Immunofluorescent studies of the INAD signaling complex and the TRPL ion channel in fly photoreceptors (*Drosophila melanogaster* and *Calliphora vicina*)

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1. Summary

The visual system in *Drosophila* has emerged as a tempting model for studying a G-protein coupled cascade. The rapid speed of propagation of a signal is achieved only when all the phototransducing components are present in the same photoreceptive compartment known as "rhabdomere". The initial approach applied in this thesis was to differentiate the different types of rhabdomeres by localising rhodopsins. The use of lectin, Wheat Germ Agglutinin (WGA), allows to label the rhabdomeres by direct fluorescence, a method which is independent of immunocytochemistry and at the same time provides a fluorescent confocal image. In order to localise the photoreceptor cell expressing a distinct rhodopsin, an elegant approach employing reporter gene flies which express the *lacZ* gene under the different rhodopsin promoters was tested for β-galactosidase expression in the photoreceptor cells. This approach also allowed to trace the photoreceptor cell axons into the optic ganglia. These three independent methods were establised inorder to study the distribution of the components comprising the INAD signaling complex.

The organisation and localisation of the proteins responsible for phototransduction is provided by the INAD signaling complex that functions to assemble these proteins in the rhabdomeres. With respect to the study of the INAD signaling complex, a mutant of a larger fly species *Calliphora rpa*, is studied which was formely reported to be a *norpA* mutant. In context with the immunocytochemical studies of this thesis it is subsequently shown to be an equivalent of the *Drosophila inaD*^{1} null mutant. The lack of INAD protein expression is shown to result in the mislocalisation of the major ion channel, TRP, while PLCβ is below the detection limit. The disorganisation of the signaling complex is shown to result in a light-age-dependent degeneration of the rhabdomeres indicating the importance of the correct organisation and localisation of the INAD signaling complex for photoreceptor maintainance. The activation of the INAD signaling complex finally leads to the activation of two ion channels TRP and TRPL. An issue which has been less investigated so far is if the ligands of the INAD signaling complex and the channel proteins are subject to light-regulated localisation. Analysis reveal that TRPL is subject to

light-regulated distribution while INAD and TRP localisation is light independent. Characterisation of TRPL distribution in *Drosophila* and *Calliphora* shows that it is influenced by the activation of rhodopsin, the absence of TRP in the rhabdomeres and independent of the downstream activation of PLCβ.

2. Introduction

Two classes of photoreceptors have emerged during the course of animal evolution: ciliary photoreceptor, which are represented by vertebrate rods and cones, and the microvillar or rhabdomeric photoreceptors typical of arthropods and most molluscs. Whilst the phototransduction cascade in rods and cones has been understood in great detail, major uncertainties still persist in invertebrate phototransduction due to the high complexity and adaptational mechanisms adopted. Besides, the apparent diversity and the fact that the general class of mechanism (phospholipase-C-activated Ca^{2+} influx) is not fully understood. However, the fly eye has proved to be an invitable model to implement different approaches for the study of phototransduction due to its splended properties that it shares with other invertebrates classes. The great complexity of the fly eye with its distribution and arrangement of photoreceptor comparments has invited much attention of workers to use this system to find out signaling pathways unknown so far. One such signaling pathway known as the "visual pathway" has been studied in fly eyes because of its rapid speed of working, besides being known as the fastest G-protein coupled pathway.

Phototransduction specially in *Drosophila*, serves as a model system for the dissection of G-protein-coupled signaling cascades which displays exquisite sensitivity and specificity. The transduction machinery which is housed in a specialised compartment of the photoreceptor cells, the rhabdomere, is sensitive to single photons of light, and in addition, it is finely regulated to ensure sensitivity and speed over a broad dynamic range. The complete understanding of invertebrate phototransduction may require cloning and sequencing of all the gene products involved in this process, besides physiological studies which will determine the effect of their loss or misfunction. Therefore, at the present time, *Drosophila* phototransduction is a tempting model to apply multidiciplinary approaches involving genetic, molecular, biochemical and electrophysiological techniques to the same cell.

2.1 The visual system of *Drosophila*

The *Drosophila* visual system is composed of compound eyes and ocelli. The ocelli are simple eyes which are located in the vertex of the head and express a violet-sensitive rhodopsin, Rh2 (Cowman *et al*., 1986; Feiler *et al*., 1988; Pollock and Benzer, 1988). Each of the two compound eyes is made up of 750 ommatidia or unit eyes (Fig 1A). Inturn, each ommatidium consists of 20 cells, eight of which are photoreceptor neurons (Fig 1B, C & D). Each photoreceptor cell has a specialised organelle consisting of a stack of microvilli (approximately 60, 000) designated as rhabdomere (Fig 1E). The rhabdomere is where the phototransduction machinery is housed and it may be seen as the equivalent of vertebrate rod outer segments discs (Fein *et al*., 1982).

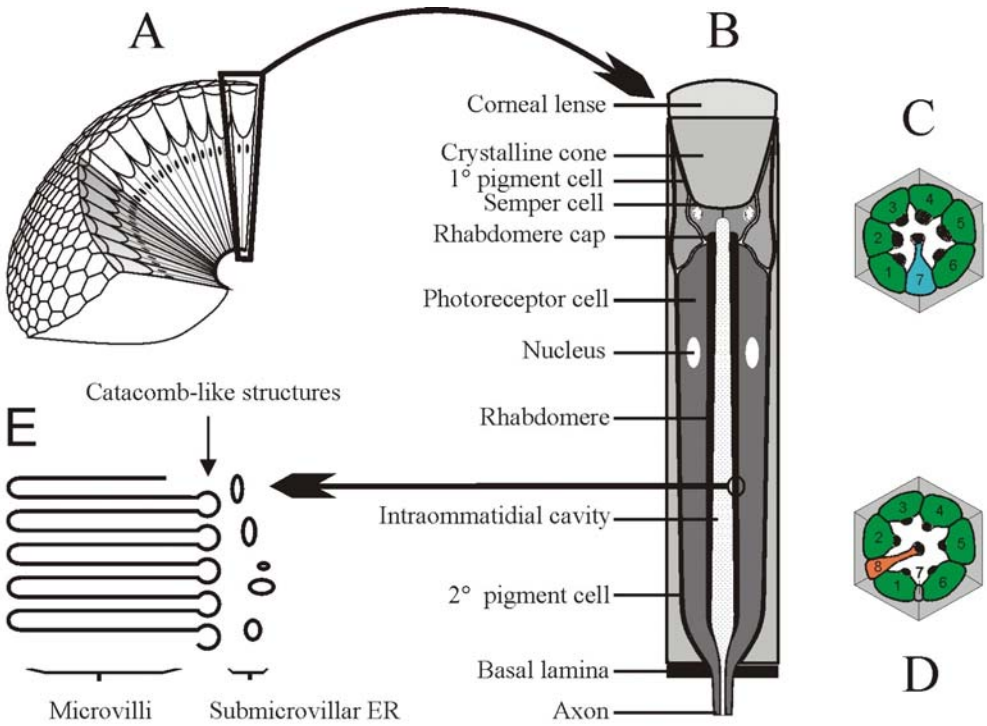

Fig 1: The compound eye of *Drosophila*

(A) Schematic drawing of a compound eye of *Drosophila,* (B, C , D) a longitudinal and cross section through an ommatidium, (E) structural representation of the rhabdomere. (modified from Paulsen *et al*., 2001a).

The eight photoreceptors can be divided into three classes based on their spectral sensitivity, position of the rhabdomere within the ommatidium, and synaptic connections in the optic lobes (Hardie, 1983; Franceschini, 1985). The R1-6 cells represent the major class of photoreceptors in the retina and express a blue-absorbing rhodopsin, Rh1, which has an absorption maximum at 478 nm (Fig 2A) (Stavenga *et al*., 1983; Paulsen, 1984; Zuker *et al*., 1985; O'Tousa *et al*., 1985; Feiler *et al*., 1988).

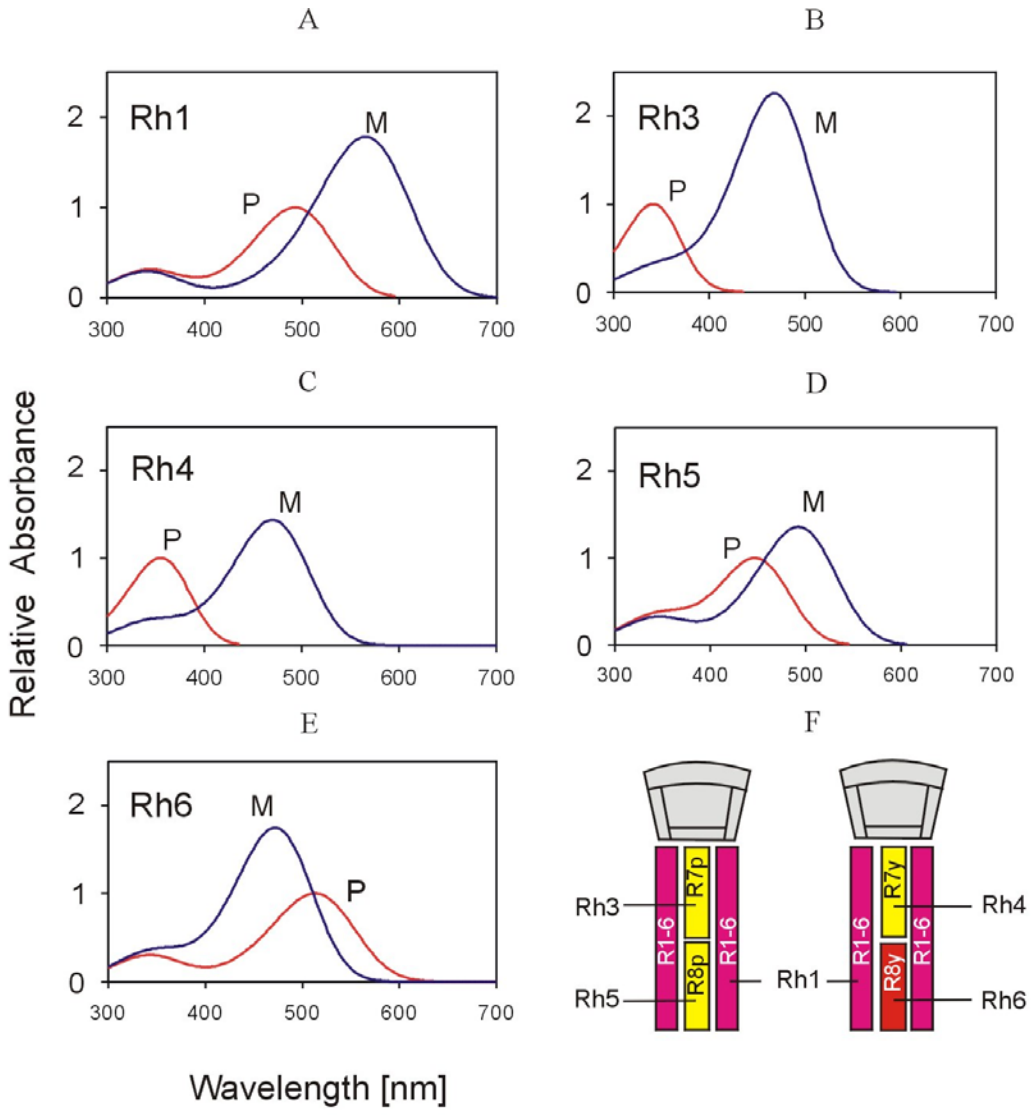

Fig 2: Absorbance spectra of five different visual pigments expressed in the *Drosophila* compound eye

Shown are the absorbance spectra of rhodopsins (P) and metarhodopsins (M). (F) Schematic representation showing the expression pattern of the rhodopsins in the central photoreceptor cells. (Figure adapted from Henrich, 1999).

The axons of these cells synapse in the first optic lobe, the lamina. The other two classes of photoreceptors, R7 and R8 are defined according to the position of their rhabdomeres within the center of the intraommatidial space. Recent evidence obtained by studying the rhodopsin expression pattern revealed that R7 / R8 photoreceptors are functionally divided into two major groups. The R7 cell which is located distally in the retina expresses either one of two opsins, Rh3 or Rh4, with absorbance maxima at 345 nm and 375 nm (Fig 2B & C). The Rh3 and Rh4 opsin genes are expressed in non-overlapping sets of R7 cells (Fryxell and Meyerowitz, 1987; Montell *et al*., 1987; Zuker *et al*., 1987; Feiler *et al*., 1992) which are ultraviolet-sensitive and whose axons synapse in the second optic lobe, the medulla. The R8 photoreceptor cell is located proximally in the retina, just beneath the R7 cell and expresses either one of two opsins namely Rh5 and Rh6 which are expressed in non-overlapping subsets of R8 cells (Chou *et al*., 1996; Huber *et al*., 1997). Rh5 is maximally excited at 437 nm (Fig 2D) and Rh6 at 508 nm (Fig 2E) (Salcedo *et al*., 1999).

2.2 The rhodopsin cycle in the visual system of *Drosophila*

The switching on of the visual cascade in *Drosophila* photoreceptors begins with the activation of the receptor molecule, rhodopsin, by light. By absorbing a photon, the 11-*cis* form of the chromophore (11-*cis* 3-hydroxyl retinal) isomerizes to the *all-trans* configuration and rhodopsin is transformed into an active metarhodopsin state. The change induces conformational changes in the protein thus enabling it to bind heterotrimeric G-proteins, continuing the phototransduction cascade. Once bound to activated rhodopsin, G-proteins are activated and this results in the propagation of the light induced signal downstream of the phototransduction cascade, ultimately resulting in the depolarisation of the photoreceptor cell. Deactivation of the visual cascade is brought about by several mechanisms ; interaction of metarhodopsin with arrestins (Dolph *et al*., 1993), interaction of Gα with PLCβ (Bähner *et al*., 2000), by control of a not yet defined channel, and absorption of a second photon of light by metarhodopsin to regenerate rhodopsin. The carboxy terminal of *Drosophila* metarhodopsin is phosphorylated by rhodopsin kinase at several serine and / or threonine residues within the cytoplasmic C-terminal tail (Doza *et al*., 1992, Vinós *et al*., 1997), after arrestin has bound to the metarhodopsin (Bentrop *et al*.,

1993). This however is essential to quench metarhodopsin activity (Byk *et al*., 1993; Dolph *et al*., 1993). Though two variants namely arrestin 1 (Arr1) and 2 (Arr2) are expressed in the eye of *Drosophila*, the functional significance of these two visual variants is not yet known (Scott and Zuker, 1997). The dissociation of arrestin from rhodopsin is followed by rhodopsin dephosphorylation by a photoreceptor cell-specific Ca^{2+} dependent phosphatase encoded by the *rdgC* locus subsequently resulting in its regeneration back to the active state (Fig 3).

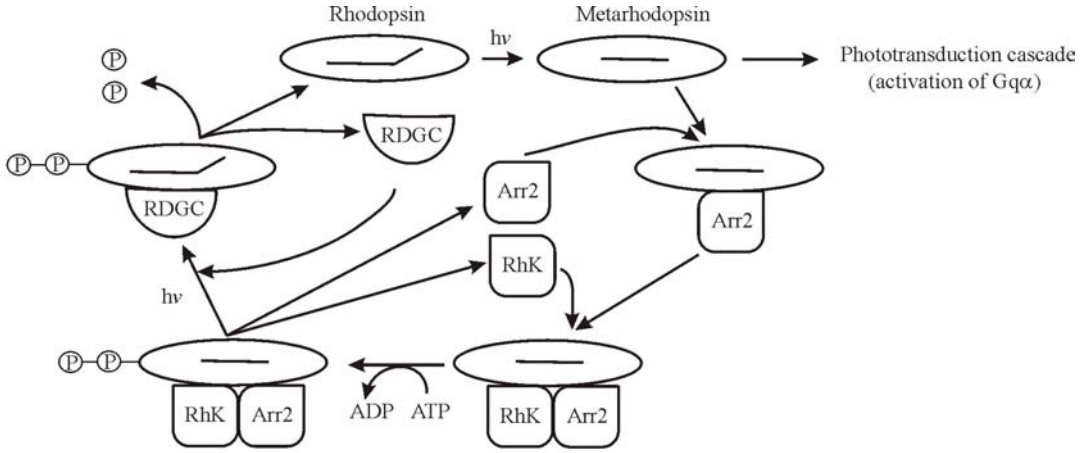

Fig 3: Activation and inactivation of *Drosophila* visual pigment

H*v*, photon capture; Arr2, arrestin 2; RhK, rhodopsin kinase; RDGC, retinal degeneration C protein (= rhodopsin phosphatase). (Refer text for further details) (modified from Paulsen *et al*., 2001a).

2.3 The phospholipase Cβ (PLCβ) activated visual cascade and the INAD signaling complex in fly phtoreceptors

The visual cascade in the fly eye has been reported to be a PLCβ activated pathway. The activation of G-protein by rhodopsin results in the exchange of bound GDP for GTP and seperation of the α-subunit from the βγ-subunit. The inactivation of G-protein takes place by the reassociation of $G\alpha_e$ with $G\beta\gamma_e$ following the hydrolysis of the bound GTP to GDP by intrinsic GTPase activity (O′Day *et al*., 1997).

The activated G-protein is responsible for the activation of a phospholipase Cβ (PLCβ), which in turn catalyses hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) to inositol-1,3,5-trisphosphate (IP_3) and diacylglycerol (DAG) (Devary *et al*., 1987; Bloomquist *et al*., 1988; Lee *et al*., 1994; Scott *et al*., 1995), which eventually leads to Na^+ and Ca^{2+} influx as a result of opening of the cation influx channels (Hardie, 1991; Ranganathan *et al*., 1991; Hardie and Minke, 1992), a mechanism not fully understood till date (Fig 4).

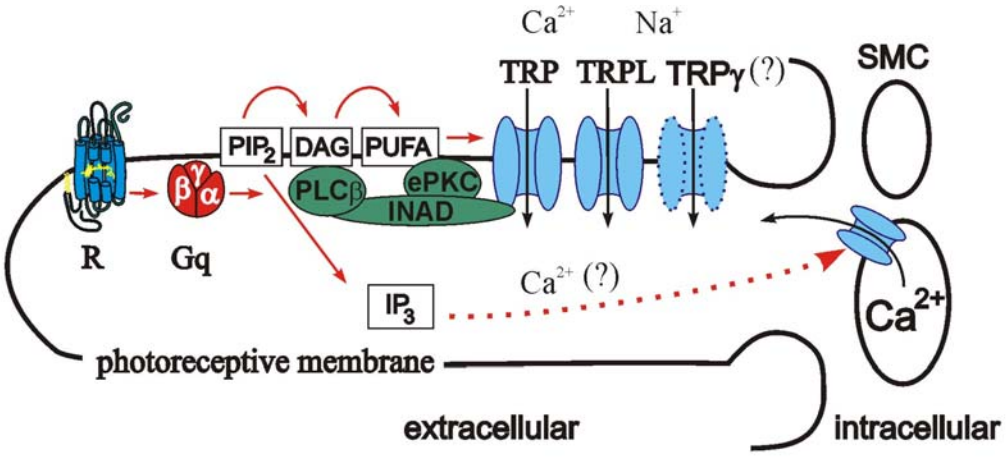

Fig 4: Schematic representation of the visual cascade in *Drosophila* photoreceptors

R, rhodopsin; Gq, visual G protein; PIP_2, phosphatidylinositol-4-5-bisphophate; PLCβ, phospholipase Cβ; ePKC, eye protein kinase C; DAG, diacylglycerol; PUFA, polyunsaturated fatty acid; IP_3, inositol trisphosphate; TRP, transient receptor potential; TRPL, transient receptor potential like; TRPγ, transient receptor potential γ subunit; SMC, submicrovillar cisternae. (Refer text for further details) (Figure modified from Paulsen *et al*., 2001a).

The activation of rhodopsin to the generation of receptor potential takes just a few tens of milliseconds and less than 100 msec to terminate the response (Ranganathan *et al*., 1995). Therefore, phototransduction in *Drosophila* has been characterised to display two unique properties: first, it's the fastest known G-protein signaling cascade, taking only a few milliseconds to go from activation of rhodopsin to opening of the light-sensitive channels, and second, this transduction pathway displays tremendous sensitivity to light by responding to single photons of light. How is such a speed of signaling and specificity achieved? Is it a random collision or an arranged array of the signaling molecules in the

phototoreceptors. Studies in the visual system of *Drosophila* have indicated that INAD (inactivation-no-afterpotential D) to be responsible for the organisation of signaling proteins into macromolecular transduction complex. In *Drosophila* and the blowfly, *Calliphora erythrocephela*, INAD functions as a protein scaffold, bringing together different components of the phototransduction cascade, assembling them (Huber, 2001) and localising them to the rhabdomeres of photoreceptor cells (Tsunoda and Zuker, 1999), facilitating speed and efficiency of vision (Ranganathan and Ross, 1997). The assembly of signaling proteins into supramolecular complexes has been recognized to hold signaling pathways at synapses, for pathways controlling development and for visual transduction pathways (Gomperts, 1996; Pawson and Scott, 1997; Craven and Bredt, 1998; Fanning and Anderson, 1998, 1999; Paulsen *et al*., 2001). Besides functioning as a modular multivalent PDZ protein, INAD has been shown to interact with different components of the same pathway (Tsunoda *et al*., 1997). Functional studies have claimed that the integration and association of phototransduction proteins into a supramolecular signaling complex is a prerequisite to localise and sustain the members of the cascade to the rhabdomeral membrane (Chevesich *et al*., 1997; Tsunoda *et al*., 1997; Tsunoda *et al*., 2001), the generation of reliable single photon responses (Scott and Zuker, 1998a) and for correct response termination (Shieh *et al*., 1997; Adamski *et al*., 1998; Wes *et al*., 1999). Analysis have shown that the *inaD* gene product consists of five homologous domains belonging to a conserved family of novel protein-interaction modules known as PDZ domains (Tsunoda *et al*., 1997; Shieh and Niemeyer, 1995).

The name PDZ domain was designated according to their occurrence in the post-synaptic density protein, PSD-95 (Cho *et al*., 1992), the tumor suppressor protein disc large, Dlg, of *Drosophila* (Woods and Bryant, 1991) and the zonula occludens protein, ZO-1 (Willott *et al*., 1993). The identified components of this INAD signaling complex are INAD, the ion channel TRP (Shieh and Zhu, 1996, Huber *et al*., 1996a), the norpA encoded phospholipase C (Huber *et al*., 1996a; Chevesich *et al*., 1997), and the eye-specific protein kinase C (Huber *et al*., 1996a; Xu *et al*., 1998) (fig 5).

Additional evidence has shown that rhodopsin (Chevesich *et al*., 1997; Xu *et al*., 1998), the second ion channel TRPL (Xu *et al*., 1998), the unconventional myosin NINAC (Wes *et al*., 1999) and calmodulin (Chevesich *et al*., 1997; Xu *et al*., 1998) are constitutive members of the complex or are bound to INAD transiently. NINAC has been proposed to interact with PDZ1 of INAD, while calmodulin appears to occupy a binding site which is located in the region between PDZ1 and PDZ2 (Xu *et al*, 1998). Rhodopsin was found to bind to PDZ3 and PDZ4 (Xu *et al*., 1998) while PDZ1, PDZ2 and PDZ3 were found to bind to ePKC (Adamski *et al*., 1998). TRP (Tsunoda *et al*., 1997; Shieh *et al.,*1997; Xu *et al*., 1998) has been proposed to interact with PDZ3, however, Xu *et al*. (1998) has suggested it also to bind to PDZ4. PDZ3 and PDZ4 have been proposed to bind to TRPL (Xu *et al*., 1998) while PLC binds to PDZ5 (Tsunoda *et al*., 1997; Shieh and Zhu, 1997) (fig 5).

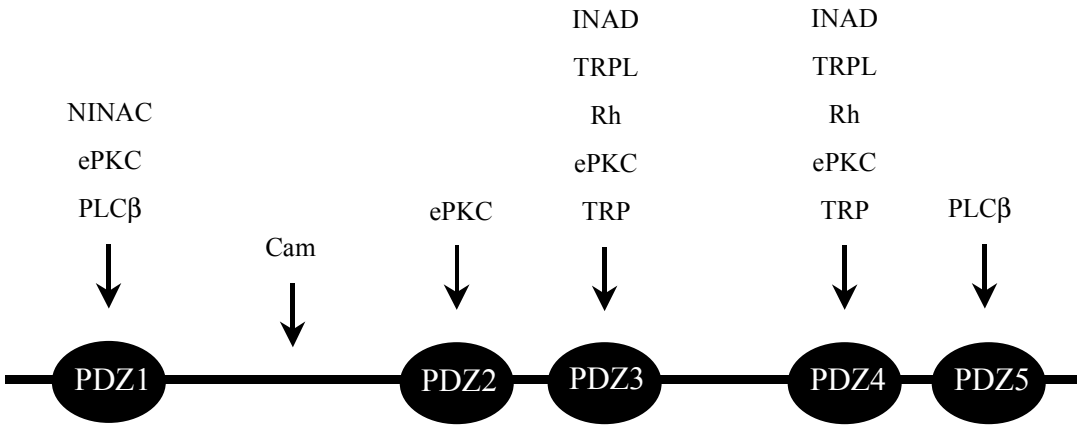

Fig 5: Structure of the PDZ domain INAD

The binding sites for the various INAD ligands are indicated by arrows (refer text for details).

Therefore, INAD seems to coordinate the recruitment of components involved in both activation (PLCβ and TRP) and deactivation (ePKC) (Smith *et al*., 1991; Huber *et al*., 1996b; Tsunoda *et al*., 1997). Tsunoda *et al*. (1997) demonstrated that TRP, ePKC and PLCβ which are bound to INAD are mislocalised and / degraded in INAD null mutant, indicating the importance of this protein for the stability of the core complex components. Similar results were reported for the *Drosophila inad* ^{P215} mutant (Chevesich *et al*., 1997)

and for a mutant of the larger fly species *Calliphora* which also lacks the INAD protein (Huber *et al*., 2000). As hypothesised, the phosphorylation of TRP may be responsible for the INAD-TRP association localising them to the rhabdomeres and facilitating Ca^{2+} feedback regulation through TRP. But evidence has shown that TRP localisation is unaffected in *inaC* null mutants, indicating that ePKC-mediated phosphorylation is not critical for the TRP-INAD interaction (Chevesich *et al*., 1997). Recent findings indicate that INAD and TRP are targeted independently to the rhabdomeres (Tsunoda *et al*., 2001) but the interaction of both in the rhabdomeres is needed for retention of the signaling complex (Li and Montell, 2000). The preassembly of INAD signaling complexes could be a strategy adopted to minimise the number of stray signaling components in the rhabdomeres while maximising the number of complete functional complexes (Tsunoda *et al*., 2001). It is likely that INAD along with the association of TRP serves to assemble, localise and organise phototransduction proteins to specialised microdomains of the microvillar membrane.

2.4. Ion channels in the visual system of *Drosophila*

The TRP protein family constitutes a novel class of ion channels which have been implicated in calcium signaling. Prototypical members of this class have been first identified in the visual system of *Drosophila* where they have been shown to be responsible in generating the light-activated conductance (Hardie and Minke, 1992, 1993; Niemeyer *et al*., 1996). Since then, *Drosophila* visual transduction has emerged as a related simple system in which to study the function of TRP channels. These TRP homologues belong to the STRPC ("Short TRP channels") subfamily of channels. STRPC channels are Ca^{2+}-permeable cation channels that are activated subsequent to receptor-mediated stimulation of different isoforms of phospholipase C. Three structurally related channel proteins of the *Drosophila* visual system which are encoded by the genes transient receptor potential (*trp*) (Montell and Rubin, 1989), transient receptor potential like (*trpl*) (Phillips *et al*., 1992) and a third, TRP-related subunit (*trpγ*) (Xu *et al*., 2000) have so far been identified. These channels have been shown to be highly enriched in the phototransducing organelle of the photoreceptors, the rhabdomere (Niemeyer *et al*., 1996;

Chevesich *et al*., 1997; Xu *et al*., 2000), besides, TRP has also been observed to be expressed in antennae and may be involved in the olfactory response or in the development of olfactory receptor cells (Störtkuhl *et al*., 1999). Genetic and electrophysiological experiments have shown that the light-activated conductance in *Drosophila* photoreceptors is composed of two ion channels encoded by the *trp* and *trpl* gene (Hardie and Minke, 1992; Niemeyer *et al*., 1996). TRP mutants exhibit severely impaired photoresponses (Cosens and Manning, 1969; Pak, 1970; Minke *et al*., 1975; Minke, 1982) while a *trpl*:*trp* double mutant is totally unresponsive to light (Scott *et al*., 1997; Reuss *et al*., 1997) although a small amount of residual current which is detected could either result from a third channel or a small amount of functional TRP or TRPL protein (Niemeyer *et al*., 1996). This residual response was later attributed to result from a small residual amount of functional TRP present in the *trpl*:*trp* mutant (Reuss *et al*., 1997). TRP and TRPL channels share structural features which are also detected in vertebrate voltage-gated calcium channels like the six putative transmembrane domains S1 to S6 (fig 6).

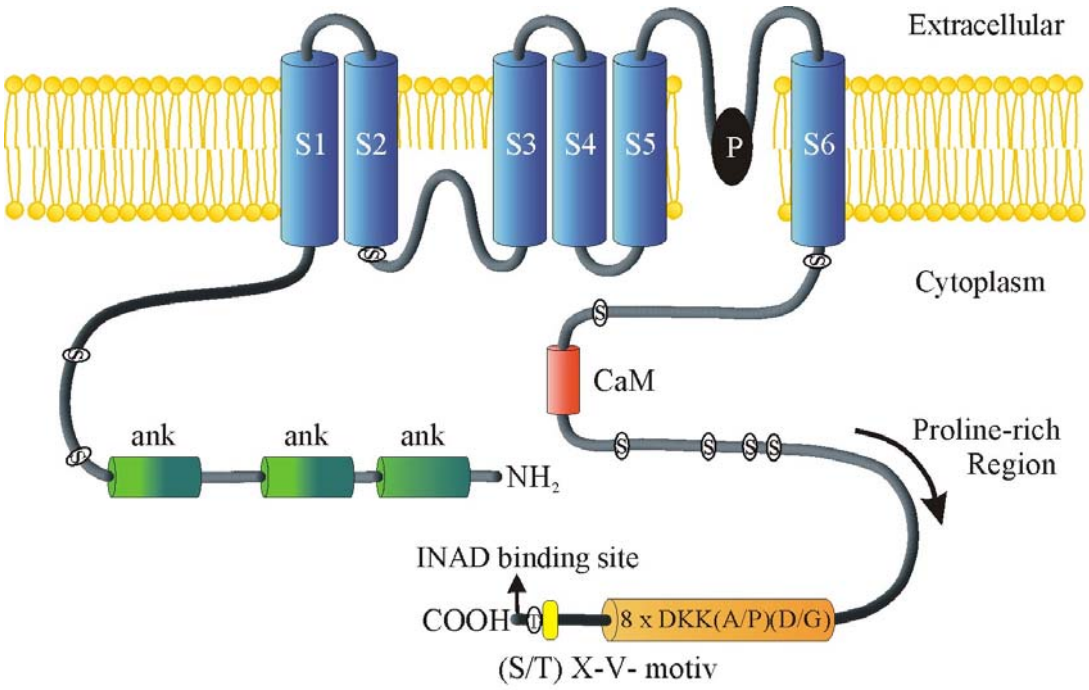

Fig 6: A model of the TRP channel in *Drosophila*

The conserved ePKC phosphorylation sites are indicated by circles labeled with S (serine) or T (threonine). S1 - S6, transmembrane domains; P, putative pore-loop; ank, ankyrin repeats; CaM, putative calmodulin binding site. (modified from Paulsen *et al*., 2001b).

The highest sequence similarity exists between transmembrane domains S4, S5 and S6 which include the putative pore-forming region (between S5 and S6). S4 of TRP and TRPL lack a positive charge that acts as voltage gated channels (Phillips *et al*., 1992). Other common features shared by TRP and TRPL are the three ankyrin repeats which are located at the intracellularly N-terminal region and are involved in protein-protein interactions which could be responsible to anchor the channel to cytoskeletal proteins (Hofmann *et al*., 2000). The mechanism of activation of both of these channels in photoreceptors cells is not yet known (Leung *et al*., 2000) however, it has been shown that *Drosophila* TRP and TRPL channels can be activated by polyunsaturated fatty acids (PUFAs) (Chyb *et al*., 1999). Three mamalian TRP homologues, TRP3, TRP6 and TRP7 appear to be activated by exogenous application of diacylglycerols (DAGs) (Okada *et al*., 1999; Hofmann *et al*., 1999). The TRP channel has been shown to be highly calcium-permeable but the TRPL channel is nonspecifically cation-selective (Hardie and Minke, 1992; Niemeyer *et al*., 1996; Reuss *et al*., 1997).

The similarities shared between TRPγ and many *Drosophila* and vertebrate TRP protein include three to four ankyrin repeats, ≥40% sequence homology over approximately 700 residues that extend from the N-terminus to a highly conserved region immediately C-terminal to the transmembrane segments (Xu *et al*., 2000). As reported by Xu *et al*. (2000), the coassembly of TRPγ and TRPL produces a channel that can be activated through stimulation of PLC. However, several questions concerning to the requirement for the expression of two or more classes of channels in the fly visual system are not yet solved or are discussed highly controversely (Scott and Zuker, 1998b; Montell, 1998; Hardie, 2001). Although it is possible that *trpl* encodes a subunit of a light activated channel, findings by Leung *et al*. (2000) demonstrates that the TRPL channels may have a role in sustaining the photoreceptor response during prolonged illuminations and in adaptation to dim light stimuli. The TRPL channel has been shown to be localised to the rhabdomeres (Niemeyer *et al*., 1996; Chevesich *et al*., 1997) and to bind to INAD (Xu *et al*.,1998), though the latter has yet to be confirmed by other authors since TRPL localisation is unaltered in $inaD^{1}$ null mutants (Tsunoda *et al*., 1997). The correct localisation of phototransduction proteins to the rhabdomeres which form supramolecular,

multimeric signaling complexes has been hypothesised to be of prime importance for the propagation of the light response and for signal termination.

2.5 Outline of the thesis

Fly photoreceptors express different rhodopsins in R1-6, R7 and R8 cells with a paired expression of Rh3 / Rh5 and Rh4 / Rh6 in a variable expression pattern. The correct localisation of the receptor protein (rhodopsin) as well as of other key players of the phototransduction cascade in the rhabdomere is a prerequisite in understanding the principles of visual transduction in *Drosophila*. The correct localisation / detection of the proteins comprising the INAD signaling complex is important inorder to study the functional role and consequence from the lack of the INAD signaling complex in fly photoreceptor cells. The following approaches were undertaken towards understanding the functional role played by the INAD signaling complex in fly photoreceptors. To obtain information on the mechanism underlying INAD signaling complex and possible light dependent processes on the localisation of the INAD signaling complex members and channels, mutants with defect in the phototransduction cascade as well as flies that have been genetically manipulated to ectopically express rhodopsins were used.

In order to establish a method to localise and label the rhabdomeres and the photoreceptor cells, three independent methods were adopted. **(1)** a reliable immunocytochemical method to localise rhodopsin, **(2)** a method independent of rhodopsin or immuno labeling is applied inorder to label the rhabdomeres by a fluorescent marker which would give a confocal image in the same plane as the labeled protein under investigation, and **(3)** in addition an approach is applied wherein reporter gene flies are used which express the *lacZ* gene under the different rhodopsin promoters. The primary method to label the rhabdomeres / photoreceptor cells by immuno or direct labeling with the use of confocal microscopy techniques is established for studying the INAD signaling complex. Due to the absence of INAD in *Drosophila* photoreceptors, core proteins of the signaling complex like TRP, ePKC and PLCβ are either degraded or mislocalised at an early stage. The *Calliphora rpa* mutant which turned out to be a *Calliphora* INAD null

mutant is characterised for the distribution of the key components of the INAD signaling complex. Speculations are that Rh1, TRPL, NINAC and Calmodulin which are localised to the rhabdomeres might bind transiently to the signaling complex. Therefore, it can be postulated that the transient binding and localisation of these proteins to the rhabdomeres might be subjected to light-regulation or activation of the phototransduction cascade. To investigate this, wild type *Drosophila* and *Calliphora* were dark and light raised and analysed for the distribution of the INAD, TRP and TRPL. Since TRPL is subjected to a light-regulated distribution within the photoreceptors, *Drosophila* and *Calliphora* flies that have a defect in the phototransduction cascade are investigated for analysing the light-regulated distribution of TRPL.

3. Material and Methods

3.1 Material

The chemicals used were of the highest available quality and were obtained from Sigma (Steinheim, Germany), dianova (Hamburg, Germany), Mo Bi Tec (Göttingen, Germany), Roth (Karlsruhe, Germany), Fluka (Neu-Ulm, Germany), Bio-Rad (München, Germany), and Merck (Darmstadt, Germany), unless otherwise mentioned.

3.1.1 Buffers and solutions for biochemical experiments

1x SDS-PAGE buffer:
4% (w/v) Na-Dodecylsulfate (SDS), 65 mM Tris-HCl pH 6.8, 1% 2-Mercaptoethanol (added to the protein extract just before loading it on the SDS-PAGE gel).

TBS-T (Tris-buffered saline with Tween):
150 mM NaCl, 50 mM Tris-HCl pH 7.3, 0.1% Tween 20.

Na-Phosphate buffer (0.2M):
0.2 M NaH_2PO_4 solution and 0.2 M Na_2HPO_4 were mixed and titrated to pH 6.5.

Blocking solution for western blots:
5% (w/v) Skim Milk Powder in TBS-T.

Bromophenol Blue (0.01%):
72% (v/v) glycerol, 0.01% (w/v) Bromophenol blue, prepared in distilled water.

3.1.2 Solutions and buffers for immunocytochemical experiments

10 X PBS buffer (Phosphate-buffered saline):

1750 mM NaCl, 84.1 mM Na_2HPO_4, 18.6 mM NaH_2PO_4 dissolved in double distilled water and titrated to pH 7.3.

PBS-S (Phosphate-buffered saline with BSA and Saponin):

1% (w/v) BSA (Bovine Serum Albumin), 0.1% (w/v) Saponin in 1x PBS buffer.

Blocking solution:

0.1% (w/v) Ovalbumin (dried egg white), 0.5% (v/v) Fishgelatin (Teleostean gelatin from cold water fish skin) in 1X PBS pH 7.4. The solution was filtered through 0.45 µm sterile filter (Schleicher und Schuell, Dassel, Germany) and stored at -20°C. Working solution was stored at 4°C.

X-Gal staining solution:

a) Stock solution: 10 mM Phosphate buffer pH 7.2, 0.3% (v/v) Triton X-100, 150 mM NaCl, 1 mM NaCl, 3 mM $K_4[Fe(II)(CN)_6]$ x $3H_2O$; 3.1 mM $K_3[Fe(III)(CN)_6]$.

b) 8% (w/v) X-Gal solution prepared in Dimethylformamide (J. T. Baker Chemicals B. V., Deventer, Holland).
   Just prior to use, 100 µl of stock solution and 2.5 µl of X-Gal were mixed.

Fixitative solution:

10% (w/v) Paraformaldehyde in 1x PBS. Store aliquots at 4°C.

Mounting medium (Mowiol 4.88):
25.6 % (w/v) Mowiol 4.88 (Polysciences, Inc., Warrington, USA) (added in small portions with constant stiring), 2.5 % (w/v) n-Propyl-Gallate dissolved in 1x PBS. After stirring overnight at room temperature 51 % (v/v) glycerine was added. The solution was centrifuged at 15.000 rpm for 30 minutes and stored in aliquots at 4°C.

3.1.3 Fly stocks

Calliphora erythrocephala (*vicina*)

Calliphora erythrocephala Meig., *chalky* mutant (Langer, 1962) and the *rpa* mutant (Torkkeli *et al*., 1989, 1991) hereafter designated as "wild type" and "*rpa* mutant" were used. To generate the *rpa*/*chalky* double mutant, *rpa* males (pigmented eyes) were crossed with chalky females. The resulting progeny of the F1 generation were crossed to themselves. *Chalky* males and females appearing in this cross (F2 generation) were immediately seperated and selected after eclosion. These white-eyed (*chalky*) flies were tested for photoreceptor degeneration by deep pseudopupil method. The flies which showed photoreceptor degeneration were crossed individually to estabilish two independent lines. These lines which consistently showed white eye colour and photoreceptor degeneration were propagated and used in this thesis. The larvae of both wild type *Calliphora* and *rpa* mutant were raised on a vitamin A-rich diet obtained from bovine liver in order to increase the rhodopsin content in the retina (Paulsen & Schwemer, 1979). The flies were propagated under 12 hour light / 12 hour dark cycle at 25°C, unless otherwise mentioned.

Drosophila melanogaster

The designated "wild type" flies are white eye mutant from the fruitfly *Drosophila melanogaster*. The flies were propagated on cornmeal diet under 12 hour light / 12 dark cycle at 24°C, unless otherwise mentioned. The transgenic flies used are designated as P (Rh1 Δ Phos.), P (Rh1 + 3) and Gγ. In P (Rh1 Δ Phos.), a mutated Rh1 gene is expressed

under the control of Rh1 promoter in a *ninaE*^{oI17} background. These transgenic flies express Rh1 rhodopsin in R1-6 photoreceptors which lack all the phosphorylation sites of the C-terminal region (Schillo, 2001). In P (Rh1 + 3), the Rh3 rhodopsin is expressed under the control of Rh1 promoter in a *ninaE*^{17} background (Feiler *et al*., 1992), i.e these transgenic flies express Rh3 rhodopsin in R1-6 photoreceptors, besides also in a subpopulation of R7 cells. In Gγ, the *Drosophila* visual Gγ subunit which has a cystine mutated to glycine in the farnesylation site was coupled to a *c-myc* tag and expressed under the control of Rh1 promoter region in wild type background. Therefore its expression is restricted to R1-6 photoreceptors (Schulz, 2001).

The reporter gene flies expressing a bacterial gene (*lacZ*) from *E. coli* under the control of the various rhodopsin promoters are displayed below:

Table 1: Fly strains expressing *lacZ* / *taulacZ* under the various rhodopsin promoters are given below

Fly strain	Promoter	Gene fused	Designation	Reference
Rh1 [w; P (Rh1. 833 *lacZ*)]	Rh1	*lacZ*	P (Rh1. 833*lacZ*)	Huber & Paulsen, 1998
Rh3 [w;; P(Rh3. 343 *lacZ*)]	Rh3	*lacZ*	P(Rh3. 343 *lacZ*)	Fortini & Rubin, 1990
Rh4 [w; P (Rh4. 1900 *lacZ*)]	Rh4	*lacZ*	P (Rh4. 1900 *lacZ*)	Fortini & Rubin, 1990
Rh5 [wy;; (Rh5. 2800 *taulacZ*, y+)]	Rh5	*taulacZ*	P (Rh5. 2800 *taulacZ*)	Steve Britt (Denver, USA, personal communication)
Rh6 [yw; P (Rh6. *taulacZ*)]	Rh6	*taulacZ*	P (Rh6. *taulacZ*)	Steve Britt (Denver, USA, personal communication)

The *Drosophila* mutants which have a defect in the phototransduction cascade and which are used in this thesis are summarised below:

Table 2: *Drosophila* mutants used in this thesis

Mutant	Mutation	Primary defect	Reference
trp^{343}	Unspecified	Null mutant of TRP	Pak, 1979
$trpl^{302}$	Amber nonsense mutation at position 302	Null mutant of TRPL	Niemeyer *et al*., 1996
$norpA^{P24}$	Deletion from 28bp (bp 2710-2737)	Null mutant of PLCβ	Pak *et al*, 1970
$inaC^{P209}$	Point mutation at G279A	Null mutant of ePKC	Pak, 1979; Smith *et al*., 1991
$inaD^{1}$	Amber nonsense mutation at position 811	Null mutant of INAD	Tsunoda *et al*., 1997

3.1.4 Software and hardware:

SDS-PAGE coomasie gels were photographed by using a video camera and Biocapt Software, Version 99.02s (Microsoft, Redmond, USA). Western blots were scanned with a Sharp scanner (JX 330, Hamburg, Germany) and assembled in "Corel Photo Paint 9" and "Corel Draw 9" (Corel, Ottawa, Canada). Statistical calculations were performed by "Excel 97" (Microsoft, USA). Confocal microscopic images were obtained by using a Leica Confocal Microscope (hereafter designated as " CLSM ") (LSM-SP, Leica, Bensheim, Germany). The immunocytochemical preparations were scanned using Leica TCS NT Version 1.6.582 (Heidelberg, Germany) and Leica Confocal Software Version 2 Build 0585 (Heidelberg, Germany) software. The images were croped and assembled in Adobe Photoshop 6.0.1 (Adobe Systems, USA) and PowerPoint (Microsoft,

USA). Whole mount images of eyes of *Drosophila* stained for β-galactosidase and images of deep pseudopupil of *Calliphora* were obtained with a digital camera (Leica DC 200) (Leica Microsystems AG, Heerbrugg, Germany) and a Leica DC Viewer program (Leica Microsystems AG, Heerbrugg, Germany). X-Gal stained sections were viewed with a Zeiss Axiovert 35 microscope (Carl Zeiss, Oberkochen, Germany) and photographed with a Contax 167 MT camera (Yashica Kyocera, Hamburg, Germany) by using a Fujichrome Sensia II 100 Film (Fuji Photo Film Co., LTD., Japan). The slides were scanned with a SprintScan 35 scanner (Polaroid, Taiwan).

3.1.5 Wideband filter and cutoff filter boxes

Commercially available wideband filter and cutoff filter (Corning NetOptic, USA) sheets were fabricated into boxes of 44 x 30 x 15 cm^3 which are used for the study of light-regulated TRPL localisation. Fig 7 shows the transmission spectra of wideband and cutoff filters.

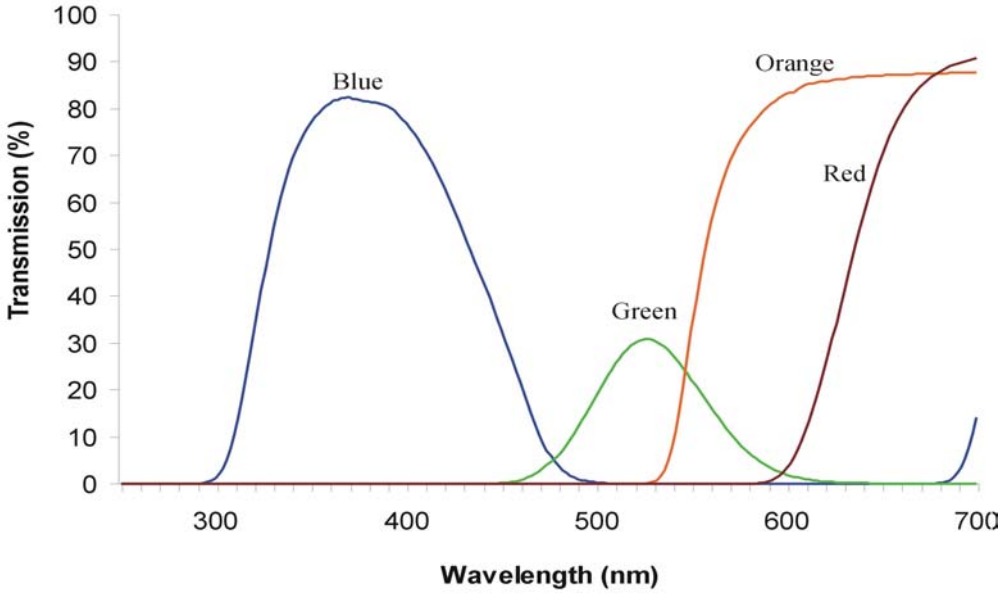

Fig 7: Spectral transmission of wideband filters (blue & green) and cutoff filters (orange & red).

3.1.6 Primary and secondary antibodies

All polyclonal primary antibodies against the phototransduction cascade proteins of *Drosophila* and *Calliphora* were generated by expressing the mouse DHFR containing the carrier protein in *E.coli*. Monoclonal mouse antibodies α-DmRH1 antibody were obtained from Developmental Studies Hybridoma Bank (University of IOWA, USA), α-DmRH3, α-DmRH4, and α-DmRH5 were generous gift by Prof. Dr. Steve Britt (University of Denver, USA). Anti-β-galactosidase antibody was obtained from Mo Bi Tec (Göttingen, Germany).

Table 3: Antibodies / Antisera used in this thesis. Dm-*Drosophila melanogaster*; Cv-*Calliphora vicina*; WB-Western blot; IC-Immunocytochemistry

Antiserum / Antibody	Species	Antigen (amino acids)	Host species	Dilution	Reference
α-DmTRP	*Drosophila*	906-1275	Rabbit	1:1000 (WB) 1:20 (IC)	Bähner, 2001
α-DmTRPL	*Drosophila*	1083-1097	Rabbit	1:1000 (WB) 1:20 (IC)	Bähner, 2001
α-DmINAD	*Drosophila*	281-550	Rabbit	1.1000 (WB) 1:20 (IC)	Bähner, 2001
α-DmRH1	*Drosophila*	237-258	Rabbit	1:1000 (WB) 1.20 (IC)	Bentrop *et al*., 1997
α-DmRH1 (4C5)	*Drosophila*	Unspecified	Mouse	1:20 (IC)	de Couet *et al*., 1987 (Developmental Studies Hybridoma Bank, Iowa, USA)

α-DmRH3	*Drosophila*	359-380	Mouse	1:20 (IC)	Steve Britt (University of Denver, USA)
α-DmRH4	*Drosophila*	352-366	Mouse	1:20 (IC)	Chou *et al*., 1999
α-DmRH5	*Drosophila*	353-367	Mouse	1:20 (IC)	Chou *et al*., 1999
α-DmRH6	*Drosophila*	231-260	Rabbit	1:20 (IC)	Huber *et al*., 1997
α-β-Galactosidase (A-11132)	*E. coli* β-Galactosidase	-	Rabbit	1:40 (IC)	Fagotto *et al*., 1999 (Mo Bi Tec, Göttingen, Germany)
α-DmPLCβ	*Drosophila* , *Calliphora*	66-231	Rabbit	1:1000 (WB) 1:20 (IC)	Bähner, 2001
α-CvTRP	*Calliphora*	671-1183	Rabbit	1:1000 (WB) 1.20 (IC)	Huber *et al*., 1996a
α-CvTRPL	*Calliphora*	Last 300 amino acids	Rabbit	1.1000 (WB) 1:20 (IC)	Paulsen *et al*., 2000
α-CvINAD	*Calliphora*	262-542	Rabbit	1:1000 (WB) 1:20 (IC)	Huber *et al*., 1996b
α-CvePKC	*Calliphora*	312-685	Rabbit	1:1000 (WB)	Sander, 1996
α-CvRH1	*Calliphora*	333-371	Rabbit	1:1000 (WB) 1:20 (IC)	Sander, unpublished

Table 4: Secondary antibodies used in this thesis

Species Specificity	Host Species	Fluorochrome	Company
Rabbit (Whole molecule) F (ab')$_2$	Goat	FITC	Sigma, Germany
Mouse IgG	Goat	Cy^{TM5}	dianova, Germany

3.2 Biochemical methods:

For western blot analysis, one week old (unless specified) *Calliphora* and *Drosophila* fly heads were dissected under binocular (Zeiss Stermi 2000) (Zeiss, Wetzlar, Germany) with a cold light source (Schott KL 750) (Schott, Mainz, Germany).

3.2.1 Purification of anti-*Drosophila* TRPL and anti-*Drosophila* Rh1 antiserum

Following purification of α-DmTRPL (Bähner, 2001), and α-DmRh1 (Bentrop *et al*., 1997) antiserum by column chromatography, these antisera had to be additionally purified to eliminate unspecific labeling that was observed in immuocytochemical experiments. Heads of *trpl*^{302} and *ninaE*^{oI17} were homgenised in SDS-PAGE buffer (1 µl / 1 head) and allowed to stand for 10 minutes. To obtain a purified protein extract, the homogenate was centrifuged for 10 minutes at 14.000 rpm, 4°C. The supernatent was pipetted on a Immuno-Blot-PVDF membrane (0.2 µm) (Bio-Rad, München, Germany) and incubated for 2-3 hours. The membrane was blocked for 4 hours in 2% (w/v) BSA in 1X TBS (150 mM NaCl, 50 mM Tris-HCl pH 7.3) and washed in TBS two times 10 minutes each before incubating it in the respective primary antiserum overnight. The membrane was then discarded and 0.04% (w/v) NaN_3 was added to the antibody for long term storage.

3.2.2 Preparation of photoreceptor membranes from *Calliphora*

The heads of male *Calliphora* flies were cut from the rest of the body. With the help of a razor blade, the ventral side of the head was cut open and the retina was dissociated from the rest of the brain by using a pair of forceps. The dissected retinae were then kept in ice cold distilled water causing the retinula cells to swell. The retinula cells were then broken apart by slowly and repeatedly pipetting each retina through a fine pipette. The resulting mixture containing retina's were centrifuged for 10 minutes at 1000g, room temperature. The tracheae and basement membranes which floated in the supernatant were discarded (Paulsen, 1984)

To the pellet, 500 µl of distilled water, 26 µl of Percoll (Amersham Pharmacia Biotech, Freiburg, Germany), and 24 µl 50 mM Na-Phosphate buffer pH 6.2 was added and centrifuged for 10 minutes at 12.000 rpm, 4°C. The rhabdom pieces which were of different lengths concentrated as a band at the top of the gradient. To obtain purified photoreceptor membranes, the isolated rhabdoms were pipetted out. An equal volume of 50 mM Na-Phosphate buffer pH 6.2 was added and the sample was centrifuged for 10 minutes at 55.000 rpm, 4°C. The supernatant was removed and the pellet was stored at - 80°C.

3.2.3 Extraction of photoreceptor proteins from *Calliphora*

To the isolated rhabdoms, SDS buffer containing 1% β-Mercaptoethanol (1µl / retina) was added and allowed to stand for 10 minutes. To obtain a purified protein extract, centrifugation was performed for 10 minutes at 55.000 rpm, 4°C. Bromophenol blue was added (2 µl / 10µl of sample extract) to the sample just before loading it to the polyacrylamide gel.

3.2.4 Preparation of *Drosophila* heads / eye cups for extraction of heads / eye proteins

50 *Drosophila* were anaesthesised with CO_2 and the heads were cut apart from the rest of the body and placed in an 1.5 ml eppendorf containing 100 µl SDS-PAGE buffer (2µl/head) and β-Mercaptoethanol (1%) (Serva, Heidelberg, Germany). For preparing protein extracts from P (Rh1 + 3), eye cups were dissected with a razor blade and extracted in SDS-PAGE buffer (1µl SDS-PAGE / 1 eye cup). The heads / eye cups were then homogenised with a plastic pestle and allowed to stand for 10 minutes at room temperature followed by a brief centrifugation (~ 1 minute 14.000 rpm, 4°C). To obtain a purified protein extract, the supernatent was once again centrifuged for 10 minutes at 55.000 rpm, 4°C. The resulting pellet was discarded and the supernatant stored at -80°C. Just prior to loading the sample on the polyacrylamide gel, bromophenol blue (2µl / 10 µl extract) was added to the extract.

3.2.5 Preparation of SDS-PAGE gels

The composition of 8% and 12% Seperating and Stacking polyacrylamide gels are given in table 3. Distilled water was added on top of the Seperating gel during the polymerisation process. The gel was allowed to polymerise for 10-15 minutes at 50°C. After polymerisation, the distilled water was removed with the help of Gel blotting paper (GB 002, Schleicher & Schuell, Dassel, Germany) and substituted by Stacking gel, simultaneously inserting a polystyrene comb and allowing it to polymerise in the same way as below.

Table 5: Composition of Seperating and Stacking gels for SDS-PAGE

Constituents	Seperating gels (8 % / 12 %)	Stacking gel (6 %)
Acrylamide	8 % / 12 % (w/v)	6 % (w/v)
Bisacrylamide	0.08 % / 0.12 % (w/v)	0.06 % (w/v)
Tris-HCl	0.375 M pH 8.8	0.125 M pH 6.8
SDS	0.1 % (w/v)	0.1 % (w/v)
APS (Ammonium persulphate)	0.05 % (w/v)	0.1 % (w/v)
TEMED (Tetramethylenediamine)	0.075 % (w/v)	0.15 % (w/v)

3.2.6 Sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE)

The analytic seperation of protein extracts were carried out on 8% or 12 % homogenous (table 3) acrylamide gels which were 0.75 mm thick, 5 cm long (Seperating gel) and 1 cm long (Stacking gel) using a discontinous buffer (Stacking gel buffer-0.5 M Tris / HCL pH 6.8, 0.4% (w/v) SDS; Seperating gel buffer-1.5 M Tris / HCL pH 8.8, 0.4% (w/v) SDS; and Running buffer-0.25 M Tris/HCL pH 8.3, 1% (w/v) SDS), according to Laemmli system (Laemmli, 1970). The electrophoresis of the protein samples was carried out by using a “2050 Midget” electrophoresis unit (Amersham Pharmacia Biotech, Freiburg, Germany) for 1 hour at constant 20 mA. A 10 kDa molecular weight standard marker (Gibco-BRL, Karlsruhe, Germany) was used as a size marker.

3.2.7 Coomassie staining of SDS-PAGE gel

To visualise the protein pattern, the gel was stained for 40 minutes in 0.2 % (w/v) Serva Blue R (Serva, Heidelberg, Germany), 50 % (v/v) methanol, 10 % (v/v) acetic acid at room temperature. The gel was finally destained in 25 % (v/v) methanol, 10 % (v/v) acetic acid.

3.2.8 Transfer and immunological detection of proteins from SDS-PAGE gel

After SDS-PAGE electrophoresis, the proteins were transfered electrophoretically from the gel to a Immuno-Blot-PVDF membrane (0.2 µm) (Bio-Rad, München, Germany) in a Semi-Dry Transfer Cell (Bio-Rad, München, Germany) for 1 hour at constant current (0.8-1 mA / cm^2). Just prior to the above use, the PVDF membrane was equilibrated for 5 minutes in blot buffer (50 mM Tris/HCL pH 8.0; 0.1 % (w/v) SDS; 20 % (v/v) Methanol). After electrophoretic transfer, the membrane was equilibrated in TBS-T for 5 minutes and the unspecific binding sites were blocked for 1 hour in 5 % Skim Milk Powder in TBS-T. Prior to incubation with the primary antibody, the membrane was washed three times for 10 minutes in TBS-T. The membrane was incubated in the primary antibodies (see table 3) for approximately 12 hrs. The membrane was then washed three times in TBS-T for 10 minutes each, followed by incubation with either Protein A / Alkaline Phosphatase-Conjugate (Sigma, München, Germany) (1:1000 in TBS-T) or Goat anti-Mouse Alkaline Phosphatase-Conjugate (Sigma, München, Germany) (1:25000 in TBS-T) for 1-2 hours. Following incubation, the membrane was washed three times in TBS-T for 10 minutes and equilibrated briefly in equilibrium buffer (50 mM Tris-HCl pH 9.5; 150 mM NaCl; 10 mM $MgCl_2$). The protein bands were visualised through a chromogenic reaction with 300 µg/ml NBT (4-nitro-blue-tertazolium chloride) and 150 µg/ml BCIP (5-bromo-4-chloro-3-indolyl phosphate). The reaction was terminated by washing the membrane in distilled water.

3.2.9 Staining of PVDF membrane with Amido black

The PVDF membrane containing the molecular weight marker was stained for 5 minutes in 0.1 % (w/v) Amido black; 45 % (v/v) methanol; 10 % (v/v) acetic acid and destained in 25 % (v/v) methanol; 10 % (v/v) acetic acid.

3.3 Immunocytochemical methods

For performing immunocytochemical experiments in this thesis, *Calliphora* and *Drosophila* heads / eyes were dissected under a binocular (Zeiss Stermi 2000) (Zeiss, Wetzlar, Germany) with a cold light source (Schott KL 750) (Schott, Mainz, Germany). For studying light-regulated TRPL localisation in *Calliphora* or *Drosophila*, eyes were dissected under different light condition pertaining to the experimental approach (refer fig 7). A 21 x 10 x 10 cm^3 dark box was used for dark kept flies while light kept flies were kept under a white fluorescent light (Philips TLD 36W- 1/25) (Philips, France) for approximately 12 hours at a light intensity of 500-1000 Lux. The dark raised and light raised flies are hereafter designated as "Dark kept" and "Light kept" flies. For studying TRPL distribution in blue, green, and orange light, wild type and P (Rh1 + 3) flies were kept in coloured boxes (refer 3.1.5) for 12 hours and eyes were dissected pertaining to the light condition.

3.3.1 Preparation and processing of heads or eye sections for immunocytochemistry

Fly heads or dissected eyes were fixed in 2 % (w/v) paraformaldehyde (except for detecting β-galactosidase by X-gal staining, heads were fixed in 1 % (w/v) paraformaldehyde; 2 % (v/v) glutaraldehyde in 1x PBS) for 2 hours. This was followed by three subsequent washes in 10 % (w/v) sucrose in distilled water (Roth, Karlsruhe, Germany) and two washes in 25 % (w/v) sucrose in distilled water for 15 minutes each. The heads / eyes were then infiltrated with 50 % (w/v) sucrose in distilled water overnight at room temperature. The heads / eyes were embeeded in boiled calf liver and covered with Tissue Tek (Polysciences, Eppelheim, Germany). The embeeded heads / eyes were then jerk freezed in melting isopentane (Fluka, Neu-Ulm, Germany) that was cooled by liquid nitrogen. The specimen were stored at -80 °C in 50 ml falcon tubes containing isopentane.

Heads / eyes were sectioned at 10 µm thickness in a cryostat at -20 to -25 °C and obtained on coverslips precoated with 0.01 % (v/v) aqueous Poly-L-lysine (prepared in distilled water) (Sigma, Deisenhofen , Germany). The cryosections were encircled with a

Pap-Pen (Polysciences, Eppelheim, Germany) and incubated in 2% (w/v) paraformaldehyde for 10-15 minutes followed by two washes in 1x PBS buffer (for X-Gal staining, sections were briefly incubated with 1x PBS). For staining the rhabdomeres and plasma membranes in *Drosophila* and *Calliphora*, the sections were incubated with Wheat Germ Agglutinin (WGA) coupled to either FITC (fluorescein isothiocynate) or Tetramethylrhodamine (Mo Bi Tec, Göttingen, Germany) (0.1 mg / ml in 1X PBS) for 4 hours at room temperature followed by 3 washes in 1x PBS, 5-7 minutes each and mounted in Mowiol 4.88 (Polysciences, Warrington, USA).

For antibody staining, the sections were blocked depending on the primary antibody used.

a. 0.2 % (v/v) Triton X-100 (Serva, Heidelberg, Germany) in blocking solution (for labeling Rh1, Rh4, Rh5 & Rh6 rhodopsins in *Drosophila*). Incubation time: 30 minutes.

b. PBS-S (for labeling TRP, TRPL, INAD, Rh3 in *Drosophila* and Rh1, TRP, TRPL, INAD, PLCβ in *Calliphora*). Incubation time: 1 hour.

c. Blocking solution (refer 3.1.2) for anti-β-galactosidase labeling. Incubation time 30 minutes.

After blocking the unspecific binding sites, the sections were incubated overnight at 4°C with the primary antibody diluted in PBS-S (for Rh1, Rh4, Rh5, Rh6, and β-galactosidase were diluted in blocking solution). The sections were subsequently washed three times in 1x PBS, 5-7 minutes each. For single, double or triple immunocytochemical labeling, secondary antibodies were diluted 1:150 in blocking solution, mixed with WGA coupled to tetramethylrhodamine (WGA-TMR; 0.1 mg / ml) (Mo Bi Tec, Göttingen, Germany) and centrifuged for 10 minutes at 14000 rpm, 4°C. The sections were incubated with the secondary antibody for 5-7 hours at room temperature and finally washed three times in 1x PBS, 5-7 minutes each, mounted in Mowiol 4.88 and examined with a confocal laser scanning microsope (LSM-SP) (Leica, Bensheim, Germany). Confocal images were

obtained by using a Leica TCS NT Version 1.6.582 program for which the settings are displayed in table 6.

Table 6: Software settings used for obtaining confocal images

Fluorochrome	Excitation (nm)	Emission (nm)	Potentiometer settings	Offset settings
Fluorescein (FITC)	488	500-550	750-850	-50 to -90
TRITC / WGA-TMR	568	580-630	580-680	-20 to - 40
Longwavelength / Cy5	647	665-800	700-800	-20 to -30

3.4 Whole mount staining for β-galactosidase

The heads from reporter gene flies which were 3-5 days old (Post-eclosion) were cut off from the rest of the body with a razor blade. The heads were cut into two halves between the eyes, placed in 1.5 ml eppendorfs containing X-Gal staining solution and incubated for 4-5 hours at room temperature. The heads were gently washed in 1x PBS to remove excess of X-Gal stain upon blue colour development.

3.5 Deep pseudopupil method

Calliphora flies were anaesthesised on ice and fixed with an insect pin (0.25 mm) to a microscope slide. The deep pseudopupil was viewed with a Zeiss Stermi SV 11 (Carl Zeiss, Oberkochen, Germany) binocular with 2.5X magnification using indirect cold light source (Schott KL 750) (Schott, Mainz, Germany). Pictures were obtained and processed as described in 3.1.4.

4. Results

4.1 Characterisation of antibodies and lectin labeling

In this thesis, localisation of phototransduction proteins as well as dynamic processes such as synthesis and light-regulated influence of proteins involved in phototransduction are studied primarily by monitoring immunofluorescence by confocal laser scanning microscopy. The term "labeling" in this thesis applies for a procedure that involves detection of primary antibody interaction with a secondary fluorescently coupled antibody or by a primary reaction of a fluorescent-lectin with a glycoprotein.

A prerequisite for the differentiation between mitochondrial proteins and the visualisation of the major photoreceptor compartments requires it to have a set of methods at hand which specifically allows to detect the expression of individual proteins at the cellular level. To study proteins assembled into the INAD signaling complex, the initial approach was to visualise the rhabdomeric compartments primarily by fluorescently labeling the glycoproteins expressed in the photoreceptors which interact with lectins in particular WGA (Wheat Germ Agglutinin), by using rhodopsin and β-galactosidase (from reporter gene flies) expression. The different rhabdomeres or subcellular compartments within an ommatidia are identified on the basis of their rhodopsin expression which is followed either by detecting the expression of the *lacZ* reporter gene or directly by labeling with specific markers.

4.1.1 Specificity of antibodies directed against *Drosophila* phototransduction proteins tested by western blots

In this immunolabeling experiment monoclonal as well as polyclonal antibodies have been tested. The polyclonal antibodies against *Drosophila* phototransduction proteins comprised of antiserum against *Drosophila* Rh1 (Bentrop *et al*., 1997), *Drosophila* Rh6 (Huber *et al*., 1997), *Drosophila* INAD (Bähner, 2001), *Drosophila* TRP (Bähner,

2001), and *Drosophila* TRPL (Bähner, 2001) which are presented in context with the present study. The monoclonal antibodies employed were directed against *Drosophila* Rh3, Rh4 and *Drosophila* Rh5 (Chou *et al*., 1999). In order to reduce background staining in immunocytochemistry, the *Drosophila* anti-TRPL and anti-Rh1 antibodies had to be additionally purified by preabsorption against a PVDF membrane containing SDS-extracted proteins from a homogenate of $trpl^{301}$ and $ninaE^{ol17}$ mutant heads. To demonstrate the specificity of these antibodies, *Drosophila* tissue containing 17µg of protein (equivalent of 1.5 heads) were subjected to SDS-PAGE on 8%/12% polyacrylamide gels. Fig 8A lane 1 shows the coomasie stained protein pattern indicating that an equal amount of protein was loaded on each lane.

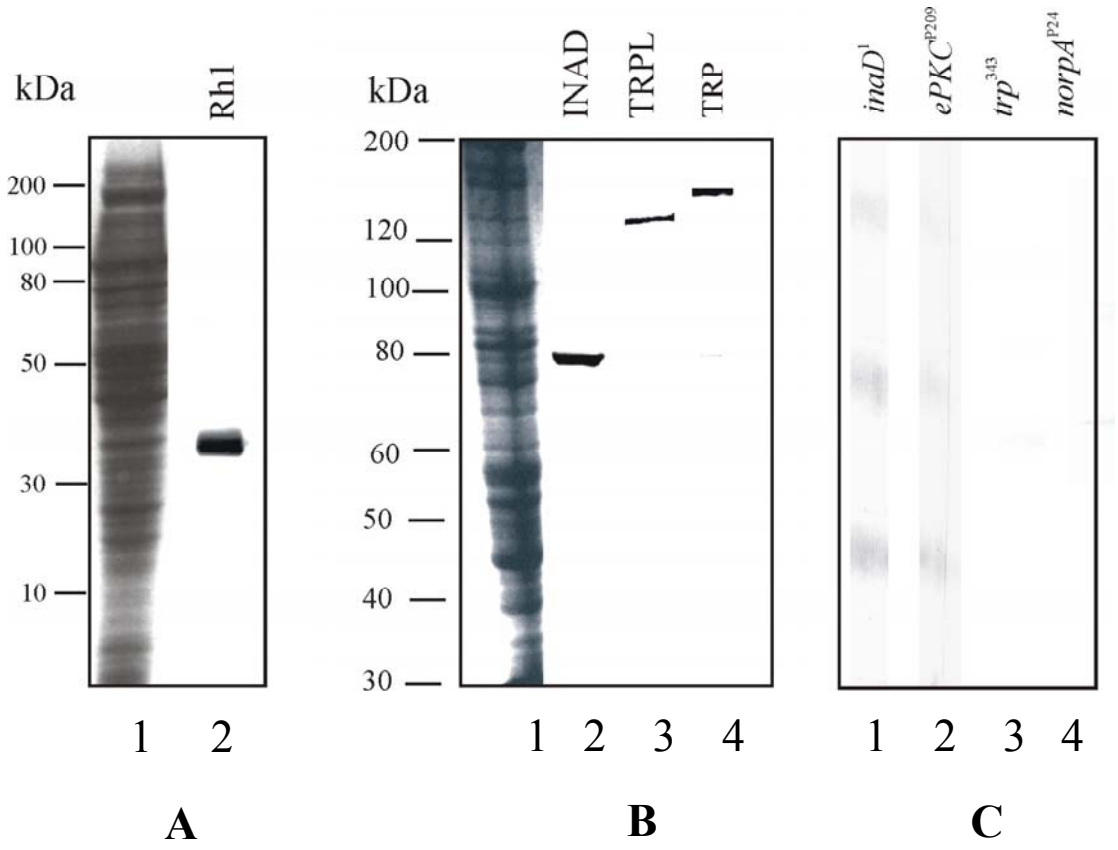

Fig 8: Characterisation of primary antibodies used against proteins of the phototransduction cascade of *Drosophila* by western blots

Proteins were extracted from eyes of *Drosophila* by SDS-buffer and seperated by SDS-PAGE. 17 µg of protein (equivalent to 1.5 eye cups) were loaded in each lane and analysed by western blot. Cross reactivity of the primary antibody was detected by Protein A alkaline phosphatase conjugate. (A) wild type *Drosophila*, lane 1 (12%) coomasie gel, lane 2 Rh1 band. (B) wild type *Drosophila*, lane 1 (8%) coomasie gel, lane 2, 3, 4 detection of the respective protein bands as indicated. (C) analysis of eye protein extract from *Drosophila* mutants as indicated for: lane 1 INAD; lane 2 ePKC; lane 3 TRP; lane 4 PLCβ.

α-DmRh1 with one major band at 33kDa is detected (Fig 8A lane 2) which shows that fly rhodopsins have a higher mobility on SDS-PAGE gels than expected from the molecular mass of 42kDa (Bentrop *et al*., 1997). Western blot analysis for phototransduction proteins of higher molecular weights yielded the results shown in Fig 8B. Major bands at 80 kDa (α-DmINAD), 130kDa (α-DmTRPL), 140kDa (α-DmTRP) (Fig 8B lane 2, 3, 4) confirms the fact that the antiserum used detects specifically their antigen as observed previously (Tsunoda *et al*.,1997). Specificity of the antibodies was confirmed with head extract from *Drosophila* mutants which do not express the respective gene (Fig 8C).

4.1.2 Specificity of antibodies directed against *Calliphora* phototransduction proteins tested by western blots

The antibodies directed against *Drosophila* phototransduction proteins sometimes cross-react with their counterparts in the *Calliphora* compound eye. In many cases, the homology between the phototransduction proteins of the *Drosophila* and the *Calliphora* eye is not high enough to give reliable signals with anti-*Drosophila* antibodies in *Calliphora* eye tissue. In this case, antiserum had to be generated against these proteins recombinately expressed from the *Calliphora* gene. The antibodies generated against *Calliphora* phototransduction proteins were characterised by western blot analysis. The polyclonal antibodies generated against *Calliphora* TRPL (Paulsen *et al*., 2000), *Calliphora* TRP (Huber *et al*., 1996a), *Calliphora* INAD (Huber *et al*., 1996b), *Calliphora* ePKC (Huber *et al*., 1998), *Calliphora* Rh1 were directed to detect the C-terminal region of the respective protein. *Drosophila* anti-PLCβ antibody showed a reliable cross-reactivity with *Calliphora* PLCβ (Huber *et al*., 2000). Inorder to test the specificity of these antibodies, protein extracts were prepared from isolated *Calliphora* rhabdomeral membranes as described by Paulsen (1984). As shown in fig 9A lane 1 and B lane 1, equal amounts of protein extracts were loaded onto each lane. Protein extracts were seperated by SDS-PAGE on 8% and 12% polyacrylamide gels and subjected to western blot analysis. As expected, the antibodies specifically recognise their antigen by displaying a major band at the correct molecular weight that had been calculated for the respective protein 33 kDa

(α-CvRh1), 80 kDa (α-CvINAD), 85 kDa (α-CvePKC), 140 kDa (α-CvTRP), 130 kDa (α-CvTRPL) and 135 kDa (α-DmPLCβ).

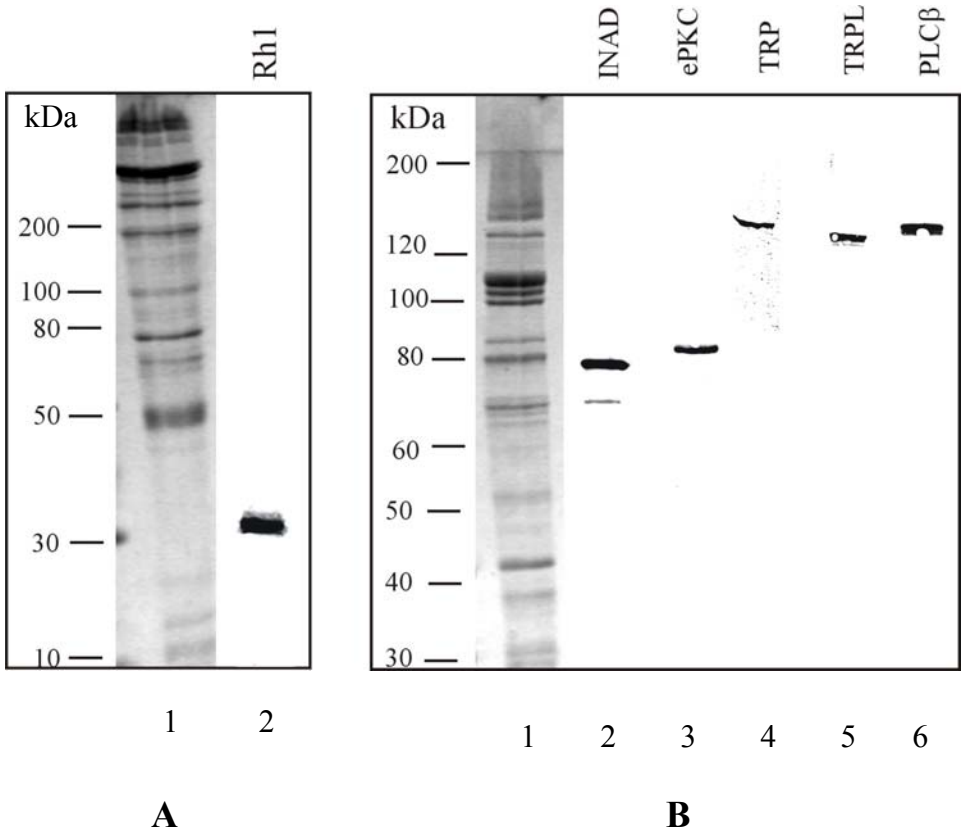

Fig 9: Characterisation of antibodies against proteins involved in the phototransduction cascade in *Calliphora*

Proteins of purified photoreceptor membranes which were obtained from wild type *Calliphora* were seperated by SDS-PAGE and subjected to western blot analysis. (A) lane 1 pattern (12%) gel; lane 2 Rh1 band at 33 kDa. (B) lane 1 pattern (8%) gel; INAD, ePKC, TRP, TRPL, PLCβ are detected at 80 kDa, 85 kDa, 140 kDa, 130 kDa and 135 kDa respectively.

4.1.3 Labeling of fly rhabdomeres by lectins

In general, most of the proteins involved in the phototransduction cascade will be localised and detected, which at the same time will provide information on the state of rhabdomeric compartment. However, if a phototransduction protein is missing due to a mutation or is internalised, the availability of a independent marker for identifying rhabdomeres by fluorescent microscopy is essential. Although rhabdomeres can readily be

identified in transmission images of cross sections through *Drosophila* eyes, transmission images are not ideal for use in combination with confocal microscopy because they do not represent confocal images. To obtain confocal images of rhabdomeres, lectins which bind to specific sugar molecules of glycoproteins present in the rhabdomeral membranes represent versatile primary detection reagents. Paulsen and Bentrop (1986) reported that Concanavalin A has a high affinity for proteins present in isolated rhabdomeres. A test approach with different lectins showed that lectin WGA (Wheat Germ Agglutinin) was found to be particularly useful for labeling the rhabdomeres in histochemical sections. This is revealed by CLSM images of longitudinal and cross sections of wild type *Drosophila* eyes in which WGA is observed to label the rhabdomeres (Fig 10A, B, C, D). Cross sections through wild type *Drosophila* eyes labeled with α-DmRh1 and WGA confirms that WGA colocalises with Rh1 to the outer R1-6 cell (Fig 10E & F). Besides, the border of the intraommatidial matrix that is bonded to the rhabdomeres displays a WGA positive signal. In cross sections through eyes of wild type *Calliphora*, WGA labels all the rhabdomeres i.e. the outer as well as the central rhabdomeres which is also confirmed by Rh1 labeling using double fluorescent labeling technique (Fig 11A, B, C, D).

Thus, the use of lectin WGA labeling supports to reliably identify the photoreceptor cell type and the identification of position within the ommatidia by fluorescence. Apart from identifying the number of rhabdomeres within a ommatidia, it also allows to monitor rhabdomeric degeneration. The colocalisation of WGA with Rh1 labeling (Fig 10 & 11) shows that WGA labeling does not interfer with Rh1 labeling, indicating its further use in double and triple labeling immunocytochemical techniques. Moreover, if the experiment requires triple labeling of a section i.e., in addition to the use of a monoclonal and a polyclonal antibody, one is dependent on a non-immune marker, for which, WGA can be used.

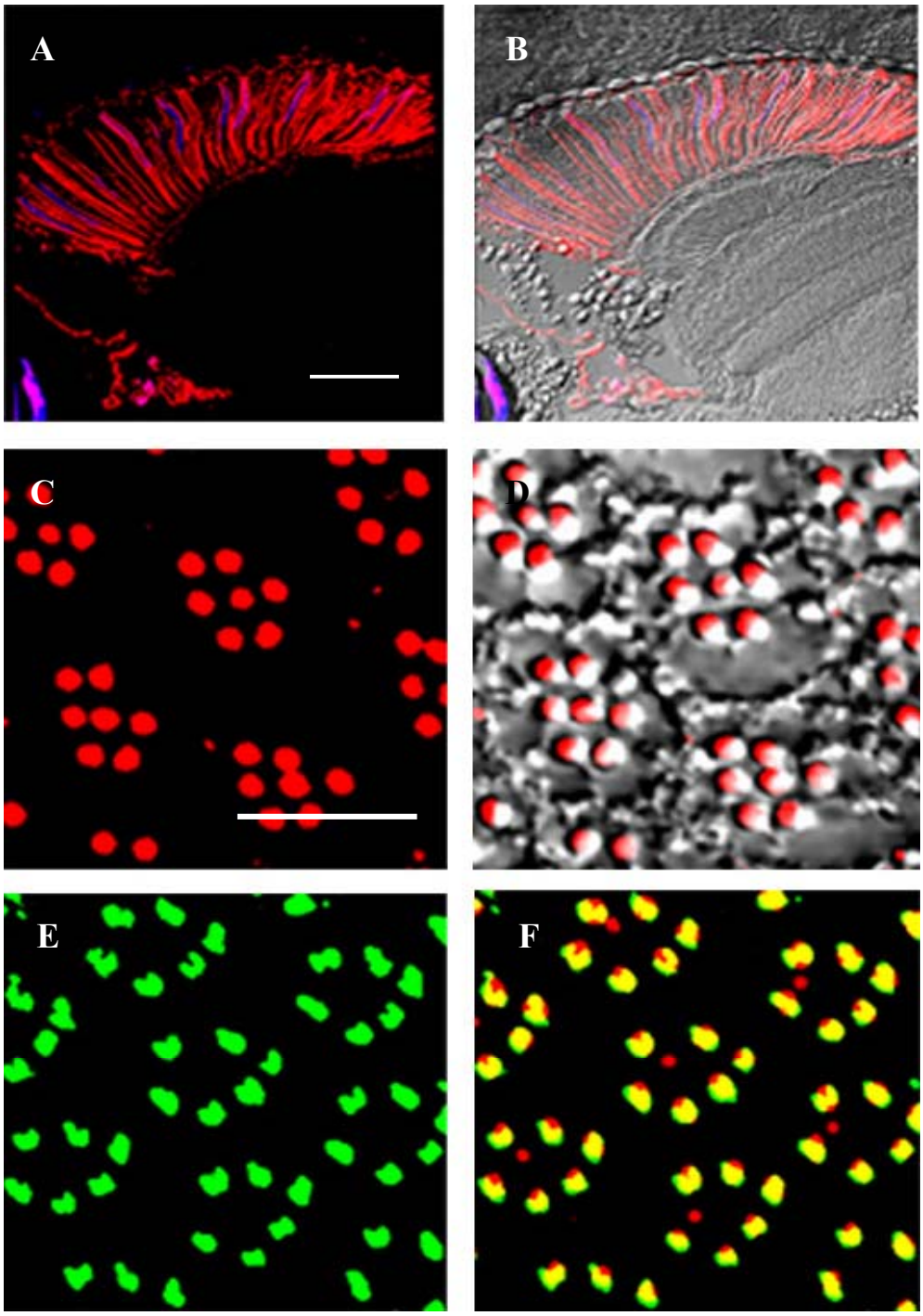

Fig 10: The lectin WGA labels rhabdomeres of the compound eye of *Drosophila*

The lectin Wheat Germ Agglutinin (WGA) coupled tetramethylrhodamine (TMR) (red colour) (0.1mg / ml) was used to label sections through the eyes of wild type *Drosophila.* 10µm thick (A) longitudinal sections labeled for α-DmRh3 (blue) and WGA-TMR (red) showed an even distribution of WGA over the entire rhabdomeres (C) cross sections probed for WGA labeling. (B) and (D) show overlay of WGA labeling with the corresponding transmission image. Cross sections though eyes of wild type *Drosophila* were labeled with α-DmRh1 and WGA-TMR (red colour). (E) Rh1 is detected by FITC (green colour) coupled secondary antibody. (F) overlay image of Rh1 labeling with WGA showing colocalisation of WGA in R1-6 cells (yellow colour). Scale bar in (A)-(B): 50µm; (C)-(F): 10µm.

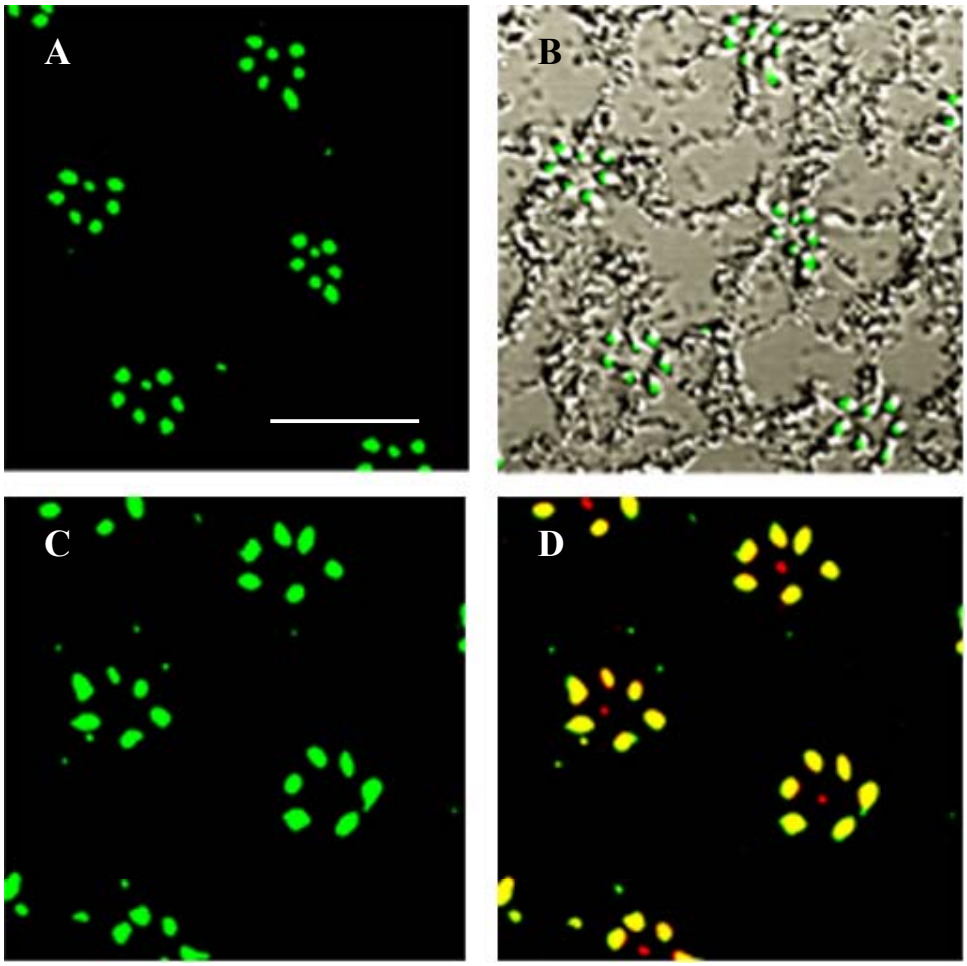

Fig 11: Rhabdomeres in *Calliphora* showing lectin WGA labeling

Wheat germ agglutinin (WGA) coupled Oregon Green (OG) (green colour) and Tetramethylrhodamine (TMR) (red colour) were used to label sections through eyes of wild type *Calliphora.* 10µm thick cross sections were probed with (A) WGA-OG (green colour) (0.1mg / ml) and (C) WGA-TMR (red colour) (0.1 mg / ml) & α-CvRh1 antibody. α-CvRh1 is detected by Goat anti-rabbit FITC (green colour) coupled secondary antibody. (B) show overlay of WGA labeling with corresponding transmission image. (D) overlay image showing colocalisation of WGA with Rh1 labeling indicated by yellow colour. Scale bar: 10µm.

4.1.4 Immunocytochemical localisation of rhodopsins for the characterisation of rhabdomeric compartments in the compound eye of *Drosophila*

As already reported, each ommatidium is an assembly of 12 accessory cells and eight photoreceptor cells (Hardie, 1986; Wolff and Ready, 1993). It was formerly mentioned that spectral, morphological, and genetic criteria divide the eight ommatidial photoreceptors just into three distinct cell type: cells R1-6, cell R7, and cell R8 (Harris *et*

al., 1976; Heisenberg and Wolf 1984). However, one of the criterias (expression of rhodopsin) turned out that the central cell types, R7 and R8, can be furture differentiated into two pairs according to the pattern of rhodopsin expressed in them. Fig 12 shows the example in which longitudinal sections through wild type eyes were probed with α-DmRh1, α-DmRh3, α-DmRh4, α-DmRh5 and α-DmRh6 antibodies.

As expected from the known localisation of rhodopsins (see introduction), Rh1 rhodopsin is observed to be localised in the R1-6 photoreceptor cells that extend throughout the entire retina (Fig 12A & B). Rh3 and Rh4 rhodopsins localise to the distal half of the retina and label subsets of the R7 photoreceptor cells judged by their location in the retina (Fig 12C, D, E, F). By employing double labeling immunocytochemical technique, Rh5 and Rh6 are observed in the distal half of the retina in subsets of the R8 photoreceptor cells (Fig 12G & H).

To localise the plane of section within the compound eye of *Drosophila*, two approaches can be employed namely; labeling for the rhodopsins expressed in the R7 / R8 cell or the positional entry of the R7 / R8 cell within the ommatidium. To investigate this, cross sections through the eyes of wild type *Drosophila* flies were incubated with α-DmRh3, α-DmRh4, α-DmRh5 and α-DmRh6 antibodies. As reported from previous workers (see introduction), Rh3 and Rh4 are expressed in non-overlapping subsets of R7 cells which enter the ommatidia between the R1 and R6 cell (Fig 13A & B). Rh5 and Rh6 are observed to be present in subsets of non-overlapping R8 cells which has already been reported by previous workers (Fig 13C & D). The positional location of the R8 cell within the ommatidia is between R1 and R2 cell.

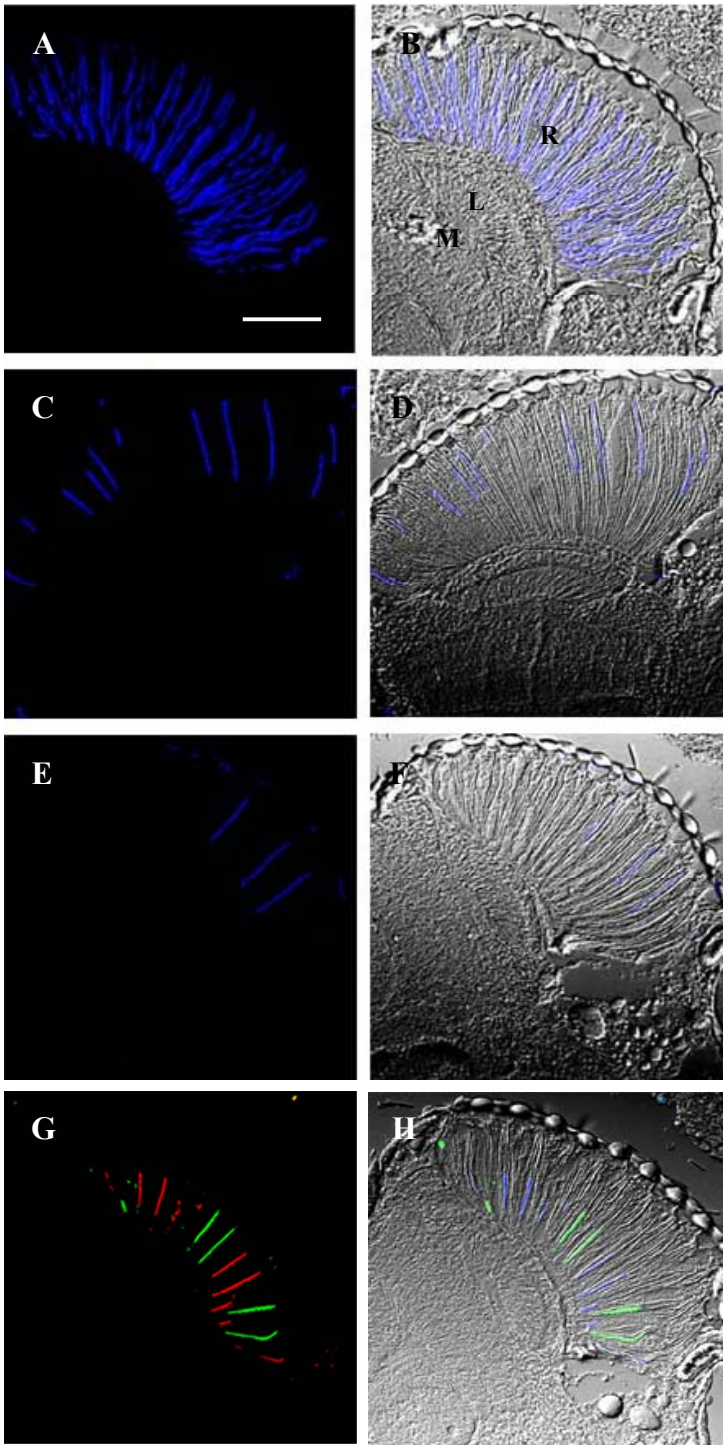

Fig 12: Localisation of rhodopsins for the characterisation of rhabdomeric compartments within the compound eye of *Drosophila*

10µm cryosections were probed with antibodies directed against the different rhodopsins expressed in the adult compound eye of *Drosophila melanogaster*. The primary antibodies used were detected with sheep anti-mouse CY5 (blue) except for the primary antibody against Rh6 rhodopsin which was detected by goat anti-rabbit FITC (green). Longitudinal section through adult fly head showing the localisation of (A) Rh1 rhodopsin in the entire retina while (C) Rh3 & (E) Rh4 rhodopsin in the proximal half of the retina. Localisation of (G) Rh5 and Rh6 rhodopsin in the distal half of the retina. (B), (D), (F) and (H) show an overlay of the fluorescent images with the corresponding transmission images. Note: the red fluorescence in (G) is displayed as blue in the corresponding overlay. R-retina; L-lamina; M-medulla. Scale bar: 50µm.

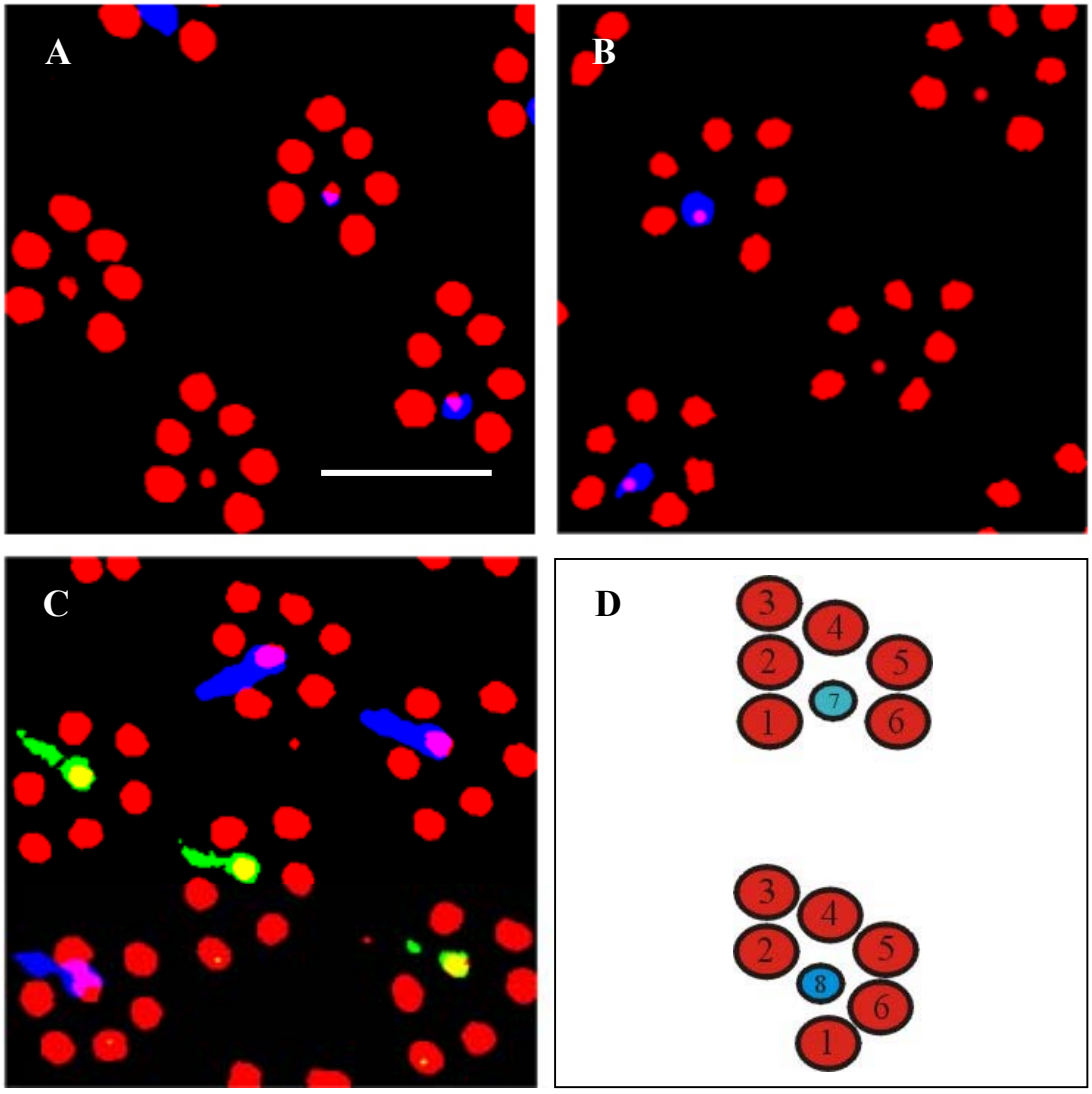

Fig 13: The photoreceptors of central R7 and R8 cells express the rhodopsin paired as Rh3 / Rh5 and Rh4 / Rh6 in non-overlapping subsets in the compound eye of *Drosophila*

10µm thick cross sections through eyes of wild type *Drosophila melanogaster* were probed with primary antibodies against the rhodopsins expressed in the R7 and R8 cells. Seen above are confocal overlay images for the localisation of (A) Rh3 (blue) with WGA-TMR (red) (B) Rh4 (blue) with WGA-TMR (red) (C) Rh5 (blue) and Rh6 (green) with WGA-TMR (red). (D) schematic representation displaying the positional entry of the R7 and R8 cell within a ommatidia. Scale bar: 10µm.

4.1.5 Characterisation of photoreceptor types by rhodopsin promoter driven reporter gene expression

As shown above, rhabdomeric compartments can be differentiated by the type of rhodopsin they express. As rhodopsin localisation is confined to the rhabdomere, only section at the level of the rhabdomere will reveal the ommatidial organisation. An elegant

alternate approach to characterise the type of photoreceptor cell and which allows to identify photoreceptor cells axons projecting into the optic lobes is to use reporter gene construct flies. In these flies, the bacterial gene, *lacZ*, is fused to the various rhodopsin promoters. Three of the transgenic flies investigated here had the *lacZ* gene fused to the respective promoter regions of Rh1 [w; P (Rh1. 833 *lacZ*)], Rh3 [w;; P(Rh3. 343 *lacZ*)] and Rh4 [w; P (Rh4. 1900 *lacZ*)] while in two reporter gene construct flies the *taulacZ* fusion construct was combined with the Rh5 [wy;; (Rh5. 2800 *taulacZ*)] and Rh6 [yw; P (Rh6. *taulacZ*)] promoter regions (Steve Britt, personal communication) (Fig 14).

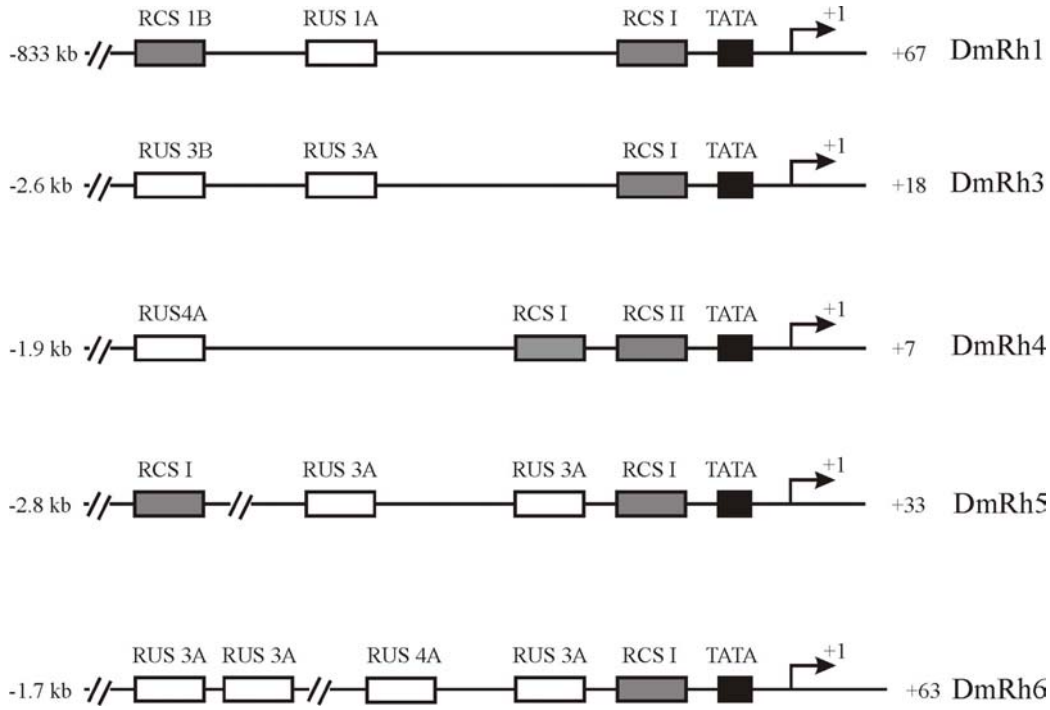

Fig 14: Schematic structure of *Drosophila* opsin promoters

Shown above are opsin promoter regions of the rhodopsins expressed in the compound eye of *Drosophila melanogaster*. The putative *cis*-acting elements including TATA-box (TATA) and rhodopsin core sequences (RCS I & II) are enclosed in solid boxes while distal conserved sequences specific to each promoter type (RUS elements) are enclosed in open boxes of Rh1 to Rh6 (DmRh1-DmRh6) opsin promoters.

To test the *lacZ* gene expression in whole mount preparations, heads were dissected into half and incubated in X-gal staining solution. The eyes that express the reporter gene under the Rh1 promoter region show an intense blue staining indicative of *lacZ* expression in the whole eye (Fig 15A). In P (Rh3. 343 *lacZ*) and P (Rh4. 1900 *lacZ*) blue staining in almost the whole eye except for the outer periphery of the eye (Fig 15B& C) is observed.

In P (Rh5. 2800 *taulacZ*) and P (Rh6. *taulacZ*), the staining pattern is restricted to the central region of the eye as shown in fig 15D & E. The presence of R8 cells at the distal half of the retina results in less spreading of the *lacZ* gene product, β-galactosidase, to the proximal half of the eye. As a negative control, eyes from wild type fly heads were also incubated with the chromogenic X-gal staining solution. Fig 15F shows in this case no β-galactosidase staining detectable in any part of the eye.

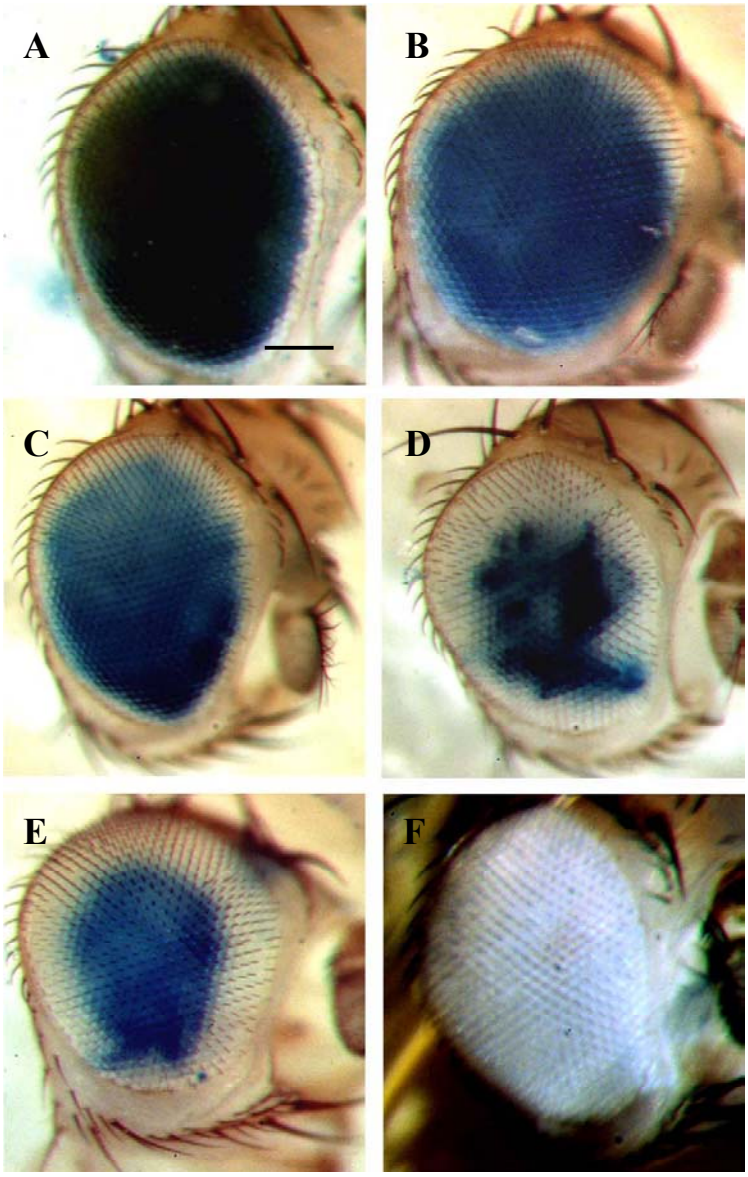

Fig 15: Detection of the bacterial *lacZ* gene by X-gal assay

The heads of transgenic *Drosophila* expressing the bacterial *lacZ* gene were cut into two halves and incubated in the chromogenic X-gal staining solution for 5-7 hrs at 24°C. Detection of β-galactosidase in transgenic *Drosophila* which express *lacZ* gene under the control of the (A) Rh1, (B) the Rh3, (C) the Rh4, (D) the Rh5, and (E) the Rh6 promoter region are shown. (F) wild type negative control. Scale bar: 100µm.

The whole mount result from reporter gene flies confirms that the expression of the product of the *lacZ* gene can be monitored in the whole eye, facilitating the study of gene expression / localisation.

To explore the spatial distribution of β-galactosidase, longitudinal sections through heads of these flies were analysed by histochemical staining using X-gal staining solution. Thus with the P (Rh1. 833 *lacZ*) construct, β-galactosidase expression in the retina and the first optic ganglion, the lamina is detected. The staining of the lamina is a result of the diffusion of β-galactosidase along the photoreceptor neurons of the Rh1 expressing R1-6 cells (Fig 16A) which make synaptic connections exclusively to the lamina, a observation which has also been reported by Mismer and Rubin (1987). Longitudinal sections through P (Rh3. 343 *lacZ*) and P (Rh4. 1900 *lacZ*) heads show that within the retina, both lines exhibit staining of R7 photoreceptors, as judged by their characteristic morphology and localisation to the distal half of the retina (Fig 16B & C). Throughout the sections, most of the retinal β-galactosidase is expressed in only a subset of the R7 cell population. As observed in Fig 16B & C it is possible to follow the thread like axonal projections of these cells which emerge from the R7 cell bodies, traverse the proximal retina and the lamina, cross over each other at the optic chiasma and enter the second optic ganglion, the medulla, to make their synaptic connections with second order neurons. The precisely aligned synaptic terminal of these axons at a single layer of the medulla is especially evident, as is their characteristic swelling prior to entering this optic ganglion. Within the medulla neuropil, the stained axonal terminals of Rh3 & Rh4 expressing R7 cells form a broken line punctuated by gaps. Apart from the reported splitting of R7 and R8 cells into subpopulations, these results confirm earlier findings by Fortini and Rubin (1990).

Longitudinal sections through the heads of P (Rh5. 2800 *taulacZ*) and the P (Rh6. *taulacZ*) line show that both lines exhibit a staining of R8 photoreceptors as judged by their localisation to the proximal half of the retina (Fig 16D & E). Since these two lines express the *taulacZ* fusion behind the Rh5 and Rh6 promoter region, β-galactosidase is detected in the axons that emerge from the R8 cell bodies, which traverse the retina and cross over each other at the optic chiasma before entering the medulla where they synapse

with second order neurons. Within the medulla, the stained axonal terminals of the Rh5/Rh6 expressing R8 cells form a broken line interrupted by gaps that presumably reflect the presence of unstained synaptic terminals of Rh6/Rh5 expressing R8 photoreceptor neurons (Fig 16D & E). The negative control (Fig 16F) in this case shows no β-galactosidase staining in the retina, lamina and medulla.

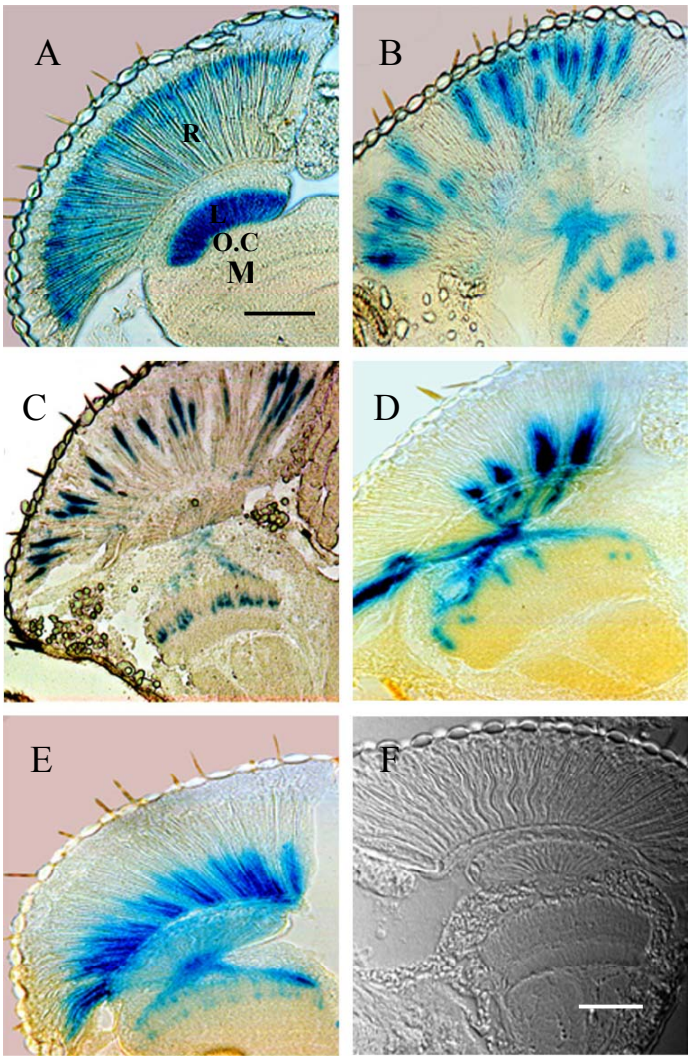

Fig 16: Histochemical analysis of *Drosophila* transformants expressing the *lacZ* gene

Cryostat sections (10µm) of transgenic *Drosophila* heads were prepared and stained with the chromogenic X-gal stain as described in materials and methods. Longitudinal sections were assayed for β-galactosidase activity in (A) P (Rh1. 833 *lacZ*), (B) P (Rh3. 343 *lacZ*), (C) P (Rh4. 1900 *lacZ*), (D) P (Rh5. 2800 *taulacZ*), (E) P (Rh6. *taulacZ*) and (F) negative wild type control. R, retina; L, lamina; OC, optic chiasma; M, medulla. Scale bar: (A, B, C, E):100µm; (D & F): 50µm.

By studying reporter gene construct flies, it is possible to have an information on the projections of the axons of the photoreceptors into the optic lobes. This makes it possible to visualise the distribution of soluble phototransduction proteins which could diffuse along the photoreceptor cell axons. Besides this, the onset of photoreceptor cell degeneration can be monitored which begins in the optic lobes. By tracing the neuronal pathways and their synaptic connections to the optic lobes one can easily monitor light-regulated localisation and internalisation of the phototransduction proteins in fly photoreceptors.

4.1.6 Immunofluorescence detection of β-galactosidase by confocal microscopy in flies expressing the *lacZ* reporter gene

The use of X-gal assay offers a quick analysis to study β-galactosidase activity, though prolonged incubation of the sections results in an unspecific spreading of the stain over areas of the eye. To confront this hurdle, the use of an anti-β-galactosidase antibody offers an alternative approach for investigating the localisation of β-galactosidase in the fly eyes. Longitudinal sections through heads of reporter gene flies were probed with anti- β-galactosidase antibody which inturn was detected by a FITC-coupled goat anti-rabbit secondary antibody.

In the transformant P (Rh1. 833 *lacZ*), an intense labeling is observed in the entire retina and in the lamina (Fig 17A). P (Rh3. 343 *lacZ*) and P (Rh4. 1900 *lacZ*) exhibit a staining of R7 photoreceptors as judged by its localisation to the distal half of the retina, and also in the axons that emerge from these R7 cells and terminate in the medulla (Fig 17B & C). Unlike these, in P (Rh5. 2800 *taulacZ*) and P (Rh6. *taulacZ*), labeling is observed only in the proximal half of the retina that corresponds to a subpopulation of the R8 photoreceptor cells. The axons emerging from these R8 photoreceptors are distinctly labeled which synapse in the medulla (Fig 17D & E). These results are in confirmation with the localisation of β-galactosidase as seen with X-gal assay.

The reporter gene immunocytochemical assay proves to be an alternate approach to suitably identify photoreceptor compartments and to trace the axonal pathway to the optic lobes. These flies also show that a gene (in this case, *lacZ*) can be suitably expressed under the control of various rhodopsin promoter regions and targetted correctly to the photoreceptor cell specific for that rhodopsin.

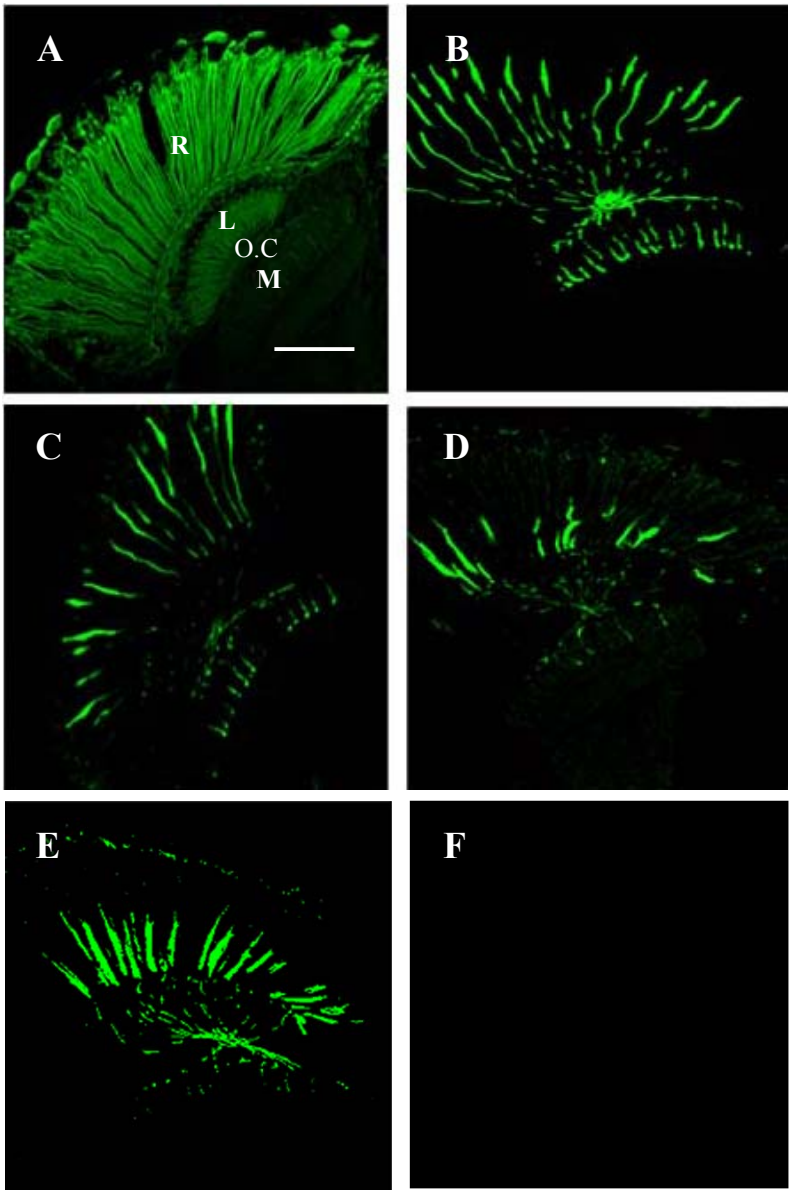

Fig 17: Spatial detection of β-galactosidase activity by confocal laser scanning microscope

Longitudinal sections through heads of transgenic *Drosophila* expressing the bacterial *lacZ* gene were investigated for β-galactosidase activity by immunocytochemistry using an anti-β-galactosidase antibody (1:80). The primary antibody was detected by a FITC-coupled (green colour) Goat anti-rabbit secondary antibody. Seen above are confocal images showing fluorescent labeling in (A) P (Rh1. 833 *lacZ*), (B) P (Rh3. 343 *lacZ*), (C) P (Rh4. 1900 *lacZ*), (D) P (Rh5. 2800 *taulacZ*), (E) P (Rh6. *taulacZ*), and (F) negative control. R-retina; L-lamina; OC-optic chiasma; M-medulla. Scale bar: 40µm.

The detection of β-galactosidase activity in the axons shows that the expression of the *lacZ* gene can be used to trace axonal pathways and synaptic connections in the optic lobes signifying that the protein expressed is stable which allows it to be used to visualise the photoreceptor compartments by light microscopy and confocal microscopy methods.

The stable expression of *lacZ* gene under the Rh1 promoter can be used as a control for ectopically expressing Rh3 rhodopsin under the control of Rh1 promoter (see material and method). These transgenic flies [P (Rh1 + Rh3)] (generous gift from Steve Britt, University of Denver, USA) are used during the further course of this thesis for studying light influenced changes in the localisation of the components comprising the INAD signaling complex and the TRPL channel. To confirm the ectopic expression of Rh3, western blot analysis was performed by using a anti-DmRh3 monoclonal antibody. As expected, a major protein band at 33kDa is labeled in western blots by cross-reaction with the α-DmRh3 antibody (Fig 18A lane 2). Confocal microscopic analysis through eyes of these transformed flies shows that Rh3 rhodopsin is localised according to ectopic expression in R1-6 rhabdomeres and is also present in subsets of R7 rhabdomeres (Fig 18B & C).

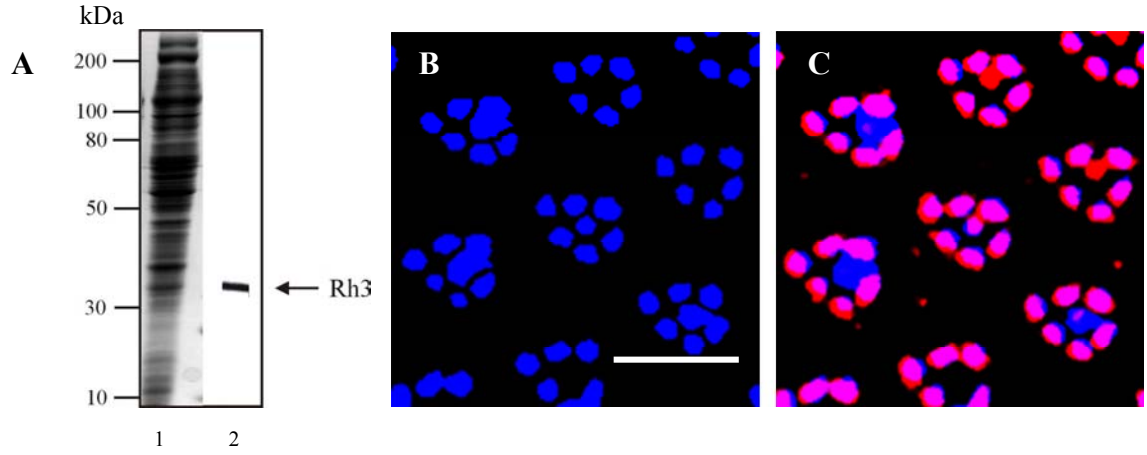

Fig 18: Rh3 rhodopsin is ectopically expressed under the control of Rh1 promoter in *Drosophila*

Analysis of transgenic *Drosophila* that ectopically express Rh3 under the control of Rh1 promoter. For western blot analysis, 68µg of protein extract (equivalent to 20 eye cups) from ectopic *Drosophila* flies was loaded in each lane. (A) a major band is observed at 33kDa for α-DmRh3 (lane 2). Lane 1 shows a coomasie staining to indicate equal loading of the protein. Cross sections through eyes of these transgenic flies were also analysed for the localisation of Rh3 by immunocytochemistry. Seen above are confocal images showing the localisation of (B) Rh3 (blue) in R1-6 rhabdomeres and in subsets of R7 rhabdomeres. (C) overlay image showing colocalisation of Rh3 with WGA-TMR (pink). Scale bar: 10µm.

4.1.7 Analysis of *Drosophila* mutants with mutations in the genes coding for proteins assembled with the INAD signaling complex

The absorption of a photon of light by rhodopsin ultimately results in the downstream activation of phototransduction proteins which are housed in the rhabdomeric compartments. A valuable tool for the genetic dissection of the INAD signaling complex are the *Drosophila* mutants that have a defect in the phototransduction cascade. A number of these mutants have been used here to study light influenced localisation of some of the phototransduction proteins in *Drosophila*.

Inorder to implement this approach, it is important to know if the rhabdomeres are intact and if every ommatidia has the correct number of rhabdomeres. This is because in one of the strains of *trp* mutant, the R7 rhabdomere was missing (Minke, personal communication). Therefore, cross sections of $norpA^{P24}$, $inaD^{1}$, trp^{343} and $ePKC^{P209}$ *Drosophila* mutant eyes were analysed for the localisation of Rh3 rhodopsin. As observed in wild type (refer Fig 13A), Rh3 is localised in subsets of R7 photoreceptors. No observeable degeneration is seen in the outer and central photoreceptors (Fig 19A, B, C, D) nor any abnormality with respect to number of photoreceptors as indicated by WGA and Rh3 labeling.

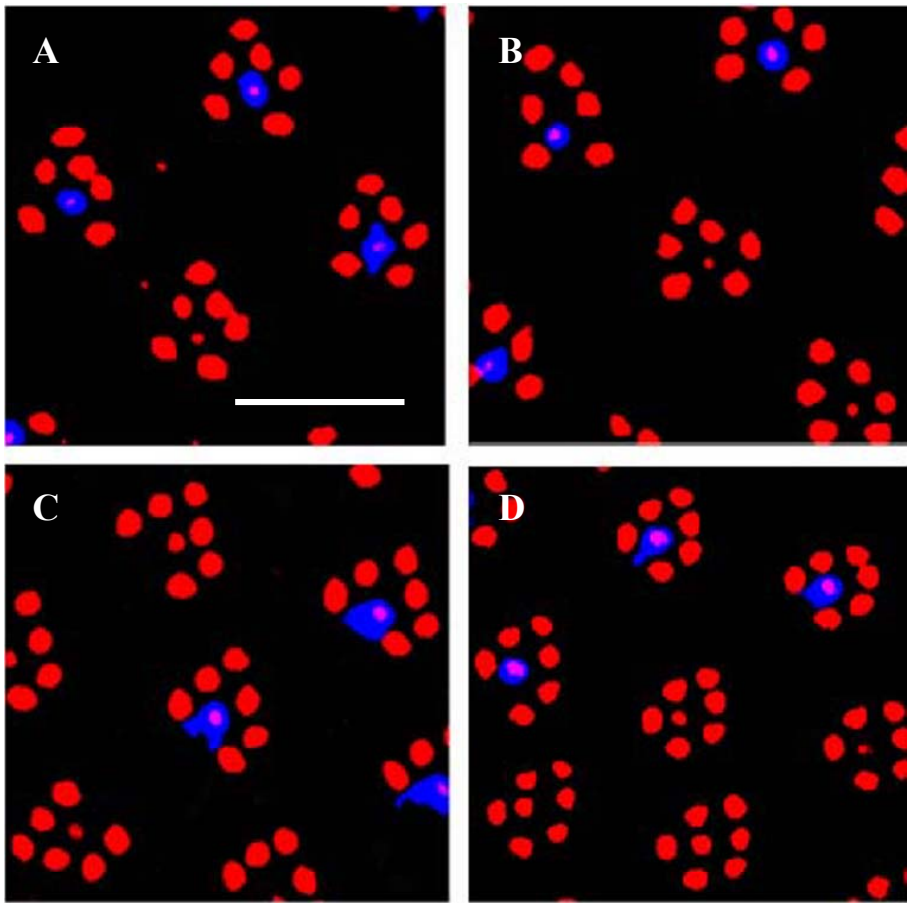

Fig 19: Rhodopsin is correctly localised in *Drosophila norpA*, *inaD*, *trp* and *ePKC* mutants

Localisation of Rh3 rhodopsin in the compound eyes of *Drosophila* mutants that lack key proteins involved in the phototransduction cascade. 10µm thick cryosections through 3 to 5 days old mutants eyes were probed with monoclonal mouse α-DmRh3 antibody which was detected by sheep anti-mouse CY5 (blue) secondary antibody. WGA-TMR (red) was used to label the rhabdomeres. Overlay of red and blue appears pink. The mutants used are (A) $norpA^{P24}$ (B) $inaD^{1}$ (C) trp^{343} and (D) $inaC^{P209}$. Scale bar: 10µm.

Analysis of the localisation of rhodopsins in wild type *Drosophila* provides means not only to visualise the intact rhabdomeric compartments but is a prerequisite for experiments starting at the downstream activation of the phototransduction cascade. Since rhodopsin expression is subjectable to variations i.e. carotenoid deprivation results in a largely reduced level of rhodopsin which is accomplished by a reduction in the size of the rhabdomeres (Boschek and Hamdorf, 1976; Harris *et al*., 1977; Paulsen & Schwemer, 1979; Schinz *et al*., 1982) and also blocking opsin synthesis (deCouet and Tanimura, 1987), the above observation helped to exclude this possibility.

4.2 The INAD signaling complex in *Calliphora erythrocephela*

The INAD signaling complex is important for assembling and localising the phototransduction proteins in the rhabdomeres to facilitate rapid signaling. Though *Drosophila* has proved to be a useful model for genetical manipulations, on the other hand, isolated microvillar photoreceptor membranes can be obtained in larger quantities from the blowfly *Calliphora*. This is an important requirement to have an information on the quantification of the amount of signaling proteins. The eyes of *Calliphora* can also be used for *in vivo* labeling of newly synthesised proteins (Huber *et al*., 1996a).

To gain an insight into the distribution of the proteins in the photoreceptor cells that are assembled into the signaling complex, it is of prime importance to be able to label these proteins and for detecting their cellular distribution in the fly eye. To implement this approach for studying the INAD signaling complex, the *rpa* mutant of *Calliphora* is explored because the biochemical analysis showed that both INAD and PLCβ are absent, and TRP is reduced (Huber *et al*., 2000) in this mutant. The mutant classified as receptor potential absent (*rpa*), arose spontaneously in a laboratory stock and was reported to show reduced phospholipase C activity, a age-dependent degeneration of the rhabdomeres, and a reduced intracellulary recorded impulse response to light stimuli (Torkkeli *et al*., 1989, 1991; McKay *et al*., 1994). The mutant was thereafter thought to represent a *norpA* mutant. Therefore, the *rpa* mutant was analysed for the immunocytochemical localisation of the INAD signaling complex inorder to confirm if the loss of PLCβ is a primary or secondary effect.

4.2.1 Distribution of the proteins comprising the INAD signaling complex in wild type *Calliphora* and *rpa* mutant

As reported for the *Drosophila* visual system, three of the binding proteins TRP, PLCβ and ePKC require the interaction with INAD for localisation to the rhabdomeres (Chevesich *et al*., 1997; Tsunoda *et al*., 1997). Evidence in *Drosophila* had shown that in *inaD*^{1} null mutant, TRP, PLCβ and ePKC are mislocalised in adult photoreceptors (Tsunoda *et al*., 1997). To investigate if the lack of INAD results in the absence of PLCβ in the *rpa* mutant, cross sections through heads of young (3 to 5 days old) wild type *Calliphora* and *rpa* mutants were analysed for the localisation of INAD, TRP, PLCβ, and Rh1 (control). As expected, in wild type *Calliphora* INAD, TRP, PLCβ and Rh1 localise to the rhabdomeres (Fig 20A, C, E & G) as observed in wild type *Drosophila*. However, in the *Calliphora rpa* mutant, no immunofluorescent labeling is observed for INAD (Fig 20B), but some residual TRP is observed in the rhabdomeres with most of it being mislocalised to the cell bodies (Fig 20D). PLCβ is below the detection limit of the method (Fig 20F). Rh1 is unaffected and localises to the outer rhabdomeres (Fig 20H).

Similarly for *Drosophila inaD*^{1} null mutant pupae it is shown that TRP, ePKC, and PLCβ are targeted to the rhabdomeres (Tsunoda *et al*., 2001). To investigate if these findings hold true also for *Calliphora*, pupae of wild type *Calliphora* and *rpa* mutant were analysed. Immunocytochemistry of cross sections through heads of wild type pupae reveal that TRP, PLCβ, and Rh1 localise to the rhabdomeres (Fig 21A, C & E). Though TRP localises to the rhabdomeres and in the plasma membrane (Fig 21B) in the *rpa* mutant, no labeling can be detected for PLCβ (Fig 21D). Control labeling with Rh1 labeling is the same as observed for young *rpa* flies (Fig 21F)

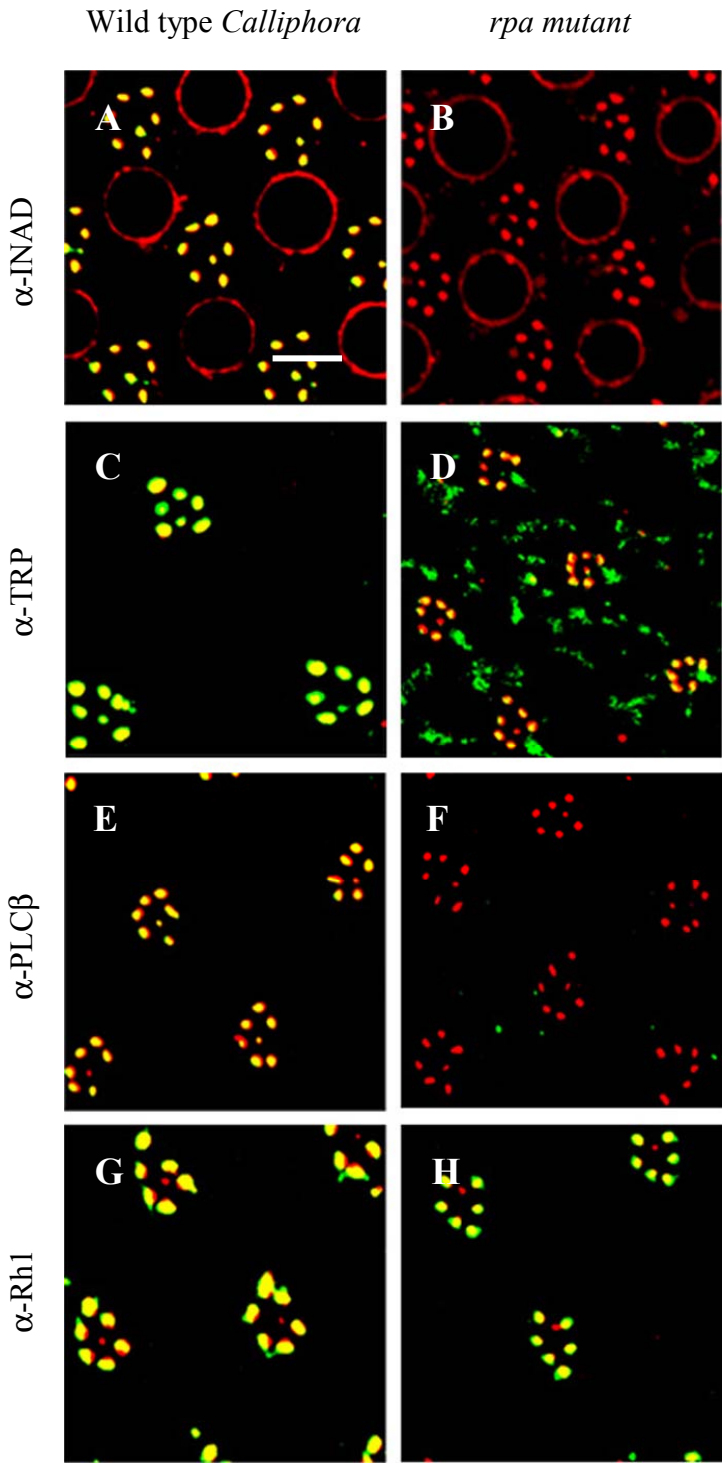

Fig 20: TRP is mislocalised in the *Calliphora rpa* mutant photoreceptors

10µm thick cryosections through heads of 3 to 5 days old wild type *Calliphora* (*Chalky* mutant in *Calliphora*) (A, C, E, G) and *rpa* (B, D, F, H) mutants were probed with α-CvINAD, α-CvTRP, α-DmPLC and α-CvRh1 antibodies (as indicated to the left). Each primary antibody was seperately detected by Goat anti-rabbit coupled FITC (green) secondary antibody. WGA-TMR (red) was used to label the rhabdomeres. Seen above are confocal overlay fluorescent images with WGA-TMR (yellow) for the labeling of INAD (A, B), TRP (C, D), PLCβ (E, F) and Rh1 (G, H). Scale bar: 10µm.

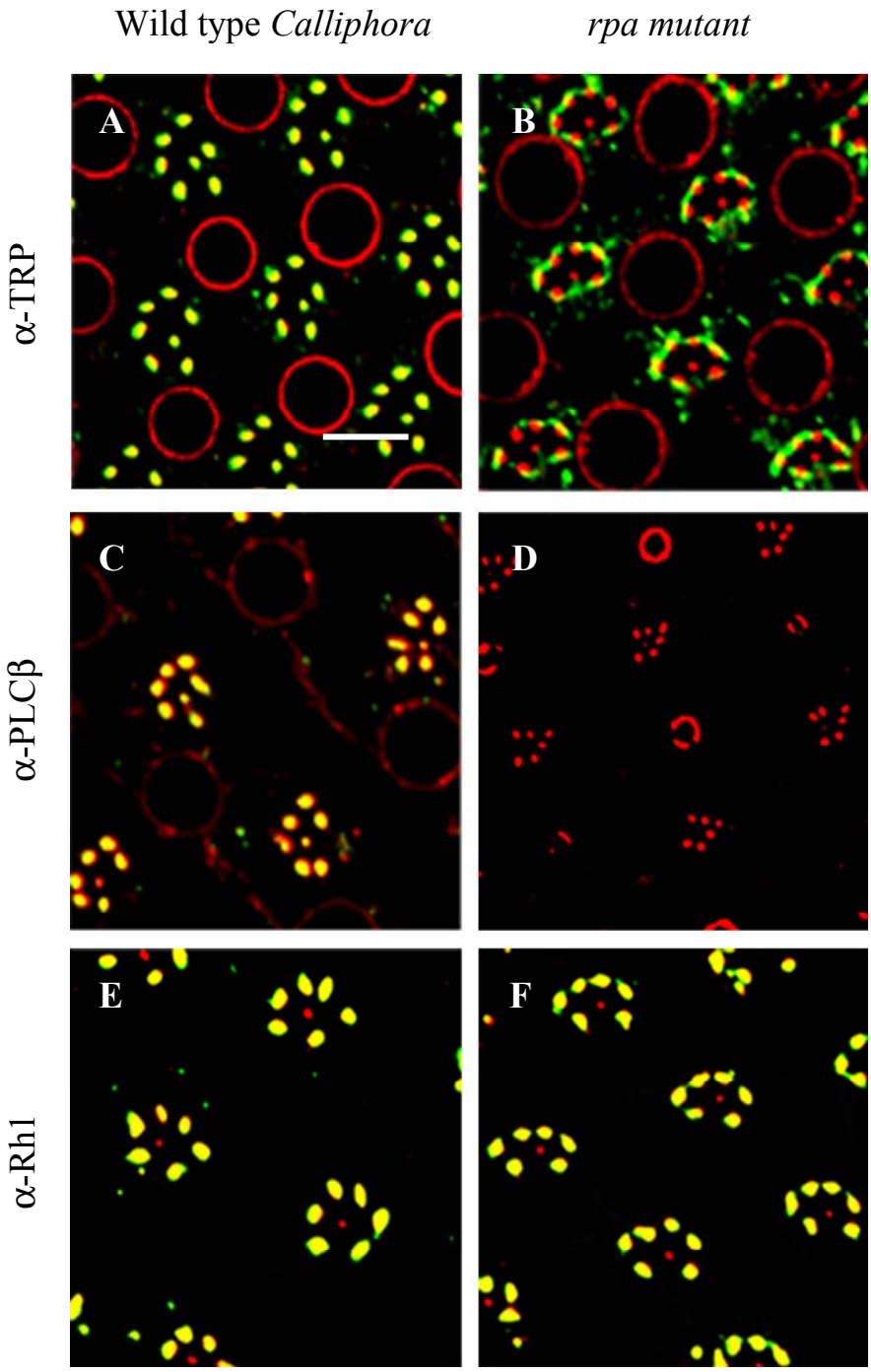

Fig 21: TRP is mislocalised in the *Calliphora rpa* pupae photoreceptors

10µm thick cryosections through eyes of pupae of wild type *Calliphora* (A, C, E) and *rpa* (B, D, F) mutants are probed with α-CvTRP, α-DmPLC and α-CvRh1 antibodies (as indicated to the left). Goat anti-rabbit coupled FITC (green) secondary antibody was used. WGA-TMR (red) was used to label the rhabdomeres. Seen above are confocal overlay fluorescent images with WGA-TMR (yellow) for the labeling of TRP (A, B), PLCβ (C, D), and Rh1 (E, F). Scale bar: 10µm.

In *Drosophila inaD*^{1} null mutant, the levels of TRP, PLCβ and ePKC are reduced to 10% of the wild type at 10 days post-eclosion (Tsunoda *et al*., 1997). Though PLCβ is already below the detection limit in the *Calliphora rpa* pupae, the distribution of TRP in old *rpa* flies was investigated. For this, 10 to 12 days old wild type *Calliphora* and *rpa* mutant were dark reared upon eclosion. As a control, 10µm thick cryosections through eyes of wild type and *rpa* mutant were probed with α-CvRh1 antibody. The result reveals that Rh1 is unaffected and localises to the outer rhabdomeres in both wild type and *rpa* flies (Fig 22C & D). Though TRP labeling is detected in the rhabdomeres in wild type flies (Fig 22A), it is below the detection limit with this method in the *rpa* mutant (Fig 22B). Accordingly, the *rpa* mutant resembles with respect to the absence of the INAD signaling complex a *inaD* null mutant rather than a *norpA* null mutant, a finding comparable to the *Drosophila inaD*^{1} mutant.

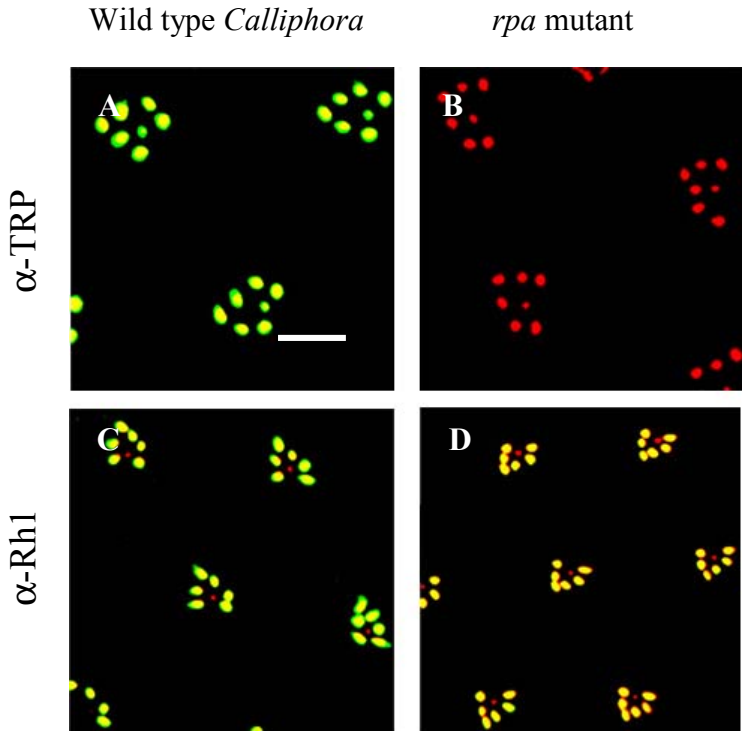

Fig 22: TRP activity is not detected in eyes of old *Calliphora rpa* flies

Double-fluorescent labeling of cross sections though eyes of 10 to 12 days old wild type *Calliphora* (A, C) and *rpa* (B, D) flies probed with α-CvTRP and α-CvRh1. Goat anti-rabbit FITC coupled secondary antibody was used. WGA-TMR (red) was used to label the rhabdomeres. Shown above are confocal overlay images for the localisation of TRP and Rh1 as indicated. The yellow colour indicates colocalisation of the respective protein with WGA. Scale bar: 10µm.

4.2.2 Immunochemical detection of proteins of the INAD signaling complex in young wild type *Calliphora* and *rpa* mutant by western blots

Western blots performed inorder to test the presence / absence of the INAD signaling complex proteins shows as expected, that the 80kDa protein band detected by α-CvINAD antibodies in wild-type flies (refer Fig 9B lane 2) is missing in extracts obtained from the *rpa* mutant (Fig 23 lane 2).

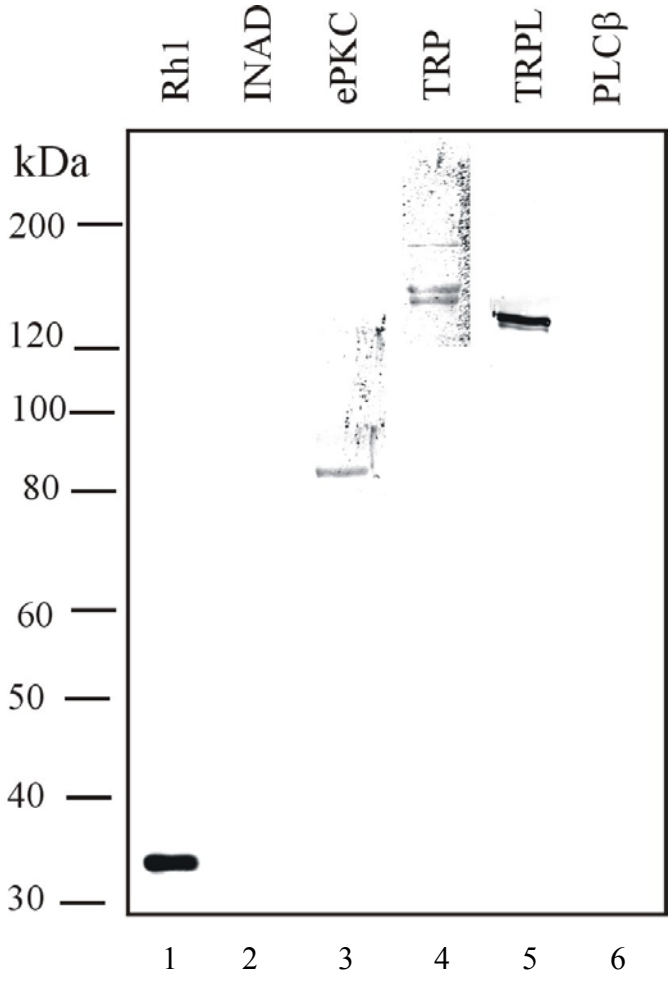

Fig 23: TRP, ePKC and PLCβ are affected in the *Calliphora rpa* mutant

Proteins of purified photoreceptor membranes obtained from *Calliphora rpa* mutant were sperated by SDS-PAGE and subjected to western blot analysis. For seperating Rh1, 12% polyarylamide gel was used while INAD, ePKC, TRP, TRPL and PLCβ were seperated on a 8% gel. After blotting, proteins were detected with alkaline phosphatase-conjugated protein A. The proteins bands were visualised through a chromogenic reaction with 5-bromo-4-chloro-3-indolyl phosphate/4-nitro-blue-tertazolium chloride.

Though PLCβ is detected in extracts of wild-type flies (Fig 9B lane 6) the corresponding extracts obtained from *rpa* flies fail to display a band (Fig 23 lane 6). Further, ePKC and TRP are significantly reduced in extracts from *rpa* flies (Fig 23 lane 3 & 4) when compared to wild type flies (Fig 9B lane 3 & 4). Extracts obtained from *rpa* mutants show no decrease in level of Rh1 expression (Fig 23 lane 1) when compared with *Calliphora* wild-type extracts (Fig 9A lane 2). These observation thereby explain that besides INAD, PLCβ is also absent while TRP is greatly reduced in the *rpa* mutant.

4.2.3 Light dependent degeneration of rhabdomeres in *rpa* mutant

Previous analysis reveal that *Drosophila inaD* and *trp* mutants show degeneration of photoreceptor cells in a light-dependent manner. The *rpa* mutant was monitored for the degeneration of photoreceptor cells by using the "deep pseudopupil" method since, the *rpa* mutant can be compared as an equivalent of the *Drosophila inaD* mutant. A convenient optical invasion free method for visualising rhabdomeres in fly eyes is the deep pseudopupil technique (Franceschini and Kirschfeld, 1971; Franceschini, 1975). The deep pseudopupil allows to monitor the degeneration of photoreceptor cells in *rpa* flies which were light and dark reared. Light reared wild type flies were used as control. By using cold light source and indirect illumination, 3 to 5 days and 12 to 15 days old dark reared *rpa* flies show a dark pseudopupil (Fig 24A & B) while 12 to 15 days old *rpa* flies which were continously kept in light show no pseudopupil (Fig 24C). As a control, 12 to 15 days old wild type *Calliphora* flies which were continously kept in light show a dark pseudopupil (Fig 24D). The image of individual rhabdomeres disappears from the pattern represented by the deep pseudopupil in 12 to 15 days old light reared *rpa* flies because of the degeneration of some rhabdomeres. Therefore loss of deep pseudopupil indicates a loss of architecture of the compound eye.

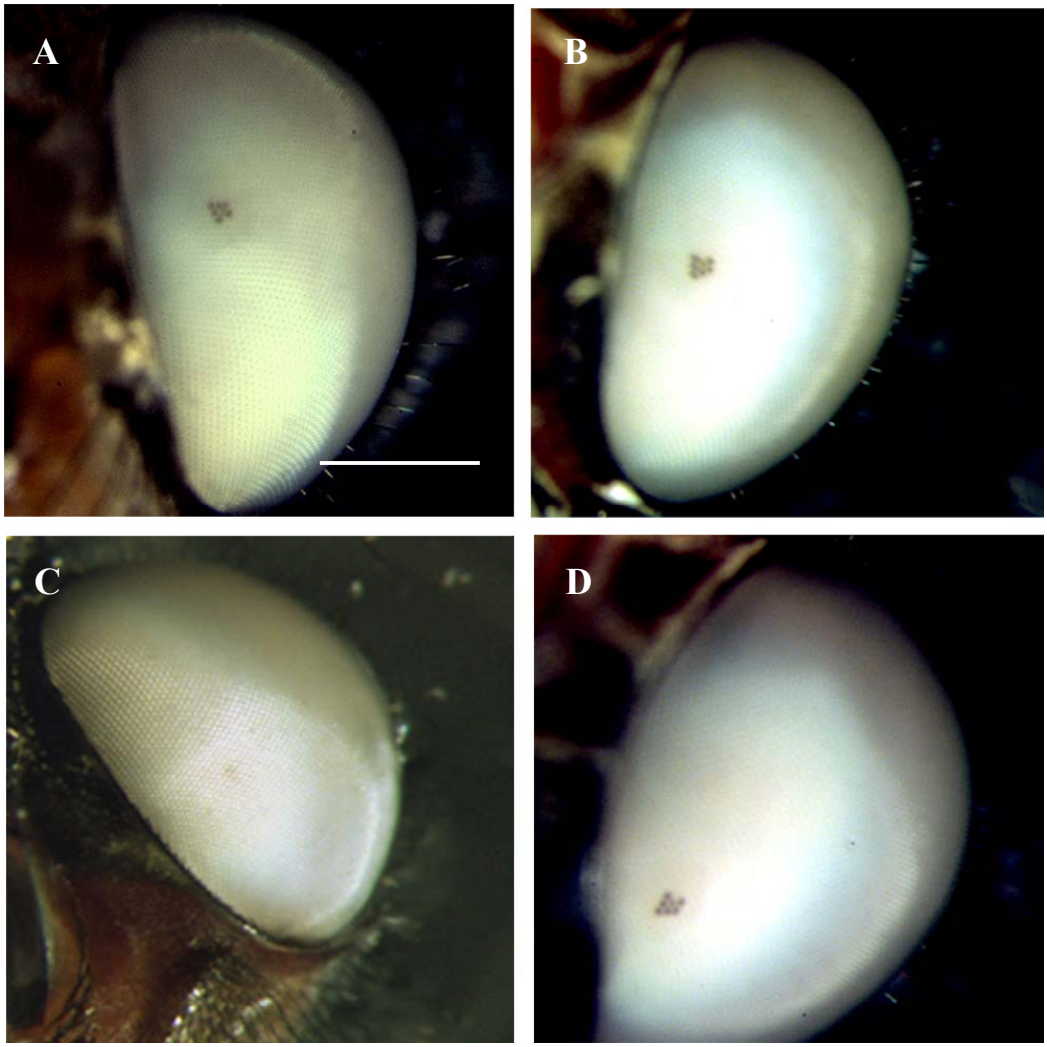

Fig 24: Loss of deep pseudopupil in *Calliphora rpa* flies subjected to continous light

Wild type and *rpa Calliphora* were reared in continous light and darkness and monitored for the deep pseudopupil by indirect illumination. (A) 3 to 5 days old dark-reared *rpa* flies (B) 12 to 15 days old dark-reared *rpa* flies (C) 12 to 15 days old light-reared *rpa* flies (D) 12 to 15 days old light-reared wild type flies. Scale bar: 500µm.

Subsequently, the time course for the degeneration of photoreceptors was also assayed by observing the loss of deep pseudopupil for a population of wild type flies kept in continous light and *rpa* flies kept in continous light and darkness. Based on the observations (table 8), a degeneration index was calculated by assigning values 1, 0.5 and 0 to flies which display strong dark pseudopupil, weak pseudopupil and no pseudopupil. The values were added for each time point and divided by the number of flies inspected. It is observed that approximately 60% of total *rpa* flies show degeneration of the rhabdomeres within 10 days of eclosion. This degeneration is light dependent since the *rpa* mutant maintains a deep

pseudopupil at least until 18 days after eclosion when reared in the dark. Wild type flies are unaffected by continous light and continue to show a deep pseudopupil until 18 days (Fig 25).

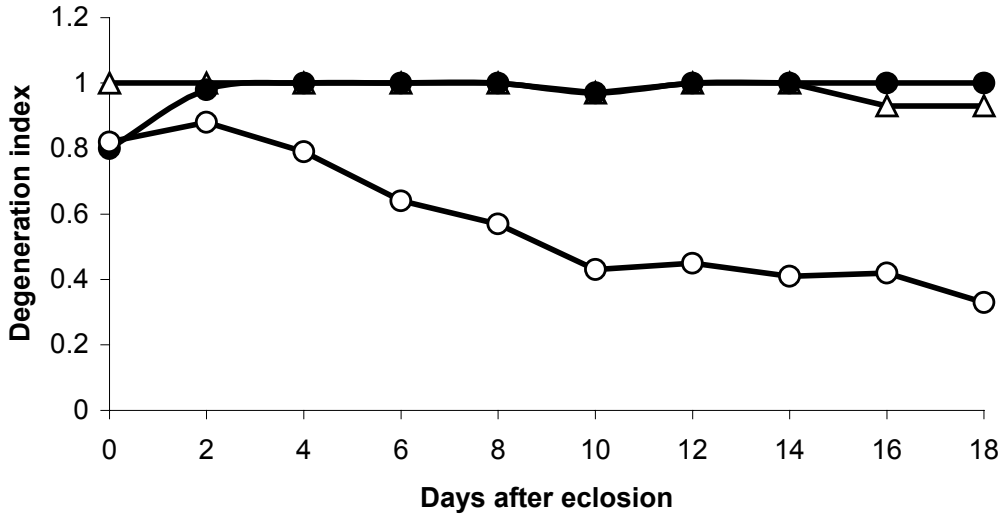

Fig 25: Light dependent loss of deep pseudopupil in *Calliphora rpa* mutant

Time course of light dependent loss of deep pseudopupil in the *Calliphora rpa* mutants. Twenty *Calliphora* wild type (Δ) and *rpa* flies (•) (o) were reared in continous darkness (•) or light (o) (Δ) and inspected for the presence of a deep pseuodopupil. Time after eclosion is plotted against a degeneration index (refer text). Around 60% of the *rpa* flies show a total loss of the deep pseudopupil upon exposure to continous light. Dark reared wild type and *rpa* flies remain unaffected until 18 days after eclosion.

To observe the degeneration of the rhabdomeres within the ommatidia by direct fluorescence, cross sections through wild type and the *rpa* mutant were labeled with WGA coupled to Oregon Green. Eyes of 3 to 5 days old and 12 to 15 days old *rpa* flies which were continously dark-adapted show that all rhabdomeres are present in young and old dark-adapted flies (Fig 26A & B). However, 12 to 15 days old *rpa* flies which were continously kept in light clearly exhibit a loss of microvillar membranes in the rhabdomeres of R1-6 cells, though the central rhabdomeres formed by R7 cells are less affected (Fig 26C). Wild type flies which were continously light-adapted show bright fluorescence for WGA labeling in all the rhabdomeres (Fig 26D). The result confirms that the *rpa* mutant undergoes a degeneration of the photoreceptors which is age-light-dependent.

Table 7: Results for the deep pseudopupil count undertaken for wild type *Calliphora* and *rpa* flies

1. Wild type *Calliphora* raised in light condition

Days after eclosion	(A) Flies with dark pseudopupil	(B) Flies with weak pseudopupil	(C) Flies without pseudopupil	% with pseudopupil	Degeneration index (X) = (1xA+0.5xB+0xC)/n
0	20	0	0	100	1
2	18	0	0	100	1
4	19	0	0	100	1
6	18	0	0	100	1
8	18	0	0	100	1
10	16	1	0	100	0.97
12	16	0	0	100	1
14	15	0	0	100	1
16	12	2	0	100	0.93
18	11	2	0	100	0.93

2. *Calliphora rpa* flies raised in dark condition

Days after eclosion	(A) Flies with dark pseudopupil	(B) Flies with weak pseudopupil	(C) Flies without pseudopupil	% with pesudopupil	Degeneration index (X)= (1xA+0.5xB+0xC)/n
0	11	8	0	100	0.8
2	20	1	0	100	0.98
4	19	0	0	100	1
6	18	0	0	100	1
8	18	0	0	100	1
10	14	1	0	100	0.97
12	16	0	0	100	1
14	15	0	0	100	1
16	13	0	0	100	1
18	4	0	0	100	1

3. *Calliphora rpa* flies raised in light condition

Days after eclosion	(A) Flies with dark pseudopupil	(B) Flies with weak pseudopupil	(C) Flies without pseudopupil	% with pseudopupil	Degeneration index (X)= (1xA+0.5xB+0xC)/n
0	11	6	0	100	0.82
2	15	5	0	100	0.88
4	13	9	0	100	0.79
6	9	10	3	86	0.64
8	9	7	6	72	0.57
10	5	8	8	62	0.43
12	6	8	8	64	0.45
14	6	5	10	52	0.41
16	6	4	9	52	0.42
18	4	4	10	44	0.33

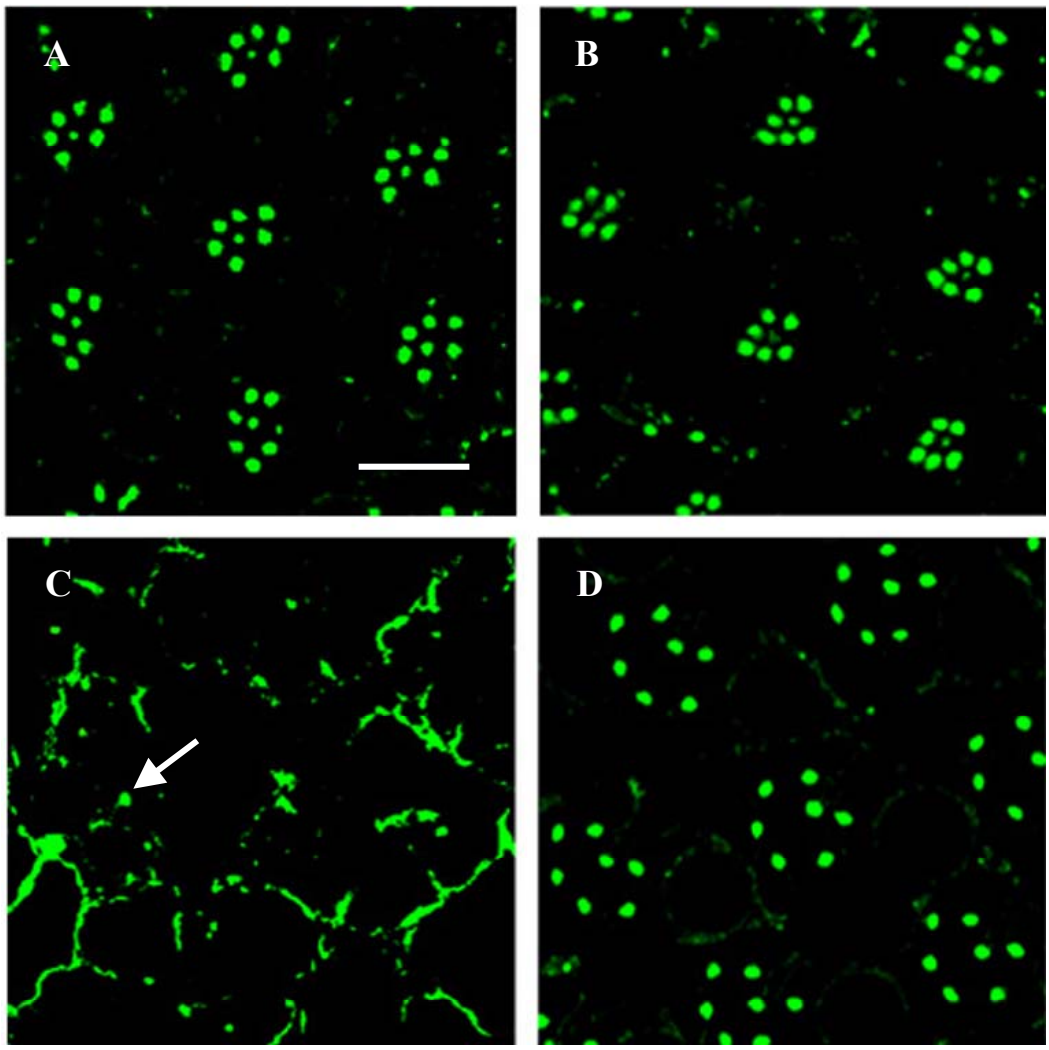

Fig 26: Lectin labeling for monitoring the light dependent degeneration of rhabdomeres in the *Calliphora* *rpa* mutant

Direct fluorescent labeling of cross sections through wild type *Calliphora* and *rpa* flies probed with lectin WGA coupled to Oregon Green (green). Seen above are confocal images of (A) 3 to 5 days old dark reared *rpa* flies (B) 12 to 15 days old dark reared *rpa* flies (C) 12 to 15 days light reared *rpa* flies and (D) 12 to 15 days light reared wild type flies. The arrow in (C) indicates a rhabdomere of a R7 cell which is unaffected by the light condition. Scale bar: 10µm.

The *Calliphora rpa* mutant can thus be characterised as an equivalent of a *Drosophila inaD*[1] null mutant because besides INAD, the other core components of the INAD signaling complex like TRP and PLCβ are affected or degraded in an age-dependent manner. Rh1 is unaffected which has also been shown in the *Drosophila inaD*[1] mutant (Tsunoda *et al*., 1997). Xu *et al*. (1998) has reported that Rh1 and TRPL may be bound to INAD transiently, but this however has not been confirmed by other authors. The reason

for TRPL to interact transiently with INAD may be regulated by light-dependent Ca^{2+} influx inducing conformational change in INAD which inturn could alter the INAD-TRPL interaction.

4.3 Assembly of the INAD signaling complex in fly photoreceptors

A view point which has largely been neglected in the initial studies is whether or not the members of the INAD signaling complex as well as Ca^{2+} channels subunits such as TRPL are subject to light triggered changes in their localisation within the photoreceptor cell. Therefore, by keeping flies under different light conditions, immunocytochemistry was used to follow the fate of INAD, TRP and TRPL in the photoreceptor cell.

4.3.1 Light-dependent distribution of INAD, TRP and TRPL in the compound eye of *Drosophila* and *Calliphora* monitored by immunocytochemistry

To investigate if INAD, TRP and TRPL distribution are subject to light influenced distribution, young (3-5 days old) *Drosophila* wild type flies were initially kept in white light (500-1000 Lux) or in the dark for 12 hrs. The eyes of dark kept flies were dissected in red light while eyes of white light kept flies were dissected under white light and fixed (refer 3.3.1) in the same relevant condition. 10µm cross sections of fly eyes exposed to dark and light conditions were probed with α-DmINAD, α-DmTRP and α-DmTRPL primary antibodies. Confocal microscopic analysis reveals that in dark kept fly eyes, INAD, TRP and TRPL are localised to the rhabdomeres in all the photoreceptor cells (fig 27A, C, E). Cross sections through eyes of light exposed flies shows that INAD and TRP are also localised to the rhabdomeres of the photoreceptor cells (fig 27B, D). In light kept flies, the situation changes dramatically with a strong fluorescent signal that is obtained in the cell body indicating that a change in the distribution of TRPL has occured. TRPL is no longer present in the rhabdomeres but is rather localised to the cell bodies of the photoreceptor cell (fig 27F). To investigate if the light-regulated TRPL localisation is age dependent, newly eclosed (less that 1 hour old) and old (10-12 days old) wild type *Drosophila* flies were kept in dark or in light respectively. Confocal microscopy results

reveals that the light-dependent localisation of TRPL is independent of the age of the inspected flies (fig 28).

TRPL localisation was also studied in wild type *Calliphora* to investigate whether or not the light-dependent distribution of TRPL is restricted to *Drosophila*. Young (3-5 days old) wild type flies of *Calliphora* were kept in white light (500-1000 Lux) or dark for 12 hrs. Eyes of these dark and light kept flies were dissected in red and white light respectively. Immunocytochemical evidence from cross sections through eyes of wild type flies reveals again that INAD, TRP and TRPL localise to the rhabdomeres (fig 29A, C & E) in dark kept flies. Though INAD and TRP localise to the rhabdomeres in light kept flies, TRPL once again is not present in the rhabdomeres (fig 29F) but is distributed to the cell bodies of the photoreceptor cells. In older dark or light kept wild type *Calliphora* (10-12 days old), TRPL localisation is the same as observed in young wild type *Calliphora* flies (fig 30). Therefore it can be concluded that the light effect observed on TRPL distribution within the photoreceptors is independent of the age of a fly but is a general phenomena which is not limited to *Drosophila*.

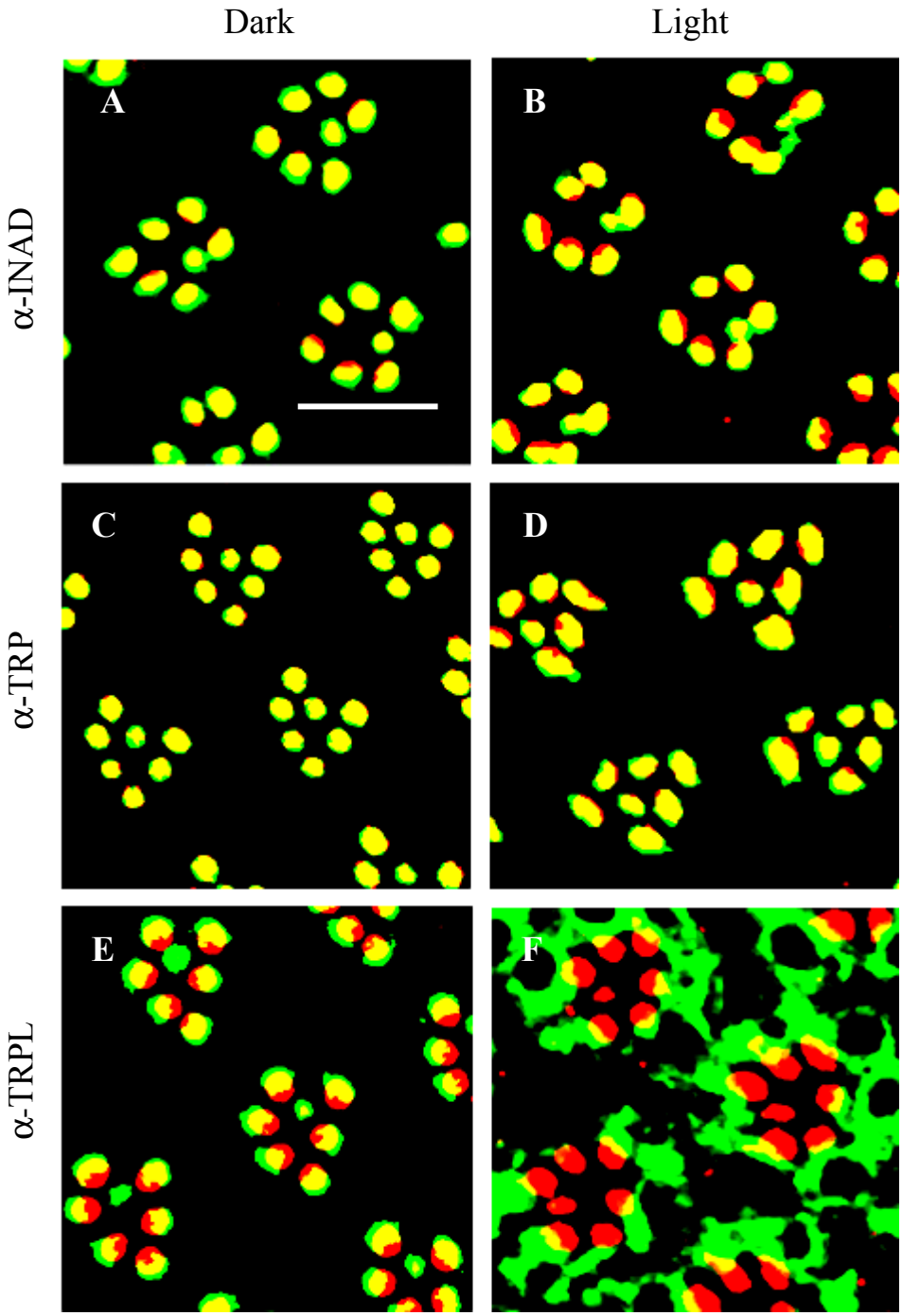

Fig 27: TRPL but not INAD and TRP localisation is influenced by light in photoreceptors of wild type *Drosophila*

Double-fluorescent labeling of cross sections through eyes of 3 to 5 days old wild type *Drosophila* kept in dark (A, C, E) and light (B, D, F) for 12 hours. Sections were probed with α-DmINAD, α-DmTRP and α-DmTRPL antibodies as indicated. Goat anti-rabbit FITC coupled secondary antibody (green) was used to detect the respective primary antibodies. WGA-TMR (red) was used to label the rhabdomeres. The yellow colour is a result of the colocalisation of the respective protein with WGA-TMR. Scale bar: 10μm.

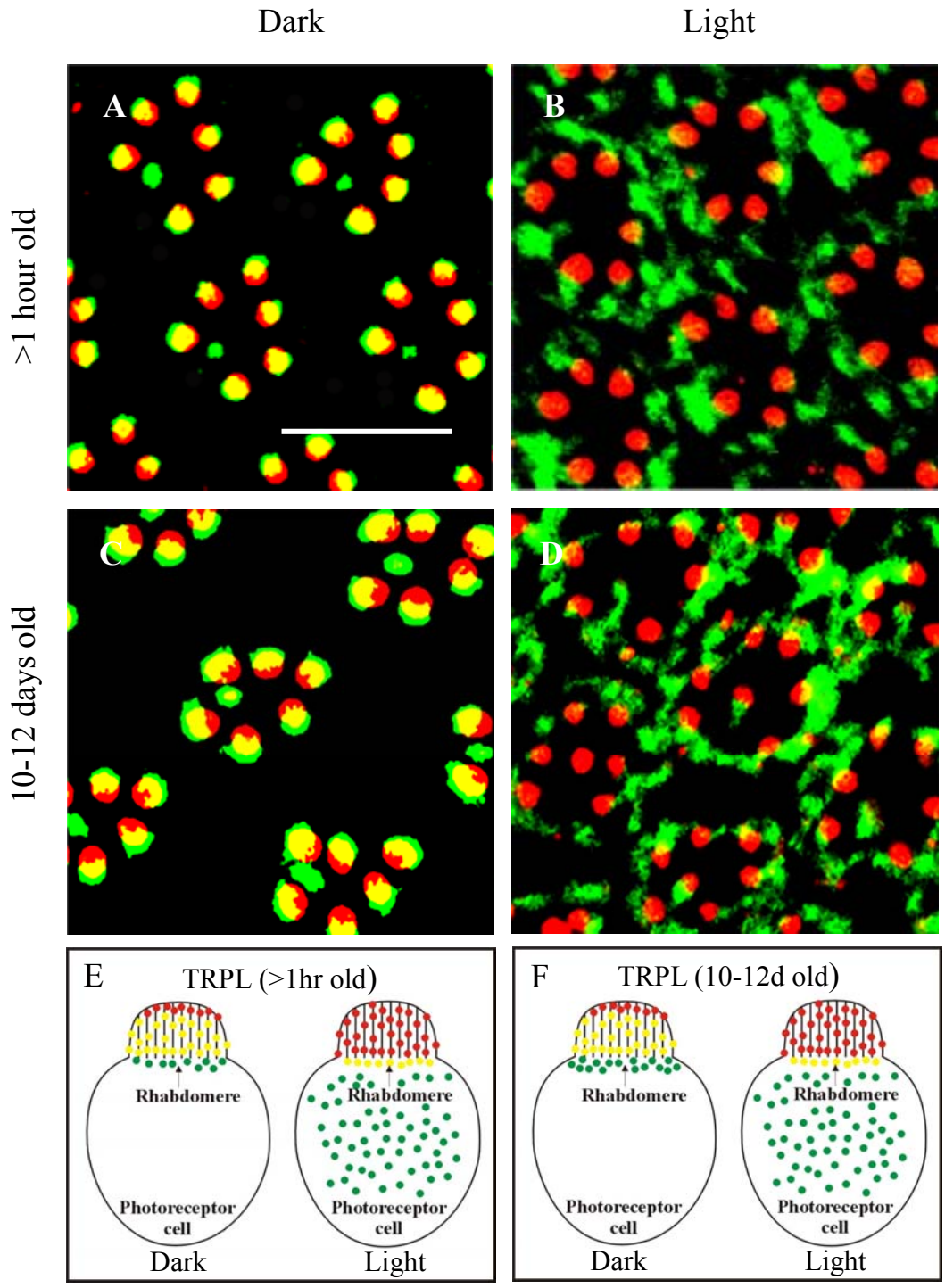

Fig 28: Light-regulated TRPL localisation is independent of the age in wild type *Drosophila*

Shown above are confocal images for the localisation of TRPL (green) in dark (A, C) and light (B, D) kept newly eclosed (> 1 hour old) and 10 to 12 days old wild type *Drosophila*. The yellow colour indicates a colocalisation of TRPL with WGA-TMR (red) which was used to label the rhabdomeres. (E) & (F) schematic representation of a single photoreceptor corresponding to the above images. Scale bar: 10µm.

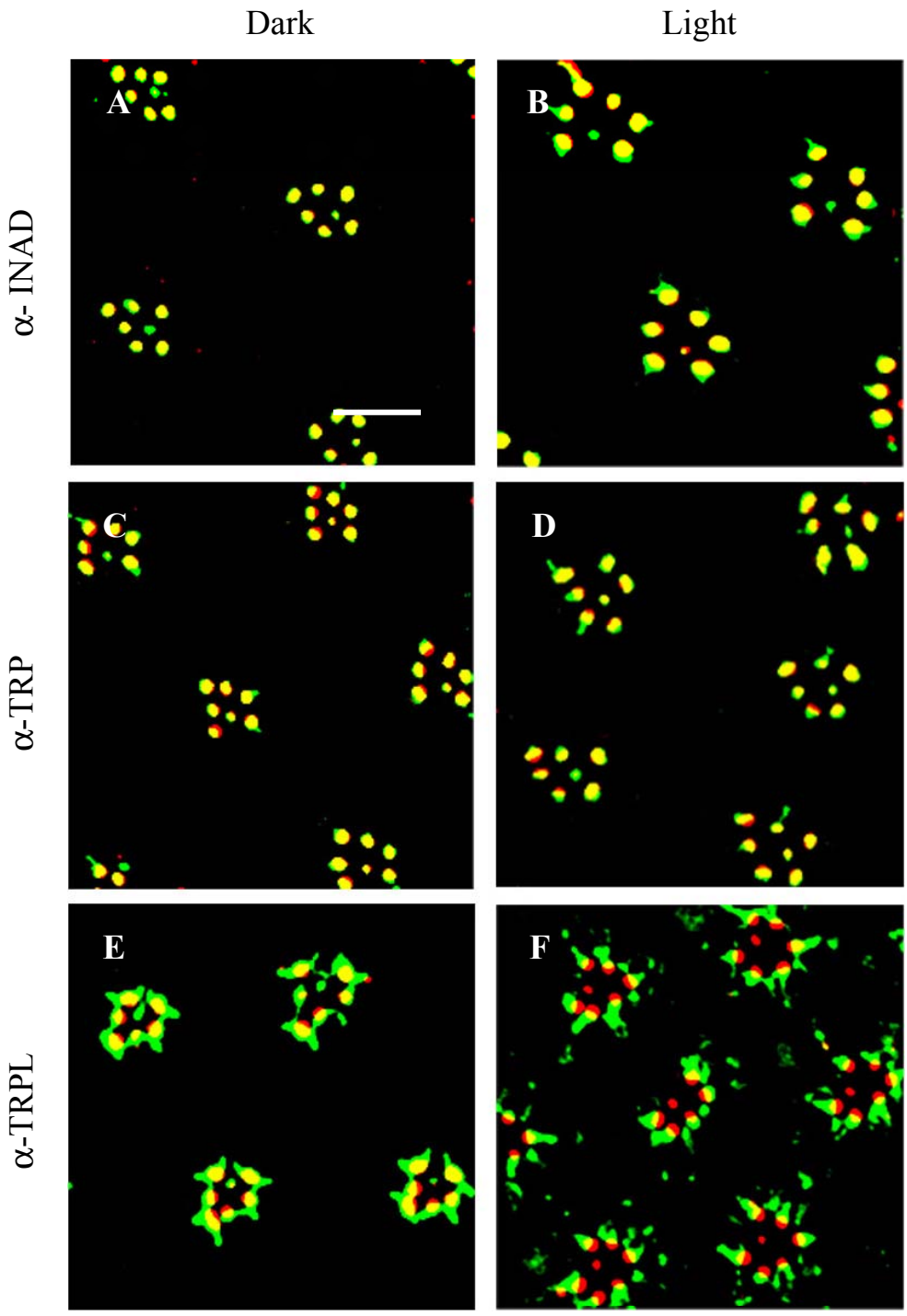

Fig 29: TRPL localisation is influenced by light in wild type *Calliphora* photoreceptors

Immunofluorescent labeling of cross sections through eyes of 3 to 5 days old dark (A, C, E) and light (B, D, F) kept wild type *Calliphora* flies probed with α-CvINAD, α-CvTRP and α-CvTRPL antibodies as indicated. Goat anti-rabbit FITC coupled secondary antibody (green) was used to detect the respective primary antibodies. WGA-TMR (red) was used to label the rhabdomeres. The yellow colour indicates colocalisation of the respective protein with WGA. Scale bar: 10µm.

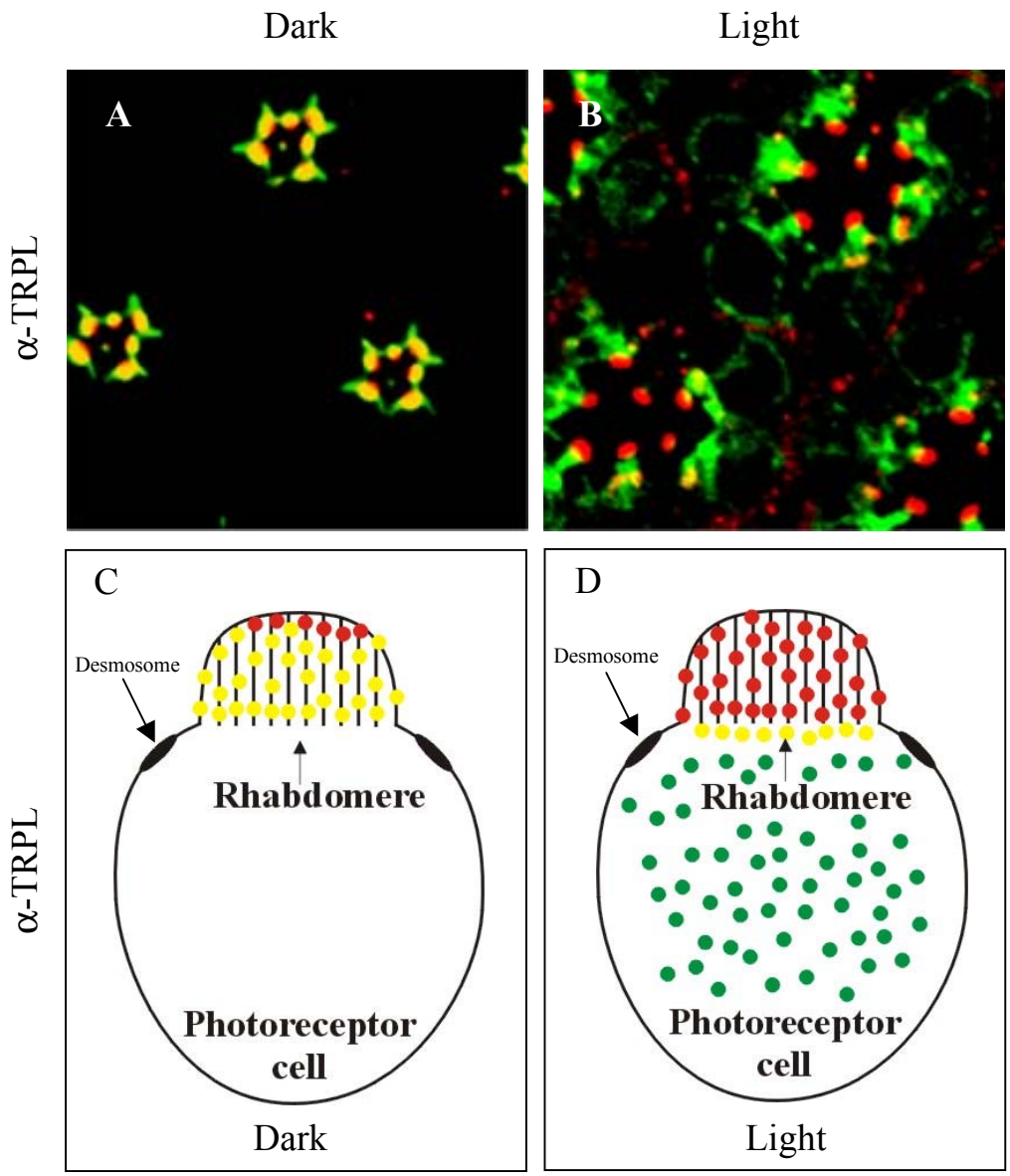

Fig 30: TRPL localisation is independent of age in wild type *Calliphora*

Fluorescent labeling for the localisation of TRPL (green) in dark (A) and light (B) kept (10 to 12 days old) wild type *Calliphora*. The yellow colour indicates the colocalisation of TRPL with WGA-TMR (red). (C) & (D) schematic representation of a single photoreceptor corresponding to the above images. Scale bar:10µm.

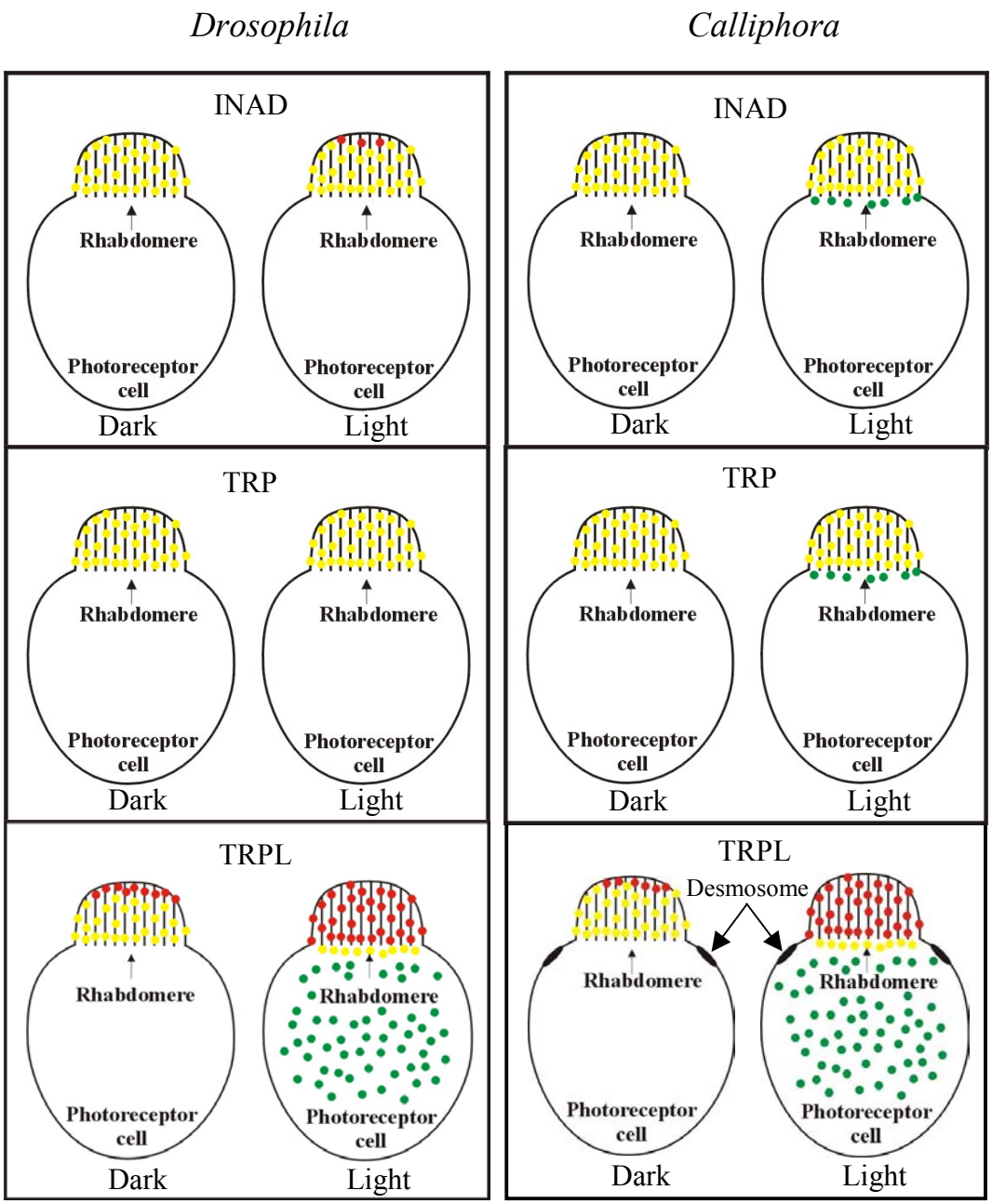

Fig 31: Schematic representation of INAD, TRP and TRPL localisation in young wild type *Drosophila* and *Calliphora* photoreceptors

The green colour indicates the localisation of the respective protein while the yellow colour indicates colocalisation of the respective protein with WGA-TMR.

4.3.2 TRPL distribution in *Drosophila* photoreceptors exposed to different wavelengths of light

As reported above, TRPL localisation in fly photoreceptors changes in response to an exposure of eyes to white light. It is reasonable to assume that the light effect is triggered by photoabsorbance of the rhodopsin present in the rhabdomeres. Another possibility would be that the light receptor influencing TRPL distribution is not the rhodopsin responsible for activation of the transduction cascade but another not yet identified pigment. As light activation of rhodopsin results in the activation of ion channels TRP and TRPL which are the ion channels associated with the phototransduction cascade, some fundamental questions remained unanswered for example

(1) does TRPL localisation in dark and light kept flies depend on the activation of the receptor protein, rhodopsin, and if yes,

(2) are the quantitative effects dependent on the amount of rhodopsin transformed into metarhodopsin, and

(3) the distribution of TRPL and TRP channel proteins which have been proposed to form homo- or heteromultimers in *vivo*, in flies exposed to different wavelengths of light.

The *ninaE* mutant (rhodopsin gene absent) would provide a candidate to answer these questions. However, previous workers have reported that mutations in the *ninaE* gene causes atrophy (Kumar & Ready, 1995) resulting in a rapid decrease in rhabdomere size at eclosion and a progressive deterioration of rhabdomere structure over time (Leonard *et al*., 1992; Kumar & Ready, 1995). Therefore *ninaE* mutant cannot be used to study light influenced TRPL localisation. Hence, an alternative set of experiments was designed which made use of wild type flies and transformed flies which ectopically express Rh3 rhodopsins. The rhodopsin namely Rh1 and Rh3 in wild type and P (Rh1 + 3) *Drosophila* absorb maximally at 478nm and 345nm (Salcedo *et al*., 1999). Taking advantage of the spectral properties of these rhodopsins, wild type *Drosophila* and transgenic flies that

ectopically express Rh3 under the control of Rh1 promoter, P (Rh1 + 3) (refer Fig 18), were subjected to different light conditions to study TRPL and TRP distribution within the photoreceptors. To have a detailed insight into the distribution of TRPL and TRP in different light conditions with respect to rhodopsin activation, *Drosophila* flies were kept in different light conditions. The lights to which flies were exposed had been choosen to shift the rhodopsin / metarhodopsin equilibrium in R1-6 cells (Fig 32).

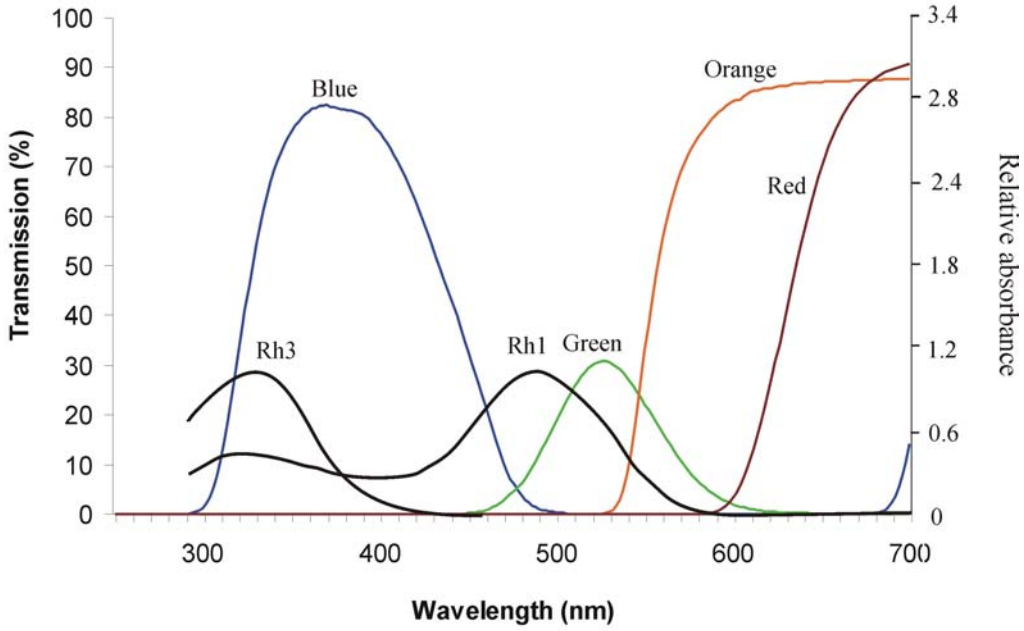

Fig 32: Spectral transmission of wideband filters (blue & green) and cutoff filters (orange & red) along with spectral absorbance for Rh1 & Rh3 rhodopsins (Rhodopsin absorbance curves adapted from Henrich, 1999)

Initial experiments to confirm light influenced distribution of TRPL in P (Rh1 + 3) were undertaken wherein these transgenic flies were kept in light or dark conditions as described previously. Cross sections through eyes of dark kept flies probed with α-DmTRPL and α-DmTRP antibodies reveals that both TRPL and TRP localise to the rhabdomeres (Fig 33A & C). In light kept flies, most of the TRPL detected in the photoreceptors is located in the cell bodies (Fig 33B). Some TRPL positive labeling is also observed in submicrovillar membrane areas. TRP is present in all the photoreceptors and is located within the rhabdomeres (Fig 33D).

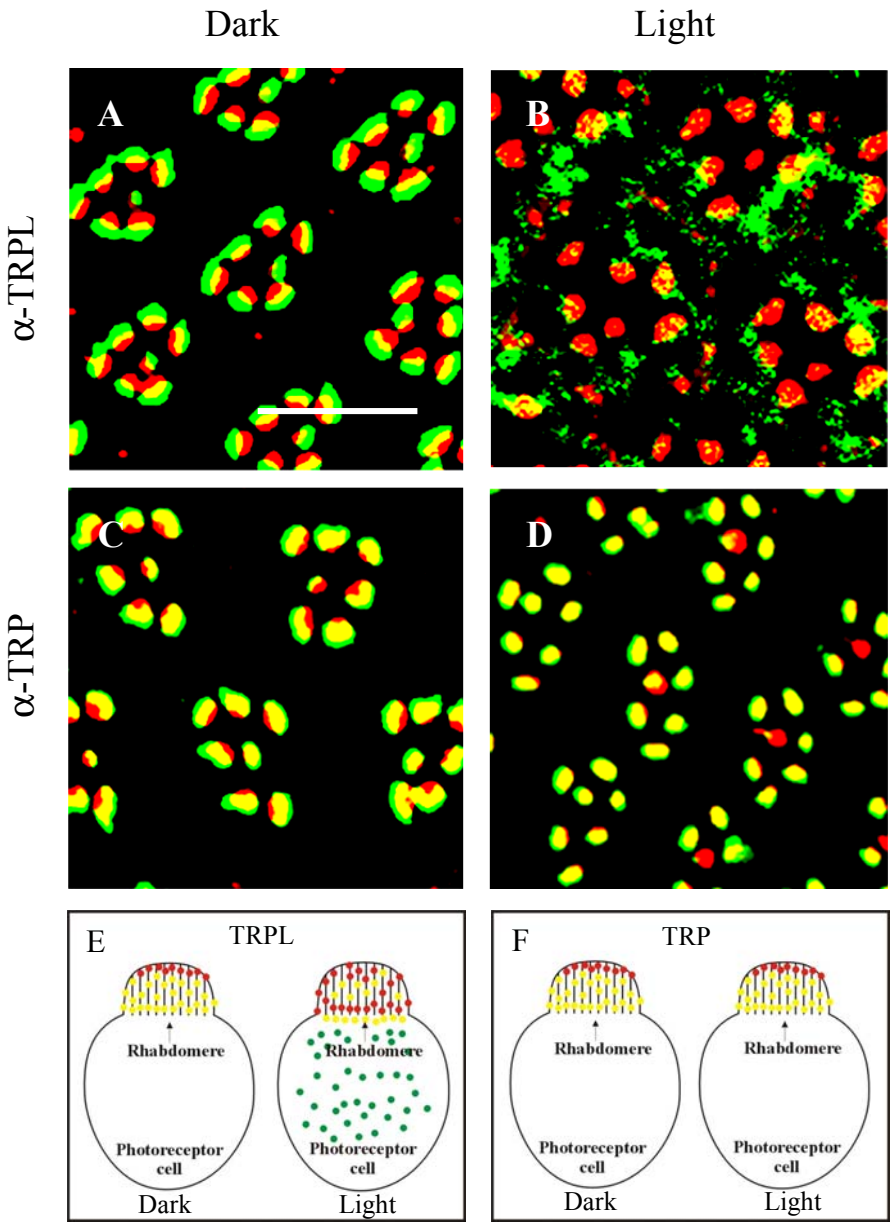

Fig 33: A fraction of TRPL is influenced by light in transgenic *Drosophila* ectopically expressing Rh3 under Rh1 promoter

Transgenic flies ectopically expressing Rh3 under the Rh1 promoter were dark (A, C) and light (B, D) kept for 12 hours. Shown above are confocal images for the localisation of TRPL and TRP as indicated. The yellow colour indicates colocalisation of the protein with WGA-TMR. (E) & (F) schematic representation of a single photoreceptor corresponding to the above images. Scale bar: 10µm.

A. Effect of blue-light exposure

Wild type and P (Rh1 + 3) *Drosophila* were kept in blue light for 12 hours (refer fig 32). Cross sections through eyes of these flies which were probed with α-DmTRPL and α-DmTRP antibodies reveal that in wild type flies, TRPL localises to the rhabdomere of the R7 cell which is situated in the center of the rhabdom and the cell bodies (Fig 34A), while TRP localises to the rhabdomeres (Fig 34C). In P (Rh1 + 3), TRPL distributes both to the rhabdomeres and the cell bodies (Fig 34B) but TRP is detected only in the rhabdomeres (Fig 34D).

B. Effect of green-light exposure

Confocal microscopic analysis of cross sections of wild type *Drosophila* kept in green light for 12 hours reveals that TRPL is present in the cell bodies and in the central R7 rhabdomere (Fig 35A) while the localisation of TRP is changed. TRP no longer localises to the rhabdomeres but is rather mislocalised to the cytosol of the cells (Fig 35C). Since TRP is mislocalised, cross sections of wild type *Drosophila* were also probed with α-DmINAD to check for the distribution of the scaffolding protein, INAD. Analysis show that INAD is also mislocalised to the cytosol (Fig 35E). Due to the inactivation of Rh3 in P (Rh1 + 3) flies, TRPL like TRP and INAD is distributed to all the rhabdomeres (Fig 35B, D, E). This could indicate that TRPL protein distribution may be influenced by the activation of rhodopsin which could also have other effects with respect to TRP and INAD distribution.

C. Effect of orange-light exposure

In wild type *Drosophila* kept in orange light for 12 hours, TRPL is absent in the R1-6 rhabdomeres as expected, which could be due to the activation of Rh1 rhodopsin. Since orange light does not activate Rh3 / Rh4 rhodopsins, the rhabdomeres of the R7 cells display a fluorescent labeling for TRPL (Fig 36A). Besides the presence of TRP and INAD in the rhabdomeres of R7 cells, TRP and INAD are found to localise at the base of the

rhabdomeres of the outer photoreceptor cells (Fig 36C, E). In P (Rh1 + 3) flies exposed to orange light for 12 hours, TRPL protein is detected in the rhabdomeres of the ommatidia but some of them show a reduction in TRPL labeling (Fig 36B). TRP and INAD localise to the rhabdomeres of all the photoreceptor cells (Fig 36D, F).

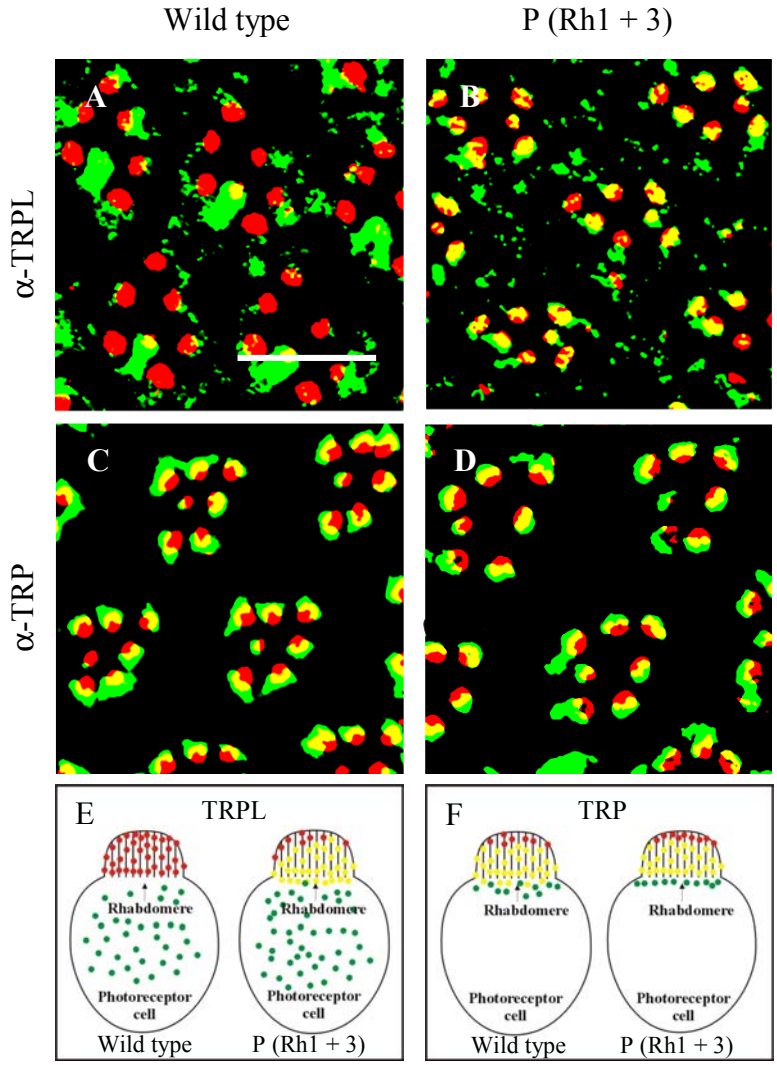

Fig 34: TRPL is localised to the R7 rhabdomere in wild type *Drosophila* kept in blue light

Confocal fluorescent labeled images for the localisation of TRPL (green) (A, B) and TRP (green) (C, D) in wild type and P (Rh1 + 3) *Drosophila* kept in blue light for 12 hours. The yellow colour indicates a colocalisation of the protein with WGA-TMR (red) which is used to label the rhabdomeres. (E) & (F) schematic representation of a single photoreceptor corresponding to the above images. Scale bar: 10µm.

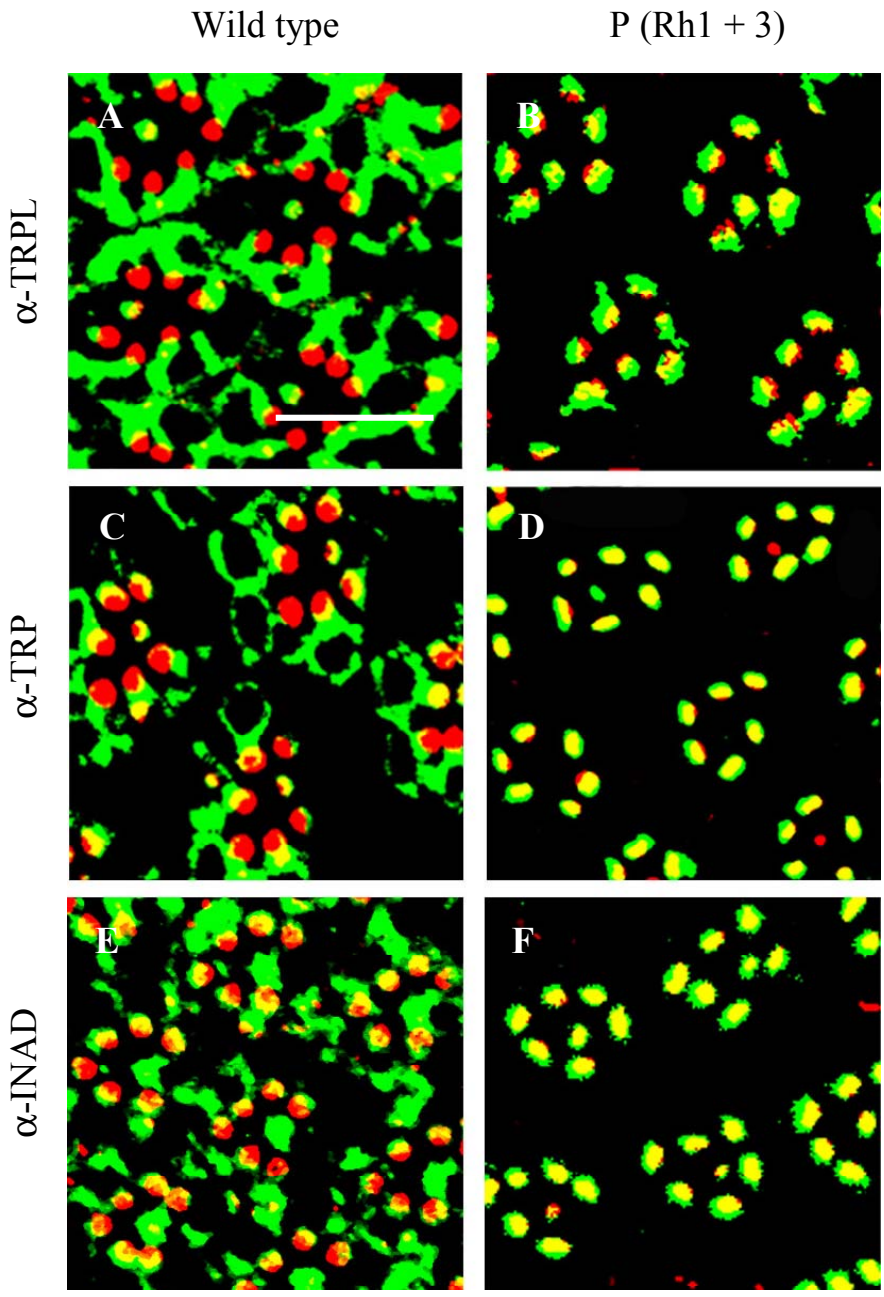

Fig 35: TRPL is localised to all the rhabdomeres of the ommatidia in P (Rh1 + 3) *Drosophila* exposed to green light

Double-fluorescent labeling for the localisation of TRPL (green) (A, B), TRP (green) (C, D), and INAD (E, F) in wild type and P (Rh1 + 3) *Drosophila* kept in green light for 12 hours. Seen above are overlay images displaying the colocalisation of the protein with WGA-TMR (yellow). Scale bar: 10µm.

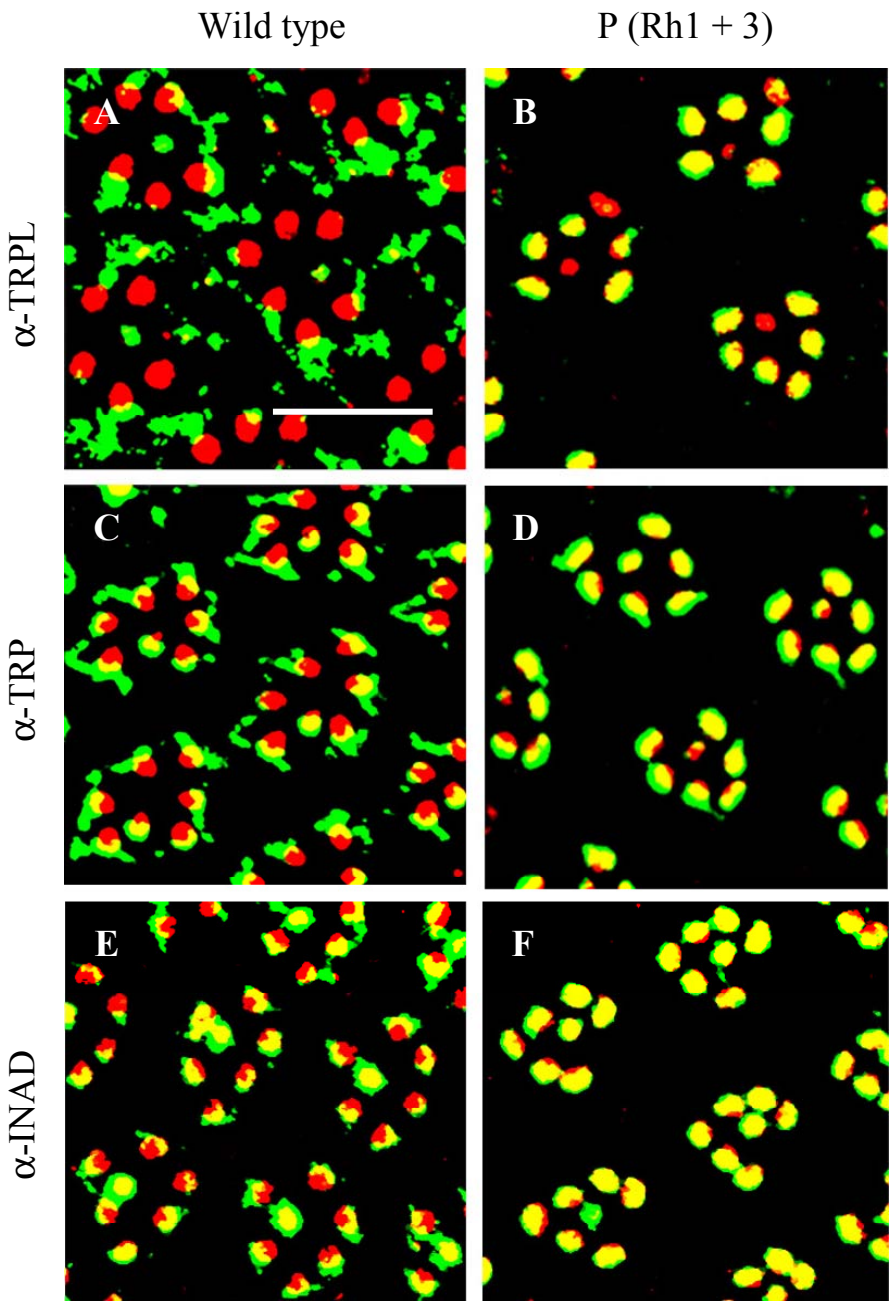

Fig 36: TRPL is localised to the rhabdomeres in P (Rh1 + 3) *Drosophila* exposed to orange light

Wild type flies and P (Rh1 + 3) *Drosophila* were kept in orange light for 12 hours. Confocal fluorescent images showing the localisation of TRPL (green), TRP (green), and INAD (green) in wild type (A, C, E) and P (Rh1 + 3) (B, D, F) as indicated. The yellow colour indicates colocalisation with WGA-TMR. Scale bar: 10µm.

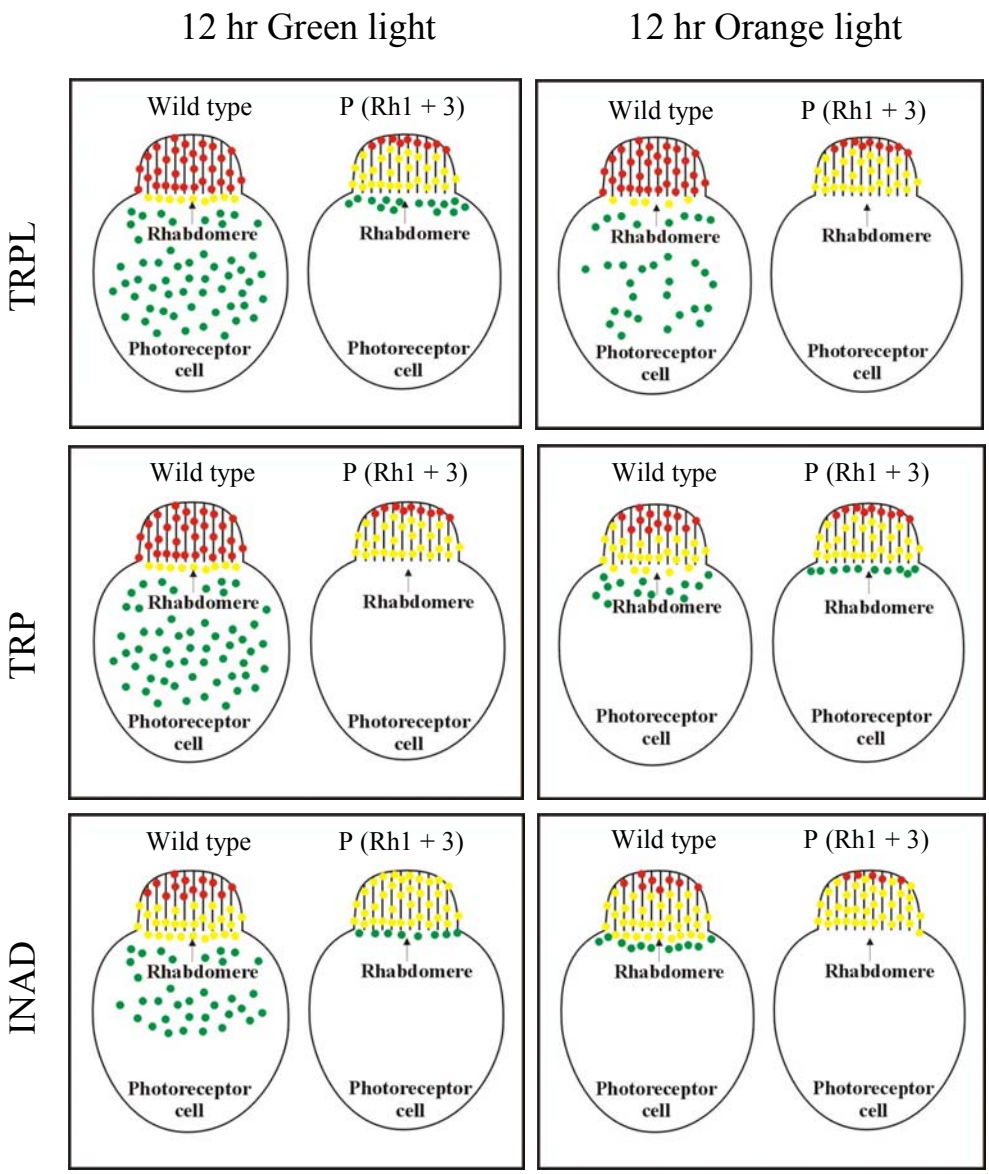

Fig 37: Schematic representation of TRPL, TRP and INAD distribution in wild type and P (Rh1 + 3) *Drosophila* photoreceptors exposed to green and orange light

The green colour indicates the distribution of the respective protein as indicated, while the yellow indicates the colocalisation of the respective protein with WGA-TMR.

D. Effect of 12 hours orange-light exposure followed by 2-6 hours dark exposure

Taken together, it can be postulated that the light-regulated distribution of TRPL may be influenced by the activation of rhodopsin. Since orange light kept P (Rh1 + 3) flies express a reduced level of TRPL in some rhabdomeres, it is of interest to learn whether or not down-regulation of TRPL expression could be influenced by keeping the flies in the dark.

To explore this, wild type and P (Rh1 + 3) *Drosophila* were kept in orange light for 12 hrs followed by 2 hours in the dark. Immunofluorescence labeling shows that in wild type flies, some labeling for TRPL is still observed in the cell bodies, though most of it localises to the rhabdomeres (Fig 38A). This may indicate that a time period of 2 hours dark exposure is insufficient to localise all the TRPL protein molecules in the rhabdomeres. However, under these condition, TRP and INAD both localise to the rhabdomeres within 2 hours of dark exposure (Fig 38C & E). In P (Rh1 + 3) some rhabdomeres still show a reduced expression of TRPL protein (Fig 38B) while TRP and INAD localise to the rhabdomeres (Fig 38D & F).

To find out if the expression of TRPL in the rhabdomeres is influenced by the time interval of the dark period exposure, wild type and P (Rh1 + 3) *Drosophila* were kept in orange light for 12 hours followed by 6 hours in the dark. Confocal microscopic results reveal that in this case the amount of TRPL is much higher in the rhabdomeres in wild type flies compared to flies which were kept in the dark for 2 hours (Fig 39A). This gives an understanding that within a time interval of 2-6 hours dark exposure of wild type *Drosophila*, most of the TRPL molecules distribute from the cell bodies to the rhabdomeres. In P (Rh1 + 3), TRPL localises to the rhabdomeres, but does not show an increase in the TRPL content (Fig 39B). Both TRP and INAD localise to the rhabdomeres in wild type and P (Rh1 + 3) fly strains (Fig 39C, D, E, F).

Therefore it can be speculated that though apparently TRPL expression is down-regulated in P (Rh1 + 3) flies kept in orange light (Fig 36), keeping these flies for upto 6 hours in the dark has no effect with respect to the expression of TRPL protein level in the rhabdomeres (Fig 40). However, a significant difference with respect to TRP and INAD localisation is seen when wild type *Drosophila* are exposed to dark for 2 hrs after orange light illumination. Although rhodopsin activation may be responsible for the distribution of TRPL in the photoreceptors of wild type and P (Rh1 + 3) *Drosophila*, the possibility that other light activated pigments which act independently of the rhodopsin activated pathway cannot be excluded. To dissect the mechanism or pathway responsible for the distribution of TRPL in the photoreceptors, it is essential to learn whether or not it is dependent on the visual cascade or the influence of the any of the components associated with the visual cascade.

4.4 Analysis of the distribution of TRPL in fly photoreceptors having a defect in the phototransduction cascade

With respect to the light-regulated TRPL localisation within the different compartments of the photoreceptors a number of questions can be put forth in order to understand the mechanism affecting the distribution. In the following, it is investigated whether or not the differences in TRPL localisation is dependent on **(1)** The rhodopsin cycle **(2)** The switching on of the phototransduction cascade **(3)** The presence / absence of any member of the phototransduction cascade **(4)** Assembly of phototransduction proteins into the signaling complex. The best possible approach to answer these questions is obtained by the analysis of the various *Drosophila* mutants that have a defect or lack the key proteins of the phototransduction cascade.

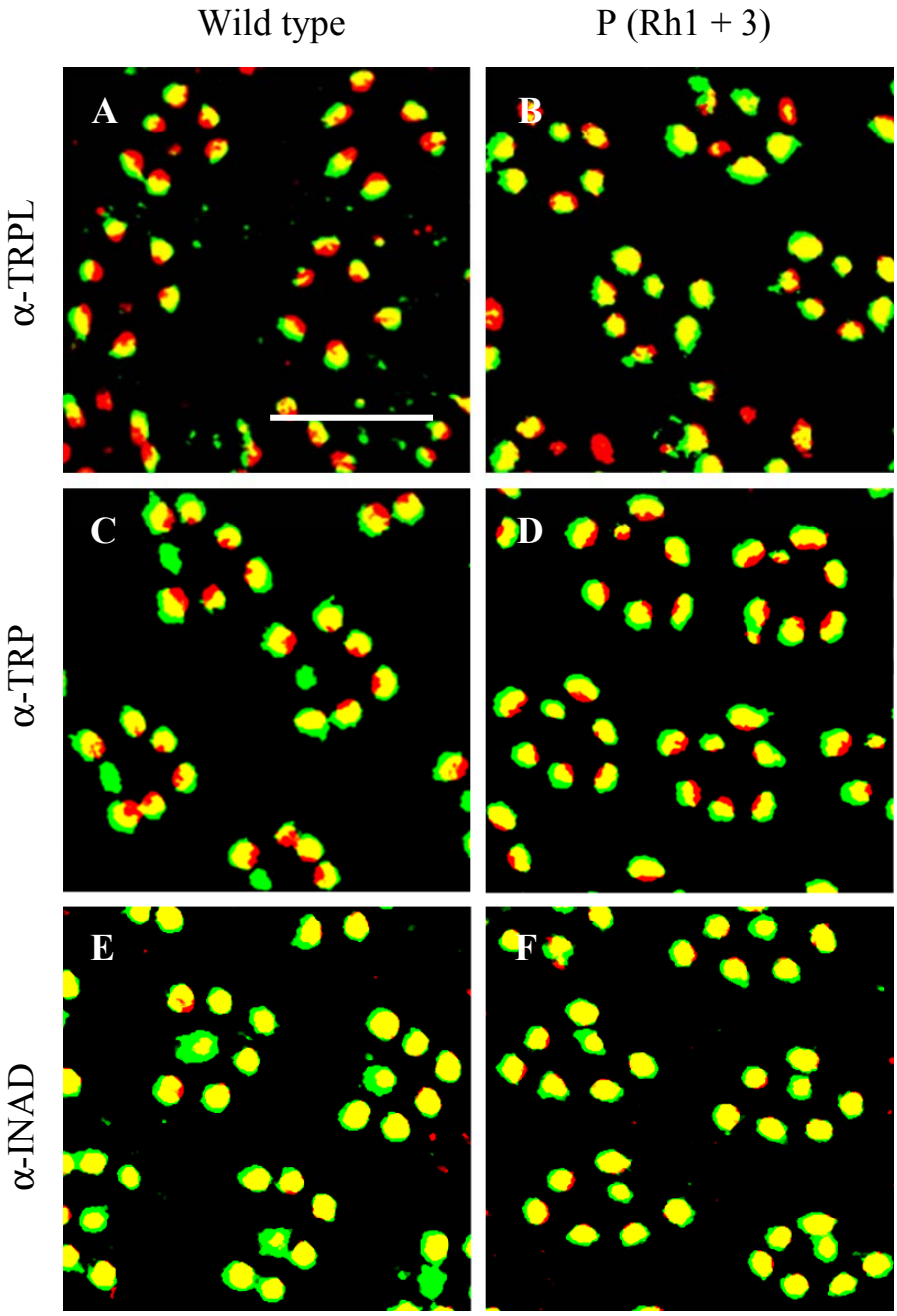

Fig 38: TRPL, TRP and INAD localise to the rhabdomeres after 12 hours orange light followed by 2 hrs of dark kept wild type *Drosophila*

Wild type and P (Rh1 + 3) *Drosophila* were kept in orange light for 12 hours followed by 2 hrs dark exposure. Seen above are confocal fluorescent images showing the localisation of TRPL (green), TRP (green), and INAD (green) in wild type (A, C, E) and P (Rh1 + 3) (B, D, F) *Drosophila*. The colocalisation of the respective protein with WGA-TMR is indicated in yellow. Scale bar: 10µm.

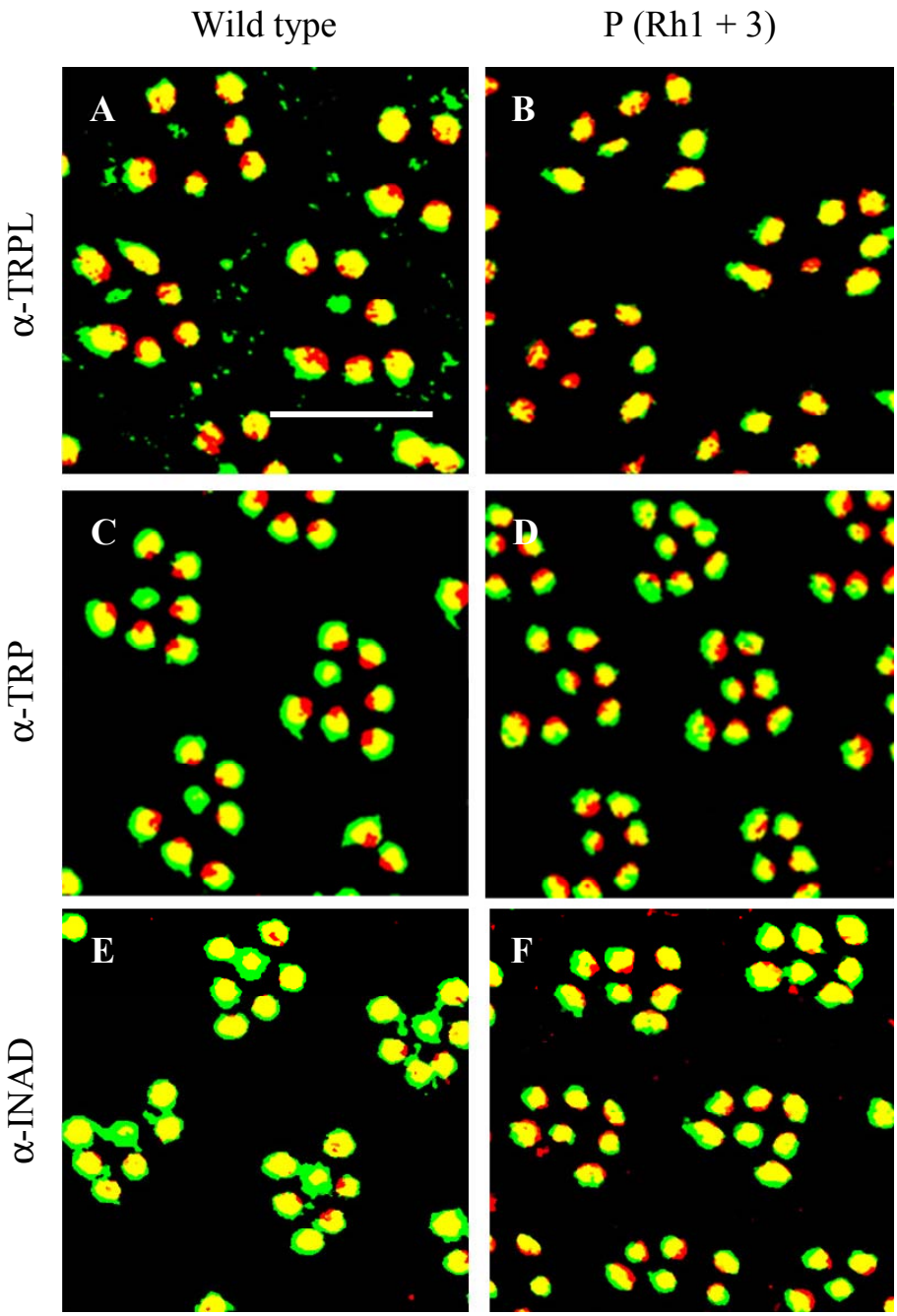

Fig 39: TRPL is localised to the rhabdomeres after 12 hours orange light followed by 6 hrs of dark kept wild type and P (Rh1 + 3) *Drosophila*

Wild type and P (Rh1 + 3) *Drosophila* were kept in orange light for 12 hours followed by 6 hours dark exposure. Seen above are confocal fluorescent images showing the localisation of TRPL (green), TRP (green), and INAD (green) in wild type (A, C, E) and P (Rh1 + 3) (B, D, F). The colocalisation of the respective protein with WGA-TMR is indicated in yellow. Scale bar: 10µm.

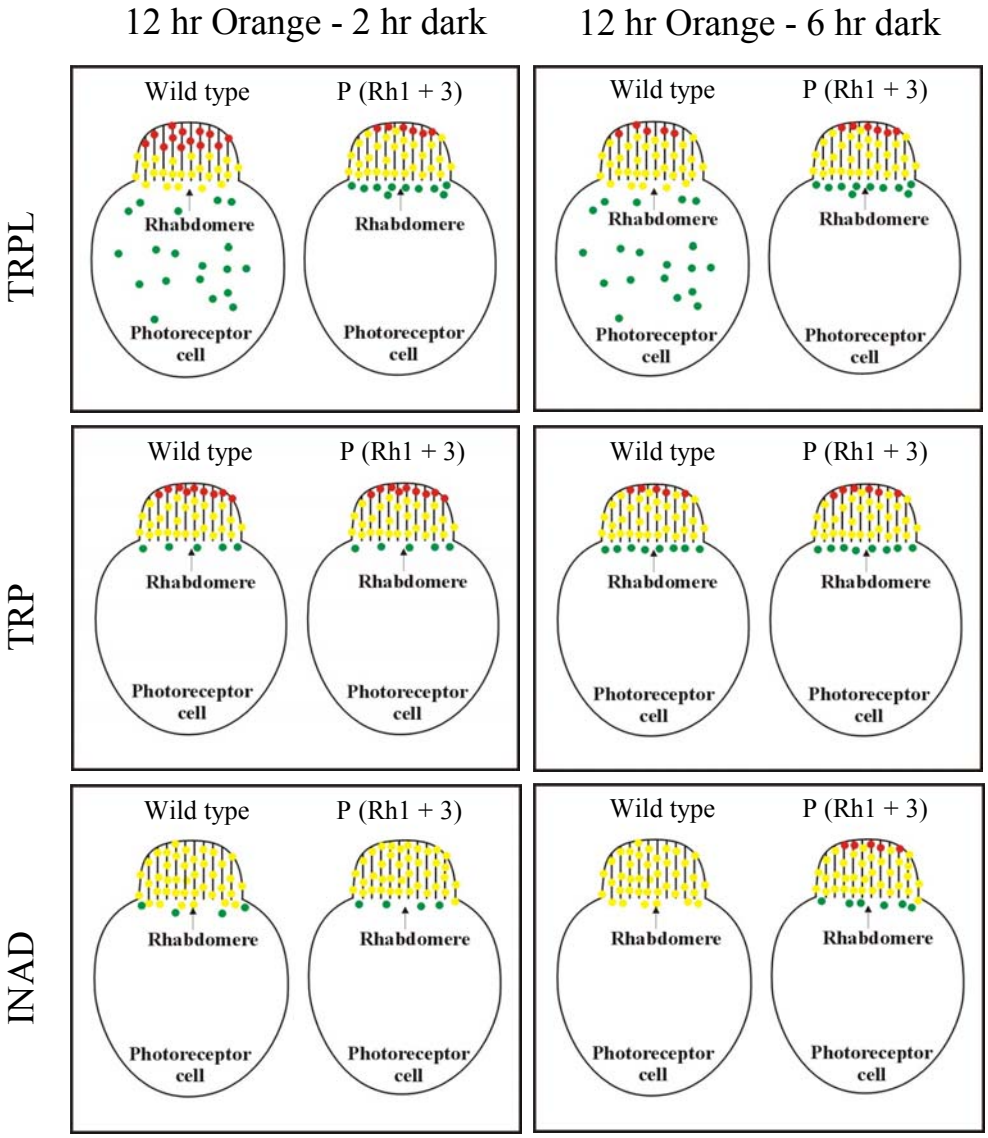

Fig 40: Schematic representation of TRPL, TRP and INAD distribution in photoreceptors of wild type and P (Rh1 + 3) *Drosophila* kept in 12 hrs orange light followed by 2-6 hrs dark adaptation

The green colour indicates the distribution of the respective protein, while the yellow indicates the colocalisation of the respective protein with WGA-TMR.

4.4.1 Influence of phosphorylation of rhodopsin on the distribution of TRPL channel

The initial step in rhodopsin phosphorylation (Byk *et al*., 1993) is brought about by a rhodopsin kinase phosphorylating it on the carboxy-terminal serine and threonine residues. It has been reported that transgenic flies synthesing a truncated form of rhodopsin which lacks its carboxy terminus have no apparent defect in deactivation but show a complete loss of rhodopsin phosphorylation (Vinos *et al*., 1997). To investigate if the light-regulated localisation of TRPL is independent / dependent on the phosphorylation of rhodopsin, a transgenic *Drosophila* strain was used that expresses Rh1 rhodopsin which lacks all its phosphorylation sites on the carboxy terminus, P (Rh1 Δ Phos.), due to site directed mutation of all putative phosphorylation sites (Ser/Thr to Ala). These transgenic flies were dark and light kept as described previously and cross sections through eyes were probed with α-DmTRPL and α-DmTRP antibody. Immunocytochemical analysis of dark kept P (Rh1 Δ Phos.) flies shows that both TRPL and TRP localise to the rhabdomeres (Fig 41A & C) as in wild type flies. In light adapted mutants, no significant change of TRPL localisation is observed when compared to wild type flies kept under similar conditions. TRPL is seen to distribute to the cell bodies of the photoreceptor cells (Fig 41B) while TRP remains associated with the rhabdomeral membranes (Fig 41D). This would imply that TRPL distribution in the photoreceptor cells is independent of the post-translational modification of rhodopsin which is brought about by its phosphorylation.

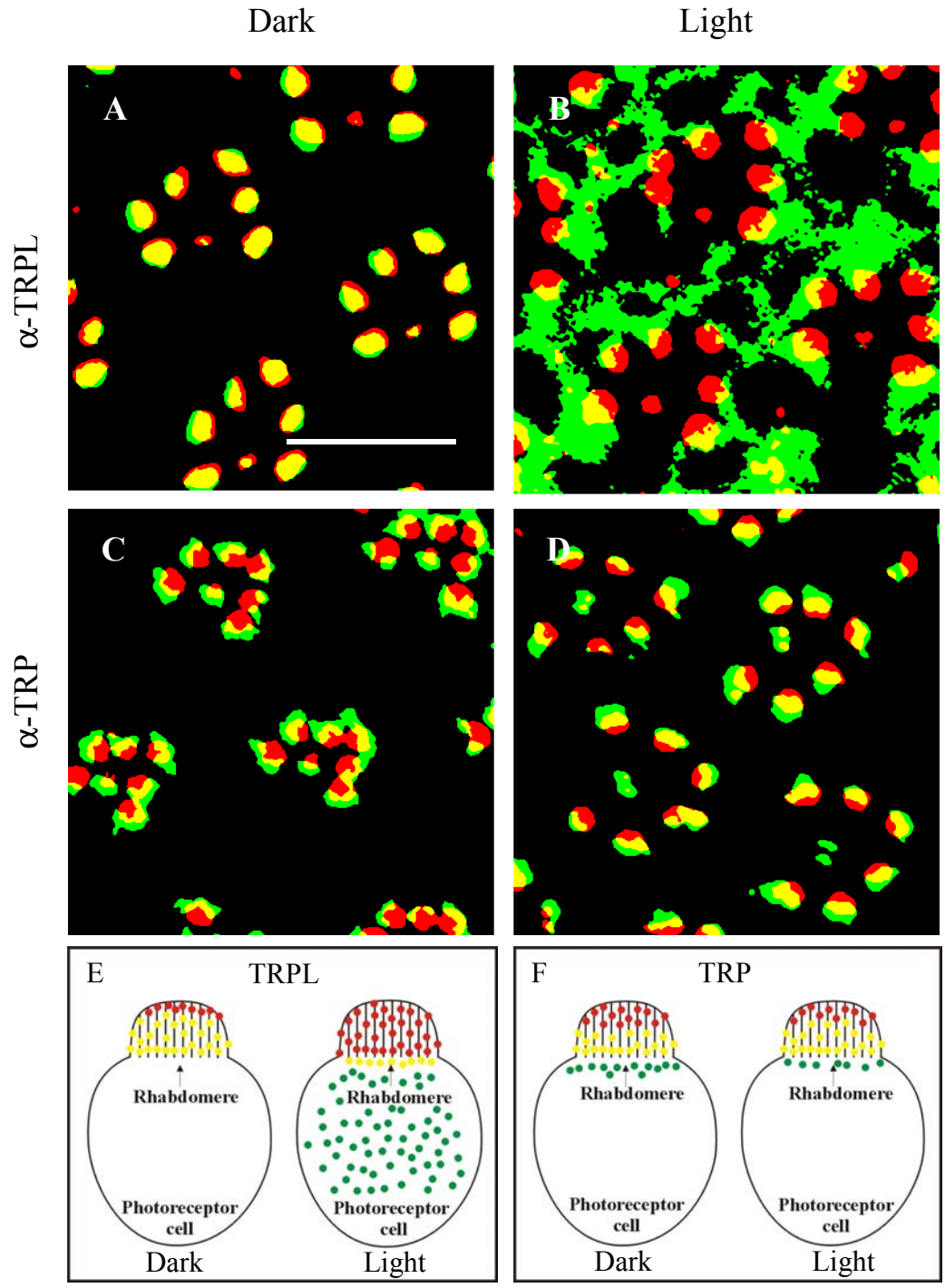

Fig 41: TRPL localisation is unaffected by the phosphorylation of Rh1 rhodopsin

Transgenic *Drosophila* expressing Rh1 which lacks all the phosphorylation sites on the C-terminus were dark and light kept for 12 hours. Presented above are fluorescent images for the localisation of TRPL (green) and TRP (green) in dark (A, C) and light (B, D) kept flies as indicated. The yellow colour displayed indicates a colocalisation of the protein with WGA-TMR (red). (E) & (F) shows a schematic representation of a single photoreceptor corresponding to the images above. Scale bar: 10µm.

4.4.2 Effect of switching on the phototransduction cascade on the distribution of TRPL channel

The *Drosophila norpA* gene encodes a phospholipase Cβ (PLCβ) that is mandatory for photoreception. Strong mutations in the *norpA* gene of *Drosophila* have long been known to abolish the light-evoked photoreceptor potential, rendering the fly blind (Hotta and Benzer, 1970; Pak *et al*., 1970). These observations support the proposal that the phototransduction pathway in invertebrates occurs via a phospholipase C-mediated signaling pathway and that in *Drosophila* PLCβ is an essential component for phototransduction (Payne, 1986; Bloomquist *et al*., 1988; Shortridge, 1991). To investigate the effect of downstream activation of the members of the phototransduction cascade on TRPL localisation, $norpA^{P24}$ flies were tested. In dark exposed $norpA^{P24}$ mutants, TRPL and TRP distribution is similar to that observed in wild type flies (Fig 42A, C). As expected, TRP localises to the rhabdomeres in light kept $norpA^{P24}$ mutants (Fig 42D) but TRPL distributes to the cell bodies (Fig 42B) which indicates that the light-regulated TRPL distribution is independent with respect to the activation of the phototransduction cascade downstream of PLCβ.

4.4.3 TRPL distribution in transgenic flies overexpressing a mutated visual Gγ

The heterotrimeric G-protein in the visual cascade of *Drosophila* consists of three subunits namely α, β, γ. Gα has been shown to be involved in the activation of PLCβ (Bähner *et al*., 2000), while the β subunit has been reported to play an essential role in terminating the photoresponse in *Drosophila* (Dolph *et al*., 1994). Several invertebrate G-protein subunits that may participate in phototransduction have been identified (Running Deer *et al*., 1995) which could influence TRPL distribution upon rhodopsin activation. As a Gqα mutant was not available and the absence of Gqβ and Gqγ mutant, the localisation of TRPL was investigated in a transgenic line in which the visual Gqγ-subunit was overexpressed under the control of the Rh1 promoter (Schulz, 2001). In this transgenic *Drosophila* line, the mutated Gγe forms a complex with Gβe with the result that the membrane association of Gβe and Gγe is altered (Schulz, 2001). To investigate the

possible effect of membrane alteration of Gβe and Gγe on TRPL distribution, these transgenic flies were tested for the localisation of TRPL and TRP in dark and light conditions. In dark and light condition, TRPL and TRP localisation is indistinguishable from wild type flies (Fig 43) indicating that though the association of Gβe and Gγe is affected (Schulz, 2001) the light dependent distribution of TRPL remains unaffected.

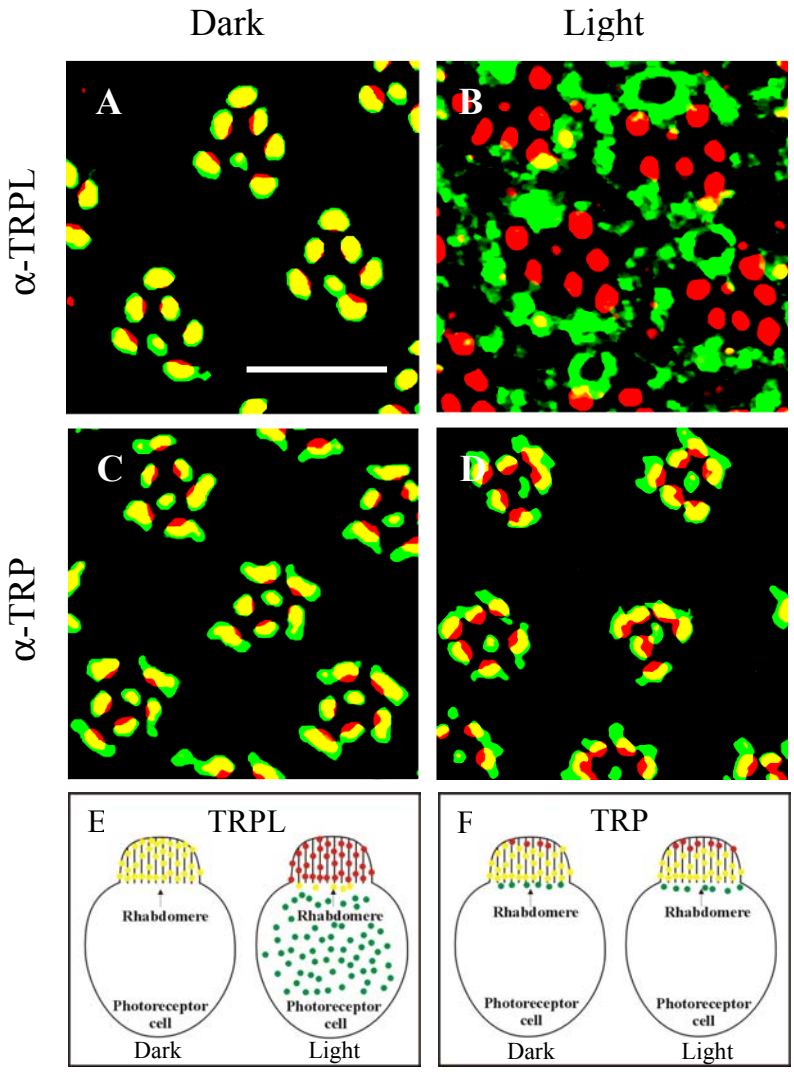

Fig 42: TRPL localisation is not influenced by the downstream activation of PLCβ

Drosophila norpA^{P24} mutants were kept in dark (A, C) or light (B, D) and cross sections through eyes were probed for TRPL and TRP localisation. Seen above are confocal fluorescent images for the localisation of TRPL (green) and TRP (green). The yellow colour indicates colocalisation of the protein with WGA-TMR labeling. (E) & (F) schematic representation of a single photoreceptor corresponding to the images above. Scale bar: 10µm.

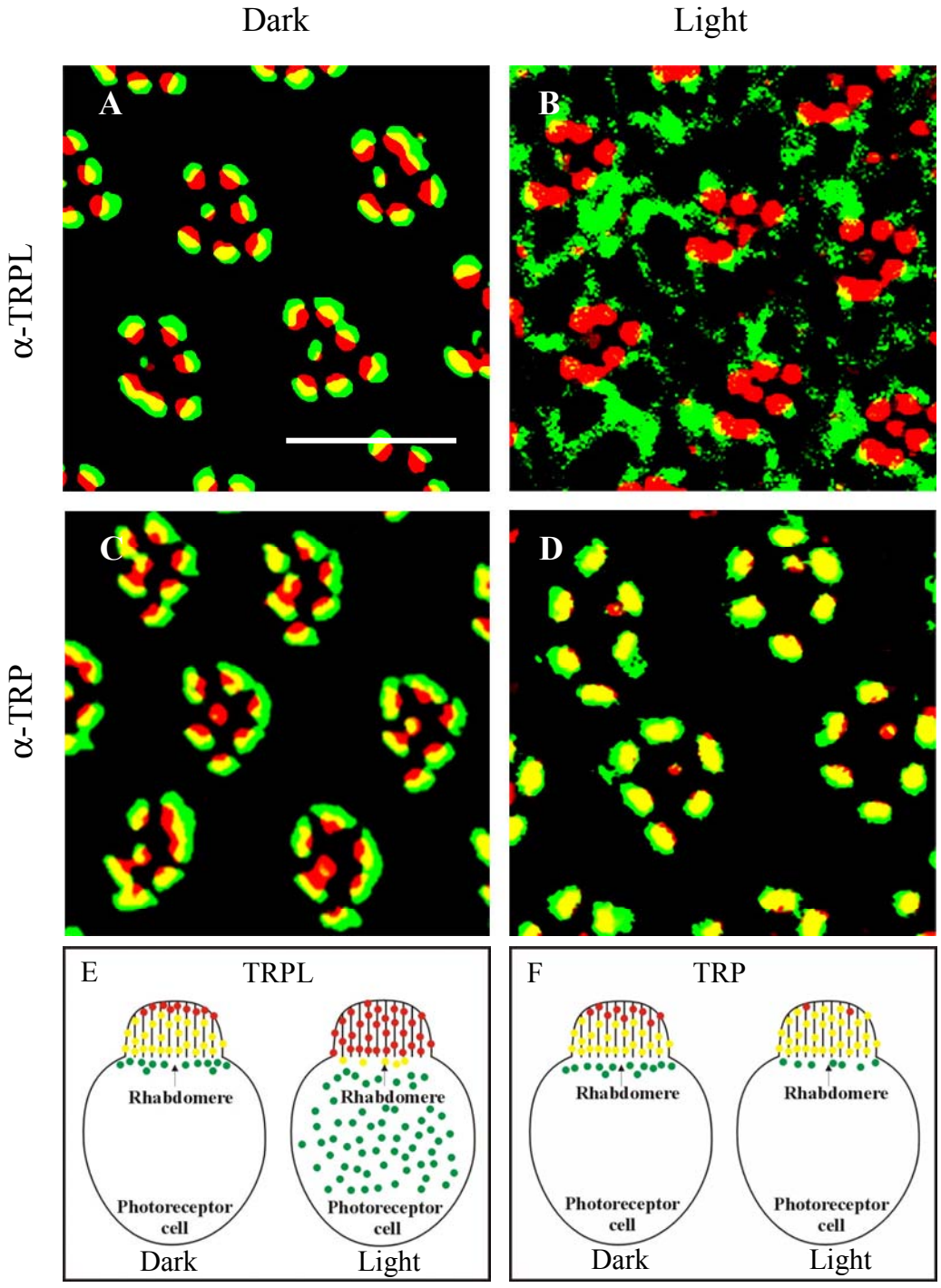

Fig 43: Localisation of TRPL in transgenic *Drosophila* overexpressing the visual Gγ-subunit

Confocal microscopic images showing double fluorescent labeling for TRPL (green) and TRP (green) in dark (A, C) and light (B, D) kept transgenic *Drosophila* overexpressing the visual Gγ under the Rh1 promoter. The yellow colour displayed indicates colocalisation of the protein with WGA-TMR (red). (E) & (F) shows a schematic representation of a single photoreceptor corresponding to the images above. Scale bar: 10µm.

4.4.4 Distribution of TRPL in *ePKC* mutant

It has been shown that the eye specific protein kinase C (ePKC) is involved in the phosphorylation of the TRP ion channel (Huber *et al*., 1998; Liu *et al*., 2000). ePKC has also been physiologically shown to be involved in negative feedback regulation of the visual transduction in *Drosophila* photoreceptors (Smith *et al*., 1991; Hardie *et al*., 1993). *InaC* mutants show a slow deactivation in response to light and are unable to adapt to different light intensities (Hardie *et al*., 1993). Therefore, to investigate if the phosphorylation of TRP could be responsible for the distribution of TRPL, $inaC^{P209}$ mutants were subjected to dark and light condition and analysed for both TRPL and TRP distribution. Immunocytochemical evidence shows that both TRPL and TRP localise in a similar way as that observed in wild type flies (Fig 44), and are unaffected by the ePKC catalysed phophorylation of TRP.

4.4.5 Localisation of TRPL in TRP mutants

The light response in *Drosophila* phototransduction is mediated by TRP and TRP homologous channels (TRPL & TRPγ). Together with TRP and TRPγ, TRPL has been reported to co-localise in the microvillar photoreceptor membrane (Niemeyer *et al*., 1996; Xu *et al*., 2000). The light-induced currents in *Drosophila* visual system cannot be composed solely of TRP channels because *trp* mutants still display a response to light stimuli (Niemeyer *et al*., 1996). Studies have shown that TRP is mislocalised in $inaD^{1}$ mutants without affecting the distribution or levels of TRPL expression (Tsunoda *et al*., 1997). Earlier reports indicate that TRP and TRPL may form heteromultimeres in heterologous systems (Scott and Zuker, 1998b) though questions remained to be answered if these channels form homo- or heteromultimers *in vivo* (Reuss *et al*., 1997; Xu *et al*., 1997).

To explore the role of TRP channel on the light-regulated localisation of TRPL in the photoreceptors, trp^{343} mutants were dark and light adapted and analysed for TRPL localisation. As expected, in dark kept flies, TRPL localises to the rhabdomeres as in wild

type *Drosophila* (Fig 45A). But a significant change in TRPL distribution is observed in light kept flies. Interestingly it is seen that TRPL no longer localises to the cell bodies but to the rhabdomeres (Fig 45B), a situation which is not observed in wild type *Drosophila*. Based on this observation it can be assumed that the absence of the major ion channel, TRP, has an impact on the distribution of TRPL within the photoreceptors in a light-regulated manner.

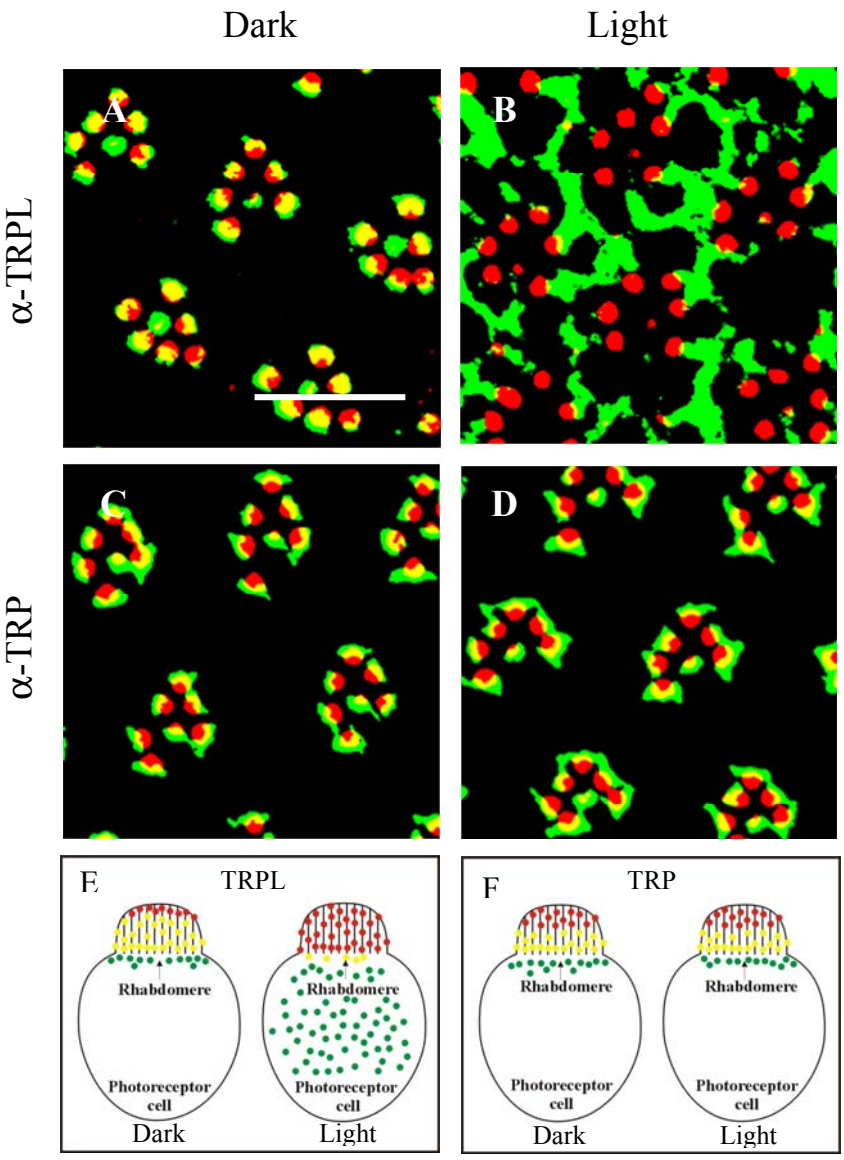

Fig 44: Localisation of TRPL in cross sections of *Drosophila inaC*^{P209} eyes

Confocal fluorescent images showing the localisation of TRPL (green) and TRP (green) in dark (A, C) and light (B, D) kept *inaC*^{P209} flies. The yellow colour shows a colocalisation of the protein with WGA-TMR (red) which was used to label the rhabdomeres. (E) & (F) schematic representation of a single photoreceptor corresponding to the images above. Scale bar: 10µm.

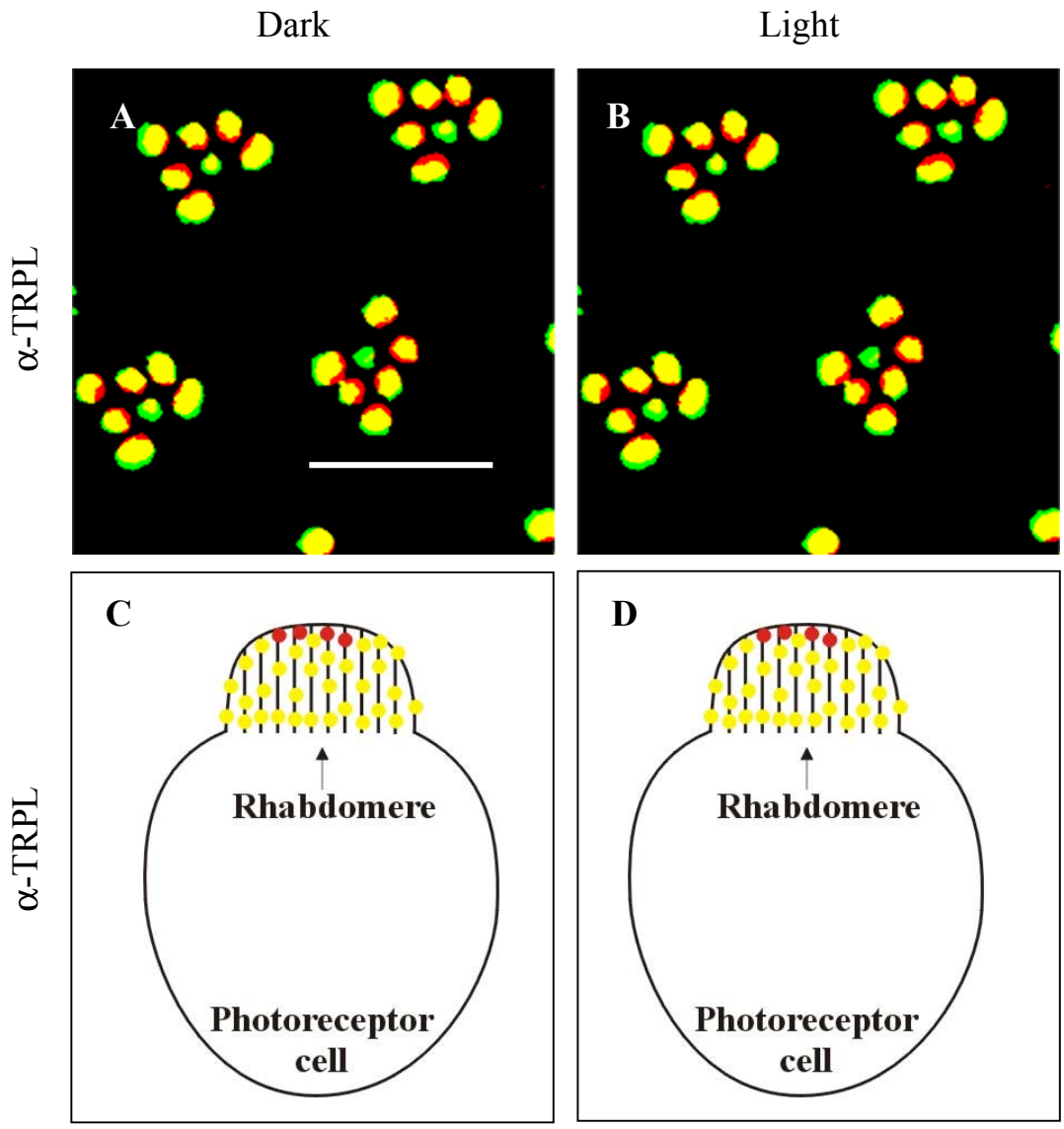

Fig 45: Light-regulated localisation of TRPL is dependent on the presence of TRP

Fluorescent labeling for the localisation of TRPL (green) in dark (A) and light (B) kept *Drosophila trp*^{343} mutants. The yellow colour shows a colocalisation of TRPL with WGA-TMR labeling. (C) & (D) schematic representation of a single photoreceptor corresponding to the above confocal images. Scale bar: 10µm.

4.4.6 Study of the effect of disruption in the INAD signaling complex on TRPL distribution

It has been shown that the association of TRP and INAD is crucial for retention of the signaling complex in the photoreceptors (Li & Montell, 2000). An important observation (as above) is that TRPL localises to the rhabdomeres in *Drosophila trp*^{343} mutants kept in light conditions. Though this is different to the observation in light kept wild type *Drosophila*, but however it is of much interest to know if

1. the light-regulated distribution of TRPL depends on the association of TRP with INAD in the rhabdomeres.
2. is it the absence of TRP together with INAD which could influence TRPL distribution.

An elegant approach to answer these questions was employed by analysing a *Drosophila inaD*^{1} null mutant. 3-5 days old *inaD*^{1} mutants were kept in dark or light and cross sections through eyes were probed with α-DmTRPL and α-DmTRP antibodies. Confocal microscopic anaylsis reveals that in dark or light kept flies, TRPL distribution is the same as in wild type *Drosophila*, while TRP is mislocalised to the plasma membrane as reported by Tsunoda *et al*. (1997) (Fig 46).

It has also been reported that in old *inaD*^{1} flies (10 days post eclosion) the level of TRP, ePKC and PLCβ declines drastically (Tsunoda *et al*., 1997). To confirm if the absence of TRP influences the localisation of TRPL in light kept *Drosophila trp*^{343} mutant (refer Fig 45), 10-12 days old *Drosophila inaD*^{1} mutants were investigated. As expected immunocytochemical analysis shows that TRPL localises to the rhabdomeres in dark. But however, the TRPL distribution is altered and is located in the rhabdomeres in light kept flies (Fig 47A & B). TRP is not mislocalised to the cytosol but rather distributes to the base of the rhabdomeres in dark and light kept flies (Fig 47C & D). In addition to confirmation of the earlier observation (4.4.5) it can be assumed that it is rather the absence of TRP in the rhabdomeres that influences the light-regulated TRPL localisation and not its total absence in the photoreceptors.

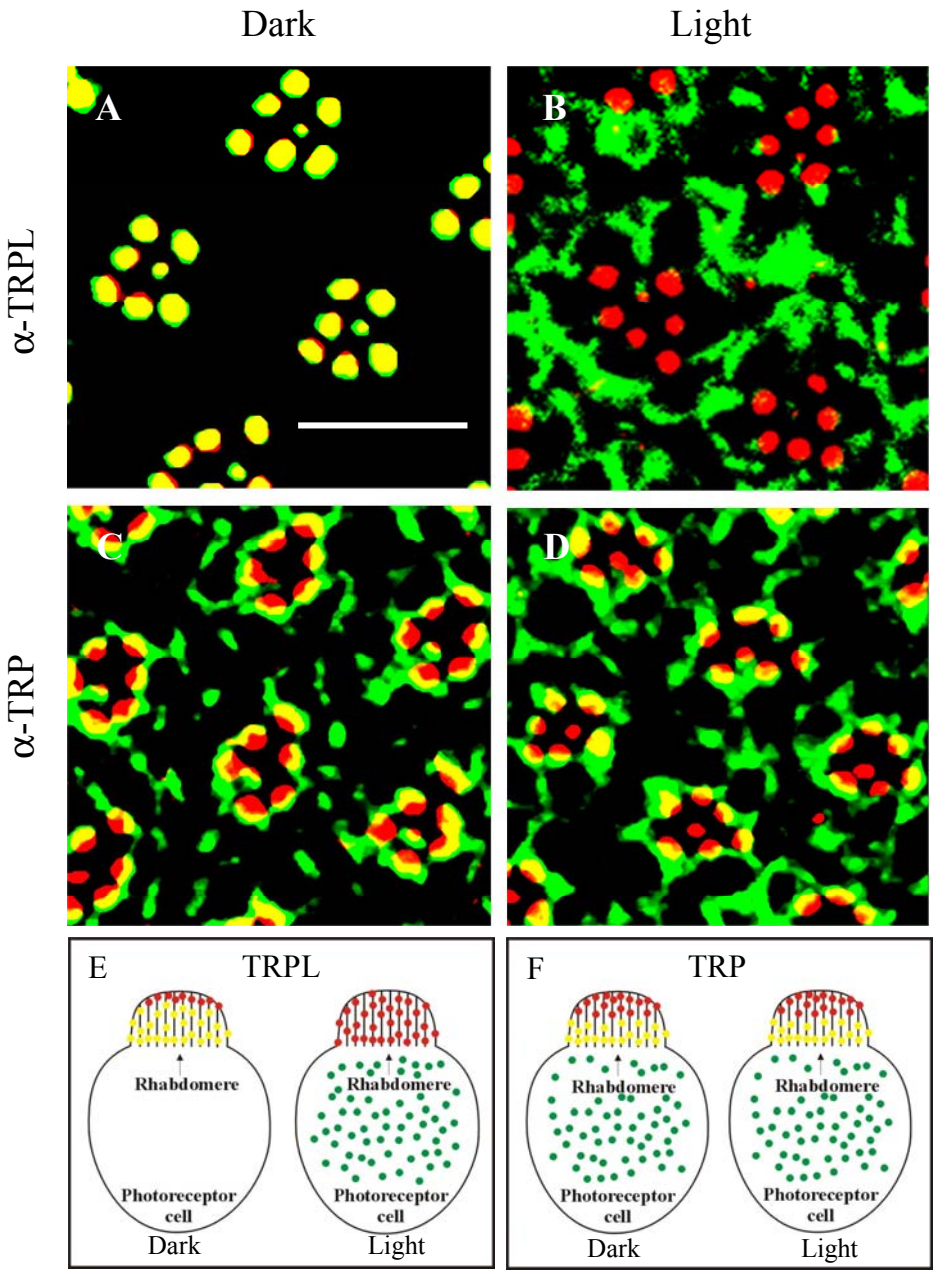

Fig 46: Localisation of TRPL in young *Drosophila inaD*[1] flies

3 to 5 days old *Drosophila inaD*[1] mutants were dark (A, C) and light (B, D) kept and cross sections through eyes were probed for the localisation of TRPL (green) and TRP (green). Shown above are confocal overlay images displaying fluorescent colocalisation (yellow) of the respective protein with WGA-TMR (red). (E) & (F) schematic representation of a single photoreceptor corresponding to the above images. Scale bar: 10µm.

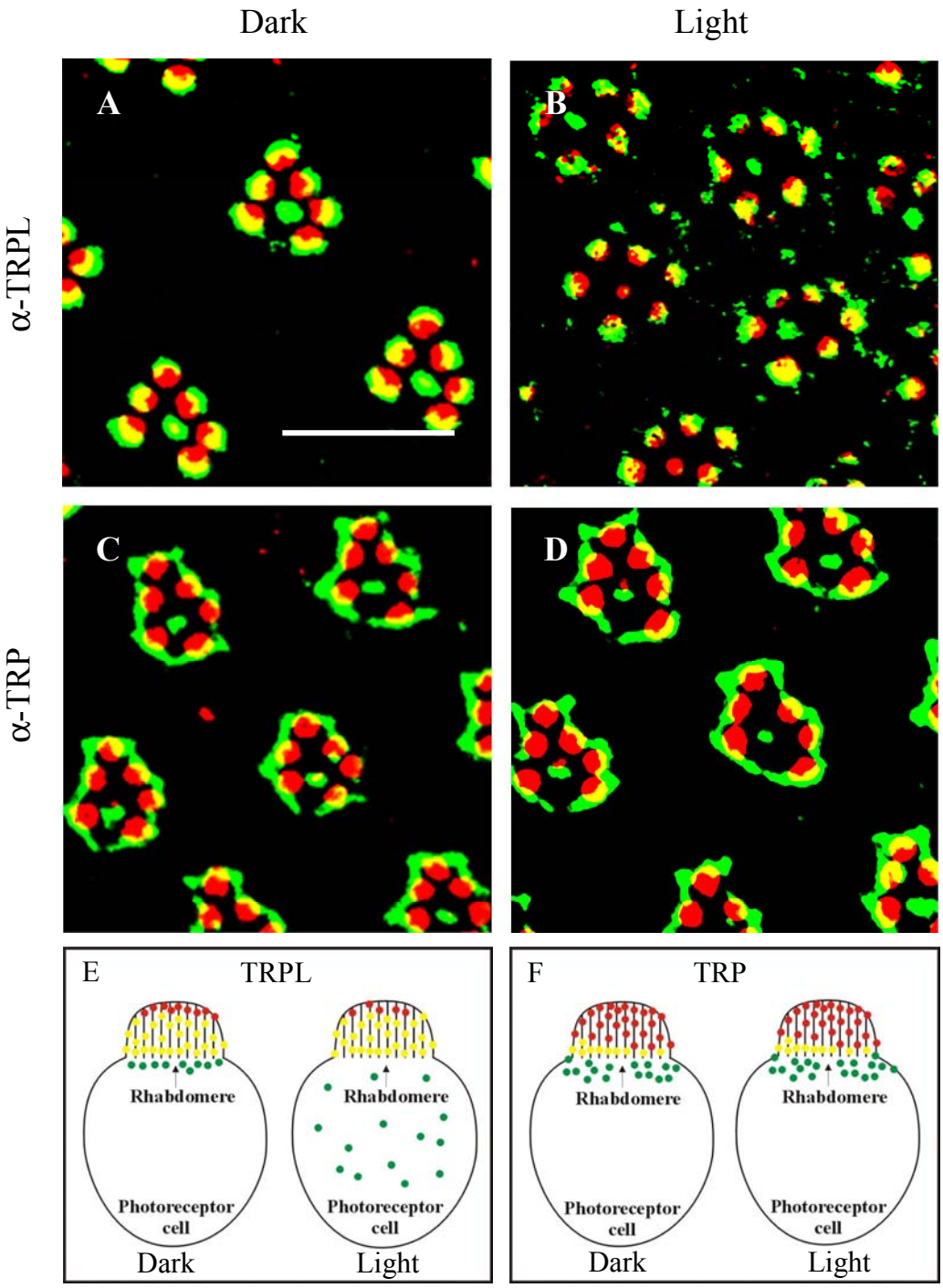

Fig 47: TRPL localisation is dependent on the presence of TRP in old *Drosophila inaD*[1] flies

Confocal fluorescent images showing the localisation of TRPL (green) and TRP (green) in dark (A, C) and light (B, D) kept old (10 to 12 days old) *Drosophila inaD*[1] flies. The yellow colour displays the colocalisation of the respective protein with WGA-TMR labeling (red) as indicated. (E) & (F) schematic representation of a single photoreceptor corresponding to the images above. Scale bar: 10µm.

To exclude non-restrictiveness of light-regulated TRP influence on TRPL distribution in *Drosophila*, both young (3-5 days) and old (10-12 days) *Calliphora rpa* flies were analysed. As reported earlier (refer 4.2.1), old *Calliphora rpa* mutants fail to show any TRP labeling. Taking adavantage of this situation, the possible role of TRP with respect to TRPL localisation was investigated in *Calliphora* flies. In young *Calliphora rpa* flies, TRPL is detected in the rhabdomeres of dark kept flies while in light kept flies the distribution of TRPL changes to some extent. It localises to the cell bodies and rhabdomeres (Fig 48A & B). As would be expected, in old *Calliphora rpa* flies (10-12 days old), TRPL localises to the rhabdomeres in dark and light conditions. This shows that the TRP influence on the light-regulated TRPL distribution is not only restricted to *Drosophila* but also observed in *Calliphora* (Fig 48C & D).

Taken together, light-regulated TRPL distribution within the photoreceptors is influenced by the absence of TRP in the rhabdomeres but independent of the presence / absence of INAD. The distribution of TRPL is independent of the downstream activation of PLCβ of the phototransduction cascade as shown in light kept *norpA*^{P24} flies. Therefore, the possibility of other G-proteins subunits associated with the visual cascade cannot be excluded. These G-protein subunits could be involved in another unidentified signaling pathway that may be light-regulated which could be responsible to influence the TRPL localisation. The distribution of TRPL protein in wild type and mutants of *Drosophila* and *Calliphora* which were exposed to different light conditions is summarised in table 8.

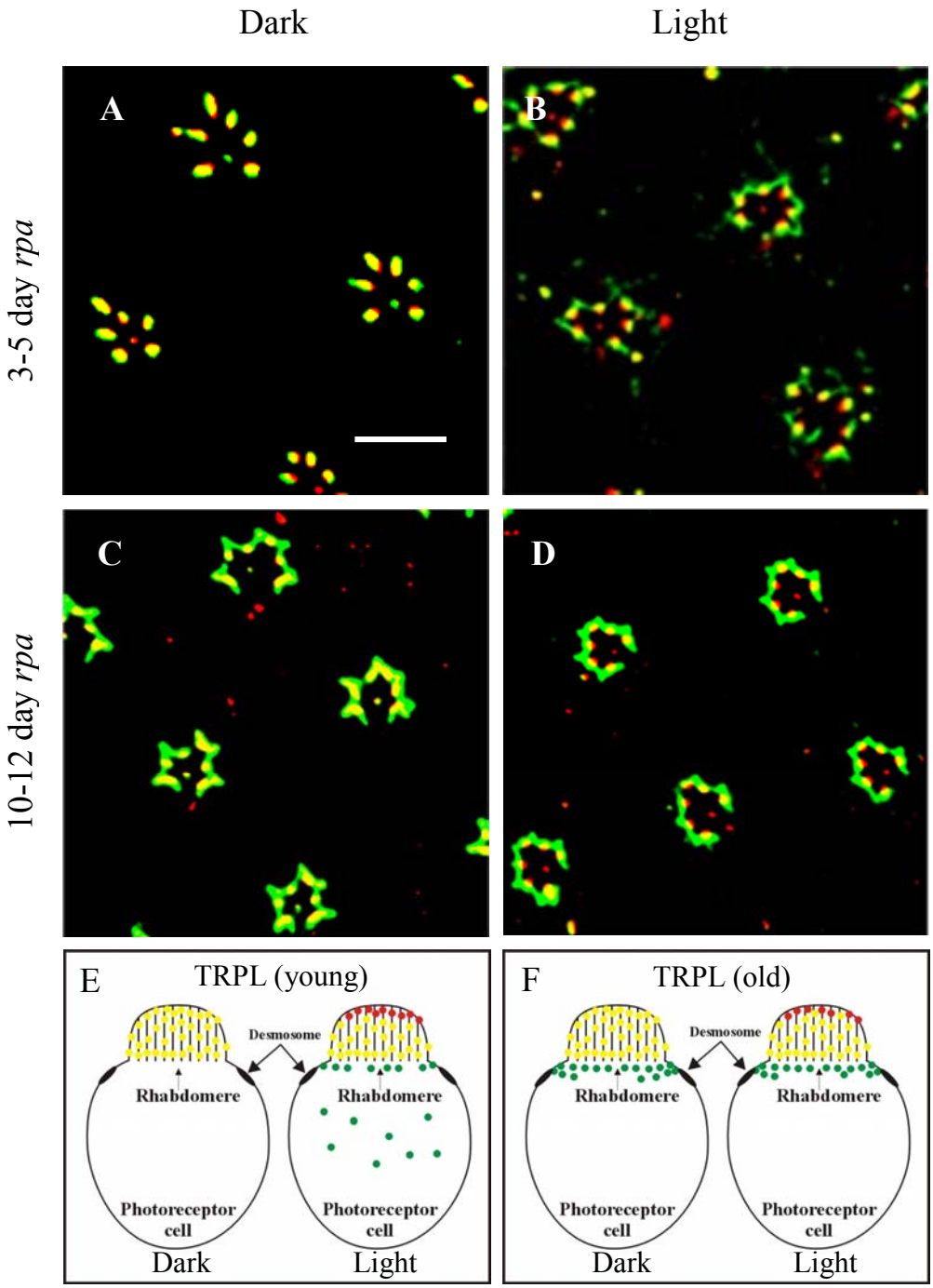

Fig 48: TRPL is localised to the rhabdomeres in light raised old *Calliphora rpa* flies

Young (3 to 5 days old) and old (10 to 12 days old) *Calliphora rpa* mutants were dark (A, C) or light (B, D) kept for 12 hours. Shown above are confocal images for the localisation of TRPL (green) as indicated. The yellow colour in the above images indicates the colocalisation of TRPL (green) with WGA-TMR (red). (E) & (F) schematic sketch of a single photoreceptor from the corresponding above images. Scale bar: 10µm.

Table 8: TRPL localisation in wild type *Drosophila* & *Calliphora*, *Drosophila* mutants and in different light conditions.

Abbrevations used: Dmwt – *Drosophila* wild type; Cvwt – *Calliphora* wild type; D-dark; L-light; + / - indicates presence / absence. All flies were raised in 12 hours dark or light condition except where specified.

Wild type (wt) or mutant	**Light condition**	**Localisation of TRPL in**			
		R1-6 rhabdomeres	**R7 rhabdomere**	**Base of rhabdomere**	**Cell body**
Dmwt (> 1hr old)	D	+	+	-	-
Dmwt (> 1hr old)	L	-	-	-	+
DmWt (3-5 d old)	D	+	+	-	-
Dmwt (3-5 d old)	L	-	-	-	+
Dmwt (10-12 d old)	D	+	+	-	-
Dmwt (10-12 d old)	L	-	-	-	+
Cvwt (3-5 d old)	D	+	+	-	-
Cvwt (3-5 d old)	L	-	-	-	+
Cvwt (10-12 d old)	D	+	+	-	-
Cvwt (10-12 d old)	L	-	-	-	+
P (Rh1 + 3)	D	+	+	-	-
P (Rh1 + 3)	L	-	-	-	+
Different wavelenghts					
Dmwt	Blue	-	-	-	+
P (Rh1 + 3)	Blue	+	+	-	+
Dmwt	Green	-	-	+	+

P (Rh1 + 3)	Green	+	+	+	-
Dmwt	Orange	-	-	+	+
P (Rh1 + 3)	Orange	+	+	-	-
Dmwt	Orange-2 hrs dark	+	-	-	+
P (Rh1 + 3)	Orange-2hrs dark	+	+	+	-
Dmwt	Orange-6 hrs dark	+	+	-	+
P (Rh1 + 3)	Orange-6 hrs dark	+	+	+	-
Mutants / transgenic *Drosophila*					
Rh1 D Phos.	D	+	+	-	-
Rh1 D Phos.	L	-	-	+	+
norpA^{P24}	D	+	+	-	-
norpA^{P24}	L	-	-	+	+
Gγ overexpressed	D	+	+	-	-
Gγ overexpressed	L	-	-	-	+
ePKC^{P209}	D	+	+	+	-
ePKC^{P209}	L	-	-	-	+
trp^{343}	D	+	+	-	-
trp^{343}	L	+	+	-	-
inaD^{1} (3-5 d old)	D	+	+	-	-
inaD^{1} (3-5 d old)	L	-	-	-	+
inaD^{1} (10-12 d old)	D	+	+	+	-
inaD^{1} (10-12 d old)	L	+	+	-	+
***Calliphora* mutant**					
rpa (3-5 d old)	D	+	+	-	-

rpa (3-5 d old)	L	+	+	+	+
rpa (10-12 d old)	D	+	+	+	-
rpa (10-12 d old)	L	+	+	+	-

5. Discussion

Phototransduction in *Drosophila* is a G-protein-coupled phospholipase C (PLC) signaling pathway that shares many similarities with other signaling pathways (Hardie & Minke, 1993; Ranganathan *et al*., 1995). In this pathway, light absorption by rhodopsin results in the activation of the visual G-protein subsequently activating PLCβ coupled downstream components. The signaling pathway eventually leads to the opening of ion channels which takes a few tens of milliseconds to go from light activation to generation of receptor potential and less than 100 milliseconds to terminate the response (Ranganathan *et al*., 1995). The INAD signaling complex has been postulated to play a major role to achieve such a speed of signaling. Though the members comprising the INAD signaling complex are assembled in the photoreceptor cell and are then targeted to the rhabdomere (Tsunoda *et al*., 2001), the association of the ion channel, TRP, with the scaffolding protein INAD is critical for the retention of the signaling complex in the rhabdomeres (Li and Montell, 2000; Tsunoda *et al*., 2001). With respect to the importance of the INAD signaling complex, the functional role in *Calliphora* is evaluated in this thesis. The other point of debate is as to why do fly photoreceptors express two or more classes of ion channels? Is the channel composition in fly photoreceptors the same or are these channels subjected to light regulation? In the discussion of this thesis, the functional importance of the INAD signaling complex in *Calliphora* photoreceptors and of the other ion channel, TRPL, in fly photoreceptor cells will be described.

5.1 Establishing methods for identifying distinct photoreceptor cells and subcellular compartments

The adult compound eye of *Drosophila* expresses five different rhodopsins in specific photoreceptor cells. Rhodopsin being the principle membrane protein of rhabdomeral microvilli (Kumar & Ready, 1995) constitutes 65% of total membrane protein of the rhabdomeres in *Calliphora* (Paulsen & Schwemer, 1979). Its transport to the photoreceptive membrane requires that it is correctly folded and post-translationally processed (Huber *et al*., 1994). In *Drosophila*, mutations in the first cytoplasmic loop of

Rh1 opsin block rhodopsin synthesis subsequently resulting in its degradation and photoreceptor degeneration in an age-dependent manner (Bentrop *et al*., 1997). The immunofluorescent studies by confocal microscopy indicates that rhodopsins expressed in the compund eye of *Drosophila* are primarily localised to the rhabdomeres (Fig 12 & 13) with a typical pattering of Rh1 in R1-6 cells and Rh3/Rh5 and Rh4/Rh6 pairs in central cells respectively. Localisation of rhodopsin to the photoreceptor membrane thus allows to differentiate the different types of photoreceptor cells by immunocytochemistry which is an important prerequisite for the interpretation of light effects on protein translocation in individual photoreceptors. Rhodopsin labeling is also of pratical importance as it allows to identify the rhabdomeral photoreceptor cell compartments in double and triple labeling studies. However, the implementation of this approach would require primary antibodies which are generated in different animals inorder to avoid cross-reaction with the secondary antibodies. Therefore, an alternate method for labeling the rhabdomeres was used here. Fluorescently coupled lectins like Wheat Germ Agglutinin (WGA) have been reported to extensively label the plasma membrane and golgi elements in honeybee photoreceptors (Baumann, 1998).

Paulsen and Bentrop (1986) observed that lectins like Concanavalin A (Con A) have a high affinity to the rhabdomeres, indicating the presence of glycoproteins in rhabdomeral membranes. Subsequent analysis using WGA to detect oligosaccharide side chains in rhabdomeral proteins suggested that N-acetyl-D-glucosamine may be present in the oligosaccharide chain(s) of fly opsin is bound by WGA (Paulsen & Bentrop, 1986). Con A binding sites have also been localised on both sides of the rod outer segments disk membranes (Nir, 1978). Analysis of *Drosophila* and *Calliphora* eyes reveals that WGA positive signal are observed in the rhabdomeres of the photoreceptor cells (Fig 10 & 11), but not in the optic lobes (Fig 10A & B), confirming the earlier observations by Paulsen and Bentrop (1986). It has been shown that Rh1 rhodopsin is glycosylated only in a nascent state (Huber *et al*., 1990). Therefore from the positive signals observed for WGA labeling in *Drosophila* and *Calliphora* eyes suggests that it is not the result of rhodopsin glycosylation but of other proteins associated with the rhabdomeral membranes. Accordingly WGA can be conveniently used to fluorescently label the rhabdomeres

independent of the amount and type of rhodopsin present in the rhabdomeres. WGA labeling in the plasma membrane of photoreceptors observed in honeybee (Baumann, 1998) as well as in rhabdomeres of fly eyes indicates that WGA positive elements may be conserved between closely related insect species. In the case of dipteran flies, WGA labeling provides a method to fluorescently label the rhabdomeres reliably in a way which is independent of immunocytochemistry and which does not affect the labeling of the phototransduction proteins (Fig 10C & D). This is very essential because key mutations in phototransduction proteins often result in retinal degeneration (Hotta & Benzer, 1970; Steele & O'Tousa, 1990) which is most easily detected at the level of rhabdomeres. The loss of the rhabdomeral structure can be conveniently monitored by WGA labeling (see fig 26). The direct fluorescent labeling of the rhabdomeres provides a confocal image which allows to monitor the distribution of the protein under study.

Besides the expression of different rhodopsins, an important feature distinguishing the outer and inner photoreceptor cells is the termination of their axons in distinct neuropiles, i.e. the R1-6 cells terminate in the first optic lobe, the lamina, while the R7 and R8 axons traverse through the lamina and form synapses in the second optic lobe, the medulla (Kirschfeld, 1971; Meinertzhagen and Hansen, 1993). This mode of projection can be distinctly seen in fly strains expressing the *lacZ* gene under the different rhodopsin promoters that allows to label the photoreceptor cells and trace the axonal terminations of the photoreceptor cells in the optic lobes (Fig 16 & 17). Similar studies for Rh1, Rh3, and Rh4 have also been reported previously (Mismer and Rubin, 1987; Fortini and Rubin, 1990). Reporter gene analysis provides an alternate method for showing Rh1 expression in R1-6 cells (Scavarda *et al*., 1983; O' Tousa *et al*., 1985; Zuker *et al*., 1985; Feiler *et al*., 1988), Rh3 and Rh4 expression in non-overlapping R7 cells, and Rh5 and Rh6 expression in non-overlapping R8 cells (Montell *et al*., 1987; Feiler *et al*., 1992; Chou *et al*., 1999). These three independent approaches allow to characterise and locate different photoreceptor cell compartments which provides a ready tool to study the distribution of the components of the INAD signaling complex which are involved in the phototransduction cascade.

5.2 Organisation and functional importance of the INAD signaling complex in fly photoreceptors

The organisation of the key players involved in the phototransduction cascade is brought about by the scaffolding protein, INAD. With respect to the members associated with INAD, the signaling complex components can be subdivided into "core" and "peripheral" proteins. This thesis focusses on the core components TRP, PLCβ, and ePKC since these proteins become mislocalised or degraded in the absence of INAD while the peripheral components like rhodopsin, TRPL, NINAC, and CaM are unaffected and localise to the rhabdomeres (Tsunoda *et al*., 1997) independent of the INAD protein. Evidence has shown that in the *Drosophila inaD*^{P215} mutant, TRP is mislocalised due to a point mutation in the third PDZ domain while PLCβ and ePKC are not mislocalised. Moreover, electroretinogram (ERG) studies of this mutant shows a phenotype of a *trp* mutant (Tsunoda *et al*., 1997) in an age-dependent manner.

Analysis of the *Drosophila inaD*^{2} mutant (mutation in the fifth PDZ domain which is involved in the interaction with PLCβ) shows a mislocalisation and subsequently decay of PLCβ while TRP and ePKC are unaffected (Tsunoda *et al*., 1997). ERG recordings performed with young *Drosophila inaD*^{2} mutant flies exhibit major defects in response kinetics, demonstrating that it is not the presence of a transduction molecule but rather its location that promotes rapid signaling. Further, in *Drosophila inaC*^{P209} (ePKC mutant) and *norpA*^{P41} (PLCβ mutant) mutant, INAD and TRP are correctly localised to the rhabdomeres, indicating that the localisation of INAD and TRP is independent of ePKC and PLCβ. Therefore, the association of INAD and TRP is critical not only for retaining INAD and TRP but also for the signaling complex in the rhabdomeres (Tsunoda *et al*., 2001). Since these findings pertain to *Drosophila*, it is of much interest to know if the situation is true with respect to the localisation of the INAD signaling complex members in *Calliphora*.

The *Calliphora rpa* (receptor potential absent) mutant was reported to express reduced levels of PLCβ, show age-dependent degeneration of the rhabdomeres and a reduced intracellulary recorded impulse response to light stimuli and was thus designated to represent a *Calliphora norpA* mutant (Torkkeli *et al*., 1989, 1991; McKay *et al*., 1994). But analysis of this mutant by confocal microscopy in this thesis reveals that it exhibits the phenotype of an *inaD* null mutant (Fig 20). The northern blots showed that the *rpa* mutant expresses normal levels of *norpA* mRNA but not of *inaD* (Huber *et al*., 2000). This failure in the expression of *inaD* mRNA could be a defect in the *inaD* gene itself or defect in a factor(s) regulating the expression of the *inaD* gene. The analysis of the core components of the INAD signaling complex reveals that due to the absence of the INAD protein, there is a mislocalisation and degradation of TRP and the absence of PLCβ at an early age (Fig 20 & 23). These findings are so far in line with observations reported for the *Drosophila* $inaD^1$ mutant (Tsunoda *et al*., 1997) which indicates that due to the absence of the INAD protein, there is a mislocalisation and degradation of TRP, PLCβ and ePKC. Taken together it suggests that INAD is one of the essential components required in order to localise the key members of the signaling complex to the rhabdomeres.

A similar requirement of binding to a PDZ domain protein for correct subcellular localisation has been reported for the Shaker channel and Fasciclin II which must interact with Discs-large (a PDZ protein) in order to localise to the *Drosophila* neuromuscular junction (Tejedor *et al*., 1997; Thomas *et al*., 1997; Zito *et al*., 1997). Likewise in *C. elegans* also, the PDZ domain proteins LIN-2, LIN-7 and LIN-10 are required to localise the EGFR-like receptor tyrosine kinase LET-23 to the basolateral side of vulval precursor epithelial cells. The absence of these PDZ containing proteins results in a loss of LET-23 dependent signaling and a defect in vulval induction (Simske *et al*., 1996; Kaech *et al*., 1998). Thus PDZ-domain-containing proteins (like INAD in fly photoreceptors) could play an important role in transport, localisation and assembly of supramolecular signaling complexes.

A total absence of PLCβ in *Calliphora rpa* should (as reported for *Drosophila*) result in the abolishment of any photoresponse (Bloomquist *et al*., 1988). Electroretinogram recordings (ERG) from the *rpa* mutant infact showed that it still displays a photoresponse indicating the presence of a small amount of PLCβ (Huber *et al*., 2000). Accordingly, *Calliphora rpa* pupae which were analysed showed that the residual PLCβ is still below the detection level, while most of the TRP localises to the rhabdomeres with a part of it distributing to the base of the rhabdomeres (Fig 21). This result is in line with the observation reported for *Drosophila inaD*^{1} pupae that TRP along with PLCβ and ePKC is localised to the rhabdomeres (Tsunoda *et al*., 2001). The possible explanation for the failure to detect PLCβ labeling in *rpa* pupae could be that its level of expression is below the required amount to be detected by immunocytochemistry. On the other hand, the difference between TRP distribution in *Calliphora rpa* pupae and *Drosophila inaD*^{1} pupae could be due to the defect in the *inaD* gene which has a more severe effect in *Calliphora* at early stage of development. Due to the absence of INAD in *rpa* flies, TRP is not detected in older flies (Fig 22), a similar observation also reported for the *Drosophila inaD*^{1} mutant (Tsunoda *et al*., 1997). Despite the differences in the onset of TRP and PLCβ mislocalisation and degradation, the *Calliphora rpa* mutant can be regarded as an equivalent of the *Drosophila inaD*^{1} mutant because not only INAD, but also the core components of the signaling complex (TRP, PLCβ, and ePKC) are mislocalised or degraded in an age-dependent manner.

Though rhodopsin has been reported to associate with the INAD signaling complex (Chevesich *et al*., 1997; Xu *et al*., 1998), Rh1 (major rhodopsin expressed in R1-6 cells) distributes to the rhabdomeres in pupae, young and old *rpa* flies (Fig 20, 21 & 22). A similar observation has also been reported in *Drosophila inaD*^{1} mutant by Tsunoda *et al*. (1997) which indicates that neither Gqα nor rhodopsin are affected by the absence of INAD. Since the *Calliphora rpa* mutant can be regarded as an equivalent of the *Drosophila inaD*^{1} mutant, further investigations were undertaken to find out if the *rpa* mutant differs in other ways from *Drosophila* mutants.

In *Drosophila trp* mutants, the R1-6 rhabdomeres undergo slow, and progressive retinal degeneration (Cosens and Perry, 1972; Stark and Sapp, 1989) while in *inaD*^{P215}, the rhabdomeres are either missing or reduced in size in flies which are reared under 12 hr light /12 hr dark cycle 25 days after eclosion (Chevesich *et al*., 1997). Earlier findings by Torkkeli *et al*. (1991) revealed that photoreceptor degeneration in the *rpa* mutant is age-dependent. Though they reported that the degeneration of the rhabdomeres is not light-dependent, the present study clearly shows a light-dependent degeneration of the R1-6 cells in approximately 60% of *rpa* flies after 10 days of post-eclosion (Fig 25). The loss of the rhabdomeral structure in observed primarily in Rh1 expressing cells while the R7 cells are less affected (Fig 26C). The possible explanation to the discrepancy with regard to the light-dependent degeneration is that the *chalky*/*rpa* double mutant used in this study is most likely more susceptible to light than the pigmented *rpa* mutant used by Torkkeli *et al*. (1989). A probable reason for the more pronounced degeneration of R1-6 cells compared to R7 cells in the *Calliphora rpa*, the *Drosophila trp* , and the *inaD*^{P215} mutant, may be that the turnover of the rhabdomeral membranes is much higher in the outer photoreceptor cells than in the central photoreceptor cells. This could be true because light stimulation of an invertebrate photoreceptor leads to an increase in intracellular calcium concentration which mediates signal transduction (Walz, 1982; Payne, 1986; Payne *et al*., 1988) and light adaptation (Brown, 1986). In addition, oxidative metabolism (Fein and Tsacopoulos, 1988), and membrane turn-over (Martin and Hafner, 1986) have been speculated to be triggered by changes in intracellular calcium. Hence in the *rpa* mutant, the outer photoreceptor cells may be more surceptible to light-dependent degeneration than the central photoreceptor cells.

Similar observations have also been reported in *Drosophila* mutants where missense mutations in several key components of the signaling cascade (e.g. rhodopsin, the TRP ion channel) and hypomorphic mutations in regulatory molecules (e.g. Arrestin 2, the RdgA diacylglycerol kinase) lead to excessive activation of the phototransduction cascade and this results in a rapid necrotic death of photoreceptor cells (Dolph *et al*., 1993; Bentrop, 1998; Raghu *et al*., 2000; Yoon *et al*., 2000). Therefore, the lack of INAD in *Calliphora rpa* mutant could result in an unregulated signaling activity which could lead to

pathologically elevated levels of intracellular calcium which could be one of the cause for degeneration in the photoreceptor cells.

The analysis of the *rpa* mutant supports the hypothesis that INAD like many other PDZ-scaffold proteins functions as an organiser of membrane-associated complexes along with TRP (Li and Montell, 2000). A similar situation exist at neuronal synapses where it has been reported that the PDZ-scaffold protein PSD-95 localisation to the synapses is dependent on the interaction with ion channels or other membrane proteins (Arnold and Clapham, 1999). Another type of interaction which may be involved is the ankyrin repeats on the N-terminus of TRP. These three ankyrin repeats could be important in linking TRP along with the INAD signaling complex to the cytoskeleton. Alternatively the interaction of INAD-TRP could unmask sites on TRP and INAD which are important for membrane anchoring. As it is reported that approximately 25% of INAD remains in the rhabdomeres of *trp* mutants, the presence of another ligand besides TRP in anchoring the complex to the photoreceptor membrane cannot be excluded. An example for such a case is provided by nonchannel membrane proteins such as CRIPT and neuroligin which bind to PDZ3 of the PSD-95 protein in the central nervous system (CNS) (Irie *et al*., 1997; Niethammer *et al*., 1998).

Similarly in *Drosophila* it has been reported that the PDZ1 of INAD may play a role in anchoring INAD to the membrane (Tsunoda *et al*., 2001). PDZ1 has been proposed to bind an unconventional myosin III which could link INAD to the actin cytoskeleton (Wes *et al*., 1999). But analysis of *ninaC* mutants show that INAD is localised normally to the rhabdomeres (Wes *et al*., 1999) suggesting that besides TRP and NINAC, other proteins associated with the INAD signaling complex could also be involved in anchoring INAD to the membrane.

5.3 Assembly of the INAD signaling complex

How a multimeric protein complex is assembled in fly photoreceptors is an essential question to be answered. The association of INAD and TRP has been shown to be

essential for retaining the signaling complex in the rhabdomeres (Li and Montell, 2000; Tsunoda *et al*., 2001; and this thesis). The fundamental questions remaining to be answered are if signaling components are preassembled and then targeted to the rhabdomeres or if there are alterations to the mechanism i.e., if they might be targeted independently to the rhabdomeres and then assembled into a signaling complex. The former possibility cannot be ruled out since Tsunoda *et al*. (2001) reported that transgenic flies expressing INAD under the control of an inducible heat shock promoter have almost wild type ERG recordings after 7 hours of inducing INAD expression. At that time point, PLCβ and ePKC coimmunoprecipitate with INAD and reach the rhabdomeres. According to Tsunoda *et al*. (2001), INAD along with the other core components are assembled into a complex and then targeted to the rhabdomeres (Fig 49B). However, as shown in *Calliphora rpa* pupae (Fig 21), and *Drosophila inaD*^{1} and *trp*^{343} pupae (Tsunoda *et al*., 2001) there is also evidence for independent targeting of the signaling complex proteins to the rhabdomeres. In these mutants, both INAD and TRP must be targeted independently along with PLCβ and ePKC (Tsunoda *et al*., 2001) to the rhabdomeres. This does not exclude that in a wild type fly a preassembly of the complex is taking place but suggests that in general, the core components of the INAD signaling complex are targeted independently to the rhabdomeres (Fig 49A). The interaction of INAD-TRP could result in the retention and assembly of the complex in the rhabdomeres. In a similar way, Arnold and Clapham (1999) reported that the permissive signal from PSD-95 protein to Kv 1.4 is required for its axonal localisation.

Thus the preassembly of macromolecular complexes is documented in the case of K_{ATP} channels (Zerangue *et al*., 1999) and mammalian T-cell receptor complexes (Klausner *et al*., 1990). Nevertheless, the independent targeting of the signaling components followed by assembly of the signaling complex in the rhabdomeres could be a strategy adopted by photoreceptor cells for organising signaling microdomains.

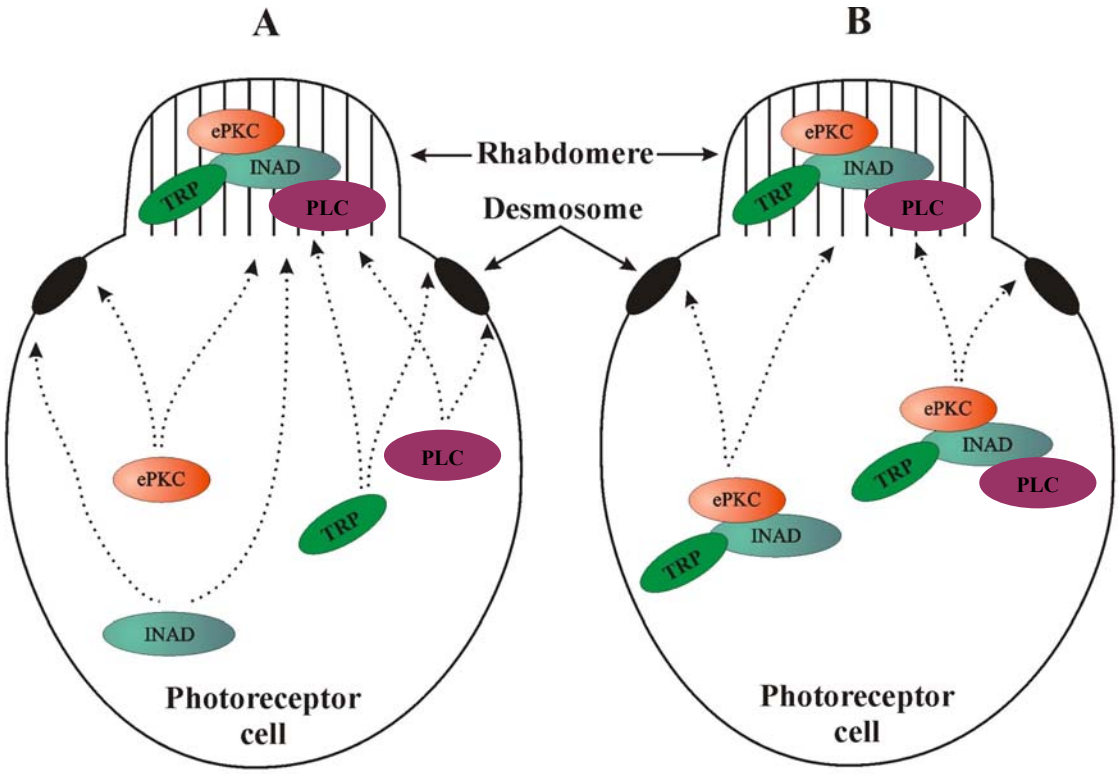

Fig 49: Schematic representation for the organisation of the INAD signaling complex in fly photoreceptors

Shown above are two hypothetical schemes for the assembly of the INAD signaling complex in fly photoreceptors. (A) the core components (INAD, TRP, ePKC, PLCβ) of the signaling complex are targeted independently to the rhabdomere (indicated by broken arrows) and are then assembled into the INAD signaling complex. (B) the ligands comprising the signaling complex are already assembled into the INAD signaling complex in the photoreceptor cell and are then targeted (indicated by broken arrow) as a whole complex to the rhabdomere.

The differences at the cellular level would be that the targeting of the individual members of the INAD signaling complex requires distinct targeting signals for each protein directed to the rhabdomere. Signaling peptides which could be responsible for the targeting process have not been identified. There may be chaperone like proteins which target the individual components to the rhabdomeres because in the *nina*A mutant, Rh1 rhodopsin is not competent for transport and this results in immature opsin accumulation in the endoplasmic reticulum (Ondek *et al*., 1992).

5.4 The functional role of TRP and TRP homologues channels in the visual system of the fly

The present state suggests that the role of the members of the TRP family is rapidly expanding. In *Drosophila* photoreceptor cells, TRP channels are considered to be responsible for the major light-activated conductance. The lack of TRP results in reduced response amplitude (Hardie and Minke, 1992; Niemeyer *et al*., 1996), lack of calcium entry (Hardie *et al*., 1993; Peretz *et al*., 1994), and the inability to sustain a steady-state current during prolonged light stimulation eventually leading to a premature termination of the light response (Cosens & Manning, 1969). Though *trp* mutants still display a response to light stimuli, *trpl*:*trp* double mutants are completely blind (Niemeyer *et al*., 1996). A residual conductance (Niemeyer *et al*., 1996) originally observed in *trpl*:*trp* double mutant can be attributed to some left over TRP channels present in this particular TRP allele used (*trpl*:*trp*^{CM}) (Reuss *et al*., 1997). A third ion channel, TRPγ may coassemble with TRPL to produce a channel that can be activated through stimulation of PLCβ (Xu *et al*., 2000). Since TRP, TRPL and TRPL-TRPγ have been proposed to constitute the light-activated conductance, the role of TRPL in visual transduction is vexing because *trpl* mutants do not display any overt phenotype. On one hand TRPL may represent true 'redundancy' while on the other, TRPL may have a specialised role that needs to be unraveled. Several studies have attempted to show that TRP and TRPL channels function as store-operated channels (SOCs), though the mechanism which gates these channels is still unknown. One hypothesis put forward is that TRPL channels may have a role in sustaining the photoreceptor response during prolonged illuminations and in adaptation to dim light stimuli (Leung *et al*., 2000) since TRPL channels are localised to the rhabdomeres in wild type flies and in mutants where there is a disruption of the signaling complexes in the rhabdomeres (e.g. *ninaE*, *norpA*, and *trp*) (Niemeyer *et al*., 1996).

Controversies continue to persist whether TRPL is a member of the INAD signaling complex. An argument against TRPL protein being a member of the signaling complex is that it is localised to the rhabdomeres in *inaD*^{1} mutants (Tsunoda *et al*., 1997), a finding

which indicates that the binding of TRPL to the signaling complex is not a prerequisite for its correct localisation. However Xu *et al*. (1998) provided biochemical evidence that both TRPL and Rh1 bind to INAD. As this result could not reproduced by other laboratories, an explanation put forth was that TRPL may interact transiently with INAD (Xu *et al*., 1998). The association of TRPL with INAD maybe regulated by light-dependent calcium influx or the phosphorylation of INAD by ePKC which could induce conformational changes in INAD, thereby altering the INAD-TRPL interaction (Montell, 1998). Cohen *et al*. (1996) showed that the Kir 2.3 channel associates physiologically with the PDZ-domain protein, PSD-95, and that the phosphorylation of Kir 2.3 at Ser-440 by protein kinase A (PKA) disrupts this interaction. The binding of Ca^{2+}/CAM to INAD is another possible mechanism that may influence the binding of INAD ligands (Xu *et al*., 1998). Alternatively, light-regulated phosphorylation of TRPL could regulate the binding to INAD, and its distribution within the photoreceptors.

A viewpoint largely neglected and less investigated till date is if the ion channel composition within the rhabdomeres is constant or subject to light-induced changes. The investigations carried out in this thesis surprisingly reveal that TRPL distribution within the photoreceptors of *Drosophila* and *Calliphora* is subjected to light-regulation, though TRP and INAD localisation is unaffected by light (Fig 27 & 29). This observation is also seen in newly eclosed and older flies (Fig 28 & 30), indicating that the light-regulated distribution of TRPL is independent of the age of the fly. Biochemical studies by Bähner (2001) showed that in isolated rhabdomeral membranes from *Calliphora* there is a dramatic difference of TRPL amounts between light and dark kept flies whereas the other components of the INAD signaling complex, namely TRP, PLCβ, ePKC, and INAD are unaffected. Accordingly it can be assumed that TRPL molecules are internalised in the light to a storage compartment within the photoreceptor cell and redistributed to the rhabdomeres in the dark (Fig 31) (Bähner *et al*., 2002). Electroretinogram (ERG) recordings performed on dark kept wild type *Drosophila* showed that the largest response amplitude is obtained with no background light while smaller responses are observed when increasing the background illumination (Bähner *et al*., 2002). With relation to this observation, wild type *Drosophila* which are kept in light for 12 hours and then dark kept

for 5 minutes show that the V-log I curves obtained without and under very dim background illumination superimpose each other (Bähner *et al*., 2002) which has also been described for *trpl* mutants (Leung *et al*., 2000). Taken together, the TRPL channels which are distributed in the dark to the rhabdomeres could contribute to the adaptation of the photoreceptors at dim background illumination as postulated by Leung *et al*. (2000). The light-modulated mechanism for the distribution of TRPL channels may be adopted by the photoreceptor cells as a control for the number of TRPL channels which can be activated by rhodopsin in the rhabdomeres which inturn could be a key feature to regulate photosensitivity of the photoreceptor cell.

5.5 Possible mechanisms for triggering TRPL distribution in fly photoreceptors

The distribution of TRPL channels in fly photoreceptors may be subjected to either vesicle transport along with other phototransduction proteins in the photoreceptors or light-modulated localisation which is dependent on the phototransduction cascade. But in this thesis, the influence of the phototransduction cascade on light-regulated TRPL distribution has been studied. As reported above (refer 5.4), TRPL distribution in fly photoreceptors depends on the light condition under which the flies were kept. The activation of rhodopsin by a photon of light results in the opening of ion channels TRP and TRPL which are associated with the phototransduction cascade and constitute the light-activated conductance. Based on the fact that TRPL channels are associated with the visual cascade, it is reasonable to put forth the following questions: **(1)** is the light-regulated distribution of TRPL triggered by rhodopsin activation, **(2)** does TRPL localisation depend on the activation of the phototransduction cascade downstream of rhodopsin, **(3)** which members of the phototransduction cascade are mandatory for light-regulated TRPL distribution. The answer to these questions will be discussed below with respect to the possible mechanism that could be responsible for triggering light-modulated TRPL distribution.

Fly rhodopsins are activated by absorption of light by the covalently bound chromophore, 11-cis-3-OH retinal (Vogt and Kirschfeld, 1984), which results in the

conversion of rhodopsin to the active metarhodopsin inturn activating the heterotrimeric G-protein. Fly photoreceptors express different rhodopsins which can be activated and deactivated by different wavelengths of light. The investigations undertaken in this thesis in order to explore the possible influence of rhodopsin activation on TRPL localisation shows that in green light illumination, TRPL localises to the rhabdomeres in P (Rh1 + 3) (transgenic flies expressing Rh3 instead of Rh1 in R1-6 cells) but not in wild type *Drosophila* (green light is absorbed by Rh1 rhodopsin) where TRPL is distributed to the cell bodies (Fig 35). Previous studies have reported that Rh1 is maximally excited at 486nm while Rh3 at 331nm (Salcedo *et al*., 1999). Thus it seems that the lack of activation of Rh3 under green light illumination results in a lack of TRPL distribution to the cell body in P (Rh1 + 3) while the activation of Rh1 in wild type triggers the mechanism responsible for localising TRPL to the cell bodies (refer fig 32). Besides this, the expression level of Rh3 rhodopsin in P (Rh1 + 3) is much less (63fmol/retina) as compared to 219 fmol/retina Rh1 in wild type *Drosophila* (Henrich, 1999). Therefore the influence of Rh3 rhodopsin on TRPL distribution in R1-6 cells could be different in relation to Rh1 influence in wild type flies exposed to blue and green light.

Though the present evidence suggests that rhodopsin activation influences TRPL distribution (Fig 35), the result obtained after green and orange light illumination of wild type flies shows that these light conditions may also have unspecific effects on cellular targeting processes (Fig 35 & 36). This is critical since the rhodopsin content of photoreceptor membranes can be down regulated by specifically depleting the 11-cis retinal chromophore by exposing flies to green light (Schwemer, 1984). Huber *et al*. (1994) reported that the chromophore is required for proper folding and processing of opsin and its targeting to the rhabdomeral photoreceptor membranes. It is likely that TRP and INAD are transported along with rhodopsin via the Golgi apparatus in the same vesicle as rhodopsin to the rhabdomeres. This assumption would explain the observations shown in fig 35 & 36 that TRP and INAD are mistargeted as they are unable to reach the rhabdomeres in wild type flies exposed to green and orange light. In support to this, wild type flies which are kept in the dark for 2-6 hrs after 12 hours orange light illumination show that both TRP and INAD along with TRPL localise to the rhabdomeres (Fig 38 &

39). Biochemical analysis of wild type *Calliphora* kept in the dark for 1-4 hours after white light illumination also showed increased TRPL protein amount in the rhabdomeres similar to a level as in dark raised flies (Bähner, 2001). However, P (Rh1 + 3) flies which show a downregulation in TRPL content in some rhabdomeres after orange light exposure (Fig 36) show no difference in the level of TRPL in the rhabdomeres even after 6 hours of dark adaptation (Fig 38). Therefore it is essential to know if there is any involvement of the post-translational modification of rhodopsin with light-regulated TRPL expression and distribution in the photoreceptor cells.

The initial step in the deactivation of the receptor molecule, rhodopsin, is mediated by arrestin binding (Bentrop *et al*., 1993) followed by its phosphorylation (Byk *et al*., 1993), by a rhodopsin kinase not yet identified on its carboxy-terminal at serine and threonine residues. The phosphorylation of rhodopsin may be the requirement for its internalisation by which "used" receptor molecules are removed from the cell membrane (Sapp *et al*., 1991). In the transformant fly P (Rh1 Δ Phos.) in which the phosphorylation sites of the Rh1 rhodopsin are eliminated, the distribution of TRPL is independent of the post-translational modification of rhodopsin by its phosphorylation (Fig 41). Therefore, if phosphorylation of rhodopsin is a prerequisite for its internalisation, it can be assumed that TRPL distribution is independent of receptor phosphorylation and its subsequent internalisation. Though rhodopsin activation has been observed to influence the light-regulated TRPL distribution, it is not clear if the phototransduction cascade downstream of rhodopsin or a part of it is involved in TRPL localisation.

To investigate if a phosphorylation of the downstream members of the phototransduction cascade has an effect on TRPL distribution, *Drosophila* ePKC mutants were analysed. This protein kinase is a key component of the INAD signaling complex and appears to catalyse the phosphorylation of TRP as well as INAD (Huber *et al*., 1996b, 1998; Liu *et al*., 2000). *Drosophila* ePKC mutants show a similar phenotype as *inaD* null mutants which is characterised by a slow deactivation of the light-induced current (Smith *et al*., 1991; Hardie *et al*., 1993; Shieh *et al*., 1995) which indicates the possibility that the functional interaction of ePKC and INAD is essential for normal visual signaling (Adamski

et al., 1998). Furthermore, ePKC is required for Ca^{2+}-mediated feedback regulation in which photoreceptor cells adjust their sensitivities over a wide range of light intensities (Hardie *et al*., 1993). It has been reported that the transient association of TRPL with INAD could result from phosphorylation of INAD which induces conformational changes in INAD to bind with TRPL (Xu *et al*., 1998). Indications are that the targeting of ion channels and enzymes to discrete plasma membrane sites is often regulated by protein phosphorylation (Cohen *et al*., 1996). The localisation of TRPL is still light dependent in *Drosophila* ePKC mutant (Fig 44) but apparently unaffected by phophorylation of the adjacent components of the INAD signaling complex. In addition, it has been proposed that the phosphorylation of TRP may affect the kinetics of this calcium channel (Huber *et al*., 1998). Even if the lack of ePKC mediated phosphorylation of TRP results in a abnormal deactivation similar to that observed in the *Drosophila inaD*^{P215} mutant the light-modulated TRPL distribution in the photoreceptors is independent of the phosphorylation of the TRP channel.

Since the light-regulated TRPL distribution is independent of the presence of one of the signaling complex members operating downstream of the phototransduction cascade, investigations were focussed on the upstream components. Fig 42 reveals that TRPL distributes to the cell bodies in light exposed *Drosophila norpA* mutants which show no electrophysiological response to light stimuli (Pearn *et al*., 1996). Therefore, it is appropriate to assume that the light-modulated mechanism responsible for TRPL distribution is independent of the activation of the visual cascade members downstream of PLCβ . In addition, the distribution of TRPL in photoreceptor cells due to the influence of Ca^{2+} influx from TRP channels can also be excluded. An example for translocation of a channel is provided by neuroendocrine cells stimulated by the neuropeptide head activator (HA). The stimulation results in the translocation of growth-factor-regulated channel (GRC) to the cell surface. Though it is postulated that the Ca^{2+} influx through this channel is triggered by HA, HA does not bind directly to GRC but requires the presence of a signaling receptor and respective messengers (Boels *et al*., 2001). It is likely that a similar mechanism operates with respect to proteins which are expressed in the photoreceptor cell and which are independent of the activation of the phototransduction cascade downstream

of PLCβ but could be the likely candidates for triggering light-regulated TRPL distribution.

The possible pathway responsible for influencing TRPL distribution could be for example either through the visual Gqβγ subunits or through other G-proteins which are not associated with the phototransduction cascade but may be activated by rhodopsin. Several invertebrate G-protein subunits that may participate in phototransduction have been identified (Running Deer *et al*., 1995). Two photoreceptor-specific G-proteins α isoforms, DGq1 and DGq2 (Lee *et al*., 1990) and a photoreceptor-specific Gβ (Yarfitz *et al*., 1991) have been cloned from *Drosophila*. Gqα of DGq1 has been shown to be involved in the activation of PLCβ (Bähner *et al*., 2000) while the β subunit is essential in terminating the photoresponse in *Drosophila* photoreceptors (Dolph *et al*., 1994). Despite the cloning of the visual Gβe subunit (Yarfitz *et al*., 1991) and the characterisation of Gβe mutants (Dolph *et al*., 1994), the precise role of the βγ complex in *Drosophila* phototransduction is not yet resolved. Overexpression of a mutated visual Gγ in *Drosophila* photoreceptors has no effect on TRPL distribution (Fig 43), though it has been shown to affect the membrane association of the Gqβ subunit (Schulz, 2001). Therefore either the visual Gqβγ subunit itself or other G-protein subunits not associated with the visual cascade but which may interact with rhodopsin could be involved with the pathway(s) triggering TRPL distribution in fly photoreceptors (refer fig 50).

The light-regulated TRPL distribution in fly photoreceptors which is independent of the activation of the members downstream PLCβ could be either influenced by the composition or the presence/absence of one of the ion channels. At present, the exact composition of the light-activated ion channels in fly photoreceptors is unclear but several observations suggest that TRPL and TRPγ function as subunits of heteromultimeric channels (Xu *et al*., 1997, 2000). TRP in contrast to TRPL and TRPγ may predominantly form homomultimers *in vivo*. In addition, TRP-TRPL heteromultimers may also exist (Xu *et al*., 1997). Consistent with this, TRPL has been reported to interact with TRP in several

in vitro assays (Xu *et al*., 1997). It is likely that wild type flies express TRP homomultimers, TRP/TRPL heteromultimers and TRPL/TRPγ heteromultimers.

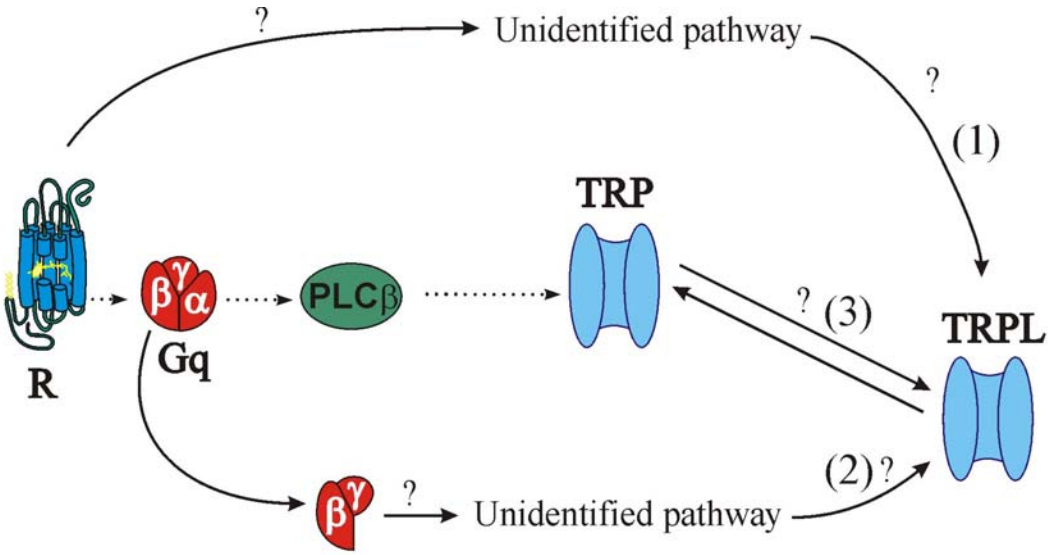

Fig 50: Hypothetical scheme displaying possible mechanisms responsible for triggering light-regulated TRPL internalisation in fly photoreceptor cells

Broken arrows indicates the phototransduction cascade in fly photoreceptors. Solid arrows indicate the possible pathways that could be responsible for triggering TRPL distribution within the photoreceptor cells. In pathway (1) rhodopsin activation could trigger TRPL internalisation by a pathway which is independent of the visual G-protein, (2) the βγ subunit from the activated Gq protein mediates internalisatiion of TRPL and, (3) indicates the interaction between TRP and TRPLwhich may be required for TRPL distribution in the photoreceptors.

In vitro assays show that TRPL homomultimers stay constitutively active hence, the mechanism regulating TRPL in *trp* mutant is vexing. Investigations in this thesis reveals that in trp^{343} mutant, TRPL no longer distributes to the cell bodies in light kept flies but rather localises to the rhabdomeres (Fig 45), a observation which is different to that observed in light kept wild type flies. A recent study has shown that TRPL is required for non-INAD-bound TRP to contribute to photoreceptor responses (Leung *et al*., 2000). Therefore it can be assumed that TRP and TRPL could heteromultimerise because TRP is at least 10-fold more abundant than TRPL in photoreceptor cells (Xu *et al*., 1997). These heteromultimers could be subjected to light-regulation and distributed to the cell bodies and rhabdomeres. It is likely that the trigger mechanism originating from the TRP channels

that form homomultimeres influences the localisation of TRP/TRPL heteromultimeric channels to the cell bodies and rhabdomeres. In *trp* mutants it is reported that the major ligands of the INAD signaling complex are either degraded or mislocalised in an age-dependent manner (Tsunoda *et al*., 2001). Therefore, it is important to know if the light-regulated distribution of TRPL in *Drosophila trp* mutant photoreceptors is due to the mislocalisation or degradation of the ligands comprising the INAD signaling complex. Several factors may be responsible for influencing TRPL distribution, some of which could be **(1)** mislocalisation / degradation of the ligands of the INAD signaling complex, **(2)** the absence of INAD itself, **(3)** the absence of TRP in the rhabdomeres / photoreceptor cells and, **(4)** the failure to form a signaling complex.

It is speculative to assume that the binding of the ligands to the INAD signaling complex could influence the distribution of the TRPL channel within the photoreceptors. But the analysis of young *Drosophila inaD*^{1} and *Calliphora rpa* flies (Fig 46 & 48) shows that the localisation of TRPL is independent of **a)** the absence of the scaffolding protein, INAD, **b)** the organisation of the INAD signaling complex and, **c)** the localisation of the core components of the signaling complex to the rhabdomeres. This observation indicates that though TRPL has been postulated to bind to the signaling complex (Xu *et al*., 1998), INAD by itself is not required for TRPL distribution within the photoreceptor cells. By analysing old *Drosophila inaD*^{1} and *Calliphora rpa* flies it has to be concluded that the degradation of the major ligands influences TRPL distribution. In these flies, TRPL no longer distributes to the cell bodies in light kept flies but is located in the rhabdomeres (Fig 47 & 48). As seen (Fig 22), and reported by Tsunoda *et al*. (1997) TRP is absent while residual PLCβ and ePKC is detected in old *Calliphora rpa* and *Drosophila inaD*^{1} mutants. Since TRP is not detected in the rhabdomeres of old *Drosophila inaD*^{1} mutants, it indicates that it is the location of TRP that influences TRPL distribution rather than its total absence in the photoreceptors.

The light-modulated distribution of TRPL could be also subjected to circadian entrainment within the photoreceptor cells. Recently Claridge-Chang *et al*. (2001) reported that the TRPL transcript is subjected to oscillations which depend on the circadian

rhythum. But biochemical analysis by Bähner (2001) showed that TRPL and TRP protein content is not subject to circadian rhythum, besides, the amount of *trpl*, *trp*, *inaC* and *inaD* mRNA is not altered by light-regulation up to 6 hours of light/dark condition (Bähner *et al*., 2002). *Trpl*:*trp* double mutants have been reported to show less TIM (*tim* gene is required for circadian behavioural rhythms in *Drosophila*) degradation in response to light pulses (Yang *et al*., 1998) which according to Claridge-Chang *et al*. (2001) indicates that oscillating TRPL function is connected with circadian entrainment. Since the TIM response to the light pulse is not completely abolished in *trp*, *trpl* and *trpl*:*trp* mutants it supports the hypothesis that circadian entrainment does not only rely on the visual system but also involves another dedicated pathway for photoreception (Yang *et al*., 1998). Earlier observations have also indicated that *Drosophila* does not display daily rhythums in photoreceptor protein biosynthesis or membrane shedding (Stark *et al*., 1988; Sapp *et al*., 1991; Chen *et al*., 1992) nor in cycling of either mRNA or protein of five major phototransduction components (Hartman *et al*., 2001). Therefore it can be postulated that though *trpl* mRNA may be subject to circadian entrainment (Claridge-Chang *et al*., 2001), the light-regulated TRPL distribution in fly photoreceptor cells is if at all little influenced by circadian rhythums.

Taken together, light-modulated TRPL distribution in fly photoreceptors seems to be subjected to rhodopsin activation but independent of the visual cascade activation downstream of PLCβ. The disruption of the membrane association between the β and γ subunits of the visual G-protein has a less pronounced effect on TRPL distribution but the total absence of Gqβγ subunits itself in influencing TRPL localisation cannot be excluded. In addition, other unidentified G-proteins expressed in the fly eye and activated by rhodopsin could also play a major role in influencing TRPL localisation. Since TRPL distribution is independent of the correct localisation of the components of the signaling complex as well as of the phosphorylation of INAD, and TRP, the absence of TRP in the rhabdomeres apparently influences TRPL distribution in the photoreceptor cell. Therefore the finding of a light-regulated TRPL distribution points to the existence of new pathways which are involved in protein targeting independent of the phototransduction cascade but

dependent on the presence of some of the members associated with the visual system. This would be essential in order to find out the importance of certain unaccounted proteins whose functional presence in fly photoreceptors has not yet been revealed.

6 References

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7 List of Abbreviations

CLSM	Confocal Laser Scanning Microscope
Cv	*Calliphora vicina*
DAG	Diacylglycerol
Dm	*Drosophila melanogaster*
Dm wt	*Drosophila* wild type
ePKC	eyespecific Protein kinase C
Gqα	α-subunit of the visual G-protein
Gqβγ	βγ-subunit of the visual G-protein
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
hrs	Hours
IgG	Immunoglobulin
inaC	inactivation no afterpotential C
inaD, INAD	inactivation no afterpotential D
IP_3	Inositol-1,4,5-trisphosphate
kDa	Kilodalton
msec	millisecond
mA	milliampere
mM	millimolar
nm	nanometre
NBT	Nitroblue Tetrazolium Chloride
ninaE	neither inactivation nor afterpotential E
norpA	no receptor potential A
PDZ	PSD-95, DLG, ZO-1
PIP_2	Phosphatidylinositol-4,5-bisphosphate
PLCβ	Phospholipase Cβ
PUFA	Polyunsaturated Fatty Acid
PVDF	Polyvinylidenedifluoride
rdgC	retinal degeneration C

R1-6	Photoreceptor cells R1 to R6 in the dipteran compound eye
R7	Photoreceptor cell R7 in the dipteran compound eye
R8	Photoreceptor cell R8 in the dipteran compound eye
Rh1	Rhodopsin 1
Rh2	Rhodopsin 2
Rh3	Rhodopsin 3
Rh4	Rhodopsin 4
Rh5	Rhodopsin 5
Rh6	Rhodopsin 6
rpa	*receptor potential absent*
SDS	Sodium-Dodecylsulfate
SDS-PAGE	SDS-Polyacrylamide-Gelelectrophoresis
trp, TRP	transient receptor potential
trpl, TRPL	transient receptor potential-like
TRPγ	transient receptor potential γ
v/v	volume per volume
WGA	Wheat Germ Agglutinin
WGA-TMR	Wheat Germ Agglutinin coupled to Tetramethylrhodamine
WGA-OG	Wheat Germ Agglutinin coupled to Oregon Green
w/v	weight per volume
wt	Wild type