Remodeling of the Cone Photoreceptor Mosaic during Metamorphosis of Flounder (*Pseudopleuronectes americanus*)

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Abstract
The retinal cone mosaic of the winter flounder, *Pseudopleuronectes americanus*, is extensively remodeled during metamorphosis when its visual system shifts from monochromatic to trichromatic. Here we describe the reorganization and re-specification of existing cone subtypes in which larval cones alter their spatial arrangement, morphology, and opsin expression to determine whether mechanisms controlling cell birth, mosaic position, and opsin selection are coordinated or independent. We labeled dividing cells with tritiated (3H) thymidine prior to mosaic remodeling to determine whether existing cone photoreceptors change phenotype. We also used in situ hybridization to identify mosaic type and opsin expression in transitional retinas to understand the sequence of transformation. Our data indicate that in the winter flounder retina the choice of new opsin species and the cellular rearrangement of the mosaic proceed independently. The production of the precise cone mosaic arrangement is not due to a stereotyped series of sequential cellular inductions, but rather might be the product of a set of distinct, flexible processes that rely on plasticity in cell phenotype.

Introduction
A major issue facing developmental biologists is how undifferentiated cells give rise to adult patterns with multiple different cell types. Retinal development has been a focus for studies on cell differentiation because photoreceptors form precise arrays or mosaics. These regularly spaced arrangements of cells have predictable cell subtypes relative to neighboring cell types. Photoreceptor phenotypes within a mosaic can be distinguished by morphology and by expression of the light-sensitive opsin photopigments. Two primary hypotheses have emerged to explain photoreceptor mosaic patterning in vertebrates: one postulating that a series of sequential inductive events specify cell fate based on preexisting mosaic position as found in *Drosophila* photoreceptor development [Raymond et al., 1995; Stenkamp and Cameron, 2002; Raymond and Barthel, 2004]; and a second hypothesis proposing that differential adhesion between cell subtypes establishes position within the mosaic based on previously determined cell fate [Galli-Resta, 2001; Mochizuki, 2002; Reese and Galli-Resta, 2002; Tohya et al., 2003]. These two hypotheses generate distinct predictions as to the order of mosaic formation and cell differentiation. The rapidity of retinal development in most retinal model systems makes distinguishing the timing of developmental events difficult. Studies of retinal development in these model systems are complemented by
Fig. 1. We categorized individual cone photoreceptors as members of either hexagonal or square mosaic regions by analyzing their nearest neighbors within a unit A1–A3 combined with the identity of adjacent mosaic units B1, B2. In these schematic illustrations, circles represent single cone photoreceptors. A1 A complete unit of the hexagonal mosaic characteristic of premetamorphic flounder retina has a central cone (drawn in gray) surrounded by six uniformly spaced single cones. A2 A complete square mosaic unit characteristic of postmetamorphic flounder retina consists of a central single cone (drawn in gray) surrounded by four pairs of regularly spaced double cones. Note that we refer to this as a complete square mosaic unit for simplicity, although the ratio of double to single cones is actually 2:1 in the retina. A3 The gray circle indicates half of a double cone in a square mosaic, which is positioned very closely to one other cone (drawn in black above the gray circle) and more distantly from other neighbors. B1 Two adjacent hexagonal mosaic units are shown as found in pre-transitional flounder retina, with the two central cones marked by the gray circles. B2 Two adjacent square mosaic units, characteristic of postmetamorphic flounder retina, differ from the adjacent hexagonal units in B1 in that the central cones of the two mosaics, drawn in gray, are not nearest neighbors.

work in vertebrates in which protracted periods of retinal patterning allow greater resolution of the temporal relationships in photoreceptor differentiation.

Species that move to a new habitat as part of their life history typically encounter changes in their visual environment and remodel their retinas to accommodate such new visual conditions [Beaudet and Hawryshyn, 1999; Evans, 2004]. Because vision can be affected by photoreceptor arrangement and spectral sensitivities, remodeling these features might improve the match between visual function and habitat, as described extensively in a variety of teleost fish species. Teleost retinal remodeling includes several possible developmental changes: (1) new photoreceptor classes may be added by new cell addition or by changes in existing cone morphology, opsin chromophore, or opsin subtype [Evans and Fernald, 1993; Evans et al., 1993; Archer et al., 1995; Hope et al., 1998; Shand et al., 1999, 2002; Novales Flamarique, 2000; Zhang et al., 2000; Haacke et al., 2001; Chinen et al., 2003; Cheng and Novales Flamarique, 2004; Mader and Cameron, 2004; Takechi and Kawamura, 2005]; (2) cell classes may be lost by cell death [Bowmaker and Kunz, 1987; Beaudet et al., 1993; Novales Flamarique and Hawryshyn, 1996; Novales Flamarique, 2000; Deutschlander et al., 2001; Allison et al., 2003], or (3) cells might move in relation to one another to form different arrangements [Evans and Fernald, 1993; Shand et al., 1999; Haacke et al., 2001; Helvik et al., 2001]. In the winter flounder, Pseudopleuronectes americanus, photoreceptors change the peak wavelength sensitivity and physical arrangement when larvae settle into deeper water, providing an excellent model for examining the relationship between photoreceptor cell birth, opsin expression, and physical position.

P. americanus progresses from a pelagic bilaterally symmetric fish to a benthic flatfish approximately two months after hatching. During this metamorphosis, important changes in photoreceptor phenotypes occur as fish transition from a monochromatic to trichromatic visual system [Evans and Fernald, 1993]. Premetamorphic larval retinas contain one photoreceptor type arranged in a hexagonal array (fig. 1). Photoreceptors in larvae are middle-wavelength-sensitive cones (λmax 519 nm) [Evans et al., 1993] that express photopigment RH2 opsin [Mader and Cameron, 2004]. In contrast, retinas of post-metamorphic flounder contain rod photoreceptors as well as single and double cones with new opsin types expressed [Evans and Fernald, 1993]. Rods appear during metamorphosis and are inserted among cones without an obvious pattern, as is typical for teleosts retinas. The cones in a postmetamorphic retina form a square mosaic, which is a repeating array of double cones surrounding one single cone (fig. 1). The single cones absorb blue light (λmax 457 nm) [Evans et al., 1993] and express SWS2 opsin mRNA [Mader and Cameron, 2004]. One partner in the double cone is maximally sensitive to green light (λmax 531 nm) [Evans et al., 1993] and expresses RH2 opsin gene [Mader and Cameron, 2004], whereas the other member of the pair can be maximally sensitive to either the same (λmax 531 nm) or longer (λmax 547 nm) wavelengths [Evans et al., 1993] and express LWS opsin mRNA [Mader and Cameron, 2004]. Why the pre- and post-metamorphic cones that contain RH2 opsin have different spectral sensitivities is unknown [Mader and Cam-
er, 2004]. Neither microspectrophotometric study found evidence for mixing opsin types nor for changes in chromophore usage [Evans et al., 1993; Mader and Cameron, 2004]. Furthermore, no other RH2 opsin sequences or alternative splice forms were identified by PCR from larval and postmetamorphic tissue [Mader and Cameron, 2004; this paper and unpubl. results using four degenerate primer pairs]. Thus, during metamorphosis the hexagonal array of single cones becomes a square array of single and double cones with new opsin genes expressed, each with distinct absorbance peaks.

How does this dramatic retinal transformation occur? Postmetamorphic mosaics may be formed from existing larval cones or from new cones. Mosaic patterning could rely on mechanisms such as opsin expression directing mosaic position, mosaic position determining opsin subtype, or independent regulation of opsin expression and mosaic patterning. Based on light and electron microscopic analysis in metamorphic retina of another flatfish, Shand et al. [1999] found that individual cones fuse to form postmetamorphic double cones, but no studies have linked new cell addition, mosaic arrangement, and photopigment expression during metamorphosis. Here we analyze the retinas of flounder as the cone mosaic in their retina is transformed during metamorphosis from a hexagonal array with a single cone type to a square array with multiple cone types. For brevity we will refer to these as ‘transitional retinas’. We labeled dividing cells with tritiated (3H) thymidine prior to mosaic remodeling to determine whether existing cone photoreceptors change phenotype. We examined morphology and used in situ hybridization to identify mosaic type and opsin expression in transitional retinas to understand the sequence of transformation and determine whether mechanisms controlling cell birth, mosaic position, and opsin selection are coordinated or independent.

Materials and Methods

Animals
Treatment of animals was in compliance with protocols approved at the institutions providing the animals (University of Oregon, Eugene, Oreg.; Stanford University, Stanford, Calif.; Marine Biological Laboratory (MBL), Woods Hole, Mass., USA; Institute for Marine Bioscience, Halifax, Nova Scotia, Canada; Marine Science Center, New Brunswick, Canada). Flounder used for autoradiography and morphology were reared at Woods Hole Oceanographic Institute (WHOI). For cloning opsin cDNA, we used tissue from larval flounder reared in captivity (8–15 days post hatch (dph); a gift of S. Douglas, Institute for Marine Biosciences, National Research Council, Halifax, Nova Scotia) and from wild-caught postmetamorphic flounder (MBL). For in situ hybridization experiments, flounder were reared in captivity (a gift from M. Costello and L. Lush, Huntsman Marine Science Centre, St. Andrews, New Brunswick).

We studied animals at several developmental stages to investigate changes in retinal structure during metamorphosis. The developmental stage in winter flounder is most accurately predicted by animal size rather than true chronological age [Williams, 1902; Chambers and Leggett, 1987]. We used lens diameter to reflect animal size because it is straightforward to measure and provides a reliable estimate of body size, and hence developmental stage, in two independent analyses (data not shown). We measured the largest lens diameter in each series of retinal sections using previously calibrated microscope images (Image 1.5, Wayne Rasband, NIH) and checked this estimate by multiplying the number of serial sections that included the lens by the section thickness, which gave the same result (not shown).

Cone Mosaic Patterns and Morphology
We assessed cone morphology to determine the location of square mosaics and double cones within transitional retina. Wild-caught winter flounder from the Woods Hole, Mass. area were kept at the Environmental Systems Lab at WHOI until gravid. Gametes were taken and eggs fertilized, then eggs developed in hatching trays in running seawater (7–10°C) on a 14 h light:10 h dark cycle. After hatching, larvae were transferred to black plastic rearing tanks (10–20 l) and fed protozoa, rotifers, copepods or brine shrimp. We observed several cohorts of larvae through the stages of hatching until metamorphosis was complete and fixed the animals for 2–3 days in Bouin’s fluid [Humason, 1979] prior to tissue processing (lens diameters 68–150 μm, 1–120 dph, n = 102 of all stages; n = 20 transitional retina, 120–140 μm lens diameters).

Fixed flounder were dehydrated through an ethanol series, embedded in resin (Immunobed, Polysciences, Warminster, Pa., USA) and sectioned on a microtome at 2–3 μm through the dorsoventral axis. The lack of skeletal calcification in flounder larvae allowed sections to be made through the entire animal, simplifying tissue orientation, which can be complex due to eye migration at metamorphosis. We made coronal sections from the nasal to the temporal pole of the eye. Depending on where the parallel sections cut through the eyecup (fig. 2), tissue sections provided either tangential views in which cone mosaic patterns (i.e., hexagonal or square mosaic arrangements) were visible, or, more centrally, radial views in which cone morphology (presence of single or double cones) was assessed. In flounder eyes used to analyze mosaic patterns and for alkaline phosphatase in situ hybridization (described below), we determined nasotemporal and dorsoventral position of tissue sections by charting the progressive appearance of both landmarks within the eye (e.g., optic nerve) as well as external structures such as the brain or nose. We took photomicrographs with a Nikon Eclipse E600 Microscope (Nikon, Melville, N.Y., USA) and Micropublisher 5.0 RTV camera (QImaging, Burnaby, B.C., Canada) and calibrated them (QCapture-Pro, QImaging).

Autoradiography
To discover whether cones present in larvae are retained after metamorphosis as members of double cones in square mosaics, we labeled dividing cells prior to the metamorphic transition and
assessed the fate of these pre-transition cones following metamorphosis. We labeled proliferating cells by treating larvae with tritiated thymidine, which is incorporated into the DNA of mitotically active cells. We dehydrated \(^{3}H\)-thymidine \((1\ g/l\ 1\ mCi/ml\ \text{ICN Pharmaceuticals, Costa Mesa, Calif., USA})\) and then rehydrated with an equal volume of seawater. Larvae were immersed in the \(^{3}H\)-thymidine labeled seawater for 1 h, and then given two 30-min rinses in non-radioactive thymidine \((1\ g/l\ \text{in seawater, Sigma, St. Louis, Mo., USA})\) to clear any residual isotope. We sampled two animals immediately in each experiment, transferred the remainder to a separate container of fresh seawater, and reared them through metamorphosis \((n = 11\ \text{labeled pre-transition and reared until transition}).\)

We processed resin-embedded sections taken from radioactively labeled tissue (prepared as described above) for autoradiography by dipping the slides in photographic emulsion (Kodak NTB-2, VWR, Westchester, Pa., USA) and exposing the emulsion to radioactive emissions from the incorporated \(^{3}H\)-thymidine for several weeks in the dark at 4°C. Slides were developed (Kodak D-19 developer, VWR), fixed (Kodak Rapid fix, VWR), and stained with cresyl violet, a non-specific cellular stain.

We viewed retinal tissue sections at high magnification (Nikon Eclipse E600 Microscope and Micropublisher 5.0 RTV camera) and identified cells dividing at the time of radiolabeling by the presence of black silver grains in the emulsion above the cell body. We considered a cell labeled if it had at least 5 times the number of silver grains over the nucleus as compared with background areas. Retinal tissue taken from animals sampled immediately following radiolabeling showed the number and location of cells undergoing mitosis at the time of \(^{3}H\)-thymidine treatment. Retinal tissue from animals that survived for extended periods following \(^{3}H\)-thymidine incorporation had cells that may or may not have retained the label. For example, cells that stopped dividing and differentiated shortly after \(^{3}H\)-thymidine incorporation would have retained their label, whereas cells that underwent several mitotic divisions would have diluted the radiolabel to undetectable levels because the label was divided among all daughter cells. Thus radioactively labeled cells observed at long survival times represented the subset of dividing cells that differentiated shortly after \(^{3}H\)-thymidine exposure.

Winter Flounder Opsin cDNA Sequences

Because flounder opsin cDNA sequences were not available when we began these experiments, we isolated opsin cDNA sequences in larval and postmetamorphic flounder. We later found these to be 99% identical to published sequences in regions of overlap (GenBank accession numbers AY631037, AY631038, AY631039), and we follow the published nomenclature throughout [Mader and Cameron, 2004]. We identified additional nucleotide sequences from the 3’ untranslated region for LWS (GenBank accession number DQ225171).

To isolate opsin cDNA sequences, we performed degenerate reverse transcription polymerase chain reaction (RT-PCR) on mRNA extracted from retina of postmetamorphic flounder and from whole larvae. Larvae were flash-frozen whole in liquid nitrogen then thawed and stored for subsequent mRNA extraction (RNALater; Ambion, Austin, Tex., USA). We anesthetized postmetamorphic flounder in 0.3% MS-222 (Sigma) prior to rapid cervical transection. Eyes were removed, lenses dissected out, and complete eyecups frozen in liquid nitrogen for mRNA isolation. Larval and postmetamorphic tissue was disrupted in liquid nitrogen in a mortar and pestle. We then added lysis solution and extracted total RNA (MicroPoly(A)Pure kit; Ambion).

We designed PCR primers by first aligning fish cone opsin sequences [Clustal W; Chenna et al., 2003 and BlockMaker], then designing degenerate primers [CODEHOP; Rose et al., 1998; http://blocks.fhcrc.org/blocks/codehop.html]. We synthesized single-stranded cDNA (SUPERSCRIPT First-Strand Synthesis System for RT-PCR; Invitrogen, Carlsbad, Calif., USA) and performed PCR (upper primers 5’TACCGCAGCGCAACAAR-ARYTNGM or GCCATGCAGTGCTCTGYYGNCNGA with lower primer 5’TGGTGTTTCCTACAGCAGTATGATN (GGRTT)). We amplified RT-PCR reactions in a thermal cycler (PTC-100; MJ Research, Boston, Mass., USA; 35 cycles of 94°C 30 s denaturation, 55°C 1.5 min annealing, and 72°C 3 min extension). PCR products were purified (GENECLEAN; Qiogene,
in 30% sucrose at 4 °C overnight for cryoprotection, and sectioned.

We fixed whole flounder (lens diameters 132–143 μm, 42–56 dph) in formalin for 24 h prior to storage in saline, placed tissue at 40 °C or dried in a vacuum chamber for 1 h and processed immediately. We targeted an average size of 200 bp probe fragments for greater access to the target DNA in the tissue. We again precipitated probes and measured yield by comparison with RNA mass standards, then diluted probes in 1× hybridization buffer (Sigma) for long-term storage.

We performed in situ hybridization on retinal tissue from flounder in metamorphic transition to describe the general pattern of individual opsin expression throughout metamorphosing retinas. We generated specific RNA probes for in situ hybridization for all opsin sequences from TOPO TA templates purified using QIAprep Spin Miniprep Kit (Qiagen, Chatsworth, Calif., USA). Plasmids were digested using restriction enzymes, purified with GeneClean, then used as template in MAXIscript In Vitro Transcription Kit (Ambion) with either digoxigenin- or fluorescein-labeled UTP (Roche Molecular Biochemicals (Roche), Indianapolis, Ind., USA). RNA probes were precipitated prior to partial alkaline hydrolysis in 60 mM sodium carbonate and 40 mM sodium bicarbonate pH 10.2 at 60 °C. We targeted an average size of 200 bp probe fragments for greater access to the target RNA in the tissue. We again precipitated probes and measured yield by comparison with RNA mass standards, then diluted probes in 1× hybridization buffer (Sigma) for long-term storage.

We cloned and sequenced the 3′ untranslated region of all cone opsins to produce in situ hybridization probes to less conserved regions of the opsin gene to avoid cross-hybridization between opsin types. We used single-stranded cDNA templates in PCR amplifications with one specific opsin primer designed from our degenerate PCR sequences and an oligo(dT) adapter using the conditions described above. Amplified products were purified (GeneClean) and either sequenced directly or cloned for subsequent sequencing (TOPO TA cloning). Note that PCR amplifications from larval and postmetamorphic tissue spanning the complete RH2 opsin sequence yielded no differences in coding or untranslated regions between these stages of fish in which cone absorption spectra differ [Evans et al., 1993; Mader and Cameron, 2004]. Opsin probes used for in situ hybridization ranged in length from 300–600 bp.

In situ Hybridization: Alkaline Phosphatase Visualization

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Preliminary experiments verified that the double label protocol did not produce any cross-reaction between digoxigenin and fluorescein detection in postmetamorphic flounder retinas. Omission of digoxigenin-labeled probes, anti-digoxigenin antibody, the initial biotinyl tyramide step, or biotinylated Alexa546 eliminated fluorescence in the red channel without influencing fluorescein label. Conversely, omitting components important for fluorescein detection preserved Alexa546 localization while removing green fluorescence. In addition, we performed pairwise combinations of in situ hybridization probes to identify co-localization of any opsins in cones of transitional or postmetamorphic fish. RH2 and LWS opsins probes were never co-localized in the same cell with each other or with SWS2 opsin expression (not shown).

**Localization of Opsin Expression within Cone Photoreceptor Mosaics**

Because retinas from animals in metamorphic transition have some regions with hexagonal mosaics and some with square mosaics, we classified the mosaic type using tangential sections of transitional retinas and identified opsins expressed within each mosaic type based on confocal sections of retinas processed with double label fluorescent in situ hybridization. Images containing separate fluorescent signals were converted to false color (Adobe Photoshop 5, Adobe Systems, Inc., San Jose, Calif., USA) and merged to combine information on positions of cones expressing opsins typical of postmetamorphic flounder (pooled LWS and SWS2 probes) and opsins expressed in both pre- and postmetamorphic animals (RH2) [Mader and Cameron, 2004]. We converted images to grayscale for mosaic classification, and marked the locations of individual cells with circles. We then categorized mosaic type by analyzing the circles alone without the original photomicrograph using criteria described below. After mosaic classification, we compared the mosaic pattern with the original color image to determine where the opsins LWS/SWS2 (postmetamorphic) and RH2 (pre- and postmetamorphic) were expressed within the mosaic.

We based mosaic classifications on the distinct spatial differences between square and hexagonal mosaics as shown in figure 1. In an ideal hexagonal mosaic, all cone cells are equidistant from one another, and each cone can be considered as a central cone surrounded by six equally spaced neighbors (fig. 1, A1; one hexagonal ‘mosaic unit’). In a square mosaic on the other hand, cone cells have one of two possible positions: cones can be central, surrounded by four pairs of double cones (fig. 1, A2; one square mosaic unit), or they can be part of a double cone, in contact with its twin and more distant from the central cone or neighboring double cones (fig. 1, A3; one double cone).

Based on these differences in cone neighbor positions, we classified mosaics in transitional flounder retinal tissue processed for double in situ hybridization into four categories: hexagonal, partial hexagonal, partial square, and square. Distortions in sectioning caused an imperfect recreation of mosaics, so classification was based on both the single mosaic components (fig. 1, A1–A3) and on the presence of neighboring mosaic units (hexagonal, fig. 1, B1, and square, fig. 1, B2) using the following criteria:

- **Hexagonal**: Two adjacent hexagonal mosaic units (fig. 1, B1)
- **Partial Hexagonal**: No double cones (fig. 1, A3)

**Results**

**Locating Square Mosaic Initiation in Transitional Retina**

To discover how re-patterning in the retina proceeds, we classified mosaic type in tangential and radial views of retinas from transitional animals to compare the first regions of the retina that become organized into square mosaics with the first regions to show opsin expression characteristic of a postmetamorphic retina (described below). We used tangential views through cone inner and outer segments to assign mosaics as either square or hexagonal, and radial views through the same eye to confirm single or double cone morphology. We examined transitional retinas and in all cases found both regions of the retina with hexagonal mosaics and areas of square mosaic containing both double and single cones (fig. 2). Single cones arrayed in hexagonal mosaics were visible in the central retina, whereas square mosaics and corresponding double cones occurred near the margins of the eyes. Figure 2 includes photomicrographs of square mosaics near the nasal and temporal margins (square mosaics present at dorsal and ventral margins are not shown). Premetamorphic fish (lens diameters less than 115 μm) had pure hexagonal mosaics.

**Cell Addition in Transitional Retina**

To determine the origin of cells in the square mosaic in transitional eyes, we identified dividing cells using tri-tiated thymidine before retinal transition (68–120 μm lens diameters) and examined mosaic structure in cohort siblings either within one day or up to 84 days after label. Just after uptake of label, cells containing 3H-thymidine were restricted to the periphery of the retina, where precursor cells divide in the marginal germinal zone (fig. 3A).
Fig. 3. Photomicrographs taken from a cohort of flounder exposed to $^3$H-thymidine that survived for either 1 day (A), 11 days (B–D), or 57 days (F–H) demonstrate that the square cone mosaic formation includes photoreceptors added before retinal transformation. Panel A is a composite image (Image-Pro Express Version5) combining 4 focal planes; all other panels are unmanipulated. Scale bars = 10 μm. A Newly divided cells in the retinas of flounders that survived for 1 day are restricted to the peripheral margin of the retina, where the only cells labeled with $^3$H-thymidine are found (asterisk). The central retina has only single cones organized into hexagonal mosaics. We found no portions of the retina that had begun the transformation associated with metamorphosis. B, C For flounder surviving 11 days, newly differentiated cells labeled with $^3$H-thymidine (between dashed and solid lines) are visible adjacent to the peripheral margin (asterisk). Retinal transformation has just begun, with a small number of double cones visible central to the zone of $^3$H-thymidine labeling (outlined by box) that differentiated prior to labeling, and therefore prior to retinal transformation. The image in B is focused on the photoreceptors whereas that in C shows the overlying silver grains indicating the $^3$H-thymidine label. D, E Higher magnification image of box in B. For clarity, an example of a double cone is outlined in E in an otherwise identical image. F For animals surviving 57 days, postmitotic cells with $^3$H-thymidine label are seen in a band of cells (white arrowhead) central to the dividing marginal cells (asterisk). Black arrow marks the location of a double cone central to the band of $^3$H-thymidine labeling, an example of a double cone formed from cells that differentiated prior to retinal transformation. G, H Higher magnification photomicrograph of the retinal section shown in F. The micrograph in G shows the photoreceptors whereas that in H is focused on the silver grains of the $^3$H-thymidine label. Double cones (black arrow marks one example) can be seen in retina that is central to the $^3$H-thymidine label in the outer nuclear layer (white arrowhead). Gray arrowheads indicate the location of the $^3$H-thymidine band in the inner nuclear layer and ganglion cell layer.
Note that no double cones were present in these pre-transition eyes. By 57 days after label, a band of $^{3}$H-thymidine labeling was visible at a distance of 100 $\mu$m from the margin, comprised of cells that differentiated within a few cell divisions after the $^{3}$H-thymidine labeling (fig. 3B). Given that new cell addition occurs at the margin, cells peripheral to this band were born after labeling, whereas more central cells were born prior to labeling. We found unlabeled double cones central to this labeled band (fig. 3C, D) and thus concluded that these double cones likely differentiated as single cones prior to retinal transformation and subsequently altered their morphology and mosaic position as the retina reorganized.

**Localization of Opsin Gene Expression in Transitional Retina**

To determine whether onset of opsin genes expressed in postmetamorphic flounder (SWS2 and LWS) occurs with the same spatial pattern as the square mosaic formation, we localized RH2, LWS, and SWS2 opsin mRNA in transitional flounder retina using alkaline phosphatase in situ hybridization. We examined both eyes from two fish at three stages to generate a series of images showing the distribution of opsin mRNA expression throughout the transitional eye. Figure 4 shows representative adjacent sections from the youngest transitional fish. Fish at all ages showed the same pattern. RH2 opsin mRNA was present throughout the photoreceptor layer. In contrast, LWS and SWS2 opsin expression was more restricted. LWS and SWS2 opsin mRNA was expressed in two patches in the retina, one in the ventronasal region (fig. 4) and one in a more dorsal and temporal position (not shown). Note that LWS and SWS2 are absent from the temporal half in the ventral retina (fig. 4) including the region near the peripheral margin.

**Double in situ Hybridization to Localize Opsin Gene Expression within the Cone Mosaic**

To determine whether opsin expression changes before or after mosaic rearrangement, we classified mosaic type in tangential sections through transitional retinas...
(fig. 1) and identified the opsin genes expressed in each mosaic type. RH2 opsin-expressing cones are found in pre- and postmetamorphic flounder, but LWS and SWS2 opsin genes are expressed only in postmetamorphic flounder retina [Mader and Cameron, 2004]. Based on the alkaline phosphatase expression patterns described above, we expected that LWS and SWS2 opsin gene expression would be found in overlapping regions. Therefore, we combined probes for LWS and SWS2 opsin mRNAs and considered the combined expression indica-
tive of postmetamorphic opsin localization. Each transitional retina had both regions that were classified as square or partially square (similar to postmetamorphic mosaics) as well as regions that were hexagonal or partially hexagonal (similar to premetamorphic retina). We predicted that if opsin expression switches prior to mosaic formation, we would find hexagonal arrays with single cones expressing LWS and SWS2 opsin genes.

We compared mosaic type with opsin expression in 16 planes of section through 6 transitional eyes to determine whether opsin type changes before or after mosaic reorganization. Square or partial square mosaics had regular spacing of cones expressing LWS or SWS2 opsin mRNA (n = 8, not shown). Partial hexagonal mosaics had regularly spaced (n = 2; not shown) or scattered cones (n = 1; fig. 5C) with LWS or SWS2 opsin mRNA. One hexagonal mosaic contained only RH2-expressing cells (not shown) whereas others contained a single LWS- or SWS2-labeled cone in an otherwise RH2-containing region (n = 4; fig. 5A, B).

Discussion

We examined cone photoreceptor cell birth, mosaic arrangement, and opsin mRNA expression during retinal transformation in the metamorphic winter flounder to understand how cell birth and differentiation are coordinated to produce precise cone photoreceptor mosaics characteristic of adults. We found that cone mosaic transformation was initiated near the peripheral margins of the retina, and these square mosaics included cones produced prior to the onset of mosaic transformation. We also showed that expression of opsin mRNA types present in postmetamorphic but not premetamorphic retinas arose in two distinct patches in the retina. The expression of new opsin types occurred, in part, within cone mosaic regions that had not yet transformed to the square mosaic characteristic of postmetamorphic retinas. We conclude that retinal changes during metamorphosis are independently regulated; existing cone photoreceptors reorganize into new mosaic arrangements and switch their opsin expression with distinctly different spatiotemporal patterns (fig. 6).

We present our data in the framework of an emerging model of photoreceptor mosaic formation in which cone cell fate determines both initial opsin protein content and cone morphology as cells differentiate, but these features are plastic and may be changed [Szel et al., 1994; Archer et al., 1995; Hope et al., 1998; Shand et al., 1999; Zhang et al., 2000; Helvik et al., 2001; Cheng and Novales Flamarique, 2004; Cornish et al., 2004; Takechi and Kawamura, 2005]. Cell spacing in mosaics might proceed via tangential cell movement, perhaps employing differential adhesion between neighboring cells based on cell type to produce square or hexagonal mosaics [Mochizuki, 2002; Reese and Galli-Resta, 2002; Tohya et al., 2003]. Uncorporated or misplaced cells might be induced either to switch fate again or be eliminated by cell death, although we did not test these possibilities. We argue below that our results do not favor an alternative model in which nearest neighbor interactions determine cone cell fate [Raymond and Barthel, 2004].

Newly Formed Square Mosaics Incorporate Existing Cone Photoreceptors

Transformation of the cone mosaic from a hexagonal mosaic with one cone type in premetamorphic flounder to a square mosaic with three different cone types in postmetamorphic flounder could in principle occur by two mechanisms: reorganization of existing cones that switch phenotypes, or insertion of new cones throughout the retina. Phenotype switching would entail coordination of both morphological changes (formation of double cones, alteration in photoreceptor shape) and molecular changes (shifts in opsin gene expression). Individual photoreceptors alter opsin content throughout development in other fish [Hope et al., 1998; Cheng and Novales Flamarique, 2004], although we do not demonstrate opsin switching directly in this paper. Previous research in another flatfish favored the hypothesis that larval cones reorganize to form the square mosaic arrangement: changes in cone morphology, including appearance of chains of cones connected by subsurface cisternae, precede square mosaic formation in the sea bream [Shand et al., 1999]. Our data complement this evidence suggesting that double cones form by fusion of existing single cones. We labeled dividing cells using 3H-thymidine prior to retinal transformation then examined retinas after transformation using 3H-thymidine presence to identify cells that underwent terminal division shortly after labeling. These 3H-thymidine-labeled cells form a ring around the retina (a band in radial section) indicating the position of the peripheral margin, where cell division occurs, at the time of label. In radial sections through the retina, cones peripheral to this band are younger than the labeled cells as they divided after the incorporation of 3H-thymidine, whereas cones central to this band are older because they likely stopped dividing before exposure to 3H-thymidine label. The unlabeled double cones we found central to the
3H-thymidine band in these postmetamorphic fish had most likely differentiated first as single cones prior to retinal transformation, and later changed from single cones in a hexagonal mosaic to double cones in a square mosaic.

Our data are most consistent with a reorganization of existing cones during metamorphosis of flatfish to produce the postmetamorphic square mosaic, congruent with the conclusions of Shand et al. [1999] in another metamorphosing fish species and the generally accepted mechanism for retinal remodeling. There are two less likely alternative explanations for our data. First, it is possible that cells born in the periphery during retinal transformation move towards the central retina where they express SWS2 or LWS opsin mRNA and become double cones. This explanation is unlikely because a large number of cones would have to migrate long distances and because we would not expect the 3H-thymidine-labeled cells to form the compact band we observe if these cells had moved through this ring. Second, new cells could be born throughout the retina during metamorphosis and differentiate in situ as double cones containing LWS and RH2 opsins or as single cones expressing SWS2 opsin mRNA. We do see 3H-thymidine-positive cells in the outer nuclear layer, but these centrally dividing cells are restricted to the ventral retina and have the morphology of rods not cones [B. Evans and R. Fernald, unpubl. observ.], consistent with the addition of rods in the central retina of in adult teleosts [Johns and Fernald, 1981]. We conclude that the precise square mosaic pattern in the postmetamorphic flounder results from reorganization of existing larval cones during metamorphosis, a reorganization which necessitates changes in opsin expression, morphological changes such as the fusion of single cones to produce double cones, and short-distance migrations to align disorganized cells.

Photopigment Expression and Cell Position Are Independently Regulated

Are the reorganization of the cone mosaic and the changes in opsin expression during metamorphosis coordinated or are these events independently regulated?
We present two lines of evidence consistent with the hypothesis that cone photoreceptor opsin expression and cone mosaic arrangement occur independently during retinal transition. First, the earliest cones expressing LWS and SWS2 opsin mRNA occur in two patches in the ventronasal and dorsotemporal retina in all transitional animals. In contrast, square mosaic formation occurred in a continuous ring close to the retinal margin throughout the retina in flounder of equivalent developmental stages based on lens diameter. These distinct spatial patterns suggest that the onset of postmetamorphic cone opsin expression and square mosaic formation occur independently (fig. 6). Second, we show LWS/SWS2 opsin expression in regions of the retina containing cones arrayed in hexagonal patterns, a mosaic type which in early premetamorphic flounder only contains RH2 opsin mRNA [Evans et al., 1993; Mader and Cameron, 2004]. This observation precludes the possibility that square mosaic formation is a necessary prerequisite to LWS and SWS2 opsin expression, and thus does not support the idea that cone mosaic position dictates opsin protein expression. Conversely, the absence of LWS and SWS2 opsin expression in the ventrotemporal retina (fig. 4), or in the far ventral or dorsal retina (not shown) suggests that the square cone mosaics in these regions express only RH2 opsin mRNA, indicating that LWS and SWS2 opsin selection are unlikely to direct mosaic organization. Because our tangential sectioning was biased toward the central and nasal portions of the retina and confocal analyses biased toward regions with LWS or SWS2 expression, we did not directly demonstrate square mosaics expressing only RH2 opsin mRNA. Moreover, the absence of detectable in situ hybridization signal using LWS or SWS2 probes in a region does not prove the absence of low levels of opsin proteins in this region, and we did not examine whether earlier markers of early cone fate might be present. Thus, we conclude that cone cell fate could play a role in mosaic formation, but that opsin expression and mosaic position likely have separate regulatory influences.

Overall, our data suggest that the precise square mosaic of cones in postmetamorphic flounder arises without a rigid sequential series of cell-cell inductions such as that responsible for Drosophila ommatidia formation as proposed by previous researchers [e.g., Stenkamp and Cameron, 2002; Raymond and Barthel, 2004]. Not only do we support the conclusion that cells shift opsin content and alter cone type during metamorphic reorganization of the cone mosaic, but we also provide evidence that cell position within the mosaic is not the cue guiding opsin selection. Shand et al. [1999] similarly conclude that cone morphological development proceeds independently from cone mosaic formation in the black bream, and Helvik et al. [2001] show that opsins restricted to double cones in postmetamorphic halibut appear in single cones of larvae. Vertebrate cone mosaic formation, in contrast to the series of fixed near-neighbor inductions in fly ommatidial development, might require individual cones to alter gene expression while jostling for position in the crystalline array of receptors on the retina.

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