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## Photopigment gene expression and rhabdom formation in the crayfish (*Procambarus clarkii*)

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**Abstract** This study examines the expression of the photopigment gene in the developing retina of the freshwater crayfish *Procambarus clarkii* (Crustacea, Malacostraca, Decapoda). Both sense and anti-sense RNA probes were used for in situ hybridization (ISH) of whole embryos collected at various stages during development. A characteristic of retinal development is the formation of screening pigment in the retinular cells of the retinal ommatidia. This pigmentation is seen as a band that begins at the lateral side of the retinal field and progresses medially. At hatching the retina is approximately 50% pigmented. ISH of whole embryos shows that expression of the photopigment gene by the retinular cells correlates with the extent of the screening pigment band in the retina and with the presence of rhabdoms within the ommatidia. Sections taken through embryos after being hybridized indicate that staining is localized in the cytoplasm of the retinular cells and in the axonal region below the basement membrane. No staining reaction was seen in the rhabdoms of older ommatidia. ISH staining was also seen at the anterior midline of the protocerebrum where extraretinal photoreceptors have been reported. The data presented here show a close correlation of opsin expression within the retinular cells of the ommatidia and the formation of the very early rhabdoms, similar to *Drosophila*. The results will be discussed in relation to recent studies in *Drosophila* that suggest rhodopsin plays a role in effecting the organization of the terminal web-like cytoskeleton at the base of the developing rhabdom microvilli.

**Keywords** Photopigment · Retina · Development · Gene · Crayfish · *Procambarus clarkii* (Crustacea)

### Introduction

In the arthropod compound eye the retina is composed of thousands of light-receptive units called ommatidia. Each ommatidium is composed of cone cells forming a lens-like structure that sits above a cluster of retinular cells whose microvilli contribute to the formation of the photopigment-containing rhabdom. Analysis of arthropod evolutionary relationships using the numerous morphological studies of adult insect and crustacean compound eyes has led to the conclusion that there is a high degree of conservation and homology in the visual systems of these two arthropod groups (Nilsson and Osario 1997; Paulus 1979, 2000). Further insight into the relation of insects and crustaceans has been gained from recent developmental studies of the visual system in a few crustaceans. These studies have reinforced the idea of conservation of the fundamental processes and patterns of retina and visual neuropil formation among the arthropods (Hafner and Tokarski 1998, 2001; Harzsch et al. 1999; Harzsch and Dawirs 1995/1996; Melzer et al. 2000; Harzsch and Wallossek 2001).

Among the arthropods, the general developmental process can have multiple larval stages or develop directly from the egg to a juvenile with no larval stages. Decapod crustaceans like the crayfish differ from *Drosophila* in that overall development is direct and they have a closed rhabdom organization rather than an open one. However, many features of early retinal development in Decapod crustaceans are similar to those of *Drosophila* (Hafner and Tokarski 1998).

The formation of the retina and eyestalk in the crayfish (*Procambarus clarkii*) has recently been described by Hafner and Tokarski (1998). The eyes develop from the paired optic primordia located at the ventral anterior end of the embryo. The retina is formed from the proliferation and differentiation of cells in the surface ectoderm covering the optic primordia. In this surface layer, postmitotic cells organize into rows of ommatidial cell clusters. During the differentiation of the ommatidia, both the retinular cells and the distal pigment cells form

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dark screening pigment within their cytoplasm. Initially, the pigmentation forms an arc-shaped band at the lateral edge of the developing retina (Hafner et al. 1982). This band is visible at about the 75% stage of embryonic development, and a rhabdom is present in ommatidia of this region. However, the younger non-pigmented region ahead of this band contains the very earliest rhabdoms. At hatching, the eye of the PO1 stage contains a pigment band covering slightly less than one-half of the retina. The retina completes its development during the subsequent postembryonic stages and becomes completely pigmented (Hafner et al. 1982; Sandeman and Sandeman 1991; Hafner and Tokarski 1998).

During formation of the rhabdom in the crayfish (*Procambarus clarkii*), Hafner et al. (1991, 1992) reported that the initial microvilli formed along the surface of the retinal cell contain an extensive core of actin filaments that extend into the cytoplasm at the base of the microvilli as rootlets. Also, zonula adherens junctions at the edge of the rhabdom link the cells making up each ommatidium, and these junctions contain actin filaments in their plaques. The zonula adherens junctions expand from distal to proximal ahead of new microvilli that will form the rhabdom (Hafner et al. 1993). The actin in the junctional complex and the actin filaments of the rootlets form a terminal web-like domain at the base of the microvilli in the crayfish.

A fundamental process of retinal development and photoreceptor differentiation in all animals is the synthesis of visual pigment opsin protein and its incorporation into the specialized light-receptive membranes. The molecular genetic events involved in retinal development and rhodopsin gene expression have been most extensively studied in *Drosophila* (Kumar and Ready 1995; Desplan 1997; Kumar et al. 1997; Triesmann 1999; Chang and Ready 2000; Friedrich and Benzer 2000). In *Drosophila*, rhodopsin 1 (rh1) gene expression is correlated with rhabdom microvillus formation during pupation. The expression of this gene also appears to be regulated by the highly conserved Pax 6 gene (Sheng et al. 1997), and the timing of its expression plays a critical role in photoreceptor morphogenesis (Kumar and Ready 1995; Kumar et al. 1997). Recently, Chang and Ready (2000) reported that during retinal formation in *Drosophila*, rhodopsin regulates the organization of the cytoskeletal network at the base of the rhabdomere by interacting with a Rho GTPase in the cytoskeletal complex. This regulatory role causes increased bundling of the actin filaments at the base of the rhabdomere and is important in preventing the collapse of the cytoskeletal network and the associated microvilli. Such a collapse of the rhabdomere is characteristic of the *Drosophila nina E* mutant.

In decapod crustaceans, the primary structure of the photopigment from *Procambarus clarkii* has been reported by Hariyama et al. (1993). The photopigment has a maximum absorbance at 530 nm (Goldsmith 1978; Cronin and Goldsmith 1982). Molecular evolution of the visual pigments of freshwater crayfish indicates that

there is little variation in the maximum wavelength (522–530 nm) among species from different genera and families (Crandall and Cronin 1997). This opsin is also most similar to the major *Drosophila* opsin (rh1).

Immunolocalization of antibodies to crayfish photopigment (*Cherax*) by de Couet and Sigmond (1985) showed staining primarily in the rhabdom regions of adult retinas. In addition, Sandeman et al. (1990) used the same antibody to identify a cluster of extraretinal photoreceptors in the brain of *Cherax*.

The combined data from *Drosophila* and crustacean retinal morphogenesis, briefly reviewed here, indicates many similarities in the organization and dynamics of the cytoskeleton in the developing rhabdom. These data raise the further question of the role of photopigment gene expression in crustacean rhabdom formation. In order to begin to answer this question, this study examines rhodopsin expression in the crayfish and its relation to retinal morphogenesis. It also establishes a basis for comparing the fundamental process of photopigment gene expression between major invertebrate groups that differ in both their general development and their rhabdom structure.

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## Materials and methods

A 900-bp photopigment probe from *Procambarus alleni* was kindly supplied by Dr. Keith Crandall (Department of Zoology, Brigham Young University, Provo, UT). Anti-sense and sense cRNA probes were transcribed from a linearized DNA template and labeled with digoxigenin (DIG) using a DIG RNA-labeling kit with Sp6 and T7 RNA polymerases (Roche Molecular Biochemicals, Indianapolis, IN).

In situ hybridization (ISH) was initially done on frozen sections of adult eyes and then applied to whole embryos of various ages using a modified procedure of Abzhanov and Kaufman (2000) (see also Rogers et al. 1997). Initial fixation of whole embryos was with 4% paraformaldehyde in 0.1 M phosphate buffer with 0.03% Tx100 for 30 min. After initial fixation, the yolk was removed from the embryos and they were placed in double aldehyde fixative (1% paraformaldehyde, 1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4) for 1 h at RT. Embryos were dehydrated in a methanol series and stored in 100% methanol at  $-20^{\circ}\text{C}$ .

For ISH, embryos were rehydrated to phosphate-buffered Tween (PBT), treated for 20–30 min with proteinase K (25  $\mu\text{g}/\text{ml}$ ), washed in buffer and postfixed in 4% paraformaldehyde. After buffer washes, embryos were preincubated in hybridization buffer (HB) at  $65^{\circ}\text{C}$  for 1–2 h (no probe). The prehybridization buffer was removed and HB plus probe was added and incubated 16–24 h at  $65^{\circ}\text{C}$  in sealed Eppendorf tubes. Hybridized embryos were washed seven times with warm HB and the last wash left overnight at  $65^{\circ}\text{C}$ . Embryos were washed two more times in HB followed by three maleic acid buffer washes and two 10-min washes in blocking buffer (Roche Molecular Biochemicals Digoxigenin labeling kit). Embryos were incubated in anti-dig antibody solution 1:1,500 in blocking buffer plus 1% goat serum and 0.1% Tween 20 for 2 h at RT, followed by buffer washes and colored using NTB/BCIP. Staining was monitored and stopped by washing in maleic acid buffer. Whole embryos were mounted in glycerol.

In order to examine the distribution of hybridization reaction cytologically, hybridized embryos with a positive alkaline phosphatase color reaction were postfixed in double aldehyde and embedded in Micro Bed (EM Science, Washington, PA). One-mi-

cron-thick sections were cut. Some sections were stained with toluidine blue and others examined unstained with either brightfield or phase-contrast light microscopy.

## Results

One measure of ommatidium differentiation in the crayfish retina is the appearance and progressive expansion of a pigment band in the retina. The pigmentation is the result of pigment granule formation in the reticular cell cytoplasm and the distal screening pigment cells of the retina. Because ommatidia form first at the lateral edge of the optic primordia and expand in a medial direction, the most mature and heavily pigmented ommatidia are found at this lateral region of the retina. This is also the region where pigmentation is first seen. The relation of pigmentation to retinal development can be seen in Fig. 1. This toluidine-blue-stained thick section shows heavy pigmentation in the cytoplasm of reticular cells forming ommatidia in the lateral and posterior region of the retina (R). In this region distinct rhabdoms (arrows) are present in the ommatidia. Pigmentation decreases and is absent in the less mature region of the retina to the right. In this region rhabdoms are not resolvable at the LM level. However, earlier work (Hafner et al. 1982, 1991) has shown that the earliest, thin rhabdoms are present here.

Figure 2 shows an anterior view of a PO1 stage hatching in which the retinas are nearly 50% pigmented. This animal was fixed for ISH and stored in 100% methanol but not processed for ISH. The extent of the pigmentation varies in relation to the percent of development. Note that retinal development is not completed at hatching. Pigmentation covers only the lateral and posterior region of the retina, while the medial portion of the retina remains non-pigmented.

Figure 3 is the no-probe, control condition processed through the ISH procedure. Here, exposure to the ISH procedure fortuitously extracts the screening pigment from the retina. This 95% embryo, seen from a dorsal view, has only a pale brownish color where the screening pigment was located in the retina. In separate control conditions using a sense RNA probe and where the coloring agent was omitted the retinas appear similar to Fig. 3.

### Correlation of ISH staining and screening pigment development

ISH of whole crayfish embryos using an anti-sense RNA photopigment probe shows photopigment gene expression localized in the most mature region of the developing retina. Although non-specific reaction is present in the legs and body of the embryos with this procedure, no background was seen in the retina or neuropils of the protocerebrum. In Fig. 4, photopigment gene expression in the retina closely correlates with the region of the reti-

na where ommatidia contain screening pigment. The similarity in the extent of ISH staining and the screening pigment band in the retina is seen by comparing Figs. 3 and 4. In these two 95% embryos, the stained region in Fig. 4 has nearly the same extent as the light brown extracted pigment band in the control. In earlier embryonic stages, the extent of the staining decreases (Fig. 5). In this dorsal view, staining occurs in the posterior and lateral retina but has not progressed as far anterior or medial. The less mature ommatidia that exist anterior and medial to the pigmented region do not appear to be stained, but it is difficult to determine the precise point where the stain ends.

### Cytological distribution of ISH

After ISH, sections of embryos embedded in Microbed (EM Sciences) show the distribution of staining within the embryonic eye. The phase-contrast image of the embryonic retina in Fig. 6 shows staining in the cytoplasm along the sides of the rhabdoms and around the reticular cell nuclei. After the ISH procedure, the absence of staining in the rhabdoms (arrows) where mRNA is not expected to be present serves as an internal control for the specificity of the stain. Staining is also seen below the basement membrane of the retina in the region of the reticular cell axons. This latter result is similar to the staining pattern in frozen sections of adult eyes, using a photopigment gene DNA probe. In this case staining was also found in the axons below the basement membrane of the retina (results not shown). Also in Fig. 6, staining intensity decreases and ends in the less mature portion of the retina medial to where rhabdoms are visible. No staining is seen in the cone cells near the corneal surface or in the lamina neuropil below the retina.

### Extraretinal photoreceptors

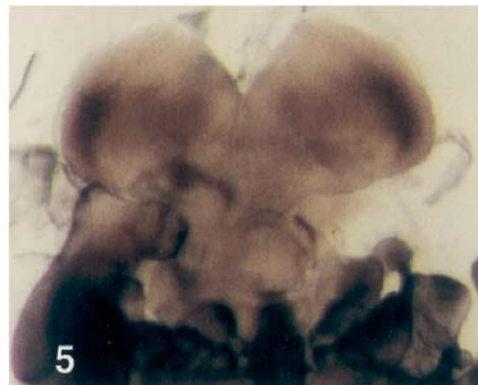
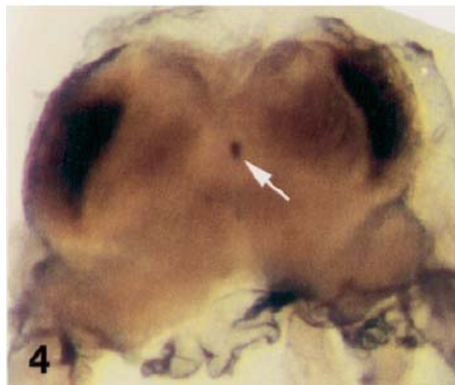
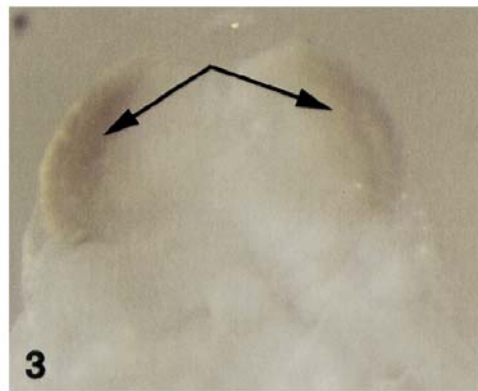
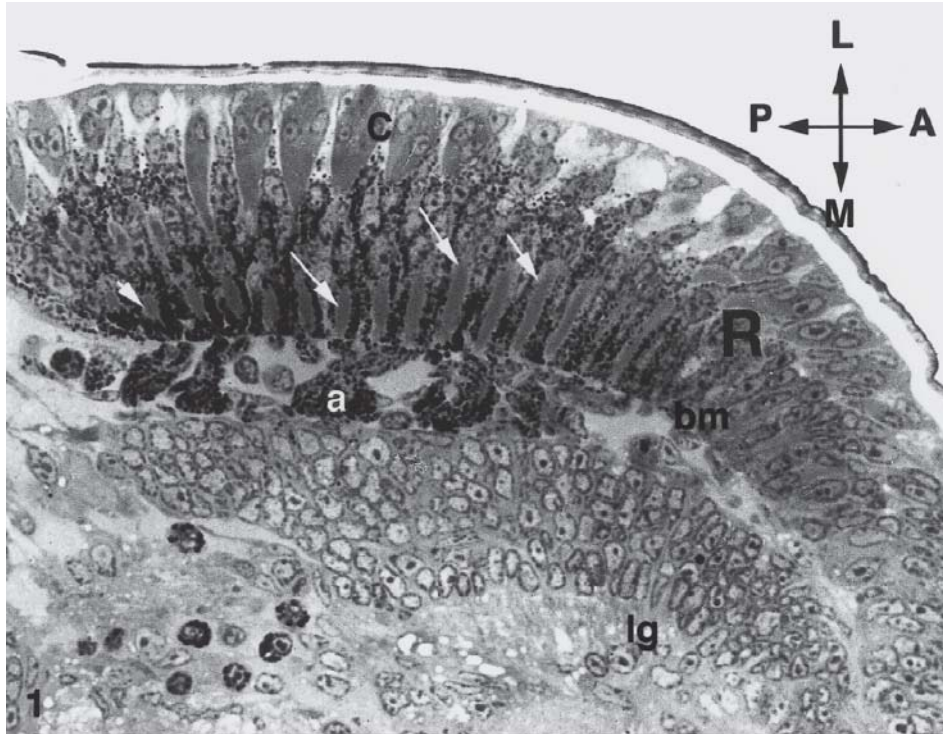
In addition to the retinal staining a small positively stained region was consistently seen at the anterior midline of the protocerebrum between the eyes (Fig. 4). Staining in this region is absent in all control conditions (Fig. 3) and in the earlier embryonic stages such as the 80–85% embryo (Fig. 5). One-micron sections through this region were not able to resolve a stained region similar to that seen in the ISH whole mounts.

## Discussion

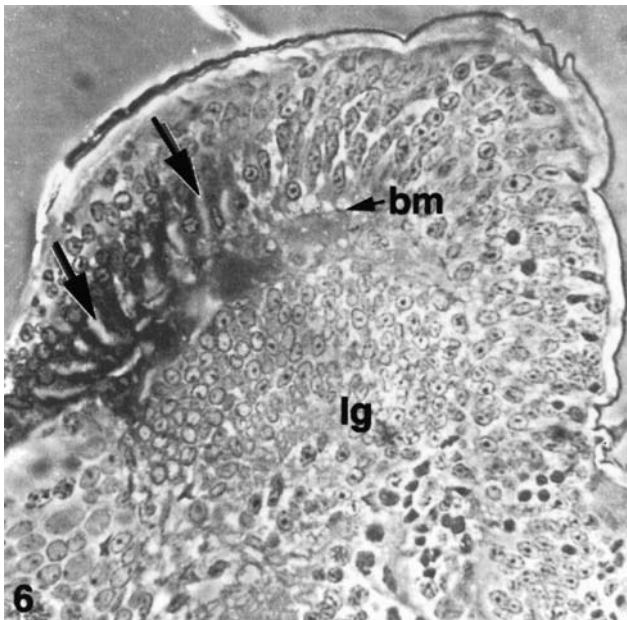
The ISH data from embryos of freshwater crayfish (*Procambarus clarkii*) show that the expression pattern of the photopigment gene in whole-mounts generally correlates with the development of screening pigment within the reticular cells of the ommatidia. The formation of both screening pigment in the cytoplasm and the

rhabdom are later events in the differentiation of the photoreceptor cells. Screening pigment appears shortly after the rhabdom begins to form but may initially not be evident as a pigment band in retinal whole-mounts.

These data also show that photopigment gene expression does not occur in the early cell clusters found at the leading edge of the forming retina or in non-photoreceptor cell types within the ommatidium.



## Rhabdom morphogenesis and photopigment expression



**Fig. 6** Phase-contrast image of a section from a PO1 stage embryo processed for ISH with an anti-sense RNA probe. The posterior and lateral portion of the retina shows dark staining in the reticular cell cytoplasm surrounding the rhabdoms (*arrows*). Rhabdoms are not stained and appear clear. The less mature retina adjacent to the rhabdom region is more lightly stained, and no rhabdom profiles are visible. Staining is also seen below the basement membrane (*bm*) (*lg* lamina ganglionaris neuropil).  $\times 512$

In the crayfish, rhabdom microvilli form along the reticular cell surfaces that face the common central space within each ommatidial cell cluster. This process begins at the distal end of the reticular cells and progresses proximally preceded by the expansion of the zonula adherens junctions between the reticular cells (Hafner et al. 1991, 1992, 1993; Hafner and Tokarski 1998). Initially, microvilli of the reticular cells interdigitate to form a loosely organized rhabdom. These very early rhabdoms, which measure approx.  $0.9 \mu\text{m}$  in diameter or smaller, are present before there is any evidence of screening pigment formation. The microvilli in these rhabdoms contain an actin cytoskeletal core that extends as a rootlet into the cytoplasm at the base of the microvilli. As the microvilli of the rhabdom become more organized, the core actin filaments decrease in number (Hafner et al. 1982, 1991, 1992; Hafner and Tokarski 1998). Because of the expected low levels of expression of photopigment in these unpigmented ommatidia where the very earliest rhabdom microvilli are forming, the present data cannot determine definitively if photopigment gene expression occurs in these earliest rhabdoms. However, based on the sectioned material from the ISH, it seems likely that the initial timing of the expression is closely related to the formation of rhabdom microvilli.

In *Drosophila*, rhabdom morphogenesis begins at 55% of pupal life (Waddington and Perry 1960; Longley and Ready 1995). At this time the apical surface of the photoreceptors begins to show a thin fringe of short microvilli adjacent to the interrhabdomeral space. By 67% of pupal life rhabdomeral microvilli have lengthened and formed an organized brush border. The first expression of rh1 is weakly detected at 60–72% of pupal life (Kumar and Ready 1995; Sheng et al. 1997). Similar results were also found for all other rhodopsins (Sheng et al. 1997). Expression of rh 1 is not detected in the third larval instar where early ommatidial cell clusters are present.

Comparing data from the crayfish and *Drosophila*, the timing of the expression in both cases corresponds closely, if not precisely, with the formation of rhabdom microvilli. In both cases elements of an elaborate cytoskeleton within and at the base of the microvilli have been reported in adult and embryonic retinas (Hafner et al. 1991, 1992; Arikawa et al. 1990; Williams 1991). Recently, Chang and Ready (2000) demonstrated that the timing of rhodopsin expression is critical because of its role in effecting the cytoskeletal organization at the base of the microvilli by interacting with the Rho GTPase, Drac 1. The present expression pattern of rhodopsin in the developing crayfish retina combined with the presence of a dynamic cytoskeletal organization at the base of the microvilli suggests that rhodopsin expression in crayfish is timed closely to the formation of the rhabdom microvilli. In this case, the orthogonal organization of rhabdomeres in the crayfish rhabdom requires an alternating pattern of microvillus formation along each reticular cell surface.

◀ **Fig. 1** Toluidine-blue-stained thick section through the retina (*R*) of a PO1 crayfish hatchling. The oldest and most mature region of the retina is at the *left* and is indicated by the dense-screening pigment within the reticular cell cytoplasm. A distal crystalline cone layer (*C*) is present above the rhabdom layer in this region (*arrows* rhabdoms). Progressing to the *right*, screening pigment in the ommatidia decreases and the rhabdoms are smaller and eventually become visible only at the EM level. The extent of the pigmented ommatidia in this section is equivalent to the pigmented region seen in Fig. 2 (*lg* lamina ganglionaris, *bm* basement membrane, *a* reticular cell axons containing screening pigment).  $\times 320$

**Fig. 2** Anterior view of PO1 stage crayfish hatchling showing partial pigmentation of the eyes, present in the lateral and posterior portion of the retinas. This control animal was not processed for ISH. The screening pigment is localized in the distal screening pigment cells and the reticular cell cytoplasm.  $\times 52$

**Fig. 3** Dorsal view of a 95% crayfish embryo processed for ISH with no anti-DIG antibody. This control condition shows that the ISH procedure has extracted the screening pigment in the retina leaving the pigmented region (*arrows*) light brown.  $\times 52$

**Fig. 4** Dorsal view of a PO1 crayfish hatchling stained with the antisense RNA photopigment probe. Staining is seen in the same portion of the retina occupied by the screening pigment. At the midline between the eyes is a small positively staining region within the protocerebrum (*arrow*). This region is similar in location to a cluster of extraretinal photoreceptors previously identified in the crayfish *Cherax* (Sandeman et al. 1990).  $\times 52$

**Fig. 5** Dorsal view of an 80–85% embryo stained with an antisense RNA photopigment probe. Staining is present in the more lateral and posterior region of the retina but has not progressed as far anterior as seen in older animals (Fig. 4). Also note that at this earlier stage no staining is seen between the eyes in the protocerebrum.  $\times 52$

What causes selective microvillus formation along the cell surface and what role rhodopsin might play in such a system remains to be determined.

#### Photopigment synthesis in reticular cell axons

In the crayfish, the extent of the expression pattern both in the developing retina and in the adult retina indicates that opsin is not only synthesized in the perinuclear and paraxonal cytoplasm but also in the proximal portions of the axons below the basement membrane. In earlier autoradiographic studies of opsin synthesis in the crayfish using both amino acid and sugar precursors, Hafner and Bok (1977) and Hafner (1984) consistently observed labeling in the region of the reticular cell axons below the basement membrane. In view of these earlier results and the present ISH staining in this region, it seems that photopigment synthesis may also take place in the distal region of the reticular cell axons.

In the adult brachyuran crab (*Hemigrapsus*), Sakamoto et al. (1996) determined the primary structures of two opsins. Using ISH, RNA probes of both opsins were localized to the reticular cell cytoplasm of ommatidia but not in the rhabdoms. No indication of staining in the axons was mentioned.

#### Extraretinal photoreceptors

The ISH data also shows a positive staining in the anterior midline region of the protocerebral ganglion. This region has been described in the crayfish species *Cherax* as containing a pigmented cluster of extraretinal photoreceptors that stain with an antibody made to crayfish retina photopigment (Sandeman et al. 1990). While the ISH staining in the protocerebral ganglion may be related to the expression of the photopigment gene in these cells, no similar staining was seen in the caudal photoreceptor, whose location in the sixth abdominal ganglion has been characterized by Wilkins and Larimer (1972, 1976). The lack of staining in the ganglion could be due to a difference in maturation rate between the rostral and caudal ends of the embryo (Scholtz 1992) or inadequate penetration of the probe into the ganglion containing these cells.

This study of photopigment gene expression in the developing crayfish retina indicates, not unexpectedly, that expression of this gene is confined to the reticular cells that form the rhabdom. It also provides further evidence that photopigment synthesis also occurs in the reticular cell axons below the basement membrane as well as in the cell cytoplasm adjacent to the rhabdom. In addition, photopigment gene expression correlates closely with the formation of the rhabdom, but further studies are needed to determine the precise timing of this relationship. Although the picture is incomplete, comparison of the initial data from the crayfish with the role and expression of rh1 in *Drosophila* tentatively supports the

hypothesis that this fundamental process is conserved among the arthropods.

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