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Behavioral Genetic Approaches to Visual System Development and Function in Zebrafish

Stephan C.F. Neuhauss

Brain Research Institute, University of Zurich and Swiss Federal Institute of Technology (ETH) Zurich, Winterthurerstrasse 190, CH - 8057 Zurich, Switzerland

Dr. Stephan Neuhauss
Brain Research Institute
ETH Zürich
Winterthurerstr. 190
CH - 8057 Zürich
Switzerland

ph. -41+ 1-635 3288
fax. -41+ 1-635 3303
neuhauss@hifo.unizh.ch

Abstract

The zebrafish is a recent vertebrate model system that shows great potential for a genetic analysis of behavior. Early development is extraordinarily rapid, so that larvae already display a range of behaviors 5 days after fertilization. In particular the visual system develops precociously, supporting a number of visually mediated behaviors in the larva. This provides the opportunity to use these visually mediated behaviors to screen chemically mutagenized strains for defects in vision. Larval optokinetic and optomotor responses have already been successfully employed to screen for mutant strains with defects in the visual system. In the adult zebrafish a visually mediated escape response has proved useful for screening for dominant mutations of the visual system.

Here I summarize visually mediated behaviors of both larval and adult zebrafish and their applicability for genetic screens, and present, the approaches and results of visual behavior carried out to date.

Keywords:

zebrafish, visual behavior, genetic screen, optokinetic response, optomotor response

In the early 1970s, phage geneticist George Streisinger, like many other geneticists of the day, thought that the time was ripe to tackle the genetics of nervous system development and function. Being an avid aquarist, he decided to start studying the genetics and embryology of the zebrafish (*Danio rerio; Brachydanio rerio* in the older literature), a tropical pet fish recommended for its hardiness. He was taken by the beauty of these lively 3 inch teleosts (bony ray-finned fish) and even more so by their offspring (Figure 1). Not only are zebrafish very fertile (an average of 100 offspring per week per pair), but the embryos are also transparent and externally fertilized, allowing unobstructed access to the researcher. Furthermore, they develop extraordinarily rapidly. The number of zebrafish researchers, similarly taken by the beauty of these fish has grown dramatically over the last decade to greater than 300 labs. At the time of George Streisinger's untimely death in 1984, not even the most avid optimists would have predicted such a growing popularity of the zebrafish as a genetic model system for various aspects of vertebrate development.

In addition to the favorable embryological features of the zebrafish, its genetic properties, as first pioneered by Streisinger, are also extremely advantageous. The identification of recessive mutants in genetic screens by the Oregon zebrafish community yielded a number of interesting mutants affecting specific aspects of vertebrate development (Kimmel et al., 1991). Two subsequent large-scale screens by the groups of Nüsslein-Volhard and Driever, whose results were described in a special issue of Development (Development 123, 1996), isolated a wealth of mutations affecting many aspects of development.

When Streisinger started his seminal work on zebrafish, he had in mind not only mutations affecting morphological events during embryogenesis, but was also particularly intrigued by the possibility of using the zebrafish for visual behavior screens. He and his colleagues were the first to use behavioral genetic approaches to investigate zebrafish visual system development .
They pioneered the use of larval optokinetic and optomotor responses to uncover a number of putative mutants defective in vision (reported in (Clark, 1981).

This review aims to demonstrate that the behavioral genetic approach to zebrafish visual system development has come of age and that these studies are beginning to bear fruit. Three decades after George Streisinger started his work on zebrafish behavioral genetics, we are starting to fulfill the promise of his insightful and seminal work.

The zebrafish visual system

The zebrafish visual system is ideal for a genetic analysis of its development. First, visual system development is extraordinarily rapid in the zebrafish embryo. Since fertilization and subsequent embryonic development take place outside of the mother, there is strong evolutionary pressure for rapid development of functional sensory systems, so that predators can be avoided and feeding can commence. Embryos hatch out of their chorions at about 3 days post fertilization (dpf) and are free swimming and actively pursuing food particles once their swim bladders are inflated at 5 dpf. At that time their yolk supply has been used up, and life depends on catching prey (protozoa and small metazoan larvae). These ecological challenges are met through an extraordinarily rapid maturation of the visual system.

Early eye development

The retina develops from an initially pseudostratified epithelium to a fully layered retina with all retinal cell types (photoreceptors, bipolar, horizontal, amacrine, ganglion, and Müller glia cells) by 60 hpf (Schmitt and Dowling, 1999). By the 4-somite stage (approximately 11 hours post fertilization (hpf)), the optic primordia emerge as evaginations of the diencephalon (Schmitt and Dowling, 1994). The ventral ganglion cells are the first to become postmitotic at about 28 hpf, followed by the cells in the inner nuclear layer (amacrine, horizontal and bipolar cells) and finally cells in the photoreceptor cell layer (Hu and Easter, 1999; Nawrocki, 1985). The first retinal ganglion cell axons reach the optic chiasm at about 32 hpf and by 65 hpf have reached their central targets, and have arborized within ten fields, including the optic tectum, their main target area (Burrill and Easter, 1994; Stuermer, 1988).

Development of light perception

The retina becomes mature enough to support visually mediated responses by 68 hpf (Easter and Nicola, 1996) and electroretinograms (ERG) have been recorded as early as 72 hpf (Branchek, 1984). The earliest visual behavior is the visual startle response (Kimmel et al., 1974). Larvae respond to a sudden decrease in illumination by a rapid body movement, presumably as an adaptive behavior to escape a looming predator. This behavioral response starts at around 68 hpf (Easter and Nicola, 1996), just at the time when outer segments of photoreceptors and synaptic ribbons have formed in the retina (Branchek and Bremiller, 1984; Schmitt and Dowling, 1999). It becomes impractical to test the visual startle response after 4 dpf, since older larvae display

spontaneous motor activity, making it difficult to distinguish between visually elicited and spontaneous body movements.

Maturation of the visual system and emergence of form vision

By 5 dpf, the outer retina is composed of a precisely patterned photoreceptor mosaic consisting of four cone types (long and short single cones, long and short double cones) interspersed with rods (Branchek and Bremiller, 1984; Raymond et al., 1995). The zebrafish retina has five different visual pigments, including a UV sensitive pigment, supporting tetrachromatic vision (Robinson et al., 1995; Robinson et al., 1993; Vihtelic et al., 1999). The retina, including the inner retina, is now mature enough to support a number of behaviors that require form vision, including the optokinetic (OKR) and the optomotor response (OMR).

Probably the most robust behavior is the optokinetic response (OKR), it is triggered by moving objects across the visual field and evokes stereotyped tracking eye movements (Figure 2A). These eye movements have two components: a smooth pursuit movement following the moving object, and a fast saccadic movement, which resets the eyes after the stimulus has left the visual field. In the zebrafish, this behavior is the earliest visual behavior to require form vision (Clark, 1981). It develops between 73 and 80 hpf, coinciding with a fully differentiated and synaptically connected retina (Easter and Nicola, 1997; Easter and Nicola, 1996).

In the optomotor response (OMR), the larva swims to follow moving visual stimuli. The simplest apparatus to evoke this responses is similar to the one used for OKR testing. The larvae are allowed to swim freely in a container surrounded by a moving drum (Bilotta, 2000; Clark, 1981). In normal larvae this behavior is fully mature about

6 days after fertilization. More subtle visual defects can be detected by varying illumination conditions (Bilotta, 2000).

Like most visually oriented animals, zebrafish larvae are phototactic. Given a choice between dark and a light, both larvae and adults have a tendency to prefer the illuminated part of their tank (Brockerhoff et al., 1995). However, this preference is not robust enough to be practical for distinguishing visual system function between mutant and normal animals.

As for many lower aquatic vertebrates, zebrafish larvae adjust the distribution of melanin pigment in their skin in response to ambient light levels. The skin contains star shaped melanophores that are filled with melanosomes (melanin granules). On a dark background, melanosomes are widely distributed throughout the melanophore's processes, giving the larva a dark appearance. In most teleosts, this cellular behavior is mediated by a direct projection of the retina to the hypothalamus, which in turn activates the secretion of two hormones from the pituitary, which acts on the melanophores (Balm and Groneveld, 1998). Many mutants lack this background adaptation ("expanded melanophore phenotype" (Haffter et al., 1996; Kelsh et al., 1996)). When rescreened for visual behavior, many of these mutants show behavioral failure (Neuhauss et al., 1999), leading to the conclusion that behavioral blindness correlates with lack of background adaptation, although not perfectly.

Besides using behavioral tests, the function and development of the retina can also be assessed by electroretinography, a measurement of summed field potential in the retina in response to light. Its development closely follows the morphological maturation of the retina. By 5 dpf, electroretinograms (ERG) containing all the components of a typical vertebrate ERG can be recorded (Bilotta et al., 2001;

Branchek, 1984; Brockerhoff et al., 1995; Hughes et al., 1998; Saszik et al., 1999; Seeliger et al., 2002).

Maturation of the adult visual system

Although the larval visual system can support a number of surprisingly complex visual behaviors, the maturation of the visual system is still ongoing well into adulthood. In a sense, visual system development is a lifelong process in teleosts, since even in the adult retina cells are constantly added at the ciliary margin. During late larval stages the photoreceptor outer segments increase dramatically in size. Although expression of rhodopsin is clearly detectable in young larvae, rod photoreceptors are not morphologically mature until relatively late stages (15 dpf; (Branchek and Bremiller, 1984)). At this stage there is little or no rod function detectable, and rods are first found to be functional in the ERG at 21 dpf (Bilotta et al., 2001). The rod contributions to the ERG spectral sensitivity function develop with age and are still not adult-like by 29 dpf, despite mature rod morphology seen anatomically (Bilotta et al., 2001). This is a clear indication that the retina is still maturing during young fry stages.

Although there is a wealth of information about the visual system of the goldfish (*Carassius auratus*), there have been comparatively few studies on adult zebrafish visual behavior.

Optomotor response in the adult

Motion detection cannot easily be assessed in adult zebrafish by the OKR, since adults are much harder to restrain in a dish, as they require water flow through the gills for sufficient oxygenation. However, the adult OMR is easily tested in an

apparatus similar to the one used for the OKR. The fish is placed inside a transparent round aquarium with an opaque central pole, surrounded by a rotating drum with black stripes (Figure 2C). Fish with intact vision tend to swim with the moving stripes (Bilotta, 2000; Clark, 1981). This assay can be modified by using light of different wavelength and intensity to determine a spectral sensitivity curve for motion detection. Such studies have revealed a peak spectral sensitivity at 500nm in the dark adapted state, which corresponds to the maximal sensitivity of the rods. In the light adapted state the maximum was at 550-600nm, an action spectrum similar to that of the long wavelength cone. Experiments using red-green striped cylinders showed that the zebrafish could not see motion unless long wavelength sensitive cones were modulated (Krauss, 2001). This indicates that the optomotor response is "color-blind", using one cone type only, as is the case in goldfish (Schaerer and Neumeyer, 1996).

Escape response

Li and Dowling developed another behavioral assay based on the escape response that fish exhibit when encountering a threatening object (Li and Dowling, 1997). As in the optomotor test, fish are placed in a round drum with clear walls and an opaque central post. A black segment on the rotating drum can elicit an escape response, causing the fish to hide behind the central pole (Figure 2D). By using different light conditions, Li and Dowling were able to measure the time course of dark adaptation and the absolute thresholds of the rod and cone systems (Li and Dowling, 1997; Li and Dowling, 2000).

Dorsal light response (DLR)

Teleosts have a tendency to turn their back to the light, since in their natural environment light almost always comes from above (Figure 2E). Fish use this dorsal light response (DLR) in conjunction with their sense of balance to determine body position in the water. Various teleosts can be tricked into tilting their body simply by lighting the tank from the side (von Holst, 1935). This simple behavior has been used to measure the spectral sensitivity and the visual sensitivity of this behavior in the adult goldfish visual system (Powers, 1978; Yager, 1968). In zebrafish the DLR is only obvious in mutants with defects in their sense of balance (Nicolson et al., 1998). The response becomes much more pronounced when the fish is placed head down in a tightly fitting water filled tube. When light is shined from the side, the fish turns its back towards the light and spins around its body axis as the light is slowly moved around the tube. This behavior is useful as a simple measure for overall light perception and can be tested using light of different brightness and wavelength.

Visual lateralisation

Preferential eye use is a peculiar feature of zebrafish visual behavior that is worth mentioning here. Similar to cerebral lateralisation in tetrapods, teleosts show visual lateralisation, revealed by preferential eye use depending on the type of visual stimulus presented (Miklosi et al., 1998). Zebrafish tend to use their right eye to look at unfamiliar objects, suggesting that the right eye is used when the stimulus requires a period of examination in order to decide on a response. The left eye is used when a familiar object is observed that does not call for a behavioral response (Miklosi and Andrew, 1999; Miklosi et al., 2001). The anatomical basis of behavioral lateralisation is currently unknown, but recent studies have revealed intriguing anatomical

asymmetries in the zebrafish brain, for instance in the diencephalic habenular nuclei and the pineal gland (Concha et al., 2000).

Genetic screens for mutations affecting larval vision

The above-mentioned visually mediated behaviors open the possibility of initiating forward-genetic screens for mutant strains defective in vision. The most favorable stage to screen for visual defects in larvae is at 5 dpf, since the visual system is mature enough to support a number of complex visual tasks, but the larva is still living off its yolk supply, allowing simple cultivation in a dish without the need for food and water changes. This property has made it feasible to conduct reasonably large screens for recessive mutations. Since this stage was the oldest stage assayed in the large scale screens for embryonic lethal mutations, a large number of available mutants can be retested for behavioral phenotypes.

An ideal behavioral assay would rely mainly on the visual system to execute the behavior and would be robust and fast enough to screen through a large number of animals (Table 1).

Feeding assay for larval vision

In a creative pilot screen, Streisinger's group developed a feeding assay for vision. They found that zebrafish larvae eat paramecia as a function of paramecium concentration and illumination intensity. In an assay for vision based on this observation, larvae are placed in a tank filled with a defined amount of green paramecia (*Paramecium bursaria*) that can easily be detected in the gut of the larvae when ingested. Normal larvae have green bellies due to the ingested paramecia. In contrast, the majority of blind larvae will not have taken up any paramecia. Several

putative mutant lines have been identified using this assay (reported in (Clark, 1981)), but have not been followed up.

Screens employing the larval optokinetic response

For screening larvae for recessive defects in vision, the OKR is a nearly ideal assay. The assay is robust and well suited for large-scale screens since a number of larvae from one clutch can be tested efficiently at the same time. The assay can also be used on mutant larvae with unrelated problems such as axial defects, as long as they do not affect the machinery required for eye movements (Neuhauss et al., 1999). These favorable properties have been used in a number of genetic screens (Brockerhoff et al., 1995; Clark, 1981; Neuhauss et al., 1999), leading to the identification of a number of mutants with defects at various stages of the visual pathway (Table 2).

For testing the OKR, larvae are typically immobilized in a petri dish filled with a non-toxic viscous fluid (e.g. 3% methylcellulose solution). The dish is placed inside a rotating drum fitted with black and white stripes. Immobilization of the larvae is necessary since body movements inhibit eye movements and allows convenient observation of the eye movements through a dissecting scope.

In a seminal screen, Dowling and colleagues used the larval OKR to screen for visually impaired mutants. They first performed a pilot screen on third-generation larvae from the Boston large-scale mutagenesis screen, screening 266 F2 families and identifying 18 recessive visually impaired mutants, with two of them lacking obvious morphological defects (Brockerhoff et al., 1995).

Neuhauss et al. (1999) used the OKR and the OMR to screen through a collection of about 450 mutant strains being kept in the Tübingen zebrafish stock center (Neuhauss

et al., 1999). Some strains could not be tested in both assays due to early embryonic lethality or an inability to swim straight in the OMR test chamber. A total of 25 visually impaired mutants were identified, with the largest fraction displaying outer-retina dystrophies. These mutants had defects in various stages of the visual pathway, including melanin deficiency, lens degeneration, lack of ganglion cells, defects in optic nerve organization and pathfinding, and inner retinal malfunction.

Both screens used secondary assays to evaluate retina structure (histology) and function (electroretinography) to analyze further the origin of the visual defect.

Mutations affecting specific cell types of the outer retina

Most of the mutants found to be behaviorally blind turned out to be defective in the survival of photoreceptors in the outer retina (Neuhauss et al., 1999)(Figure 3C). Similar results have been obtained in screens for morphological defects of the retina (Fadool et al., 1997; Malicki et al., 1996), mirroring the situation in human diseases, where outer retinal dystrophies are the most common cause for congenital blindness. These mutants will likely turn out to be valuable animal models for heritable human retinal dystrophies and their study may also contribute to our understanding of age-related macula dystrophies as well.

The OKR can easily be adapted to screen for defects in more specific aspects of visual function, such as contrast sensitivity and color. For instance, color blind fish can be identified by illuminating the drum with monochromatic light of a particular wavelength.

In such a screen, Brockerhoff et al. identified the red-blind mutant *partial optokinetic response b* (*pob*) by screening for OKR behavior under red illumination (Brockerhoff et al., 1997). Homozygous *pob* larva showed a normal response to white light but

failed to execute an OKR under red illumination. Consistent with a lack of sensitivity to red light, the mutants show a sharp drop of relative sensitivity at wavelengths longer than 550nm in the spectral electroretinogram. Subsequent histological examination showed a slight reduction in the number of nuclei in the outer nuclear layer. In situ hybridization with various opsin probes revealed a near complete lack of red opsin expression in the retina at 5 dpf. To distinguish between a lack of red cone generation and a selective loss of red cones, earlier developmental stages were analyzed, revealing the initial presence of red opsin expressing cells at 3 dpf that disappear at later stages. The behavioral defect is therefore likely due to a rapid degeneration of red cones. A molecular defect in the red opsin locus has been excluded by genetic linkage analysis, showing that the *pob* mutation identifies a new form of congenital color blindness (Brockerhoff et al., 1997).

A mutation affecting cell types in the inner retina

A cell type specific mutation affecting ganglion cells of the inner retina was also found in larval behavioral screens. In *lakritz* (meaning licorice in German, the larvae are dark due to lack of background adaptation), both OKR and OMR responses are absent. Histological analysis revealed a near complete absence of retinal ganglion cells, with a thicker inner nuclear layer (Neuhauss et al., 1999)(Figure 3B). This defect is caused by a failure of retinal ganglion cells to differentiate properly, becoming cells of the inner nuclear layer instead. Mapping the *lakritz* locus with microsatellite genomic markers placed the gene very close to *ath5*, the zebrafish homolog of the *Drosophila* basic-helix-loop-helix transcription factor Atonal. A missense mutation in the highly conserved basic-helix-loop-helix domain was found in the mutant *ath5*, and injection of genomic fragments containing the wild-type

sequence could rescue the mutant phenotype, proving that a mutation in *ath5* causes the *lakritz* phenotype (Kay et al., 2001).

Mutations affecting retinal physiology

The conceptional attraction of a behavioral screen is that it can identify mutants with functional defects. To separate functional from morphological mutants, physiological secondary screens, such as the ERG, are essential.

Two mutants, *noir* (*nir*) and *no optokinetic response a* (*noa*) display a similar characteristic defect in the ERG, namely the presence of an a-wave and absence of a b-wave over several log units of stimulation intensity (Brockerhoff et al., 1995; Neuhauss et al., 1999). This finding indicates that the photoreceptors respond to light, but that the visual signal is not transmitted to second- order cells, particularly bipolar cells. In both mutants, no overt morphological changes of the photoreceptor terminals or the outer plexiform layer have been found, at least initially. This suggests that a more subtle functional defect, for instance in synaptic transmission, causes the phenotype.

The mutant *macho* (*mao*) has a functional defect in ganglion cells, which is reflected in ERG analysis. *macho* was originally identified by a lack of touch response and an enlarged terminal arborisation field of retinal ganglion cell axons (Trowe et al., 1996). The inability of homozygous mutants to respond to visual stimuli suggested an activity related defect in the visual system. Since both retinal morphology and ERG recordings appeared normal, an outer retinal defect could be excluded. Whole cell patch clamp recordings of mutant retinal ganglion cells in a retina flat mount preparation revealed a developmentally regulated reduction of sodium current. This leads to the inability to generate overshooting action potentials in retinal ganglion

cells, causing of the lack of visually mediated responses (Fig. 4). A similar defect has been found in Rohon Beard cells of the spinal cord, primary sensory neurons in the trunk (Ribera and Nusslein-Volhard, 1998). Hence the mutation appears to specifically affect sodium conductance in a subset of sensory cells during development.

A mutation affecting optokinetic behavior

All of the mutants discussed so far affect the performance of the visual system by either preventing acquisition of the visual image or by disrupting visual signal transfer. The *belladonna* (*bel*) mutant differs in that the abnormality is not in visual perception but rather in interpretation of the visual input. *bel* displays a peculiar reversal of eye movement in the OKR assay (Neuhauss et al., 1999)(Figure 5D). In wild type larvae, rotation of the stimulating drum evokes smooth pursuit movements of the eyes in the same direction. About 50% of homozygous *bel* larvae show a reversal of eye movements, so that a clockwise movement of the stimulating drum evokes a counter-clockwise response of the eyes. The velocity of eye rotation is stimulus independent and not influenced by the speed of the rotating drum (Rick et al., 2000).

Since the mutants react to visual stimuli, albeit inappropriately, the defect is likely outside of the retina. Indeed, a combined behavioral and anatomical analysis revealed a defect in optic chiasm formation (Karlstrom et al., 1996, Rick et al., 2000). Wild type zebrafish have a completely contralateral retinotectal projection, so that axons from one eye all cross the midline and synapse in the contralateral brain. In the abnormally behaving *bel* mutants the retinal ganglion cell axons never cross the midline but rather grow towards their targets on the ipsilateral side, forming correct

topographic projections. No optic chiasm is formed, since the optic nerve never crosses the midline (Figure 5B). A perfect correlation between incorrect optic chiasm formation and OKR reversal was observed after testing the mutant larvae behaviorally followed by anatomical tracing experiments with lipophilic dye injections. Using a modified OKR assay in which only one eye is stimulated, it was shown that input to one eye drives movements of the other (contralateral) eye in the abnormally behaving *bel* mutant. In the OKR drum, the two eyes see opposite directions of stripe movement, one from temporal to nasal, the other from nasal to temporal. Hence, driving eye movements of one eye via sensory input into the other leads to a reversal of eye movements in the OKR paradigm. In the OMR, where the stimulus is presented from below and the eyes see the same direction of motion, no behavioral defect was noted in *bel* mutants (Neuhauss et al., 1999).

Screens employing the larval optomotor response

The optomotor response has also been used to screen for larvae defective in vision. The set of mutants with OMR defect largely overlap with those shwoing OKR defects (Neuhauss et al., 1999).

For large scale screening a modified OMR assay was used. A rectangular chamber was placed on an upturned computer monitor. By displaying computer-generated movies of moving stripes, larvae placed at one end of the chambers are induced to swim in the direction of the motion (Fig. 1B). After a few minutes, the distribution of larvae is determined. Larvae with an intact visual system (and the ability to swim) accumulate at one end of the chamber (Neuhauss et al., 1999; Orger et al., 2000). This assay allows fast screening of several populations in parallel, and stripes of different width and color can conveniently be generated on any personal computer. This assay

was used in an elegant study to determine the attributes of motion that are extracted by the fish visual system (Orger et al., 2000).

It should be noted that the OKR and OMR assays test overlapping but distinct features of visual function that depend (at least partially) on different regions of the brain. For instance, ablation of the optic tectum completely abolishes optomotor responses, while optokinetic behavior is left intact (Springer et al., 1977).

Genetic screens for dominant mutations affecting adult vision

Most genetic studies of zebrafish visual behavior have concentrated on finding recessive mutations at larval stages. The main reason is that after 5 dpf fish need to be fed, requiring more time and space to keep. This makes screens of reasonable size for adult recessive mutations impractical. Screens for dominant mutants in the adult visual system are more feasible, since a large number of genomes can be screened with a reasonable number of fish and effort. Many visual system defects in vertebrates, for instance retinal dystrophies, are dominant and manifest themselves only at later stages in life. Hence genetic approaches to probe the function of the adult visual system are important. . Such screens will likely play a larger role in the near future.

A screen employing the adult escape response

An adult screen for dominant visual mutations based on the visually mediated escape response has been performed by Li and Dowling (1997). They identified and recovered two dominant mutations, *night blindness a* (*nba*) and *night blindness b* (*nbb*) (Li and Dowling, 1997).

Heterozygous *nba* fish display normal visual responses before 3 months of age, but progressively become night-blind due to photoreceptor degeneration which initially affecting rods, but later also cones and likely some inner nuclear cells. The slow progressive loss of photoreceptors is reminiscent of human outer retinal dystrophies, the major cause of human heritable blindness, which displays a similar progressive loss of photoreceptors, specifically rods in the case of Retinitis pigmentosa (Gregory-Evans and Bhattacharya, 1998). Interestingly, homozygous *nba* larvae die at 5 dpf and show broad neural degeneration, arguing for a non-photoreceptor cell-specific mutation, a feature that the mutant does not share with the majority of the human diseases, which are mostly eye specific.

The *nbb* mutant also shows an age-related visual defect. In heterozygous *nbb* adults, the visual threshold fluctuates by several log units under scotopic (dark adapted) conditions, while light sensitizes the mutant fish. Thus early dark adaptation in heterozygous *nbb* fish is normal, while visual thresholds are raised after prolonged dark adaptation (Li and Dowling, 2000a). This is due to a defect in the rod system and has been shown both behaviorally and physiologically to progress with the age of the fish. Since no alterations in the ERG have been observed, the defect is likely located in the inner retina. Most interestingly, the phenotype of the mutant can be mimicked by removing the olfactory epithelium and olfactory bulb. Morphological analysis of the mutant revealed an abnormal olfactoretinal centrifugal innervation. Teleost fish retinas are innervated by centrifugal fibers that originate from terminal nerve neurons in the olfactory bulb. These fibers synapse onto dopaminergic interplexiform cells in the inner nuclear layer of the retina. In heterozygous *nbb* fish these interplexiform cells are decreased in number, and progressively decrease in aged animals. Although

the number of olfactoretinal centrifugal fibers entering the retina is not changed in the mutant, their axonal terminals in the retina is reduced and disrupted.

A similar disruption of the rod system can be achieved by pharmacologically depressing the function of the dopaminergic interplexiform cells in the retina, thus giving independent evidence of the involvement of these cells in dark adaptation (Li and Dowling, 2000b). Surprisingly, although the defect in the heterozygous adult appears to be specifically affecting one late aspect of visual function, homozygous fish are embryonic lethal with massive degeneration of the central nervous system.

Perspective

Behavioral testing of the zebrafish visual system has started to bear fruit, several decades after George Streisinger started his quest for zebrafish visual mutants. So far most behavioral assays have focused on severe defects in motion vision, with the majority of identified mutants being blind. Since young zebrafish larvae possess a rather sophisticated visual system, more advanced visual assays will undoubtedly identify mutations affecting more subtle defects of the visual system, for instance defects in fine-tuning of visual sensitivity and processing.

The phenotypic analysis of existing and new mutants will be an active area of research for many years to come. The accessibility of the zebrafish embryo will be a major asset in these studies, and a wealth of information about the structure and function of the vertebrate visual system, particularly of the retina, can be expected. Present screens have only begun to scratch the surface of the processing of visual information in the fish brain. Comparison of visual processing in the zebrafish, which lacks higher cortical processing areas, and in higher vertebrates like ourselves will be of major evolutionary interest. The fish visual system is capable of performing a

number of visual tasks, which were long thought to be exclusive to animals with a visual cortex, such as color constancy (Dorr and Neumeyer, 1996) and the perception of second-order motion (Orger et al., 2000).

The success of the zebrafish as a model for vertebrate behavior hinges on the ability to identify the molecular nature of the identified mutations. Currently this is still a time consuming task, since genes harboring mutations may have to be positionally cloned if candidate genes do not prove fruitful. Zebrafish genomic resources have grown impressively in the last few years, culminating in the zebrafish genome sequencing project initiated by the Sanger Centre. The step from mutant phenotype to the underlying molecular defect will get much faster in the near future.

In parallel, transgenic technology has progressed tremendously in the zebrafish, with an ever-growing number of stable transgenic lines being reported.

Thirty years after the zebrafish was proposed as a genetic model system for studying vertebrate behavior, the promise is being kept.

Table 1. Visually mediated behaviors in the zebrafish

Behavior	Age	Used in screens
Visual startle response	<4 dpf	No
Phototaxis	>3 dpf	No
Optokinetic response	3-7 dpf	Yes
Feeding Assay	>5 dpf	No
Optomotor response	>5 dpf	Yes
Escape response	>2 months	Yes
Dorsal light response	>2 months	No

abbreviation: dpf, days post fertilization

Table 2. Zebrafish visual behavior mutants

Name	Behavioral Phenotype	Visual System Phenotype	Other Ph
Recessive mutations affecting the larval visual system:			
belladonna (*bel*)	reversal of OKR	uncrossed optic fibers	pigment
bleached (*blc*)	defective OKR	apoptosis in all retinal cell layers, no ERG measurable	hypopigm
blumenkohl (*blu*)	defective OKR	dispersed arborization of RGCs	
dropje (*drp*)	defective OKR and OMR	abnormal ERG, late degeneration	
fading vision (*fdv*)	reduced OKR and OMR	shorter PRC outer segments, RPE disorganized, partial PRC recovery, adult viable	pale mela
grumpy (*gup*)	defective OKR	malformed lens, RGC axons disorganized	notochor
macho (*mao*)	defective OKR and OMR	dispersed arborization of RGCs, lack of sodium conductance in retinal ganglion cells	lack of to
noir (*nir*)	defective OKR and OMR	abnormal ERG, late larval retina degeneration	expanded locomoto
no optokinetic response a (*noa*)	defective OKR	ERG abnormal	
no optokinetic response b (*nrb*)	defective OKR	ERG abnormal	
no optokinetic response c (*nrc*)	defective OKR	abnormal PRC synaptic terminals, ERG abnormal	
partial optokinetic response a (*poa*)	erratic OKR	ERG reduced at bright light	
partial optokinetic response b (*pob*)	defective OKR in red light	red cones missing at 5 dpf, ERG abnormal	
pinscher (*pic*)	erratic OKR; erratic OMR	abnormal branching in optic tract	abnormal
sandy (*sdy*)	defective OKR and OMR	RPE unpigmented	no melan
sleepy (*sly*)	defective OKR	small PRC outer segments, RGC axons disorganized	notochor
steifftier (*ste*)	defective OKR and OMR	abnormal ERG	locomoto
Dominant mutations affecting the adult visual system			
night blindness a (*nba*)	no escape response at dim illumination	progressive loss of PRC	Early ons in homoz
night blindness b (*nbb*)	no escape response at dim illumination	scotopic visual threshold fluctuations, abnormal olfactoretinal centrifugal pathway	CNS deg homozyg

abbreviations: dpf, days post fertilization; ERG, electroretinogram; OKR, optokinetic response; OMR; optomotor response; PRC, photoreceptor; RGC, retinal ganglion cell; RPE, retinal pigment epithelium

Listed mutants have been tested in behavioral assays. Several mutant categories affecting visual system function are not included in this table, namely mutations affecting neurogenesis and photoreceptor survival; for review see Malicki (2000).

[a]Only those alleles are listed that have been used in behavioral assays.

References: 1, (Brockerhoff et al., 1995); 2, (Brockerhoff et al., 1997); 3, (Brockerhoff et al., 1998); 4, (Gnuegge et al., 2001); 5, (Allwardt et al., 2001); 6, (Li and Dowling, 1997); 7, (Li and Dowling, 2000); 8, (Neuhauss et al., 1999); 9, (Neuhauss et al., 2002); 10, (Rick et al., 2000); 11, (Van Epps et al., 2001)

Figure legends:

Figure 1. Zebrafish at stages where visual behavior is most commonly assayed. Adult zebrafish (A) and 5 day old zebrafish larva. Scale bar is 1 cm in A and 250 μm in B.

Figure 2. Visual behavior assays in zebrafish larvae (A,B) and adults (C-E). (A) Optokinetic response (OKR), (B) population screening for larval optomotor response (OMR), (C) adult OMR, (D) escape response, and (E) dorsal light response (DLR). Arrows indicate direction of moving grating (A-D), and movement of fish (E). Actual size of larvae and adult fish is proportionally much smaller than depicted in the diagram. For details see text.

Figure 3. Mutations affecting specific cell types. Transverse plastic section of 5 day old wild-type and mutant zebrafish (A) The wild type retina has all retina layers fully developed. (B) In the *lakritz* mutant, the ganglion cell layer is nearly devoid of cells, while the thickness of the INL is increased. (C) Photoreceptors are specifically affected with cells degenerating in the outer retina of the *oval* mutant retina, as indicated by gaps in the cell layer. Remaining photoreceptors have severely shortened or absent outer segments. The inner retina is little affected. GCL, ganglion cell layer; INL, inner nuclear cell layer; IPL, inner plexiform layer; ON, optic nerve; ONL, outer nuclear layer; OPL, outer plexiform layer; OS, outer segments of photoreceptors; RPE, retinal pigment epithelium. Scale bar, 50 μm. Modified from Neuhauss et al., 1999; printed with permission of the publisher.

Figure 4. Homozygous *macho* mutant larvae show no visual background adaptation and fail to generate overshooting action potentials. (A) Dorsal view of a wild-type

larva in bright light with contracted melanophores. (B) *macho* larvae do not adjust to background illumination and have expanded melanophores. Voltage clamp (C,D) and current clamp (E,F) whole cell patch recordings in 6 day old larvae reveal that retinal ganglion cells of *macho* mutants lack large transient inward currents (arrow in C, compare C to D) and hence are unable to generate overshooting action potentials (arrow in F), in contrast to wild-type cells (E).
(C-F) adapted from Gnuegge et al., 2001; printed with permission of the publisher.

Figure 5. Reversal of optokinetic behavior is correlated with lack of optic chiasm formation in *belladonna* mutant larvae. (A,B) Labeling the entire retinotectal projection with lipophilic tracer dyes (DiI in red and DiO in green) reveals a completely contralateral optic projection in wild-types (A), and a lack of chiasm formation in *belladonna* mutant larvae (B). Eye position is plotted over time after optokinetic stimulation (C,D). In achiasmatic larvae, optokinetic behavior is reversed (D). After tracing the entire retinotectal projection, injected eyes have been removed to allow better observation of the tecta in panels A and B. Scale is10 degrees and 5 seconds.
(A,B) adapted from Rick et al., 2000; printed with permission from the publisher.

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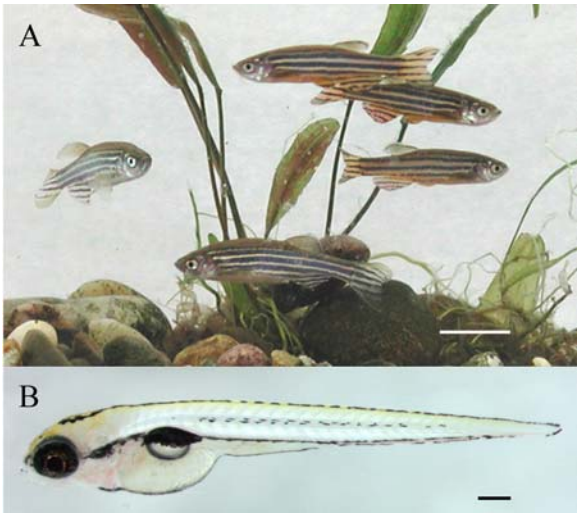

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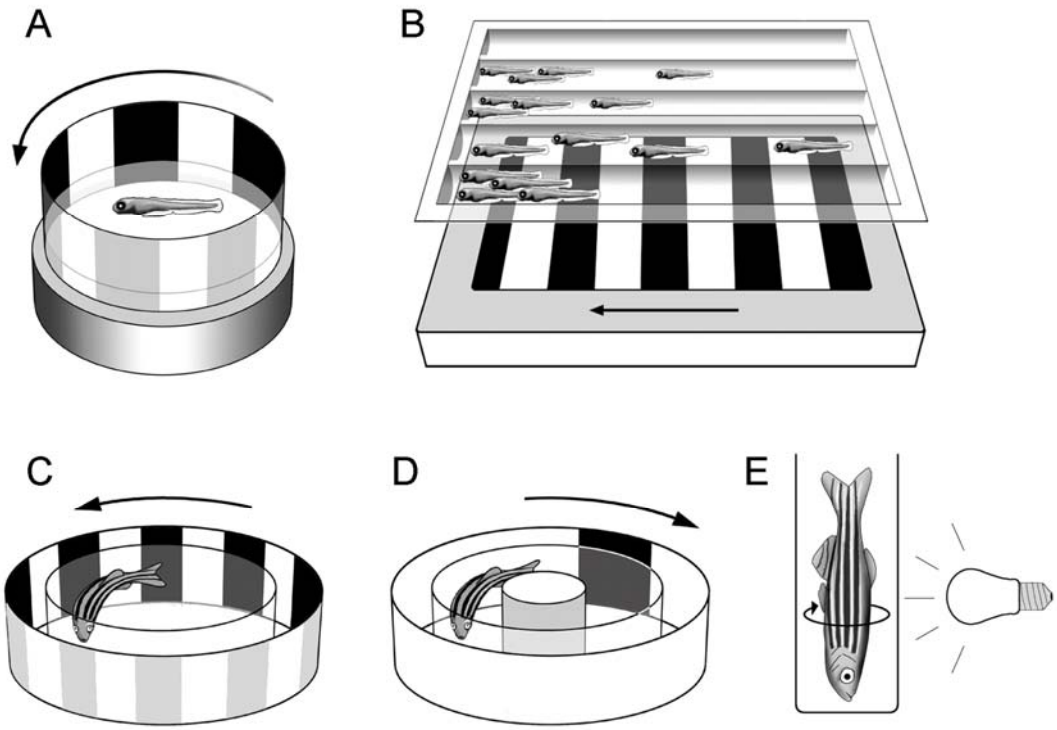

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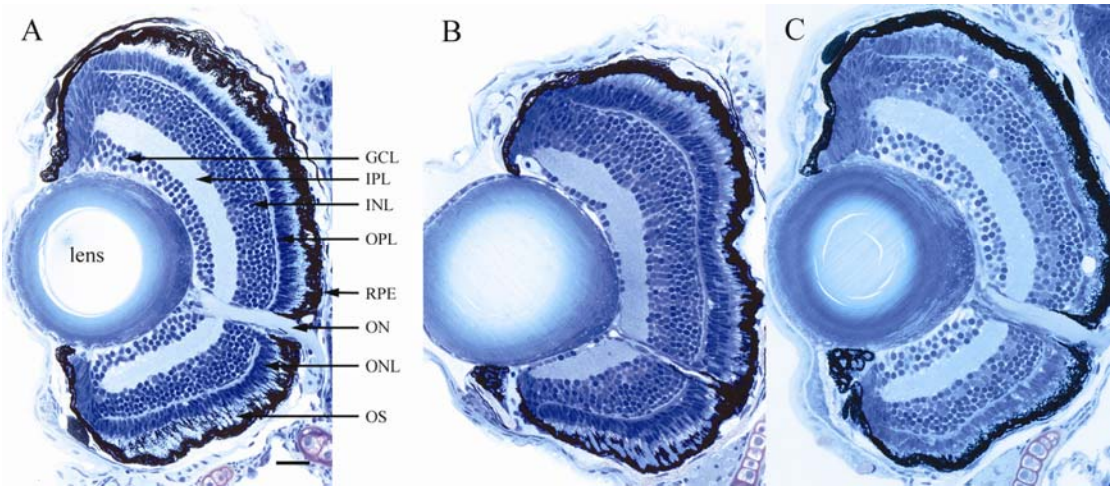

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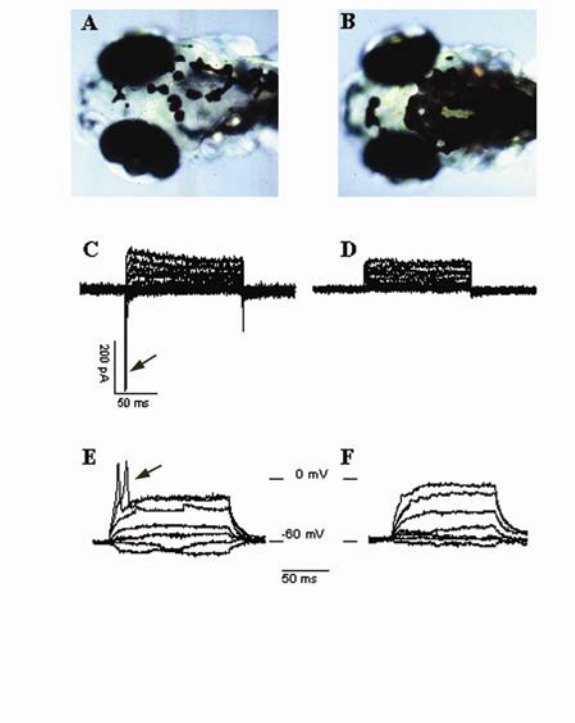

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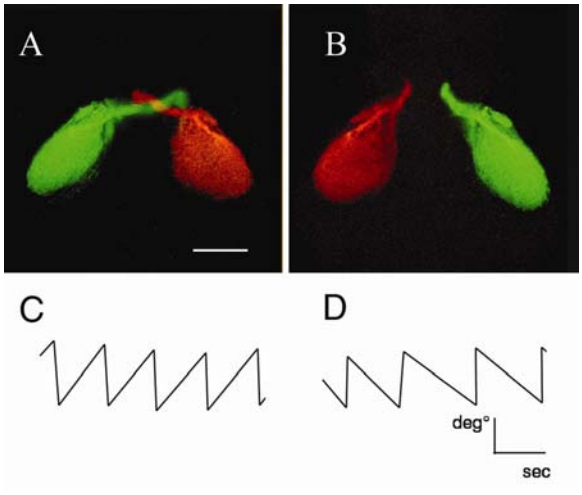

Figure 5.