Time Course and Development of Light Adaptation Processes in the Outer Zebrafish Retina

CORINNE HODEL,[1,2] STEPHAN C.F. NEUHAUSS,[1,2]* AND OLIVER BIEHLMAIER[1,2]*

[1]Department of Biology, Swiss Federal Institute of Technology (ETH), Zurich, Switzerland

[2]Brain Research Institute, University of Zurich, Zurich, Switzerland

ABSTRACT

Retinomotor movements are morphological changes in the outer retina in response to changing light conditions. They can be separated into two components: Migration of pigment granules within the microvilli of the retinal pigment epithelium (RPE) and positional changes in photoreceptor cells. These positional changes optimize exposure of the cone and rod photoreceptors to light. The aim of this study was to analyze both the time course of retinomotor movements in the adult zebrafish and the maturation of these processes in the developing fish. We show that retinomotor movements are used as a dark/light adaptation mechanism in zebrafish. In adult zebrafish, melanin granules of the RPE migrate with constant speed and reach the fully light adapted (LA) state approximately after 1h. In contrast, about two thirds of double cone outer segment movements are finished in 5min, and are fully completed in 10 to 20min. During development there are three crucial stages leading to mature retinomotor movements in response to light: at 5dpf (days post fertilization) the migration of pigment granules begins, at 20dpf the pigment granules condense in the apical part of the RPE microvilli, and at 28dpf, concomitant with the functional maturation of rods, the double cones contract as in adult retinas. Anat Rec Part A, 288A:653–662, 2006.

Key words: retina; zebrafish; light adaptation; retinomotor movement; development

The ability to adapt to changing light conditions is an essential property of all visual systems. Different mechanisms have developed during evolution in order to perform photomechanical changes during light and dark adaptation. Retinomotor movement is such a mechanism and evolved early in the evolution of vertebrates, whereas the pupillary reaction is only found in higher vertebrates.

Retinomotor movement is the morphological reaction of a fish retina exposed to various light intensities. This response to changing light conditions takes place in the outer retina and consists of migration of the granules in the retinal pigment epithelium (RPE) and positional changes of both, rod and cone outer segments. During dark adaptation (DA), cones elongate and rods contract, both mediated by contractile mechanisms in the respective photoreceptor myoid. Additionally, pigment granules of the RPE condense in the basal part of the RPE. As a result, rod outer segments (ROS) are situated closer to the outer limiting membrane, whereas the outer segments of cones are close to the pigment granules. This arrangement leads to an optimal exposure of ROS to incoming light. During light adaptation (LA), the positions are reversed: cones contract, rods elongate and the pigment granules migrate to the apical part of the RPE to expose the cone

All three authors' present address is Institute of Zoology, University of Zurich, Zurich, Switzerland

*Correspondence to: Oliver Biehlmaier or Stephan Neuhauss, Institute of Zoology, University of Zurich, Winterthurerstrasse 190, CH 8057 Zurich, Switzerland. Fax: 41-44-635-3303. E-mail: biehlmaier@hifo.unizh.ch (OB) or stephan.neuhauss@zool.unizh.ch (SCFN)

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outer segments (COS) to light as well as to shade the ROS from excessive bleaching (Ali, 1971).

Retinomotor movements are activated by external and internal factors. Light, as an external trigger, leads to retinomotor movement as long as it illuminates the eyes directly. Studies of endogenous control were done in the Blue acara (*Aequidens pulcher* (Kolbinger et al., 1996)) and very recently in adult zebrafish (Menger et al., 2005). In Blue acaras kept under a 12/12 hour light/dark cycle, rod elongation was initiated less than 1 hour before light onset and rod contraction started less than 1 hour before light offset. Furthermore, retinomotor movement of rods continued during two complete cycles of continuous darkness. During the expected light phase the rods reached about 60% of their usual light induced elongation, and during the expected dark phase the rods contracted as in the normal light/dark cycle. Thus, rods appear to require an exogenous cue to anticipate changes in light phase. In contrast, in zebrafish kept under a 14/10 hour light/dark cycle (Menger et al., 2005), rod inner segments were fully elongated within 1h after light onset, but not before the end of the dark phase as observed in the Blue acara. Rod contraction did not start before light offset. Menger and coworkers (2005) found that all cone types elongated rapidly to their maximal length during the first hour of the dark phase. Approaching the light phase, the long-single and double cones remained elongated until light onset, whereas short-single cones already began to contract during the last half of the night.

Since dopamine and melatonin are neuromodulators involved in retinal morphological changes as well as in circadian rhythm (Cahill et al., 1991; Kohler et al., 1990; Kolbinger et al., 1990; McCormack and McDonnell, 1994; Zaunreiter et al., 1998; Zawilska and Nowak, 1992), studies with dopamine- as well as melatonin-depleted retinas were performed. Surprisingly, in dopamine-depleted retinas (treated with 6-hydroxy-dopamine) of the Blue acara, rod retinomotor movement is indistinguishable from untreated dopamine-containing retinas during either a 12:12 hour light/dark cycle or continuous darkness (Kolbinger et al., 1996). Similar results were found for photomechanical changes of cones and dispersion of pigment granules (Douglas et al., 1992). Studies with different melatonin concentrations showed that melatonin is able to mimic darkness by causing cone elongation in the light. However, in the presence of exogenous dopamine the effect of melatonin on cone elongation is blocked (*Xenopus laevis,* (Pierce and Besharse, 1985)).

The motile processes are either actin- or microtubule-dependent and are triggered by cyclic adenosine 3', 5'-monophosphate (cAMP) or calcium (Burnside and Nagle, 1983).

In some species the mechanism of adaptation to changing light conditions varies with age. In metamorphosing animals like fish or amphibia, this ontogenetic phenomenon is due to a different composition of the larval and adult retina. Since the larval retina is missing rods, no retinomotor movement occurs (Ali, 1958). In salmon retinomotor movements and the migration of the RPE start simultaneously with the appearance of rods. Adult teleosts belong to a giant group of animals having duplex retinas consisting of both cones and rods. A correlation between behavior of animals and their retinal structure was found. Retinas of diurnal animals are cone-dominant, whereas rod-dominant retinas are found in nocturnal animals. A retina composed of both rods and cones allows an animal to be active at any time of the day, even 24 hours, and permits a life in different habitats (Ali, 1971). In addition to the correlation between structure and functionality, other parameters influence light/dark adaptation. Although low temperatures decrease the velocity of adaptation processes, the final arrangement of the visual cells is unaffected by temperature. The influence of temperature on the kinetics of adaptation is more pronounced during DA than during LA. Light intensity also influences the velocity of adaptation. When DA animals are exposed to light, the process of LA is faster at high light intensity than at lower intensities. Moreover, DA is faster in complete darkness than in dim light (Ali, 1971).

Here, we show that photoreceptor and RPE pigment granule migration have clearly different kinetics in LA adult zebrafish. Double COS migration is fast, while pigment granule migration is relatively slow. Additionally, we illustrate that three different developmental stages are crucial in the maturation of zebrafish retinomotor movements: At 5 dpf, a small but significant amount of pigment granule migration occurs. Strong pigment granule migration starts at 20 dpf. By 28 dpf, pigment migration and cone and rod outer segment migration are similar to that observed in the adult zebrafish.

MATERIALS AND METHODS

Fish Maintenance and Breeding

Fish were bred and crossed as previously described (Mullins et al., 1994) and kept under a 14/10 hour light/dark cycle. Embryos were raised at 28°C in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, and 0.33 mM $MgSO_4$) and staged according to development in days postfertilization (dpf). The zebrafish strains used for analysis of retinomotor movement were Tübingen (TÜ) and WIK. Adult fish were randomly selected without any knowledge of genetic background.

Fixation

For the time course experiment adult fish were DA for at least 3 hours prior to light exposure for a certain time. Prior to fixation, animals for the development experiment were exposed to either 3 hours of daylight or 3 hours of absolute darkness. For both the time course experiments on adult fish and for the developmental studies, animals older than 20dpf were anesthetized with methane sulfonate salt (Mesab, Sigma) before fixation. For standard histology they were fixed in 4% paraformaldehyde (PFA) in 0.1M phosphate buffer (PB; pH7.4) at least over night. The fixation of DA animals took place in the dark chamber under dim red light. Both LA and DA animals were sacrificed at 12:30pm (UTC+1). As a control experiment night adapted animals were fixed in the early morning two hours before light onset in their light/dark cycle (14/10).

Standard Histology

After two washes with 0.05M PB saline (PBS; pH7.4) the animals were dehydrated in a graded series of 50%, 70%, 80%, 90%, 95%, and 100% ethanol for 15 minutes each. Animals were preinfiltrated two times for 30 minutes in 1:1 Technovit7100/100% ethanol before infiltration in 1:1 Technovit 7100/dimethyl sulfoxid (Heraues Kulzer) for at least two hours. Animals were then embedded in 1:15 Technovit7100/barbibturate acid derivate, polymer-

ized for 1 hour at room temperature and for a second hour at 37°C.

Microtome sections of 3–5μm were mounted on slides (SuperFrost®Plus, Menzel-Gläser), dried on a heater at 57°C, and stained with a solution of 0.5% Toluidine blue in 1% Borax (Fluka). They were then coverslipped with Entellan (Merck).

Morphometry, Quantification, and Statistics

Toluidinblue stained Technovit7100 sections with a thickness between 3 to 5μm were evaluated using bright field optics. Double cones are the easiest to identify and exhibit the most extensive migrations. They thus are more reliable to measure as compared to either of the single cone types or the rods. Therefore, we restrained our morphological quantification of photoreceptor migration to the double cones. For adult retinas, two replicates per adaptation condition were quantified. In each fish, 9 to 37 double cones were analyzed in close proximity to the optic nerve (distance to optic nerve $\leq$ 50μm). Three different distances in the outer nuclear layer were measured (Fig. 1 B, D): Distance *a*, from Bruch's membrane to the most apical position of the granules was used to quantify the migration of the RPE. Distance *b*, from the inner edge of the outer nuclear layer (presumable rod nuclei; see Fig. 1A,C) to the double cone outer/inner segment junction, was used to quantify double cone length. Distance *c*, from the inner end of the soma to the Bruch's membrane, was a reference distance to consider interindividual as well as intraindividual differences. Distance *a* was divided by the reference distance *c* forming the RPE index, while the ratio of distance *b* to the reference distance forms the cone index. To assess the time course of LA processes in the adult retina, fish were initially DA for 3h (0min LA in Fig. 2). They were then exposed to light and dissected at specific time points (5, 10, 20, 30, 60, and 180min) after DA. Both, the RPE index, and the cone index were normalized using the normalization $y = (x - min) / (max - min)$ where *min* was the respective cone/RPE index at full DA (0min LA), and *max* was the respective value at full LA after 180min. For a closer comparison between the kinetics of the migration processes of the pigment granules and COS, we fitted a regression curve to the data by using a one phase exponential association equation: $Y=Ymax*(1-exp(-K*X))$. The regression curves as well as the statistic tests were performed in Prism 4.03 (GraphPad Software, San Diego, USA). In developing fish (i.e., 5, 20, 28dpf), three sections from two to three different fish per stage and experimental condition were quantified. In each section, 20 photoreceptors in close proximity to the optic nerve were analyzed. Thus 20 to 40 photoreceptors were analyzed per replicate. In the 25 day old retina an additional 20 photoreceptors per section were measured in the ventral zone. Significance between DA and LA retinas in each of the four stages was determined by a one way ANOVA and a Tukey post hoc test, performed in Prism.

RESULTS

Adult Zebrafish Show Retinomotor Movements Like Other Teleosts

Retinomotor movements, as described in other teleosts (Ali, 1971; Burnside et al., 1983; Burnside and Nagle, 1983), are also a feature of LA and DA in the adult zebrafish. After full LA, we found virtually all pigment granules of the retina in the apical part of the RPE to protect the ROS from bleaching by the incoming light (Fig. 1A). There was no pigment band at the basal part of the RPE as all pigment granules had condensed in the most apical part of the RPE microvilli, thus forming one band at this site. Additionally, rod myoids were elongated, thus keeping the rods behind the light absorbing barrier of the pigment granule band, whereas the cones remained embedded in the pigment band with fully contracted myoids thus providing them with a separation of each cone from its neighbors, hence increasing the resolution of the eye.

In contrast, after full DA virtually all pigment granules had condensed at the basal part of the RPE forming a pigment band that covered the RPE somata. Only a few pigment granules were left between the outer segments of the photoreceptor cells (Fig. 1C). The majority of rods were contracted and thus positioned near the outer limiting membrane to be exposed optimally to incoming light, whereas the outer segments (OS) of the elongated cones were lined up near the basal part of the retina. However, some of the rods remained in an intermediate position alongside the ellipsoids of the cones or even layed scleral to the cones. Thus, the pigment granules cleared the way for the few photons that reach the rods in a dark environment.

During Light Adaptation, Pigment Granule and Cone Outer Segment Migration Occurs at Different Velocities

In order to function in changing light conditions in the environment, it is important to adapt rapidly. Therefore we determined the time course of COS and pigment granule migration. We found that cones reached 77% of the full light-induced migration after only 5min of LA. After this rapid initial step, the migration speed decreased and the cones migrated only 13% during the next 5min (90% after 10min of LA). After 20min of LA, the cones had reached 94% of the fully LA position which was measured after 3h of LA (Fig. 2A). The statistical analysis of the data (Table 1) supports the hypothesis that most of the double COS migration is finished after 5min as the difference between 0min and 5min is highly significant ($p<0.0001$) whereas the difference between the other recorded time points is either non significant or just a tendency ($p<0.05$).

In contrast, the migration of RPE pigment granules occurred in a more even way. Every 5 to 10min, the pigment granules migrated approximately 15 to 20% of the distance to their final location in the apical RPE (Fig. 2B; 21% after 5min, 36% after 10min, 58% after 20min, 78% after 30min,). After 60min of LA the pigment granules had approximately reached their final position (Fig. 2B). Statistically (Table 1), the constant migration speed is evident in the highly significant differences ($p<0.0001$) between the 5, 10, 20, 30, and 60min time points. The non significant difference between 60min and 180min of LA indicates that the pigment granules reached their final position at 60min.

To compare the kinetics of these processes we determined the half-maximal points of cone myoid contraction and RPE pigment migration. The respective values were 2.26 min and 15.35 min. The time difference between double cone myoid contraction and RPE pigment migration in reaching 95% of their final migration distance was 45 min (15 versus 60min, respectively).

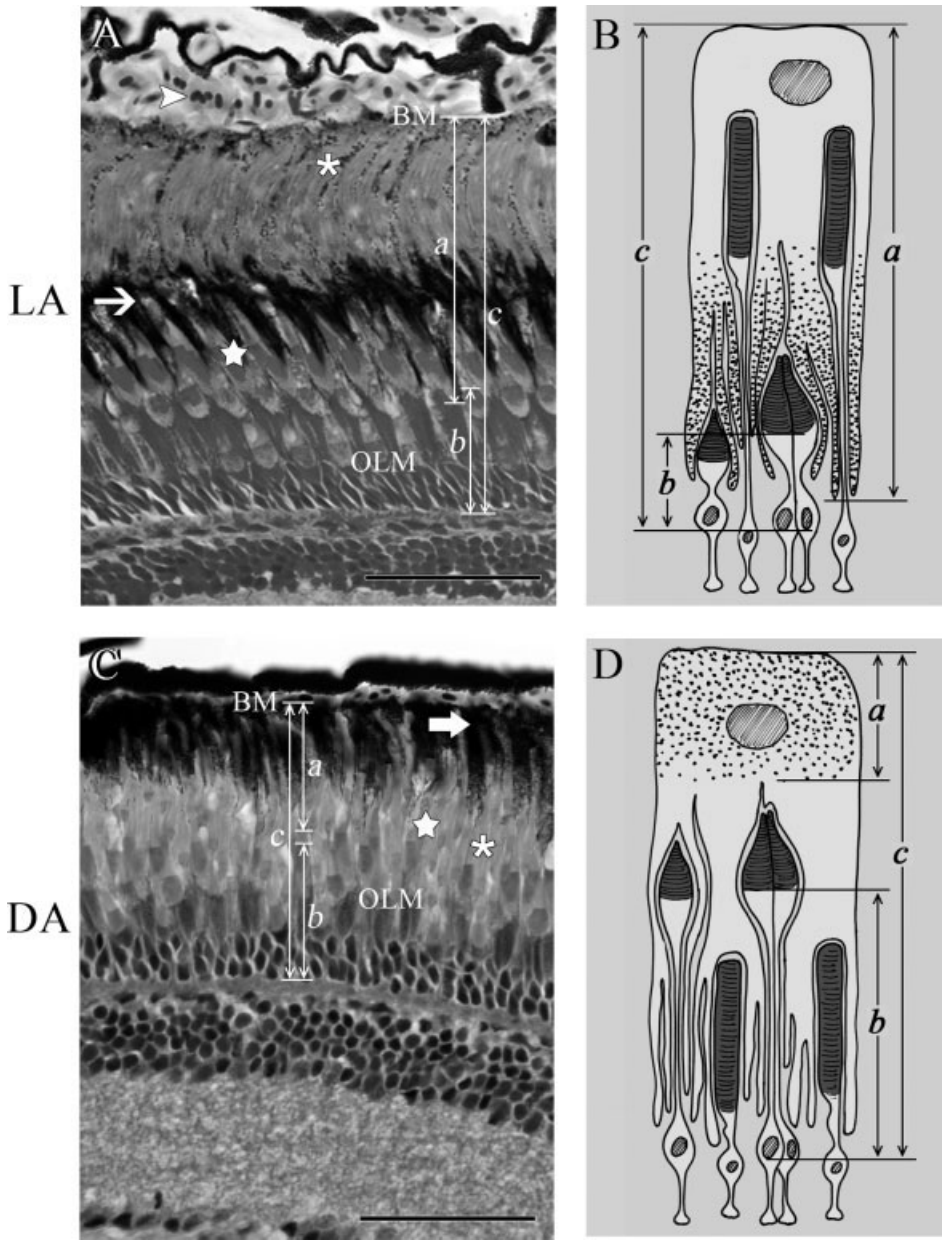

Fig. 1. Radial sections of adult LA (**A**) and DA (**C**) outer retinas and corresponding idealized schematic drawings (**B** and **D**) of RPE and photoreceptor cells. A/B: Almost all pigment granules of the LA retina migrated to the apical part of the RPE (small arrow) and; there was no longer an additional pigment band at the basal part of the retina. As a result, cell bodies of RPE and choroidal cells became obvious (arrow head). ROS (asterisk) of elongated rods were located between the basal part of the RPE and the pigment band in the apical part of the RPE. The contracted cones (star) were found in front of the pigment band exposed to incoming light. C/D: In DA specimens almost, approximately all pigment granules were condensed at the basal site of the RPE forming a broad pigment band that covered the respective cell bodies (thick arrow). Few pigment granules were found between outer segments of photoreceptors cells. Rods were contracted and the ROS (asterisk) were situated closer to the outer limiting membrane, whereas the COS (star) of the elongated cones were close to the pigment band. Distances a, b, and c denoted in B/D were used for calculating the cone and RPE indices (for a detailed explanation see methods section). BM, Bruch's membrane, OLM, outer limiting membrane. Scale bar = 50μm.

Differences Between Dark- and Light-Adapted Retinas Are Subtle at 5dpf

We were then interested to discover how these processes develop. At 5dpf, no morphological difference was observed between DA and LA cones (Fig. 3A, B). Additionally, the quantification of the morphological changes in the outer retina revealed that the cone indices (see Methods section for detailed description of the index measurements) were similar (difference of indices (DI) = 0.02; Fig.

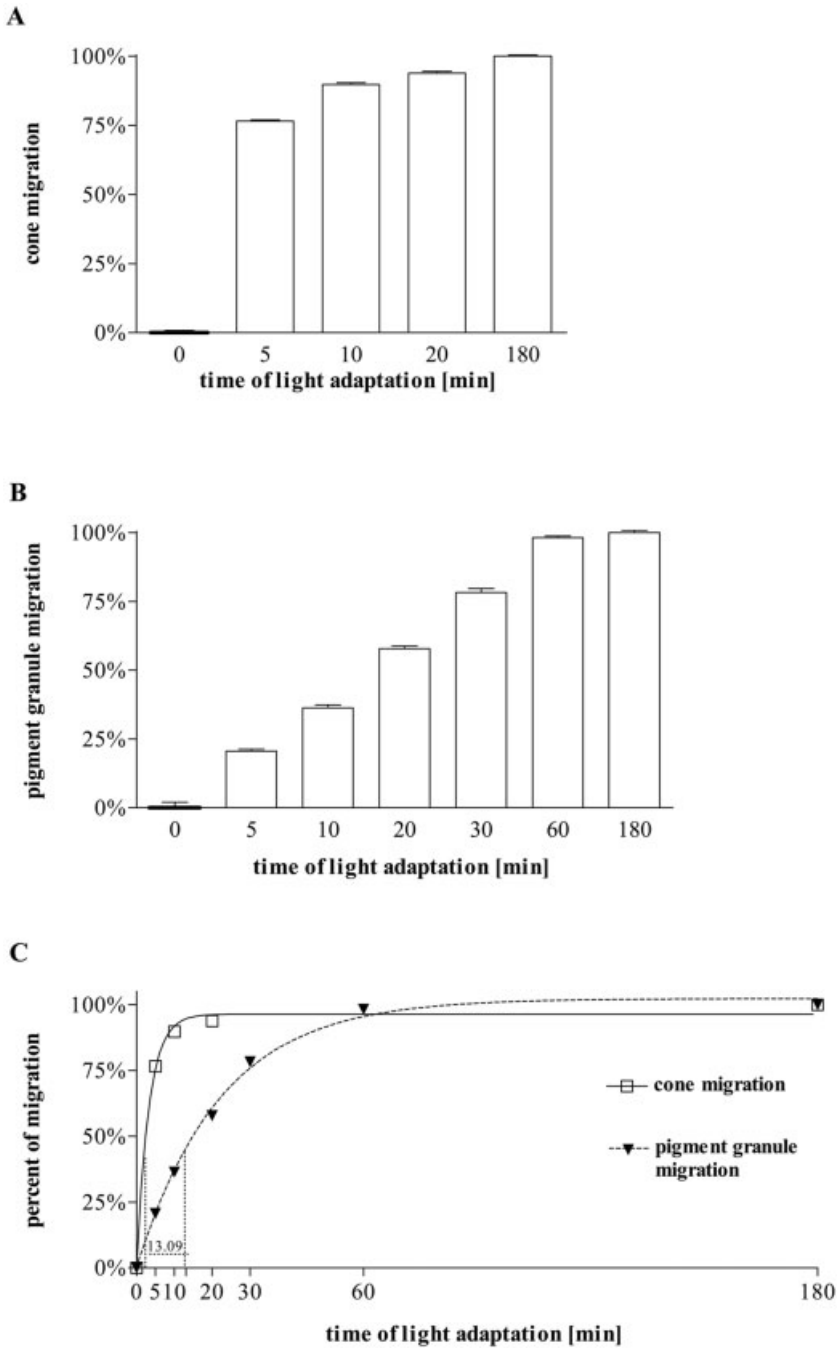

Fig. 2. Quantification of retinomotor movements at different time points of LA (between 5min and 3h of LA). The bars indicate the normalized amount of LA induced movements ± standard error of the mean **A**: **COS** migration reaches 70% already after 5min and 90% only after 10 min. **B**: Pigment granule migration is constantconstantly increasing with ongoing LA and reaches 100% after 1h of LA. **C**: The kinetics of **COS** migration are faster than those of pigment migration (difference in half-maximal migration: 13:.09 min). Significances (for A, B) see Table 1.

4A, Table 2) as the lengths of the cones were similar. The only retinomotor process observable in 5dpf zebrafish was a small but highly significant ($p<0.0001$) migration of pigment granules (DI = 0.05; Fig. 4B, Table 2). As a response to incoming light, the granules had migrated between the photoreceptors. However, a thick pigment band remained in the basal part of the RPE in the LA retina (Fig. 3B). Thus, the assumption that the DA retina remains entirely in the LA position until rods develop does not hold for the situation in the 5dpf zebrafish.

TABLE 1. Time course of light adaptation in adults: Significance between different time points of light adaptation

RPE index	0 min	5 min	10 min	20 min	30 min	60 min	180 min
0 min	-	***	***	***	***	***	***
5 min	***	-	**	***	***	***	***
10 min	***	**	-	***	***	***	***
20 min	***	***	***	-	***	***	***
30 min	***	***	***	***	-	***	***
60 min	***	***	***	***	***	-	n.s.
180 min	***	***	***	***	***	n.s.	-

ANOVA: $F(6,0.7343)=137.6, p < 0.0001$

cone index	0 min	5 min	10 min	20 min	180 min
0 min	-	***	***	***	***
5 min	***	-	n.s.	*	***
10 min	***	n.s.	-	n.s.	n.s.
20 min	***	*	n.s.	-	n.s.
180 min	***	***	n.s.	n.s.	-

ANOVA: $F(4,0.1298) = 76.89, p < 0.0001$

n.s., non significant ($p > 0.05$)

Strong Pigment Granule Migration Starts at 20dpf

Until 15dpf the outer retina of LA and DA animals exhibited a larval appearance just like the 5dpf stage described above. It was only at 20dpf that the pigment granules began to concentrate in the apical part of the RPE during LA, thereby forming a fainter second pigment band in addition to the still condensed granules at the basal site of the RPE (Fig. 3; C, D). At this developmental stage, ROS were clearly identifiable between the two pigment bands. The morphometrical analysis revealed that the differences between the cone indices of DA and LA retinas were not yet significant (DI = 0.02; Fig. 4A, Table 2) whereas the RPE indices differed with high significance ($p<0.001$; DI = 0.22; Fig. 4B, Table. 2).

Animals Older Than 28dpf Show Retinomotor Movements Like Adult Fish

From 28dpf on, both retinomotor processes were virtually mature in the adapting retina. The contraction/elongation of rod and cone myoids as well as the migration of the pigment granules were clearly observable (Fig. 3E, F) and DA retinas appeared identical in 28dpf fish (Fig. 3E) and in adults (Fig. 1C). In both stages the pigment was condensed at the basal part of the RPE. In contrast, in the LA retina there was still a small band of pigment granules in the most basal zone of the RPE forming an additional, second pigment band compared to the condensed pigment granules in the apical part (Fig. 3F). In adult retinas, all granules migrated to the apical part and formed a single pigment band. As a result, RPE cell bodies and choroidal cells became visible (Fig. 1A). Comparisons between LA and DA retinas at 28dpf revealed highly significant differences ($p<0.001$) of cone indices (DI = 0.04; Fig. 4A, Table 2) and RPE indices (DI = 0.34; Fig. 4B, Table 2). These differences were similar to the differences observed in the cone indices (DI = 0.10; Fig. 4A, Table 2) and RPE indices (DI = 0.26; Fig. 4B, Table 2) of the adults.

We then compared the retinomotor movements at 28dpf and the adult stage to find out whether they were equal. No discernible difference in cone lengths between LA retinas of 28dpf and adult specimens (cone indices LA: 28dpf 0.37 ± 0.003; adult 0.38 ± 0.006; Table 2) was observed. However, the migration of the pigment granules still differed with high significance ($p<0.001$) between animals at 28dpf and the adult stage (RPE indices LA: 28dpf 0.68 ± 0.008; adult 0.76 ± 0.013). Therefore, we conclude that the maximal contraction of cones was already reached at 28dpf, whereas the migration of pigment granules from the basal to the apical part of the RPE was not yet fully mature.

There Is a Morphological Difference Between the Central Part and the Ventral Periphery of the Developing Outer Retina

The retinas of LA zebrafish between 15dpf and 30dpf showed topographical differences in the adaptation state of their outer retinas (Fig. 5; A–C). The ventral part seemed to be further developed than the central and dorsal parts. In the central and dorsal parts of the retina, the pigment granules were condensed at the basal site as well as evenly distributed between the photoreceptor cells. In contrast, in the ventral retina the pigment granules were already forming the above described second band of pigment granules in the apical part of the RPE and nascent rods were observed between the two pigment bands. Quantification of the adaptation processes at the different retinotopic regions revealed that the cone index of photoreceptors in the ventral part was decreased with high significance ($p<0.001$) compared to more centrally located photoreceptors (ventral, 0.339 ± 0.008; and central, 0.575 ± 0.008), whereas the migration distance of pigment granules in the ventral zone was not different from the migration distance near the optic nerve (ventral, 0.70 ± 0.013; central, 0.67 ± 0.011). Thus, we conclude that the development of LA processes occurs first in the ventral part of the retina, mirroring the situation during photoreceptor development (Raymond et al., 1995).

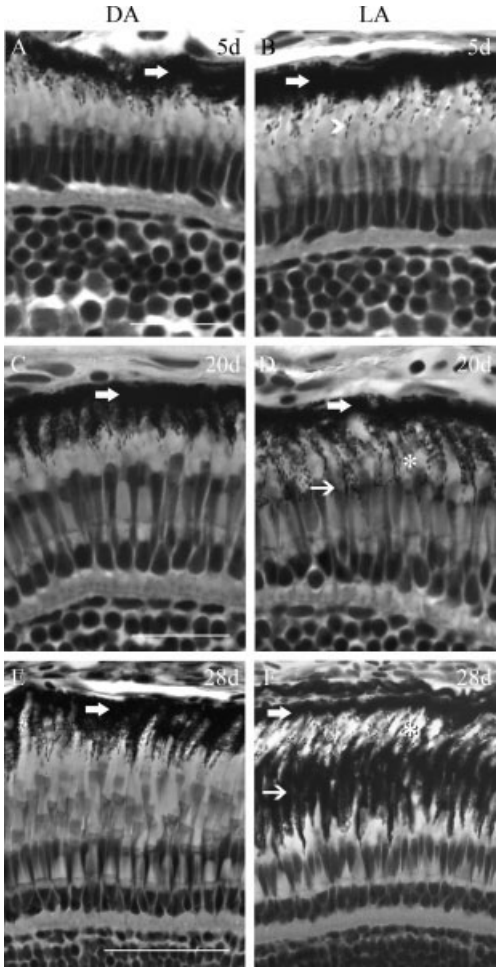

Fig. 3. Radial sections showing the outer retina close to the optic nerve of DA (left column) and LA (right column) zebrafish at 5dpf (**A, B**), 20dpf (**C, D**) and 28dpf (**E, F**). A, C, E: In DA retinas almost all pigment granules were condensed at the basal site (thick arrows) B: Already at larval stages the outer retina adapted to changing light conditions by movement of pigment granules. In LA retinas some pigment granules were found between cones (arrow head) in addition to the pigment band in the basal zone of the RPE (thick arrow). D: At 20dpf initial signs of photoreceptors retinomotor movements were observed. ROS (asterisk) could be identified between the thick pigment band at the basal site (thick arrow) and the still slight pigment band at the apical site of RPE (small arrow). F: From 28dpf on the cones contract as in adult sections (see Fig. 1). The migration of the pigment granules still differed between animals at 28dpf and adults. At 28dpf, there was still a small band of pigment granules in the most basal zone of the RPE (thick arrow) in addition to the condensed pigment granules in the apical (small arrow). ROS (asterisk) were found between these two pigment bands. Scale bars: A–D = 20 μm; E, F = 40 μm.

DISCUSSION

We showed in this study that light induced retinomotor movements in zebrafish are similar to those described in other teleosts. We specifically focused on the time course of the LA process and on the development of the two retinomotor movements, RPE pigment granule migration and double cone migration. In adult zebrafish, the time course of the adaptation processes is different. The photoreceptor movement is a very fast process and 77% of the COS reach their final destination after only 5min with the entire movement completed in 10 to 20min. In contrast, the pigment granule movement occurs at a slower pace but with constant speed and reaches the fully LA state approximately after 1h. During development, three crucial stages in retinomotor movement were revealed. At 5dpf larvae show no photoreceptor changes but there is migration of pigment granules. At 20dpf the pigment granules began to condense in the apical part of the RPE microvilli, and at 28dpf the RPE pigment granules showed virtually the same distribution as in the adults. Additionally, the cones were finally contracted as in adult retinas. Thus, the two components of retinomotor movement do not mature in parallel. The RPE granule movement develops continuously, whereas photoreceptor movement seems to require the presence of a fully functional signal from mature rods which are thought to be developed around 28dpf (Bilotta et al., 2001).

Different Time Course for Different Processes

We have a fairly precise knowledge about the time course of pigment granule movement and cone myoid contraction in fish like salmons, grunts and cichlids (Ali, 1971; Burnside and Nagle, 1983) as well as the time course of other light triggered processes in the fish retina, like spinule formation at the cone pedicle synapse (Wagner, 1980). In a recent study, Menger and colleagues (2005) described diurnal and circadian retinomotor movements in zebrafish. They show that cones are maximally contracted 1h after light onset. However, they do not describe the precise time course of retinomotor movements during the first hour of LA. The time course we found in the zebrafish retina is very similar to that described in the blue striped grunt (Burnside and Nagle, 1983). In both fish, cone myoid contraction is very rapid and full contraction is achieved after approx. 20min. The zebrafish is even faster than the grunt as its cones reach 75% of full COS migration with only 5min of LA. Concerning the RPE pigment migration, the two species show almost identical time courses that are slower than the COS migration and a plateau of full LA in 30 to 60min (Burnside and Nagle, 1983). Menger and coworkers (2005) found maximal pigment granule dispersion at midday in zebrafish with a regular light/dark cycle. This observation agrees with our findings that pigment granule migration is rather slow and occurs at a constant speed. In the atlantic salmon, the time courses are slightly delayed (Ali, 1971) but COS migration is clearly faster than RPE pigment granule migration. Thus, even though the above species live in very different habitats (zebrafish in slow moving freshwater, e.g., rice fields; grunts in costal marine water or reefs; salmons are anadromous fish in rivers and oceans) the retinomotor movements occur with similar time courses, suggesting that they are a characteristic property of fish duplex retinas.

The differences in the kinetics of the two retinomotor movements are very likely due to the different underlying cellular processes. Photoreceptor myoid contraction is thought to be mediated by sliding actin filaments (Burnside, 1978) and is thus very rapid. In contrast, RPE pigment granule migration is thought to be mediated by

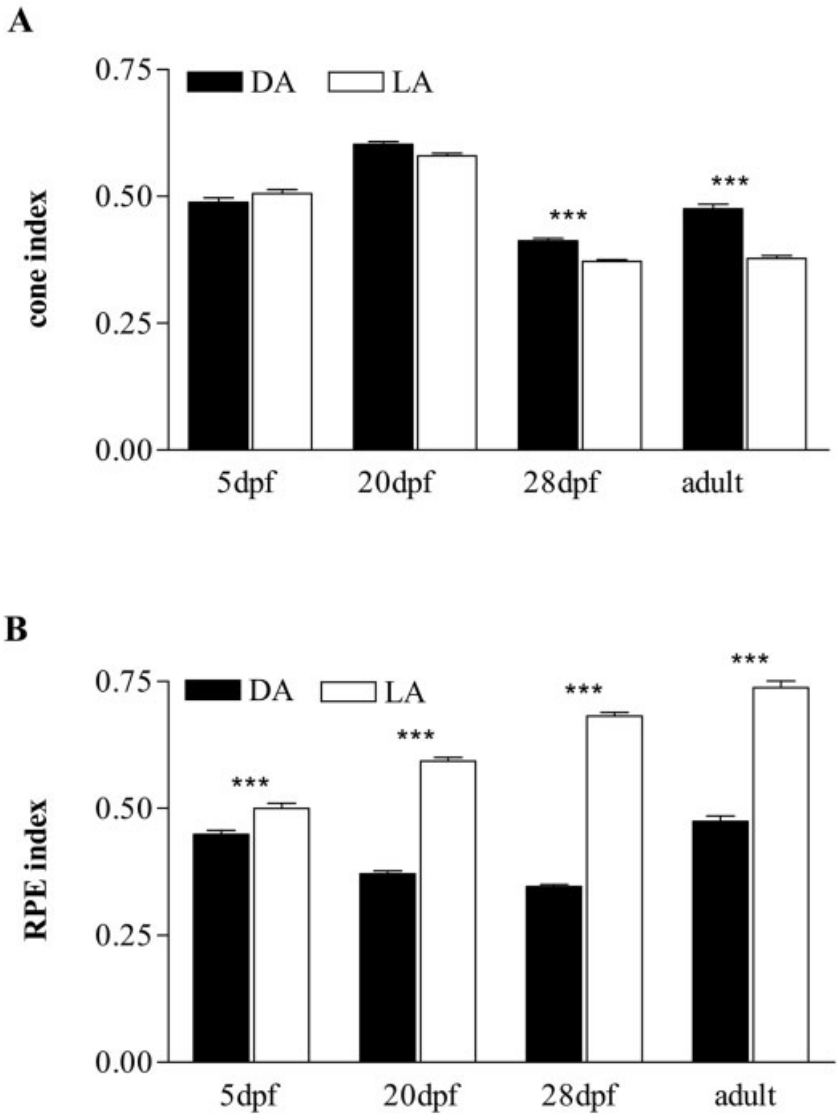

Fig. 4. Quantification of retinomotor movements at 5dpf, 20dpf, 28dpf, and at the adult stage in DA (black bars) and LA (white bars) zebrafish. The bars indicate the mean number ± standard error of the mean **A**: The cone indices in 5 and 20 day old larvae do not differ, whereas at 28dpf and in adult zebrafish the response of cones to changing light conditions differs highly significantly (triple asterisk, $P < 0.001$). **B**: The difference in migration of pigment granules between DA and LA retinas is highly significant (triple asterisk, $P < 0.001$) at all four developmental stages.

TABLE 2. Development of retinomotor movements in dark and light adapted retinas

Stage	adaptation	mean ± std. error RPE index	significance	mean ± std. error cone index	significance
5dpf	DA	0.45 ± 0.007	***	0.49 ± 0.009	n.s.
	LA	0.50 ± 0.011		0.51 ± 0.008	
20dpf	DA	0.37 ± 0.005	***	0.60 ± 0.005	n.s.
	LA	0.59 ± 0.008		0.58 ± 0.006	
28dpf	DA	0.35 ± 0.004	***	0.41 ± 0.005	***
	LA	0.68 ± 0.008		0.37 ± 0.003	
adult	DA	0.47 ± 0.010	***	0.48 ± 0.010	***
	LA	0.74 ± 0.013		0.38 ± 0.006	

n.s.: non significant ($p > 0.05$)

Rab27a and myosin VIIa as well as by other yet undefined motors like microtubules and myosin Va (Gibbs et al., 2004). These migration processes are slower than the sliding actin filaments used for cone myoid contraction and thus readily explain the slower time course of RPE pigment migration.

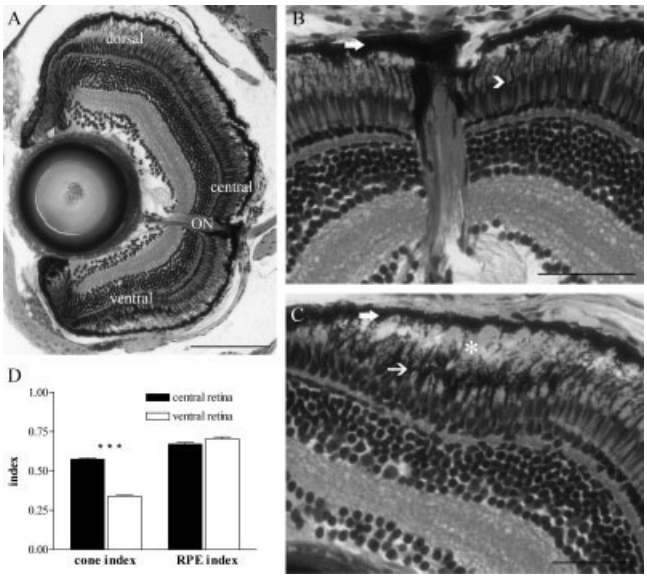

Fig. 5. Morphological differences between the central retina and the ventral periphery in the LA retina at 25dpf **A**: Radial section of a LA retina at 25dpf. High magnification of the central (**B**) and ventral (**C**) part revealed the morphological differences between these two regions of the retina B: In the central part of the retina the pigment granules were condensed at the basal zone (thick arrow) as well as being regularly distributed between the photoreceptor cells (arrow head). C: In the ventral part, the pigment granules formed a second band of pigment granules in the apical part of the RPE (small arrow) in addition to the thin band at the basal zone (thick arrow). Between these two bands, ROS (asterisk) were identified. **D**: Quantification of the outer retina in the central (black bars) and ventral (white bars) part of the retina. Cone lengths from the ventral periphery and central parts differed with high significance ($p<0.001$), whereas the RPE index did not differ between these two regions of the LA retina. The bars indicate the mean number ± standard error of the mean. ON, optic nerve. Scale bars = 100μm (A); 40μm (B,C).

Different Onset of Pigment Granule Migration and Cone Outer Segment Migration

Pigment granules migration starts early. In order to design experiments on LA and/or light damage in larval fish, and especially in order to compare these mechanisms in wild type and mutant larval zebrafish, it is indispensable to know about the onset of cone contraction and RPE pigment migration. We started the analysis at 5dpf and found that the migration distance of RPE pigment granules was already highly significantly different in LA vs. DA larvae. Branchek and Bremiller (1984) found that pigment granules do not migrate at 4dpf whereas they do at 8dpf. We show that pigment granule migration begins in larvae at 5dpf. The mechanism triggering the onset of this migration between 4 and 5dpf is currently unknown. One might speculate that RPE cells themselves harbour a specific pathway that matures shortly after photoreceptors become functional at around 4dpf triggering the onset of pigment migration in the microvilli. In order to clarify this hypothesis, we analyzed migration distances of pigment granules at 3dpf (data not shown). Surprisingly, at this stage a highly significant ($p=0.0002$) difference of pigment migration different in DA and LA larvae was already revealed. Thus, we conclude that pigment granules start to migrate concomitantly with photoreceptor OS generation at 3dpf contradicting Branchek and Bremiller's (1984) earlier assumption. Our data show that between 5dpf and 15dpf, first pigment granules were uniformly distributed between photoreceptor outer segments but did not yet reach the most apical part of the microvilli. Only at 20dpf did we detect condensed pigment granules in the most apical part of the RPE microvilli, which were not yet uniformly filled with pigment granules. As all types of photoreceptors, including the rods, are adult-like in appearance at 20dpf (Branchek and Bremiller, 1984), the now fully developed ROS need to be protected from photobleaching. Thus, we suggest that the interdigitating pigment granules between COS absorb scattered light rays to increase visual acuity at early stages of development (approx. 3dpf to 20dpf). We propose that this process is triggered by the cones themselves or by some unknown process in the RPE. In addition, RPE pigment granules form a pigment band in the apical part of the RPE microvilli in order to protect the ROS bleaching. This process is thought to be triggered by the rods.

Development of photoreceptors is mirrored in development of retinomotor movements. The development of the outer retina also influences the light-induced elongation or contraction of photoreceptors. At 5dpf all cone types are present and identifiable by opsin labeling (Vihtelic et al., 1999). However only UV sensitive single cones and red sensitive double cones show mature opsin distribution (Biehlmaier et al., 2003). Blue sensitive single cones and green sensitive double cone members remain immature concerning the respective opsin labeling (Biehlmaier et al., 2003) and their overall morphology (Branchek and Bremiller, 1984). No photoreceptors underwent positional changes at 5dpf in response to light. Since the inner segment can be morphologically divided into myoid and ellipsoid at 60hpf (Schmitt and Dowling, 1999) we conclude that the myoid does not contract even though the anatomical structures that carry out this movement are already in place. Thus we propose that the absence of functional rods at 5dpf might be the reason for the lack of COS movement.

Later in development, at 20dpf, when all photoreceptor types are adult-like with their components developed forming a regular mosaic (Schmitt and Dowling, 1999), cones still did not contract or elongate as a response to changing light conditions. However, it is known from other studies that at this stage the functional contribution of the rods is small and is seen in the immature electroretinogram (ERG; Bilotta et al., 2001).

Just 8 days later, at 28dpf, we observed maximal cone contraction with a cone index indistinguishable from the adult retina. We suggest that the increase in photoreceptor density after 24dpf (Branchek and Bremiller, 1984) leads to a stronger contribution of rods to the retinal signal and thus a more mature ERG (Bilotta et al., 2001). The maturation of COS movements thus coincides with rod maturation and is triggered by an unknown mechanism. Additionally, the remodeling of photoreceptors after 24dpf (first described in *Xenopus* (Kinney and Fisher, 1978)) due to increased photoreceptor density might be necessary to enable retinomotor movements of photoreceptors.

The Ventral Part of the Retina Develops Faster Than the Central Part

We know that the development of photoreceptors starts in the ventral part of the retina (Kljavin, 1987; Raymond et al., 1995). All types of photoreceptors are initially found in this part, before they are observed in other topographical regions of the retina. In older larvae the dorsal and central parts of the retina are mainly populated by cones, whereas the ventral part remains rich in rods. We found differences in retinomotor movements between the ventral and central part of the outer retina of LA animals at 25dpf. Both pigment migration and COS migration were different in ventral vs. central or dorsal regions. In the ventral retina cones were more contracted and the pigment granules were already condensed in the apical part of the microvilli. Contracted cones and condensed pigment granules are characteristics of mature, fully LA animals. Therefore, we suggest that, similar to photoreceptor development, the LA processes of the ventral retina precede those in other parts of the retina. Considered in a behavioral context, the rapid development of LA mechanisms in the ventral retina improves the fish's ability to hunt and eat at the bright water surface.

Taken together, these findings show that retinomotor movements are a common feature in teleosts with duplex retinas. The knowledge about the onset of these mechanisms in zebrafish can be used as a baseline for future experiments on LA processes in larval and adult zebrafish. Understanding these processes in the zebrafish paves the way for a genetic dissection of these retinomotor processes and showed help to understand the underlying mechanisms which are still largely unknown.

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