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Abstract

Optokinetic and phototactic behaviors of zebrafish larvae were examined for their usefulness in screening for recessive defects in the visual system. The optokinetic response can be reliably and rapidly detected in 5-day larvae, whereas the phototactic response of larvae is variable and not robust enough to be useful for screening. We therefore measured optokinetic responses of mutagenized larvae as a genetic screen for visual system defects. Third-generation larvae, representing 266 mutagenized genomes, were examined for abnormal optokinetic responses. Eighteen optokinetic-defective mutants were identified and two mutants that did not show obvious morphological defects, no optokinetic response a (noa) and partial optokinetic response a (poa), were studied further. We recorded the electroretinogram (ERG) to determine whether these two mutations affect the retina. The b-wave of noa larvae was grossly abnormal, being delayed in onset and significantly reduced in amplitude. In contrast, the ERG waveform of poa larvae was normal, although the b-wave was reduced in amplitude in bright light. Histologically, the retinas of noa and poa larvae appeared normal. We conclude that noa larvae have a functional defect in the outer retina, whereas the outer retina of poa larvae is likely to be normal.
A behavioral screen for isolating zebrafish mutants with visual system defects

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ABSTRACT Optokinetic and phototactic behaviors of zebrafish larvae were examined for their usefulness in screening for recessive defects in the visual system. The optokinetic response can be reliably and rapidly detected in 5-day larvae, whereas the phototactic response of larvae is variable and not robust enough to be useful for screening. We therefore measured optokinetic responses of mutagenized larvae as a genetic screen for visual system defects. Third-generation larvae, representing 266 mutagenized genotypes, were examined for abnormal optokinetic responses. Eighteen optokinetic-defective mutants were identified and two mutants that did not show obvious morphological defects, no optokinetic response a (noo) and partial optokinetic response a (poo), were studied further. We recorded the electroretinogram (ERG) to determine whether these two mutations affect the retina. The b-wave of noa larvae was grossly abnormal, being delayed in onset and significantly reduced in amplitude. In contrast, the ERG waveform of poo larvae was normal, although the b-wave was reduced in amplitude in bright light. Histologically, the retinas of noa and poo larvae appeared normal. We conclude that noa larvae have a functional defect in the outer retina, whereas the outer retina of poo larvae is likely to be normal.

Benzer (1) was the first to report that mutant Drosophila could be identified by their phototactic behavior. Subsequently, a number of nonphototactic mutants were found to have specific molecular defects in their photoreceptors (2). A phototaxis mutant that failed to respond to UV light, sevenless, lacks UV-sensitive photoreceptor cells (3); analysis of this mutant has defined the role of cell–cell interactions in onomatoid development (for review, see ref. 6).

Because there are significant differences between vertebrate and invertebrate eyes, genetic analysis of the Drosophila eye has provided only limited information about the vertebrate visual system. To apply a genetic analysis to the vertebrate visual system, we have turned to zebrafish (Danio rerio). Zebrafish are highly visual and exhibit vision-dependent behavior as early as 3 days postfertilization (pf) (4). They possess four types of cones and are tetrachromatic. Short single cones contain a UV-sensitive photopigment, whereas long single cones contain a blue-sensitive pigment; a green-sensitive pigment is in the short member of the double cones and a red-sensitive pigment is in the long member of the double cones (5). Rod photoreceptors are also present, so scotopic and photopic vision can be analyzed in this organism (7). Furthermore, early eye morphogenesis and organization of the zebrafish visual system are well characterized and similar to other vertebrates (8). Thus, information obtained from a genetic dissection of the zebrafish visual system should be applicable to other vertebrates.

Recently, two groups developed chemical mutagenesis procedures and methods for efficiently growing large numbers of zebrafish (9–12). These procedures have made it possible to conduct large-scale genetic screens in which zebrafish larvae from the third generation are analyzed for recessive mutations. Furthermore, a genetic linkage map in zebrafish is now available so that mutant genes can be isolated by positional cloning (13).

We first characterized two visual behaviors—phototaxis and optokinetic responses—in wild-type zebrafish larvae (3–19 days pf). Preliminary experiments on wild-type larvae (4) suggested that both of these assays would be useful. We then analyzed the optokinetic responses of mutagenized larvae as a primary screen for detecting recessive defects in the visual system. As a secondary screen, we recorded the electroretinogram (ERG) from larvae 5–7 days pf to identify mutations that specifically affect the retina. We describe here the feasibility of this approach for identifying mutations affecting the visual system and describe two mutants isolated on the basis of their abnormal optokinetic response.

MATERIALS AND METHODS

Animals. AB strain zebrafish were obtained originally from Oregon (14) and propagated at Harvard University by inbreeding. The AB strain maintained at the Massachusetts General Hospital was also originally obtained in Oregon and was then selected over several generations to be free of lethal mutations (9). In this study, zebrafish between 3 and 19 days pf are referred to as larvae. The water used for fish was reverse-osmosis distilled and then reconstituted for fish compatibility by addition of salts (2 g of Instant Ocean per gal; 1 gal = 3.785 liters) and vitamins (Fritz, Dallas).

Mutagenesis. The procedures for mutagenesis and for conducting crosses to identify recessive mutations in the third generation of mutagenized fish have been described (9). Briefly, male AB fish were mutagenized with N-ethyl-N-nitrosourea (Sigma) and outcrossed with wild-type females. The resulting F1 generation fish were crossed with each other or with wild-type fish to generate F2 families. Pairs of F2 siblings were then crossed to uncover recessive mutations in the F3 generation. The total number of genomes screened was determined from the total number of F2 families and the extent to which each F2 family was examined. The probability of finding a mutation in a given F2 family depends on the number of crosses performed from that family and the number of larvae examined from each cross. The number of mutagenized genomes screened per family = \( (1 - 0.75^S) \times N \), where \( S \) is the sum of fractions of crosses screened per family = \( x_1 + x_2 + \ldots \)

Abbreviations: pf, postfertilization; ERG, electroretinogram; OKN, optokinetic nystagmus.

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+ x, and a is the number of mutagenized genomes crossed into a given F2 family (value of 1 or 2); x, is the fraction of cross n that was screened (1 - 0.75^n), where L is the number of larvae screened from cross n.

One N-ethyl-N-nitrosourea-induced allele of noa was isolated. Thirty-eight of 122 larvae examined from crosses between noa-carrying control fish (F1 fish in original screen) showed no optokinetic response in white light. Of 12 noa-carrying fish were outcrossed pairwise with AB fish and 13 noa carriers were identified in the F1 generation. The optokinetic response of 442 larvae from pairwise crosses of noa-carrying F1 fish were analyzed and 119 gave no optokinetic response. Of these 119 nonresponders, only 3 were noted as not having expanded melanophores (see Results).

One pair of pao-carrying G0 fish was identified. The optokinetic responses of 57 larvae from crosses between these two fish were analyzed and 10 larvae had an abnormal partial optokinetic response. All 10 of these larvae had expanded melanophores. G0 pao-carrying fish were outcrossed with AB fish and four F1 carriers have been identified. The optokinetic responses of 26 larvae were analyzed from crosses between these F1 fish; 6 larvae had a partial optokinetic response and all 6 were darker than wild-type larvae. Additional pao larvae were selected from additional crosses between the pao-carrying F1 fish based on their unusual swimming behavior and darker pigmentation (see Results). Of 50 larvae selected in all, only had a partial abnormal optokinetic response.

Finally, the optokinetic responses of 32 larvae from a cross between noa- and pao-carrying fish were analyzed and all showed a normal optokinetic response, suggesting that the noa and pao mutations are in different genes. Furthermore, the above data suggest that both mutations are recessive.

Behavioral Assays. A useful behavioral screen must be reliable and fast because recessive defects are detected in only 25% of a given population and thus large numbers of animals must be analyzed. Also, the assay should be conducted on young fish so that the cost and labor associated with raising many larvae is minimal.

Phototaxis. The phototactic response of AB larvae was measured using a 10.5 x 3 x 4 cm (length x width x height) acrylic box with a sliding partition separating two chambers referred to as A and B. To optimize fish health, larvae used in phototaxis assays were fed beginning 5 days pf, even though feeding larvae may not be practical in a mutant screen. Two types of experiments were done. In the first type of assay, 20–30 larvae were placed in the box in ambient light (30 lux) and allowed to distribute themselves between the two chambers. After 5 min, larvae on each side were counted. Chamber A was then illuminated with white light (100 lux) and chamber B was covered. Larvae in each chamber were counted again after 1–5 min. Larvae between 7 and 14 days pf were examined by this method. In the second type of assay, 10–30 larvae were placed in chamber B in darkness for 0–2 min with the partition closed. The partition was then removed and chamber A was exposed to ambient light, while chamber B was kept dark by covering it. After 1–3 min, larvae in each chamber were counted. In control experiments, the entire box was kept dark and larvae were examined by raising the partition. Larvae between 7 and 19 days pf were examined by this method.

Optokinetic responses. The apparatus for measuring optokinetic responses is shown in Fig. 1. Ten to twenty 5- to 7-day mutagenized F3 larvae were placed in a 35-mm Petri dish containing ~4% methyleneacetic acid methyl ester (Sigma) in fish water for 2 min at room temperature. In dim red light, the anesthetized fish were covered with ~3% methyleneacetic acid anesthetic and positioned on top of a sponge so that one eye pointed toward the light source (100-W halogen; maximum illumination on head, 30,000 lux). For most recordings, a suction electrode (tip diameter, ~10 μm) was applied to the cornea and a silver ground electrode was placed in the bath. Initially, a wire electrode was used to record ERGs as described (15). Since the larvae could not be submerged by this method, a tube was attached to the fish’s mouth and anesthetic was flushed over the gills. Recordings obtained with a wire electrode were more variable and the experiments could not be sustained as long as when the suction electrode was used. Flashes (0.01–0.5 sec) produced with an electronically controlled shutter were attenuated by neutral density filters over a range of 6 log units. The responses were amplified, filtered so that a square test pulse decayed to 1/e in 0.14 sec, averaged, and recorded. Responses to dim flashes were averaged (n = 3–10). For bright flashes, only single responses were used unless it was clear that the first

FIG. 1. Apparatus for measuring optokinetic responses. D, drum; F, fiber optic; L, monochromatic light source; M, microscope; V, videocamera.
flash did not attenuate subsequent responses. When recording an intensity-response series, dim flashes were presented first.

**Histology and Immunocytochemistry.** Larvae were fixed in 2.5% glutaraldehyde/1% paraformaldehyde, dehydrated through ethanol and embedded in Paraplast (BDH) or Araldite (8). After resin polymerization, the specimens were sectioned at 1 μm, stained with an aqueous 1% methylene blue/1% azur II/1% borax solution, and examined under the light microscope. Whole-mount antibody staining was done as described (14) with the following modifications: 7-day larvae were treated with −20°C acetone for 17 min and then incubated in PBS containing proteinase K (2 μg/ml) (Sigma) for 90 min at room temperature. After this enzymatic digestion, larvae were fixed again for 30 min, incubated in blocking solution for 30 min, and continued through the remainder of the procedure. The antibody 7A11, kindly provided by Han Chang (Harvard University), was used at a 1:1 dilution.

**RESULTS**

**Phototaxis.** Initial screens for mutations that affect the visual system of *Drosophila* relied on phototactic behavior (1). To determine whether phototaxis could also be used to screen mutagenized zebrafish, we assayed zebrafish phototactic behavior by two simple protocols using a small box with two chambers separated by a removable partition. When both chambers were illuminated, 57% ± 11% of 7- to 14-day larvae were in one chamber; in contrast, if larvae were counted again after covering one chamber and illuminating the other, 85% ± 10% of larvae were in the illuminated chamber (n = 22). This demonstrates significant phototactic behavior in larvae zebrafish (P < 0.002). In the second method, when both chambers were kept dark, 36% ± 15% of larvae migrated from the starting chamber to the other chamber (n = 12). When one chamber was illuminated and the starting chamber was kept dark, 49% ± 23% of larvae migrated to the illuminated chamber (n = 36). Although scores using the second method were not as striking, this method also demonstrated significant phototactic behavior in larval zebrafish (P = 0.025). In both types of assays, there appeared to be little difference in the responses of larvae between 7 and 14 days pf. Although these studies demonstrated significant phototactic responses in larval zebrafish, the responses were not as strong or as reliable as the optokinetic response (see below); thus, we did not use phototaxis to screen mutagenized zebrafish larvae.

**Optokinetic Responses.** The optokinetic response was consistent and robust. We observed optokinetic responses as early as 3 days pf, and by 5 days pf ~98% of healthy AB zebrafish larvae (n > 100) showed a definitive optokinetic response consisting of smooth pursuit eye movements followed by rapid saccades in the opposite direction. Thus, rotating stripes elicited an optokinetic nystagmus (OKN) type response in larval zebrafish. Furthermore, the eye movements were instantly reversed when the direction of the rotating stripes was changed. Because optokinetic responses could be detected reliably by 5 days pf, and because of the efficiency of obtaining and observing these responses, we measured optokinetic responses as our primary assay of visual abilities in mutagenized larvae.

Larvae representing 266 mutagenized genomes (~13,000 F2 fish) were tested for defects in their optokinetic responses. Eighteen mutations causing optokinetic defects were identified. Sixteen of these mutants showed morphological defects in the eye and/or elsewhere and 15 of these 16 had a smaller-than-normal eye by day 5. These morphological mutants have not been examined in more detail in this study.

Two optokinetic-defective mutants did not show obvious morphological defects, although both were somewhat darker than their optokinetic-positive siblings (see below). We have analyzed these two mutants further. One showed no optokinetic response in either dim or bright white light and was therefore designated *noa* (no optokinetic response a). *noa* larvae can, however, move their eyes. They showed occasional random eye movements that were not consistent with the direction of drum rotation. The other mutant was designated *poa* (partial optokinetic response a) because it displayed abnormal eye movements (see below) in response to the rotating stripes. Both mutants moved vigorously when touched with a fine probe. However, the swimming behavior of all *poa* larvae was abnormal; they typically swam on their sides. On the other hand, *noa* larvae usually swam normally but occasionally floated on their backs.

As noted above, both *noa* and *poa* larvae appeared darker than wild-type larvae by day 5; their melanophores were chronically expanded. Normally, in the light, melanophores are small because of the aggregation of melanin-containing organelles. In the dark, melanophores expand due to melanin dispersion. In *noa* and *poa*, the melanophores did not contract even after 10 min in bright light.

To characterize further the optokinetic responses of *poa* fish, we examined the responses of 10 mutant larvae with monochromatic light at 600, 500, and 400 nm at intensities less than a log unit above wild-type threshold levels. All 10 fish showed abnormal optokinetic responses at all three wavelengths. Two types of abnormal eye movements were observed in all 10 larvae. Sporadically, *poa* fish displayed very rapid eye movements back and forth in response to the rotating illuminated stripes. These rapid eye movements seemed consistent with the direction of drum rotation but were significantly smaller than the movements observed in wild-type larvae. Furthermore, rapid eye movements were often accompanied by tail twitching and head movements. On occasion, the eyes of *poa* larvae followed the stripes in a manner similar to those of wild-type larvae. However, when the eyes were maximally turned, they did not rapidly saccade back to their original position. Instead, they remained fixed in position in the direction of rotation.

**Electroretinography.** Fish with an abnormal optokinetic response could have defects at several possible loci. The vertebrate ERG originates in the outer retina (16) and is characterized by two prominent waves. An initial corneal negative a-wave originates from the photoreceptors, whereas a larger corneal positive b-wave reflects postsynaptic activity. Electroretinography thus provides a method for localizing defects to retinal loci.

Fig. 2B shows superimposed ERG responses recorded from a *noa* larva (Right) and its OKN+ sibling (Left) to 10-msec flashes of various intensities. The ERG of the normal larvae displayed a corneal negative a-wave followed by a larger corneal positive b-wave to the maximum intensity flash (log I = 0). Both waves decreased in amplitude as the intensity of the flash decreased. In contrast, the ERG b-wave of *noa* larvae was highly abnormal; it was significantly delayed and very much reduced in amplitude. The a-wave of *noa* larvae, on the other hand, appeared quite normal. At maximum light intensity, abnormal b-wave responses were observed in 21 of 27 *noa* mutants. Normal responses were observed in 25 of 27 OKN+ siblings examined. We were unable to record any ERG response from two OKN+ larvae. Also, it should be noted that the maximum amplitude of the b-wave was quite variable (88 ± 80 for the 23 OKN+ larvae stimulated at maximum intensity); however, it was consistently larger than the b-wave amplitude of *noa* larvae (18 ± 13; P < 0.001). Both suction and wire electrodes were used to obtain these data. Fig. 2B shows superimposed ERG responses of another pair of *noa* and OKN+ siblings stimulated at the brightest light intensity for 0.5 sec. The delayed b-wave and reduced b-wave amplitude of the mutant are very obvious in this figure.

On the other hand, the ERGs recorded from *poa* mutant larvae were qualitatively normal compared to their normal...
siblings. The waveforms of the a-wave and b-wave were indistinguishable from the normal response (Fig. 3A). Average b-wave amplitudes for poa larvae were, however, somewhat smaller than for normal larvae at the highest light intensities (Fig. 3B). Maximal b-wave amplitude was 109 ± 77 μV for OKN+ larvae (n = 14) and 60 ± 29 μV for poa larvae (n = 9) (P = 0.03). Preliminary analyses of light and dark adaptation as well as recovery kinetics did not reveal any striking differences between the responses of poa and normal larvae (data not shown).

**Morphological Analysis of noa and poa.** Histological analysis of noa and poa eyes did not reveal any detectable morphological alterations of the retinas. Transverse retinal sections along the dorsal–ventral axis of the head from 5-day noa and poa larvae were compared with 5-day OKN+ larvae (Fig. 4 Left). At day 5 pf in both mutant and OKN+ larvae, the retina was fully laminated and cells in the inner and outer retina had differentiated. Photoreceptors at this stage are mostly immature and do not have substantial outer segments. Some rods can be identified in the ventral region of the retina and some short single cones can be identified scattered throughout the photoreceptor layer. Sections through the optic nerve of 5-day noa and poa larvae indicated that the nerve exited the eye at the appropriate position and had a normal morphology (Fig. 4 Right).

We also examined ganglion cell projections in noa larvae with a monoclonal antibody that recognizes the optic nerve in zebrafish. The optic nerve appeared normal in all 47 7-day larvae produced from a cross of adult fish heterozygous for the noa mutation (data not shown). Since ~25% of the population are expected to be homozygous for the noa mutation, 9–14 larvae presumably had the noa defect.

**DISCUSSION**

In this study, we showed that measuring optokinetic responses is a quick and reliable test of visual ability in zebrafish larvae and can be used to identify mutations that cause defects in the visual system. Although most fish lacking an optokinetic response had obvious morphological abnormalities and would have been detected in screens for morphological mutants (9, 10), two optokinetic-defective mutants, noa and poa, had no visible structural defects in the eye. Furthermore, the morphology of the brain and other organs as viewed under the dissecting microscope was normal in these two mutants. ERG analysis localized the defect in noa larvae to the outer retina and showed that the primary defect in poa larvae is not likely to be in the outer retina. The results demonstrate that a behavioral assay can be used to identify subtle mutations that alter visual function in zebrafish and that the ERG can then be used to define the nature and location of these mutations.
Assuming that all loci affecting optokinetic responses in the genome mutate at a rate comparable to the ones used as tester loci (∼1:1000) (9), and assuming a Poisson distribution of mutations per genome, we would need to screen 5000 genomes to identify 99.3% or 2000 genomes to identify 87% of all loci causing an optokinetic defect. Thus, in this study, analyzing 266 mutated genomes, we have conducted a small screen and should have recovered 5–10% of all genes that can mutate to optokinetic-defective phenotypes. This figure is a very coarse estimate, since good data on the relative mutability of a larger group of genes are not available for zebrafish so far. Thus, this study is a preliminary presentation of the types of mutants that should be identified through screening optokinetic responses.

The bulk of the screening presented in this study was done with white light well above threshold levels, which biased our screen toward identifying mutations that affected all classes of photoreceptors. We also can screen for mutants by using individual wavelengths at light intensities near threshold. This type of screening strategy may identify mutants with defects in specific photoreceptor types. Recently, we have begun using dim red light to screen for mutants with defects that affect only red photoreceptor pathways. Finally, by screening with light intensities near absolute threshold levels after exposure of the larvae to darkness or various intensities of background illumination, we may identify mutants that are less sensitive to light than wild-type fish or that have a defect in light or dark adaptation.

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