial slope of the a-wave has been attributed to photoreceptors. Although APB may also affect other pathways such as On transmission from postsynaptic neurons in nrc do not properly invaginate into the photoreceptor, and synaptic vesicles clump in the photoreceptor terminals.¹³ Secondary neurons that do invaginate nrc photoreceptor terminals appear to be horizontal cells. The RPE in the nrc mutant also has increased phagosomes and lipid droplets.¹³ However, photoreceptor outer segments are normal in length and the discs are ordered.¹³
cones to horizontal cells, it has no known effects on the early kinetics of the photoreceptor derived a-wave.

WT and nrc a-waves are similar in amplitude and shape in the presence of APB. In conjunction with the normal threshold sensitivity the a-wave provides additional support for normal phototransduction in the mutant. However, background illumination in the presence of APB shows that there is a light adaptation defect in nrc photoreceptors. In the presence of background light, nrc a-wave responses to a flash are attenuated compared with those of WT. Because pob, a mutant with an abnormal photoreceptor layer, has no adaptation defect (data not shown), the light adaptation defect appears to be specific to nrc photoreceptors.

Light adaptation in photoreceptor outer segments occurs through modulation of phototransduction mediated by Ca^{2+}. It seems unlikely that local changes in photoreceptor terminal would directly affect adaptation in the outer segment. Another possibility is that the nrc termini would directly affect adaptation in the outer segment and synaptic terminal.11 The appearance of phagosomes and lipid droplets within the RPE in nrc suggests that photoreceptor disc turnover may be abnormal. However, nrc phototransduction seems normal, and the outer segment morphology is normal. Alternatively, the adaptation defect may reflect a loss of intercellular feedback mechanisms dependent on synaptic transmission. Improper invagination of secondary neurons within the nrc terminal indicates possible abnormal intercellular interactions.13

In summary, a defect in an element of the photoreceptor synaptic terminal likely explains the abnormalities in nrc electrophysiology and morphology. Intriguingly, the morphologic and electrophysiological phenotypes of nrc share some striking parallels with the phenotypes of mutations associated with the dystrophin glycoprotein complex (DGC). One, disruption of dystrophin in humans and mice causes a altered b-wave similar to the nrc mutant. A mutation in dystrophin causes Duchenne/Becker muscular dystrophy. Two, disruption of laminin β2 causes morphologic defects within the retina similar to the nrc mutant. The synaptic ribbon is mislocalized, and the secondary neurons do not properly associate with the photoreceptor terminal. These studies indicate that the DGC and laminin β2 are important for normal photoreceptor synaptic development and function within the retina. Furthermore, retinal isoforms of dystrophin are important for photoreceptor synaptic function.13–15 The map position of nrc presented here will enable us to evaluate these genes as well as others as candidates. Identification of the nrc gene will give insights into photoreceptor structure and function.

Acknowledgments
The authors thank Fred Rich for providing the Iqor data acquisition program and Matthias Seliger for telling us how to use APB on zebrafish larvae to reveal the a-wave.

References


