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Rhodopsin patterning in central photoreceptor cells of the blowfly *Calliphora vicina*: cloning and characterization of *Calliphora* rhodopsins Rh3, Rh5 and Rh6

Angelika Schmitt, Andreas Vogt, Katrin Friedmann, Reinhard Paulsen and Armin Huber*

Institut für Zoologie, Universität Karlsruhe, Haid-und-Neu-Strasse 9, 76131 Karlsruhe, Germany

*Author for correspondence (e-mail: Armin.Huber@bio.uka.de)

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Summary

The ommatidia that constitute the compound eyes of flies contain eight photoreceptor cells, which are divided into two classes: the peripheral photoreceptors, R1–6, and the central photoreceptors, R7 and R8. In the fruit fly, *Drosophila*, R1–6 express the same rhodopsin (Rh1), whilst the R7 and R8 of a given ommatidium express either Rh3 and Rh5, or Rh4 and Rh6, respectively. We have studied whether this expression pattern of rhodopsins is conserved in the blowfly *Calliphora vicina*. We have cloned three novel *Calliphora* rhodopsins, which are homologues of *Drosophila* Rh3, Rh5 and Rh6, with an amino acid sequence identity of 80.7%, 60.9% and 86.1%, respectively. Immunocytochemical studies with antibodies specific for Rh3, Rh5 and Rh6 revealed that Rh3 is expressed in a subset of R7 cells, while Rh5 and Rh6 are expressed in a non-overlapping subset of R8 cells. Rh3 and Rh5 are present in most cases in the same ommatidia, which account for approximately 27% of all ommatidia, and Rh6 is found in the complementary 73%. The similarity of the rhodopsin expression pattern of *Calliphora* with that of *Drosophila* suggests that the developmental mechanism regulating the terminal differentiation of R7 and R8 cells are highly conserved between these fly species.

Key words: *Calliphora, Drosophila*, pattern formation, photoreceptor, rhodopsin, vision.

Introduction

The compound eyes of flies, in particular the fruitfly *Drosophila melanogaster*, the housefly *Musca domestica*, and the blowfly *Calliphora vicina,* represent widely recognised model systems for the analysis of various aspects of vision, for example, eye development, photoreceptor differentiation, visual transduction and visual information processing (Salcedo et al., 1999; Hardie and Raghu, 2001; Huber, 2001; Wernet and Desplan, 2004). The construction unit of compound eyes is the ommatidium, which in flies contains eight photoreceptor cells. The principal organelle of a photoreceptor is the rhabdomere, which is a stack of microvilli that contain the visual pigment and where phototransduction occurs. The set of rhabdomeres of the photoreceptors in an ommatidium is called the rhabdom. Flies have an open rhabdom, because the rhabdomeres of a fly ommatidium are separate. The rhabdomeres of photoreceptor cells R1–6 are long cylinders, spanning the full length of the retina, which are arranged in a trapezoidal pattern. The rhabdomeres of R7 and R8 are in the centre of the trapezoid. They are positioned in tandem, with R7 taking the apical and R8 the basal part, and their summed length equals the length of the R1–6 rhabdomeres.

The photoreceptor cells R1–6 and R7/R8 constitute photoreceptor classes with distinct visual functions. Anatomical, physiological and behavioural investigations suggested that photoreceptors R1–6 provide the sensory input for high sensitivity vision, image formation and motion detection (Hardie, 1985; Pichaud et al., 1999; Wernet and Desplan, 2004). The R1–6 cells express the same rhodopsin, designated Rh1, which has been cloned from *Drosophila* as well as from *Calliphora* (O'Tousa et al., 1985; Zuker et al., 1985; Huber et al., 1990). Rh1 represents over 90% of the visual pigment present in the compound eye (Hamdorf et al., 1973; Hardie, 1985; Paulsen, 1984; Huber et al., 1990; Salcedo et al., 1999).

The organization of the central photoreceptor cells R7 and R8 is considerably more complex. Kirschfeld and Franceschini (1977) first found two populations of R7 in eye-cup preparations of the housefly *Musca,* observed in transmitted light: yellow (R7y) and pale or colourless (R7p). The yellow colour is due to the presence of a blue-absorbing, carotenoid pigment in the rhabdomere of R7y, which occurs in 70% of the ommatidia; 30% has R7p. The two classes of ommatidia are distributed randomly across the eye. This is also readily recognized by epifluorescence, as the 7y rhabdomeres fluoresce bright green under blue excitation, while the R7p rhabdomeres appear black. Further microspectrophotometry and electrophysiological recordings demonstrated that the R7s were accompanied by specific R8s, thus called R8y and R8p, respectively (Hardie, 1985).

Cloning of rhodopsins Rh3–Rh6 expressed in R7 and R8

cells of the compound eye of *Drosophila* provided a means of determining in which combinations these rhodopsins are present in the various types of ommatidia (Montell et al., 1987; Fryxell and Meyerowitz, 1987; Zuker et al., 1987; Chou et al., 1996; Huber et al., 1997; Papatsenko et al., 1997). Studies using specific antibodies against *Drosophila* rhodopsins revealed that the expression of rhodopsin Rh3 in an R7 cell is coupled to the expression of Rh5 in the adjacent R8 cell, while Rh4 colocalizes with Rh6 (Chou et al., 1996, 1999; Papatsenko et al., 1997). The Rh3/Rh5 and Rh4/Rh6 ommatidia are distributed randomly in the compound eye at a ratio of about 29:71%, very similar to the ratio of ommatidia with R7p and R7y in *Musca* (Chou et al., 1999). Behavioural studies indicate that the two sets of central photoreceptors mediate colour discrimination (Troje, 1993; Pichaud et al., 1999). In the dorsal rim region of the eye, the ommatidia of *Musca* have specialized R7 and R8 photoreceptors, which are distinguishable by the larger diameter of their rhabdomeres, which are UV sensitive. This also holds for the ommatidia of the dorsal rim region of *Drosophila*, where both R7 and R8 cells express the UV rhodopsin Rh3 (Chou et al., 1999; Wernet et al., 2003). The central photoreceptors in the dorsal rim are believed to mediate detection of polarized light, in which the perpendicular arrangement of the microvilli of R7 and R8 cells plays an important role (Hardie, 1984; Wernet et al., 2003). Finally, unequivocally described for males of *Musca domestica* only, the R7 cells in the anterodorsal region of the compound eyes exhibit the same red fluorescence as R1–6 photoreceptors, because they also express Rh1. The R7 photoreceptors, called R7r, add their light signal to the R1–6 system, presumably to improve the male's chasing behaviour. The male dorsal eye area thus is called the 'love spot' (Franceschini et al., 1981; Hardie et al., 1981).

Despite the structural and functional equivalence, which is to be expected for photoreceptor classes R1–6 and R7/R8, there are some differences between the compound eyes of *Drosophila, Musca* and *Calliphora*. For example, while the eyes of *Drosophila* are composed of about 750 ommatidia, *Musca* has about 3000 and *Calliphora* has about 5200 (Beersma et al., 1977). The eyes of male *Calliphora* are much bigger than those of the female, which results in a much higher acuity in the dorsal area but not in a much higher number of ommatidia. The arrangement of optical axes, and accordingly, the spatial resolution as well as temporal photoreceptor properties are not homogeneously distributed over the ommatidial lattice of the *Calliphora* compound eye (Petrowitz et al., 2000; Burton et al., 2001). Similar properties have not yet been reported for the *Drosophila* eye. Also, there are spectral differences between the central photoreceptors of *Drosophila* (Salcedo et al., 1999) and those of *Musca* and *Calliphora* (Hardie, 1985), which we further explore in this paper.

We report the cloning of three rhodopsins expressed in the central photoreceptor cells of the compound eye of *Calliphora vicina*. We have determined the spatial distribution of these rhodopsins and the relative abundance of the ommatidia with R7y and R7p. Our results reveal a high degree of conservation between *Calliphora* and *Drosophila* in the rhodopsin expression pattern of the central photoreceptor cells.

Materials and methods

Fly stocks

Male and female *Calliphora vicina* Meig., *chalky* mutant were propagated at 25°C in a 12 h:12 h light:dark cycle and were used for the experiments at an age of 4–10 days after eclosion.

cDNA-library screening and DNA sequencing

An oligo(dT)-primed cDNA library in UniZap XR vector (Stratagene, Amsterdam, The Netherlands) produced from poly(A)$^+$ RNA isolated from retinas of *Calliphora vicina* (Huber et al., 1996) was used for the isolation of cDNA clones encoding *CvRh3*, *CvRh5* and *CvRh6*. Screening of this cDNA library was carried out using a mixture of cDNAs encoding *Drosophila melanogaster* rhodopsins (Rh3, Rh4, Rh5 and Rh6). The cDNAs were labelled with digoxigenin using the DIG DNA Labelling Mix (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instructions. The hybridization was performed in 5× SSC (1× SSC is 0.15 mol l^{-1} NaCl, 0.015 mol l^{-1} sodium citrate, pH 7.0), 0.1% laurylsarcosinate, 0.02% SDS, 1% blocking reagent (Roche Molecular Biochemicals, Mannheim, Germany) at 55°C according to standard protocols (Sambrook and Russell, 2001). Positive clones were rescreened and plasmid DNA was obtained by *in vivo* excision. DNA sequencing was carried out with an Alf-Express automated DNA sequencer (Amersham Biosciences, Freiburg, Germany) using Cy5-labelled oligonucleotide primers and the dideoxy chain termination method (Sanger et al., 1977). Different clones and subclones for each *Calliphora* rhodopsin were sequenced to obtain at least twofold coverage of each sequence.

Antibodies

Polyclonal antibodies against *Calliphora* Rh3 and Rh5 were generated against synthetic peptides coupled to keyhole limpet hemocyanin by Seqlab (Göttingen, Germany). The peptides used were based on the deduced amino acid sequence of the *Calliphora* rhodopsins: CNEKAPEASSTASTTG, corresponding to amino acids 360–374 of CvRh3, and CRERNYAASSSGGDNA corresponding to amino acids 363–377 of CvRh5. Monoclonal anti-DmRh6 antibodies that cross reacted with *Calliphora* Rh6 have been described previously (Chou et al., 1999).

Detection of rhabdomere autofluorescence

Autofluorescence analysis of R7y and R7p rhabdomeres was carried out by optical neutralisation of the cornea, essentially as described by Hardie et al. (1981). *Calliphora* heads were dissected and submerged under water in a Petri dish. Autofluorescence of central rhabdomeres was detected using a water immersion objective (Leica 20× / 0.50) and epi-

Table 1. *Similarity of* Calliphora *and* Drosophila *rhodopsins*

	CvRh1	CvRh3	CvRh5	CvRh6
DmRh1	**85.5**	34.5	31.0	51.1
DmRh3	36.3	**80.7**	40.4	30.6
DmRh4	36.4	70.5	43.0	32.3
DmRh5	32.0	42.9	**60.9**	31.5
DmRh6	52.5	30.2	28.4	**86.1**

Values are percentage amino acid identity; values for the corresponding rhodopsins from each species are in bold type.
Cv, *Calliphora vicina*: Dm, *Drosophila melanogaster*.

illumination with blue light (450–490 nm) from a Hg lamp (HBO100W), or by confocal microscopy after excitation with 476 nm and 488 nm laser light. To calculate the ratio of R7y to R7p receptors, randomly chosen areas of the compound eyes of male *Calliphora* were investigated for the presence (R7y) or absence (R7p) of green fluorescence in the central rhabdomeres. Depending on the region of the eye investigated, between 50 and 130 ommatidia were evaluated each time. Images were obtained with a Leica DC 200 camera and were processed with PhotoShop 6.0.

Immunocytochemistry

For immunocytochemical localization of *Calliphora* rhodopsins by confocal laser scanning microscopy, dissected eyes of *Calliphora* were fixed in 2% paraformaldehyde in PBS (137 mmol l^{-1} NaCl, 3 mmol l^{-1} KCl, 8 mmol l^{-1} Na_2HPO_4, 2 mmol l^{-1} KH_2PO_4, pH 7.2) for 2 h at room temperature, followed by three washes in 10%, 25% and 50% sucrose in 0.1 mol l^{-1} sodium phosphate buffer, and infiltration with 50% sucrose in 0.1 mol l^{-1} sodium phosphate buffer overnight at 4°C. The eyes were embedded in boiled bovine liver, covered with Tissue Tek and cryofixed in melting isopentane. Eyes were sectioned at 10 µm (cross sections) or 18 µm (longitudinal sections) in a cryostat at –25°C and placed on coverslips pre-coated with 0.01% aqueous poly-L-lysine (Sigma, Deisenhofen, Germany). The cryosections were fixed with 2% paraformaldehyde in 0.1 mol l^{-1} sodium phosphate buffer for 20 min, followed by two subsequent washes with 0.1 mol l^{-1} sodium phosphate buffer. For antibody staining, the sections were incubated in 0.01% Saponine in 0.1 mol l^{-1} sodium phosphate buffer (pH 7.2) for 2.5 h at room temperature, washed three times with 0.1 mol l^{-1} sodium phosphate buffer and incubated with the primary antibody diluted 1:20 in blocking solution (0.5% ovalbumin, 0.1% cold-water fish gelatine in 0.1 mol l^{-1} sodium phosphate buffer, pH 7.2) overnight at 4°C. To block antibody reactions, diluted antibodies were preincubated with 15–25 µg ml^{-1} of the corresponding peptides for 4 h at 4°C before they were applied to the sections. After incubation with antibodies the sections were washed three times in 0.1 mol l^{-1} sodium phosphate buffer and then incubated with goat anti-rabbit AlexaFluor 488 and goat anti-mouse AlexaFluor 660 antibodies (Invitrogen, Karlsruhe, Germany) diluted in blocking solution. For a concomitant staining of the photoreceptor rhabdomeres, the sections were incubated together with the secondary antibody with 0.5 pg $µl^{-1}$ rhodamine-coupled phalloidin (Sigma-Aldrich, Taufkirchen, Germany). The sections were finally washed in 0.1 mol l^{-1} sodium phosphate buffer, mounted in mowiol 4.88 and examined with a confocal laser scanning microscope (TCS-SP, Leica, Bensheim, Germany).

To obtain isolated *Calliphora* rhabdoms dissected retinas were slowly and repeatedly pipetted in 75 µl distilled water through a fine pipette (Paulsen, 1984). Rhabdoms were immediately transferred to coverslips pre-coated with 0.01% aqueous poly-L-lysine. After allowing the rhabdoms to dry on the coverslip for 30 min at 30°C, immunocytochemical detection of rhodopsins was carried out as described above for cryosections. In addition, rhabdoms were labelled with Oregon Green-coupled wheat germ agglutinin (Invitrogen, Karlsruhe, Germany), which was added together with the secondary antibody at a final concentration of 20 µg ml^{-1}.

Results

Cloning and analysis of Calliphora vicina *rhodopsins*

In order to isolate cDNA clones encoding rhodopsins of central photoreceptor cells, a *Calliphora* eye cDNA library was screened with a cDNA mixture of *Drosophila melanogaster* rhodopsins Rh3, Rh4, Rh5 and Rh6. Sequencing of the obtained *Calliphora* clones revealed that there were three different rhodopsins. By comparing the amino acid sequences they were found to correspond to Rh3, Rh5 and Rh6 rhodopsin of *Drosophila* (see Table 1). While the longest clones for Rh3 and Rh6 encompassed the entire coding region, the initially isolated Rh5 clone lacked the N-terminal portion of the coding region. Subsequent screening of the cDNA-library with a CvRh5-specific probe resulted in the isolation of a Rh5 clone, from which the missing N-terminal portion of this rhodopsin could be deduced. Out of about 30 rhodopsin clones analysed none corresponded to *Drosophila* Rh4 rhodopsin. The amino acid identity between the corresponding *Calliphora* and *Drosophila* rhodopsins is higher than 80% for Rh3 and Rh6, but for Rh5 it is only 60.9% (Table 1). Fig. 1 shows an alignment of the amino acid sequences of *Drosophila* and *Calliphora* rhodopsins, including the previously described *Calliphora* Rh1 (Huber et al., 1990). The newly isolated *Calliphora* Rh3, Rh5 and Rh6 are composed of 389, 388 and 369 amino acids, respectively, and display typical hallmarks of rhodopsins, such as a highly conserved lysine residue in the seventh transmembrane region, to which the retinal chromophore is attached; putative phosphorylation sites (Ser, Thr) near the C terminus; putative *N*-glycosylation sites (Asn-Xaa-Ser/Thr) near the N terminus, and two cysteine residues in the first and second extracellular loop, which may form a disulphide bond. In the third cytoplasmatic loop, which has been implicated in rhodopsin–G-protein interaction, a His-Glu-Lys (HEK) motif at the beginning of a conserved region is characteristic for most insect rhodopsins (Gärtner, 2000). Interestingly, the HEK motif is modified to His-Glu-Gln in

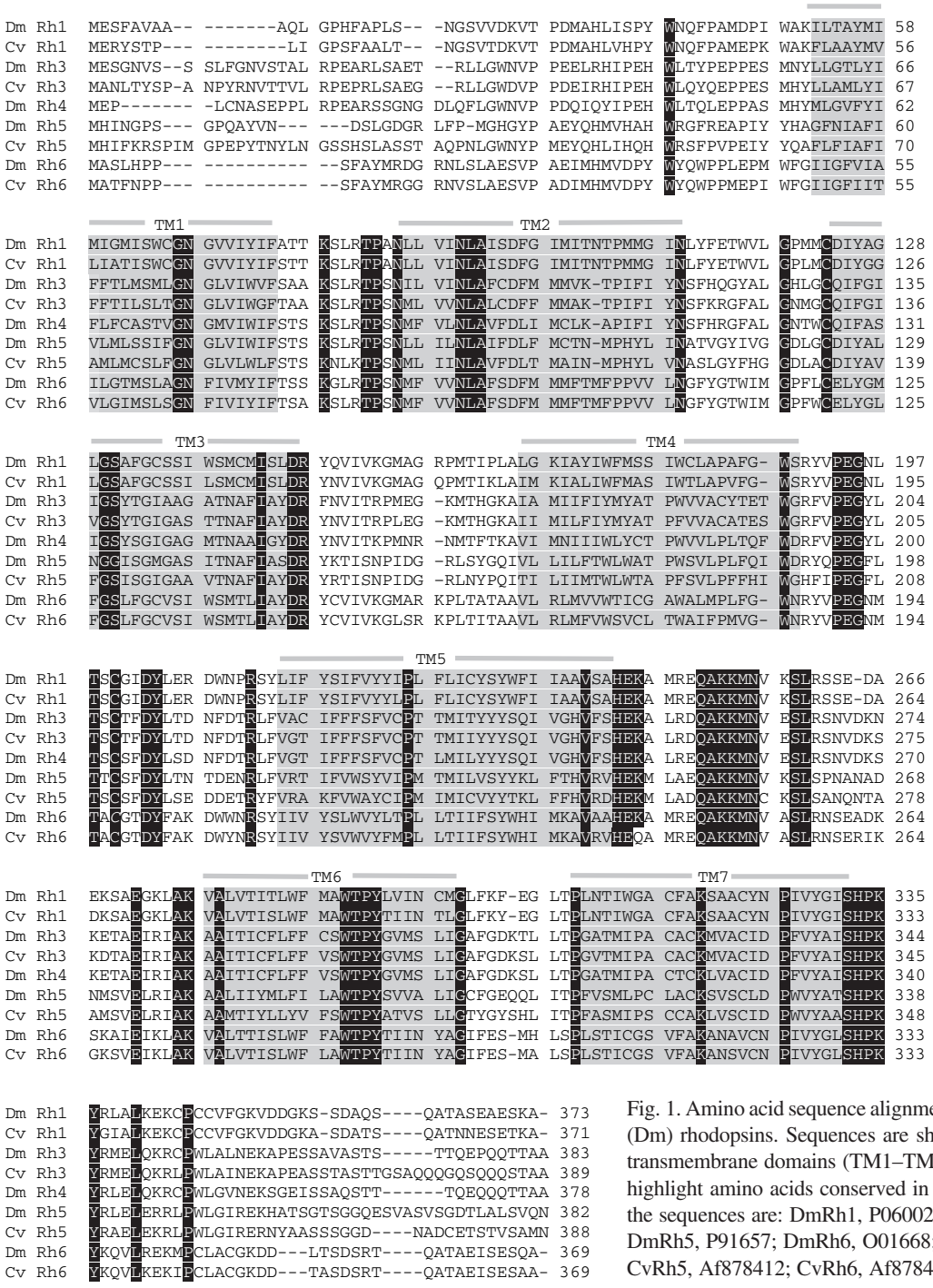

Fig. 1. Amino acid sequence alignment of *Calliphora* (Cv) and *Drosophila* (Dm) rhodopsins. Sequences are shown in single letter code. The seven transmembrane domains (TM1–TM7) are indicated in grey. Black boxes highlight amino acids conserved in all sequences. Accession numbers of the sequences are: DmRh1, P06002; DmRh3, P04950; DmRh4, P08225; DmRh5, P91657; DmRh6, O01668; CvRh1, J05596; CvRh3, Af878411; CvRh5, Af878412; CvRh6, Af878413.

Calliphora Rh6. Other insect rhodopsins in which the lysine residue of the HEK motif is substituted include two *Manduca sexta* rhodopins (Chase et al., 1997) and two *Anopheles gambiae* rhodopsins (Ano14521 and Ano01498; Hill et al., 2002).

Distribution of Calliphora *rhodopsins in the compound eye*

In larger flies, especially in *Musca*, the distribution of different types of R7 and R8 photoreceptor cells across the compound eye has been mapped precisely by evaluating the autofluorescence of the rhabdomeres (Hardie et al., 1981; Franceschini et al., 1981). Application of this method to the *Calliphora* chalky mutant revealed that autofluorescence of R7y (green fluorescence) and R1–6 rhabdomeres (red fluorescence) could be easily detected using water immersion objectives combined with conventional fluorescence microscopy or confocal laser scanning microscopy. Fig. 2 shows a fluorescence image taken from the frontal region of the compound eye, which harbours R7y and R7p rhabdomeres. To determine the ratio of R7y to R7p photoreceptors 20 images

obtained from different eyes and eye regions were analysed. The determined ratio, 68±5% R7y and 32±5% R7p, was similar to the ratio previously described for Musca (70% R7y, 30% R7p; Hardie et al., 1981). As has been shown for *Musca*, no obvious symmetry or conservation in the distribution of R7y and R7p photoreceptors between different eyes was observed. Contrary to the observations made for male *Musca*, we did not detect any evidence for a 'love spot' in the frontal dorsal region of the male *Calliphora* compound eye, which would be expected to contain red fluorescing R7 cells.

In order to compare the ratio of R7y to R7p photoreceptor cells with the expression pattern of *Calliphora* rhodopsins we employed immunocytochemistry. We used a monoclonal antibody directed against *Drosophila* Rh6, which cross-reacted with *Calliphora* Rh6 rhodopsin in sections through *Calliphora* compound eyes. The peptide used to make this antibody corresponds to amino acids 348–362 of *Drosophila* Rh6 rhodopsin, a region that is identical between *Calliphora* and *Drosophila* Rh6 rhodopsin, except for two amino acids (see Fig. 1). The cross reaction of this antibody is specific for *Calliphora* Rh6, because the reaction can be blocked with a peptide corresponding to the relevant region of *Calliphora* Rh6 (Fig. 3). As antibodies directed against rhodopsins Rh3 and Rh5 of *Drosophila* (Chou et al., 1999) showed no reaction with the corresponding *Calliphora* proteins we generated polyclonal antibodies against peptides of the C-terminal region of these *Calliphora* rhodopsins. To test the specificity of the newly generated anti-CvRh3 and anti-CvRh5 the corresponding peptides were dot blotted and incubated with the antibodies. Anti-Rh3 and anti-Rh5 reacted specifically with their corresponding peptides (not shown). In addition, immunofluorescence signals obtained with anti-Rh3 and anti-Rh5 on sections through *Calliphora* eyes could efficiently be blocked with the corresponding peptides (Fig. 3). The anti-CvRh5 antiserum reacted also with cell nuclei. The reaction of this antibody with nuclei was not specific for Rh5 rhodopsin as it could not be blocked with the Rh5 peptide. With these antibodies in hand double labelling studies of longitudinal and of cross sections through *Calliphora* eyes were carried out. For identification of the rhabdomeres the actin cytoskeleton of the rhabdomeres was also stained with rhodamin-coupled phalloidin (Fig. 4). Longitudinal sections probed with anti-Rh5 and anti-Rh6 demonstrated the localization of these rhodopsins in the basal part of the retina (Fig. 4A), suggesting that they are expressed in R8 cells. This expression pattern is in line with the initial characterization of these visual pigments as rhodopsins Rh5 and Rh6 on the basis of their homology to *Drosophila* rhodopsins, because *Drosophila* Rh5 and Rh6 are expressed in R8 cells, too (Chou et al., 1999). As is the case for *Drosophila* Rh5 and Rh6, the corresponding *Calliphora* rhodopsins are expressed in mutually exclusive sets of R8. This can best be observed in cross sections at the R8 cell level (Fig. 4B) which clearly reveal that the central rhabdomere is stained either with the anti-Rh5 or with the anti-Rh6 antibody. Double labelling experiments with antibodies directed against *Calliphora* Rh3 and Rh6 (Fig. 4C,D) showed that Rh3 is located mainly in the apical portion of the retina, suggesting its expression in R7 cells. However, in some ommatidia anti-Rh3 labelling was observed additionally in the basal portion of the retina, which may indicate that some R7 cells extend into the layer of R8 cells. Analysis of cross sections at the R7 cell level showed that the central rhabdomeres of some ommatidia are labelled by anti-Rh3 antibody while others are not. The ommatidia not labelled by anti-Rh3 are likely to express another rhodopsin in R7 cells, presumably *Calliphora* Rh4. In addition to the rhabdomeres, the rhabdomeral stalks are also labelled by anti-Rh3, anti-Rh5 and anti-Rh6 antibodies, indicating that these rhodopsins are not restricted to the rhabdomeres. However,

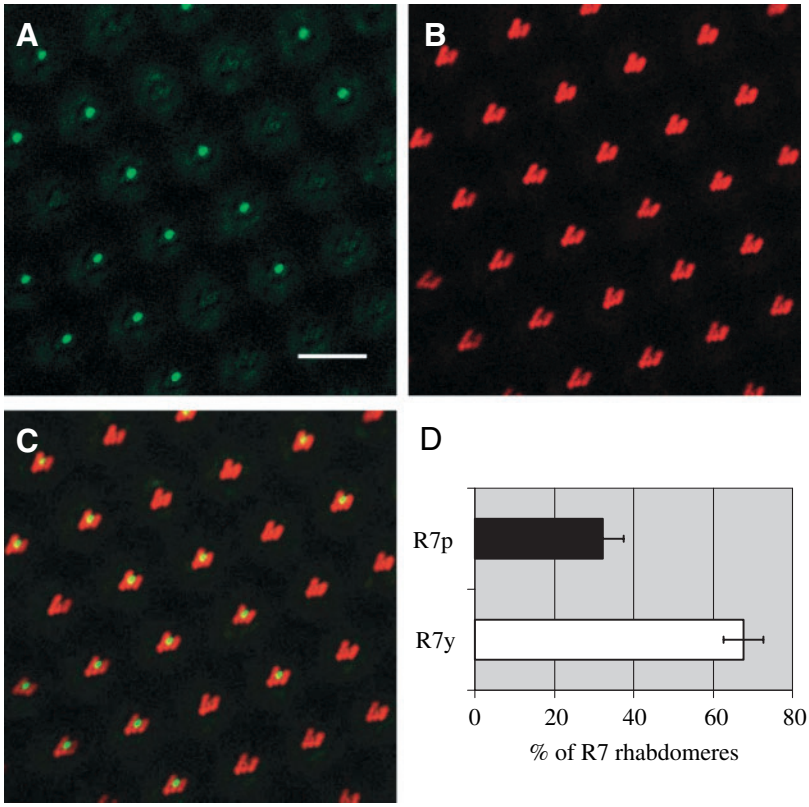

Fig. 2. Autofluorescence of *Calliphora* rhabdomeres. Confocal images of a frontal region of the intact *Calliphora* compound eye after simultaneous excitation with 476 nm and 488 nm wavelength laser light show (A) green fluorescence of R7y rhabdomeres and (B) red fluorescence of R1–6 rhabdomeres. (C) Overlay of A and B. A 20×/0.50 water immersion objective was used on a Leica TCS-SP confocal microscope. Scale bar, 20 μm. (D) Ratio of R7p to R7y rhabdomeres. Mean values were determined by evaluating the fluorescence pattern of randomly chosen regions comprising between 55 and 136 ommatidia from 20 *Calliphora* compound eyes. Bars are ± S.D.

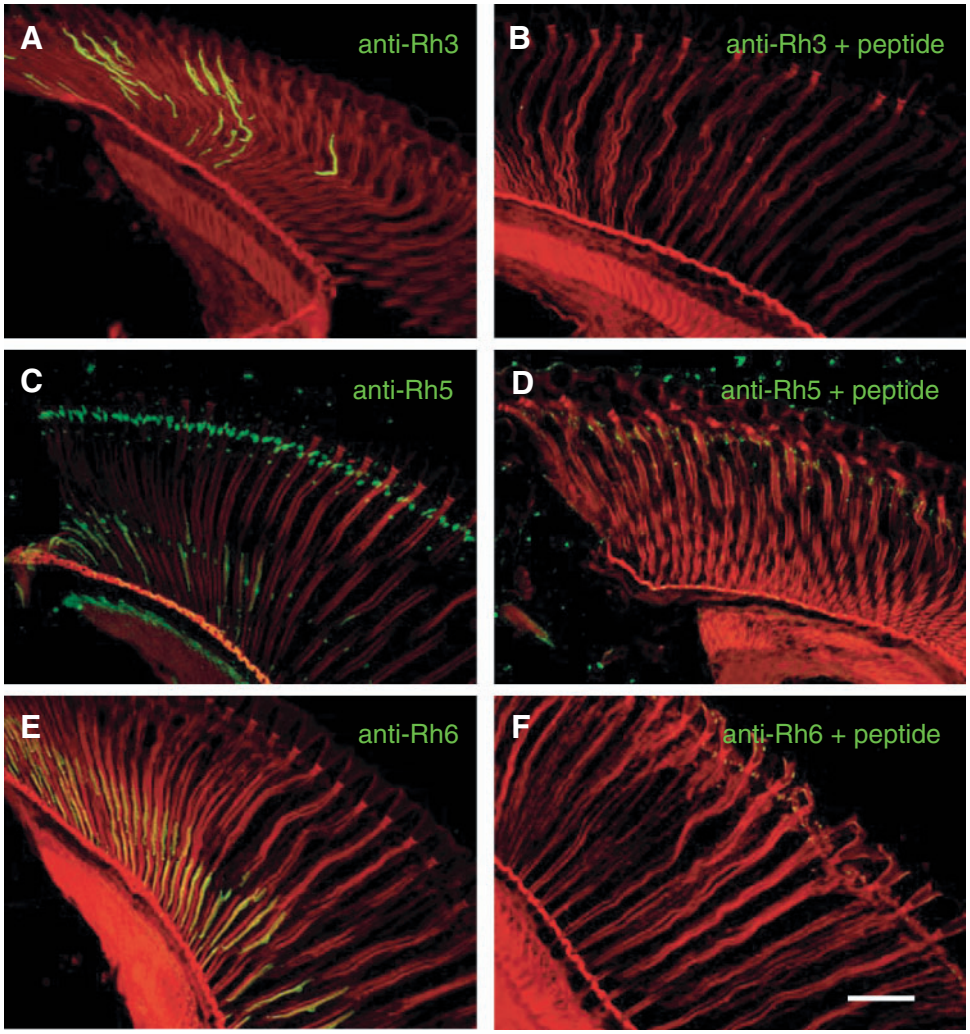

Fig. 3. Specific immunolabelling of *Calliphora* rhodopsins Rh3, Rh5 and Rh6. Longitudinal cryosections through the compound eye of *Calliphora* were probed with antibodies directed against *Calliphora* Rh3 (A,B), *Calliphora* Rh5 (C,D) and *Drosophila* Rh6 (E,F) in the absence or presence of peptides as indicated. The peptides used to block the reaction with *Calliphora* Rh3 (25 μg ml^{-1}) and Rh5 (15 μg ml^{-1}) were the same as used to generate the polyclonal antibodies. Reaction of the monoclonal anti-*Drosophila* Rh6 antibody was blocked with a peptide of the C-terminal region of *Calliphora* Rh6 rhodopsin (CGKDDTASDSRTQATA, 25 μg ml^{-1}). Indirect immunofluorescence of antibodies is shown in green. Red fluorescence: labelling of actin filaments with rhodamine-coupled phalloidin. Scale bar, 80 μm.

Rh3, Rh5 and Rh6 rhodopsin are not detected in the entire plasma membrane of the photoreceptor cells. It is likely that adherens junctions (organized by the protein Crumbs), which separate the rhabdomeral stalk from the basolateral membrane region of the photoreceptor cells (Pellikka et al., 2002; Izaddoost et al., 2002), form a barrier that cannot be crossed by the rhodopsin molecules incorporated in the rhabdomeral part of the plasma membrane.

The *Drosophila* rhodopsins Rh3/Rh5 and Rh4/Rh6 are expressed in matched pairs randomly distributed across the eye, such that ≈29% of the ommatidia contain the combination Rh3/Rh5 and 71% contain Rh4/Rh6 (Chou et al., 1999). To determine the relative abundance of Rh5- and Rh6-expressing photoreceptor cells in the *Calliphora* compound eye, we isolated rhabdoms, stained them with anti-Rh5 and anti-Rh6 antibodies, and counted the number of rhabdoms containing Rh5 or Rh6 (Fig. 5). In females 28.05±1.79% of the labelled rhabdoms contained Rh5 and 71.95±1.79% contained Rh6. In males similar values (24.95±2.47% Rh5, 75.05±2.47% Rh6) were obtained, indicating that the sexual dimorphism that results in larger eyes in the males does not affect the expression ratio of Rh5 and Rh6. The determined ratio is similar to that in *Drosophila*, i.e. roughly 30% of the rhabdoms contain Rh5 and about 70% contain Rh6. This ratio is also in line with the relative abundance of the two types of *Calliphora* ommatidia, containing either R7p/R8p or R7y/R8y photoreceptor cells (see Fig. 2). In order to be able to count the number of rhabdoms containing Rh3, isolated rhabdoms were incubated with anti-Rh3 antibodies and counterstained with Oregon Green-coupled wheat germ agglutinin. In females 39.16±8.42% and in males 37.58±7.05% of the total number of rhabdoms contained Rh3.

Discussion

Calliphora *rhodopsins*

In this paper we describe three novel cDNAs encoding rhodopsins of the blowfly *Calliphora vicina*. Based on the similarity of the deduced amino acid sequence with *Drosophila* rhodopsins, the newly cloned *Calliphora* rhodopsins correspond to the R7 cell rhodopsin Rh3 and to two R8 cell rhodopsins, Rh5 and Rh6 of *Drosophila*. For screening a *Calliphora* eye cDNA library we used a mixture of cDNA probes derived from *Drosophila* Rh3, Rh4, Rh5 and Rh6 clones. This approach should potentially allow for the isolation of the homologues of all four *Drosophila* rhodopsins present in central photoreceptor cells. However, a clone corresponding to Rh4 could not be isolated with this approach, or using *Drosophila* Rh4 alone as a probe. A formal possibility for the failure to isolate Rh4 would be that *Calliphora* expresses only one rhodopsin (Rh3) in R7 cells. Differences in the

physiological characteristics of R7p and R7y could be due to the presence or absence of screening and sensitizing pigments rather than to differential rhodopsin gene expression. Indeed, R7y photoreceptors contain screening and sensitizing pigments whereas R7p photoreceptors do not, and the yellow appearance of R7y has been attributed to the presence of a photostable blue-absorbing pigment (Kirschfeld et al., 1978; Hardie, 1985). Expression of the same rhodopsin in different spectral receptors has been reported for the violet and ultraviolet receptors of the butterfly *Papilio xuthus*, as well as for a long-wavelength absorbing rhodopsin of the small white butterfly, *Pieris rapae* (Kitamoto et al., 2000; Wakakuwa et al., 2004). However, microspectrophotometric analysis of R7 cells suggested the presence of two distinct photoconvertible visual pigments (Hardie, 1985; Hardie and Kirschfeld, 1983; McIntyre and Kirschfeld, 1981) in the two types of R7 cells. In addition, the immunocytochemical analysis presented here clearly showed that Rh3 is not expressed in all R7 cells (see Fig. 4D). Thus, as is the case for *Drosophila* the central photoreceptor cells of *Calliphora* most probably expresses a total of four distinct rhodopsin genes.

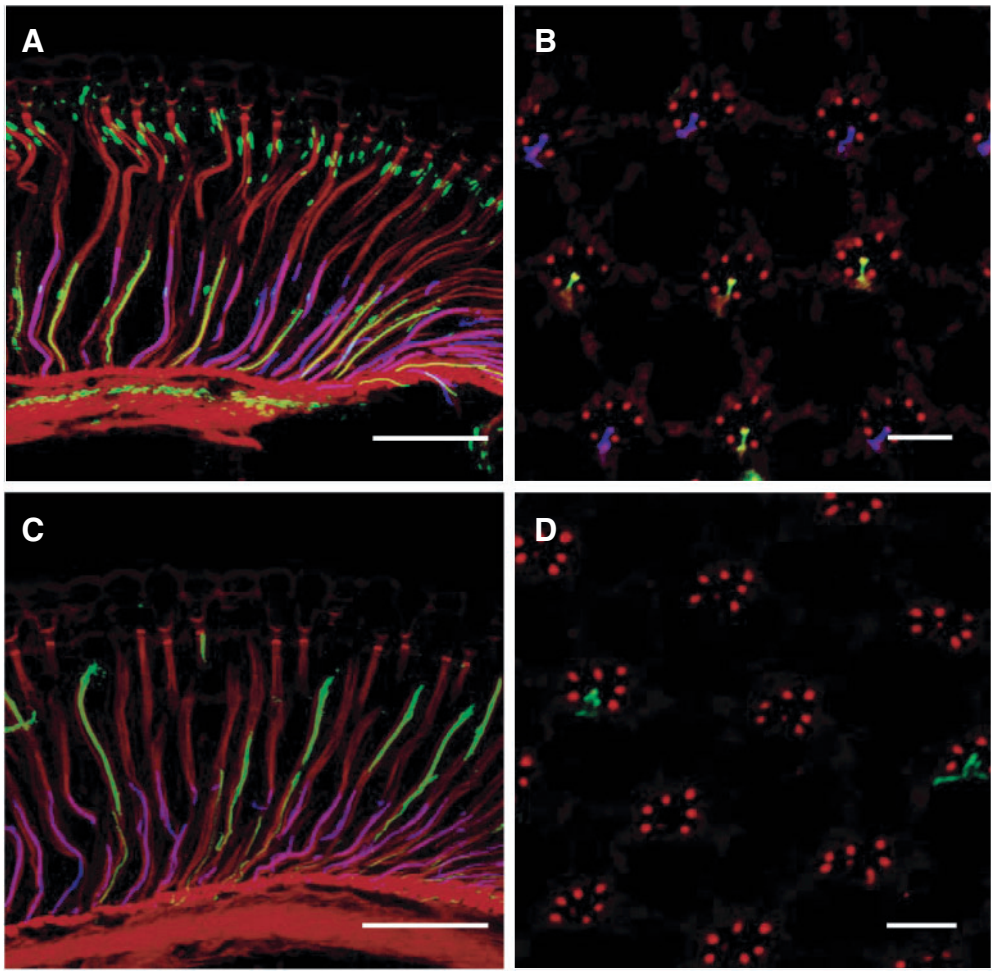

Fig. 4. Expression pattern of *Calliphora* rhodopsins Rh3, Rh5 and Rh6. Longitudinal (A,C) and cross (B,D) sections through the compound eye of *Calliphora* were labelled with antibodies specific for *Calliphora* Rh3, Rh5, Rh6 and with rhodamine-coupled phalloidin. (A,B) Labelling of Rh5 (green), Rh6 (blue) and the actin cytoskeleton of the rhabdomeres (red). The overlay of Rh6 (blue) and actin (red) labeling appears purple. Rh5 and Rh6 are detected in the basal part of the retina. The cross section in B is at the level of R8 cells. (C,D) Labelling of Rh3 (green), Rh6 (blue) and the actin cytoskeleton of the rhabdomeres (red). Rh3 and Rh6 are detected in the apical and basal portion of the retina, respectively. The cross section in D is at the R7 cell level in which Rh3 but not Rh6 is detected. Scale bars, (A,C) 80 μm; (B,D) 8 μm.

With respect to the spectral properties of rhodopsins, the absorption maxima in the rhodopsin and metarhodopsin state obtained for larger flies by microspectrophotometric measurements (summarized in Hardie, 1985) can be compared with those of *Drosophila* rhodopsins, which were determined after ectopic expression of the R7 and R8 cell rhodopsins in R1–6 cells by difference spectroscopy of extracted visual pigments and by microspectrophotometry (Salcedo et al., 1999; Feiler et al., 1992). Since insect rhodopsins with similar spectral properties typically fall into the same phylogenetic groups (Briscoe and Chittka, 2001), the conservation of the primary structure should correlate with the spectral properties. Table 2 shows that Rh3 and Rh6 of *Drosophila* and *Calliphora,* which have amino acid identity of more than 80% also display very similar spectral properties. The less well conserved Rh5 rhodopsin shows significant differences in the absorption maximum of the rhodopsin state. The highest differences in spectral properties are reported for Rh4 which in its rhodopsin state has an absorption maximum at 355 nm in *Drosophila* but 430 nm in the larger flies. It is possible that this rhodopsin is the least well conserved visual pigment between *Drosophila* and *Calliphora* which in turn could make it difficult to isolate its cDNA by homology screening. More detailed information on spectral tuning of fly rhodopsins came from studies in which amino acids conserved between rhodopsins with similar spectral properties were mutated. In a recent report it has been shown that a single amino acid polymorphism is largely responsible for *Drosophila* UV vision (Salcedo et al., 2003). In the second transmembrane domain the UV-absorbing *Drosophila* rhodopsins Rh3 and Rh4 have a

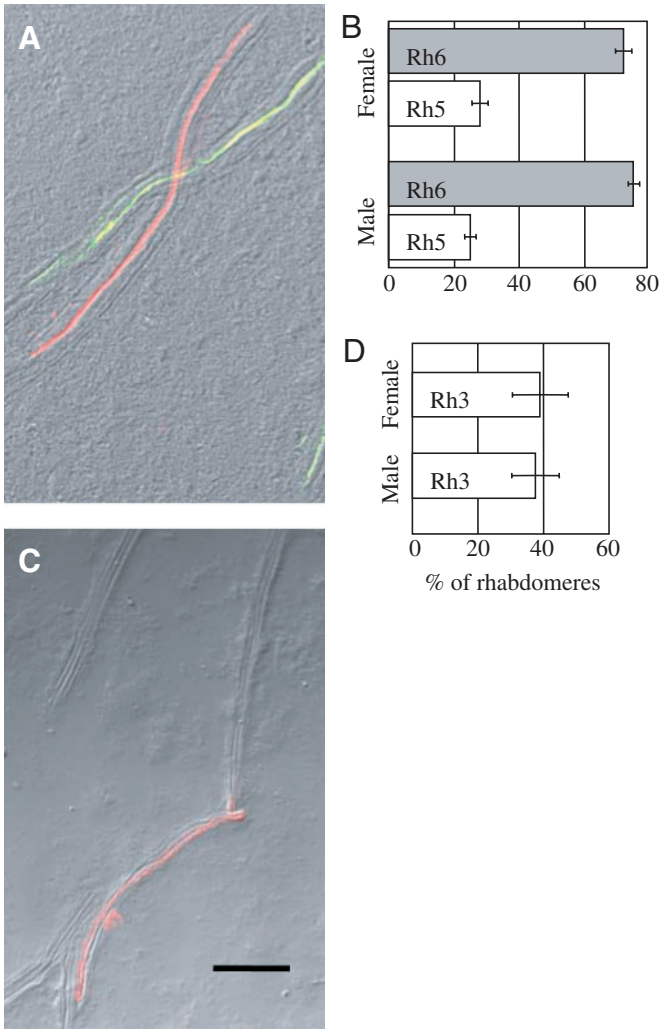

Fig. 5. Quantitative evaluation of isolated *Calliphora* rhabdoms labelled for Rh3, Rh5 and Rh6. Isolated *Calliphora* rhabdoms were double labelled with antibodies specific for Rh5 and Rh6 (A,B) or with antibodies against Rh3 and Oregon Green-coupled wheat germ agglutinin (C,D). (A) An overlay of differential interference contrast and indirect immunofluorescence images of two rhabdoms, one labelled with the anti-Rh5 antibody (red) and the other one labelled with the anti-Rh6 antibody (green). (B) The percentage of rhabdoms containing Rh5 or Rh6 was determined by counting the number of rhabdoms stained with either antibody. (C) A few rhabdoms viewed with differential interference contrast optics, one of which is labelled with anti-Rh3 (red). (D) The percentage of rhabdoms stained with anti-Rh3 was determined relative to the total number of rhabdoms stained with wheat germ agglutinin. For the quantitative evaluation, four independent preparations from both male and female flies were analysed. Values are means ± S.D. Scale bar, 40 μm.

lysine (K110 in Rh3), the mutation of which results in a large shift of the absorption maximum of Rh3 to longer wavelength. The critical lysine residue is conserved in *Calliphora* Rh3, confirming the characterization of this rhodopsin as a UV-absorbing visual pigment.

Expression pattern of Calliphora *rhodopsins*

The immunocytochemical studies on the expression pattern of *Calliphora* Rh3, Rh5 and Rh6 described here reveal a highly similar expression pattern to that described for *Drosophila*. *Calliphora* Rh5 and Rh6 are detected in non-overlapping sets of R8 cells while Rh3 is expressed in R7 cells. Furthermore, double labelling of longitudinal sections with anti-Rh3 and anti-Rh6 showed that in most cases Rh3 and Rh6 are not contained in the same ommatidium, that is, as in *Drosophila,* the expression of Rh3 and Rh6 is usually not coupled. The quantitative evaluation of immunolabelled isolated rhabdoms further supports the assumption that rhodopsin patterning is highly conserved between *Calliphora* and *Drosophila*. The ratio of *Calliphora* R8 cells containing Rh5 or Rh6 is not significantly different from the ratio determined for *Drosophila*. Although there is a sexual dimorphism in *Calliphora* resulting in larger compound eyes in the male, which is not observed in *Drosophila*, this dimorphism has no significant effect on the relative number of Rh5- and Rh6-containing R8 cells. The determined numbers for Rh3-expressing rhabdoms are about 10% higher than the values for Rh5-expressing rhabdoms, suggesting that some ommatidia express Rh3 but not Rh5. Ommatidia of the dorsal rim region, which contain Rh3 in R7 and R8 cells, may in part account for this discrepancy. In addition, besides the pairing of Rh3-containing R7 cells with Rh5-containing R8 cells, a significant number of Rh3-containing R7 cells may be paired with R8 cells containing Rh6. In *Drosophila*, coupling of Rh3 and Rh6 was reported to occur in 6% of ommatidia, whereas the combination Rh4/Rh5 was observed very rarely (0.3%) (Chou et al., 1999). In conclusion, our results suggest that the developmental mechanisms that govern coordinated rhodopsin expression in R7 and R8 cells of the fly eye are very well conserved between *Drosophila* and *Calliphora*. The similar

Table 2. *Spectral properties of* Drosophila *and* Calliphora *rhodopsins*

	Rh3/R7p		Rh4/R7y		Rh5/R8p		Rh6/R8y	
λ_{max} (nm)	R	M	R	M	R	M	R	M
*Drosophila**	331	468	355	470	442	494	515	468
Calliphora†	335	460	430	505	460	ND	520	ND
aa identity (%)	80.7		ND		60.9		86.1	

Values are absorption peak wavelengths (λ_{max}) of rhodopsin (R) and metarhodopsin (M) according to *Salcedo et al. (1999) and †Hardie (1985), as well as the amino acid identities (aa identity) of *Drosophila* and *Calliphora* rhodopsins (see Table 1). ND, not determined.

ratios of ommatidia containing R7p/R8p or R7y/R8y photoreceptors, and of the two types of ommatidia defined by their rhodopsin expression pattern in the *Calliphora* compound eye, further support the assumption that ommatidia containing R7p/R8p express the rhodopsins Rh3/Rh5 while ommatidia containing R7y/R8y express Rh4/Rh6. Detailed morphological studies of the compound eye of *Calliphora* showed that the two major types of central rhabdomeres not only differ in their fluorescence properties, but also in the relative length of R7 and R8 rhabdomeres (Smola and Meffert, 1979). This shows that terminal differentiation of R7 and R8 cell subtypes affects more than just the activation of distinct rhodopsin genes.

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