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Research report

Opsin expression in adult, developing, and regenerating newt retinas

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Abstract

Japanese common newts (*Cynops pyrrhogaster*) have an ability to regenerate their neural retina even as adults. Although extensive research has been carried out attempting to understand this retinal regeneration, the molecules characterized in newt retina are limited. We isolated cDNAs encoding three putative opsins (Cp-Rh, -LWS and -SWS1), in addition to Cp-SWS2 [Takahashi et al., FEBS Lett. 501 (2001) 151–155] from a cDNA library of adult newt retina. Our immunohistochemical and in situ hybridization studies demonstrated that Cp-Rh is selectively expressed in rods, whereas the other opsins are expressed in cones. The distribution of opsin mRNAs in normal and regenerated retinas is very similar. In both developing and regenerating retinas, Cp-Rh and its mRNA first appeared in immature rods at the beginning or just after the formation of plexiform layers. Cp-Rh was initially found isotropically in the plasma membrane, and then translocalized to the apical region along with the maturation of regenerating rods. This suggests that reorganization of the intracellular structure takes place during maturation of the regenerating newt photoreceptors. © 2002 Elsevier Science B.V. All rights reserved.

Theme: Sensory systems

Topic: Retina and photoreceptors

Keywords: In situ hybridization; Regeneration; Retina

1. Introduction

A vertebrate retina is an organ belonging to the central nerve system (CNS) and it is usually difficult to regenerate except at the embryonic stage in life. However, certain species of urodele amphibians, such as newts and salamanders, possess the ability to regenerate a functional retina from retinal pigment epithelial (RPE) cells even as adults [8,16,17,30]. After surgical removal of neural retinas from adult newt eyes, the remaining RPE cells lose their pigment granules (depigmentation), transdifferentiate into retinal progenitor cells, which further differentiate into various retinal neurons, and then finally reform a functional neural network.

It is expected that knowledge of the mechanism of such retinal regeneration would be applicable to other tissues of the CNS. Vigorous studies using molecular biological approaches have been carried out to investigate the development mechanisms. However, the molecules so far characterized in newt retina are limited [13,18,30]. Even for visual pigments, photoreceptive molecules crucial for newt vision, there are only a few reports showing the expression of putative newt rhodopsin during regeneration [3,21,22].

Rods and cones are the two types of photoreceptor cells in vertebrate retinas. Light activates visual pigments that consist of a protein moiety (opsin) and a chromophore (11-*cis*-retinal or its derivatives). Based on an analysis of their primary structures, vertebrate opsins are thought to be derived from an ancestral opsin, and have diverged into five fundamental groups; rhodopsin, a rod-specific opsin, is classified into group Rh, and other cone opsins are classified into groups LWS, Rh2, SWS2 or SWS1 [10,25,35]. It has been suggested that there is a relationship between these groups and their characteristic absorption maxima (λ_{max}) [10].

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In the present study, we isolated rod and cone opsin genes, and investigated the distribution of these opsins in normal and regenerated adult newt retinas. In addition to Cp-SWS2 (*Cynops pyrrhogaster* SWS2 group opsin) we have previously reported [32] that newts possess three kinds of opsins (Cp-Rh, Cp-LWS and Cp-SWS1) closely related to rhodopsin and the long and short wavelength-sensitive opsins of other vertebrates. All these opsins are similarly expressed in both normal and regenerated newt retinas. Furthermore, our in situ hybridization and immunohistochemical studies suggest that translocalization of Cp-Rh and its mRNA takes place in immature regenerating rods.

2. Materials and methods

2.1. Purification of newt retinal mRNA and amplification of opsin cDNAs

Adult newts (*Cynops pyrrhogaster*) were purchased from a local supplier and handled according to the Guidelines for Animal Experimentation of Osaka University. Retinas were surgically removed from newts anesthetized with 0.1% FA 100 (Tanabe Pharmacy, Osaka, Japan) and homogenized in Isogen (Nippon Gene, Tokyo, Japan). Total RNA was isolated according to the manufacturer's protocol and mRNA was purified using a Quick Prep Micro mRNA purification kit (Amersham Pharmacia Biotech).

Single-stranded cDNA was synthesized using Superscript II (Gibco) and oligo dT-primer as a primer (Pharmacia). The cDNA fragments encoding putative newt opsins were amplified with degenerate primers, VVP-F1 and VVP-R2 or SWS-F1 and VVP-R2, corresponding to the highly conserved amino acid sequences of vertebrate opsins [10,32]. Forty-five cycles of polymerase chain reaction (PCR) were carried out at 94 °C (1 min), 42 °C (1 min) and 72 °C (1 min).

2.2. Construction of retinal cDNA libraries and screenings

Construction of the retinal cDNA library was carried out as described by Hisatomi et al. [9]. Briefly, double stranded cDNA was ligated with an EcoRI–NotI adapter, inserted into the EcoRI site of Lambda–ZAPII (Stratagene), and packaged with Giga-Pack III gold (Stratagene).

The cDNA library was screened using five kinds of ^{32}P-labeled probes; three of them were amplified fragments of newt opsin cDNAs and the rest were cDNA fragments (dg1 and dg2) of gecko (*Phelsuma madagascariensis longinsulae*) opsins [33]. Plaque hybridization was performed at 55 °C with hybridization buffer containing 50 or 40% formamide [15]. After picking up a single plaque, the positive clones were transformed into plasmids by an ExAssist-SOLR system (Stratagene) and sequenced.

2.3. Preparation of developing, normal, and regenerating retinas and their frozen sections

For retinal regeneration, neural retina and lens were surgically removed from the anesthetized adult newts as described previously [14], and eyeballs were enucleated under anesthesia at 2–12 weeks after operation. The eyecups of normal (adult intact), regenerating, and regenerated newt retinas were fixed with 4% paraformaldehyde for an hour and then frozen in a 20% sucrose in 0.02 M PBS–OCT compound (2:1) [1]. Cryosections (8 μm) were dried at 65 °C for more than 2 h.

For retinal development, gravid female newts were collected in late autumn and kept in polyethylene containers at 10 °C under a natural day–night cycle. Fertilized eggs were obtained after artificial induction of spawning by injection of human chorionic gonadotropin (Gonatropin, Teikokuzouki R-3000, Tokyo, Japan) as described previously [28]. Eggs were collected and kept at about 25 °C until the embryos reached appropriate stages. Cryosections of the eggs or larvae were prepared as described previously [6].

2.4. Stages of developing and regenerating retinas

Regenerating retinas were divided into three stages (early, intermediate, and late) based on their morphological appearance, and intermediate-regenerating retina was further divided into three substages, I, II and III as described by Cheon and Saito [6]. The embryonic stages were characterized according to criteria proposed by Ichikawa and Kajishima [12].

2.5. In situ hybridization and immunohistochemistry

PCRs were carried out with VVP-F5 and VVP-R2 primers [9,10], using newt opsin cDNAs as templates. In situ hybridization was carried out as described previously [2,9,27,33]. Briefly, the amplified cDNA fragments were subcloned into pGEM3Zf and linearized by EcoRI digestion. Digoxigenin-labeled cRNA probes were synthesized with SP6 RNA polymerase. Hybridization was performed at 65 °C for 16 h in the hybridization buffer. Positive signals for opsin mRNAs were detected by using alkaline phosphatase-conjugated antidigoxygenin antibodies, and HNPP/Fast Red detection kit (Boehringer Mannheim) for fluorescent detection or NBT/BCIP kit (Boehringer Mannheim) for nonfluorescent detection.

For immunohistochemical analysis, frozen sections were blocked with 0.02 M PBS containing 3% bovine serum albumin (BSA) and 0.1% Triton X-100 for 30 min, and incubated with the antibovine rhodopsin monoclonal antibody (Rh29) [34] for 2 h at room temperature. After

washing several times with 0.02 M PBS, sections were reacted with FITC-conjugated antimouse IgG (Jackson) for 30 min at room temperature, and counter-stained with Evans Blue. The morphology of the cells was examined and photographed under either a light microscope (Olympus BX50) or a confocal laser microscopy (Olympus Fluo-view).

3. Results

3.1. Isolation and the sequences of newt opsin cDNAs

Two kinds of cDNA fragments encoding the putative opsins were amplified from a newt (*Cynops pyrrhogaster*) retinal cDNA pool. We named these cDNAs Cp-Rh cDNA and Cp-SWS1 cDNA because their deduced amino acid sequences can be classified into vertebrate opsin groups, Rh (rhodopsin) and SWS1 (short wavelength-sensitive 1), respectively. Clones containing the complete coding regions of Cp-Rh and Cp-SWS1 were isolated by screenings of cDNA libraries using the amplified cDNA fragments as probes. A third cDNA encoding the newt group, LWS (long wavelength-sensitive) opsin (Cp-LWS), was isolated by a screening of the library using group LWS opsin (dg1) cDNA of a diurnal gecko, *P.m. longinsulae* as a probe [33].

Fig. 1 shows the deduced amino acid sequences of the newt opsins. The entire amino acid sequence of Cp-Rh is very similar to rhodopsins of other vertebrates (more than 85% amino acids are identical). The deduced amino acid sequences of Cp-LWS and Cp-SWS1 appear to be most closely related to tiger salamander red-sensitive cone opsin (92.6% identity) and ultraviolet-sensitive cone opsins (93.0% identity), respectively [20,36]. We also found in the library a group, SWS2 opsin (Cp-SWS2) cDNA [32], which is very similar to green rod opsin of the bullfrog (Fig. 2). Each opsin has amino acids critical for its function. However, the cDNA encoding group Rh2 opsin was not found in this newt library, even though the group Rh2 opsin (dg2) cDNA of the diurnal gecko was used as a probe [33].

```
Cp-Rh   [AB043890]                       MNGTE   10
Cp-LWS  [AB043891]     MAYSWNSGAYAARRRYDDED   25
Cp-SWS1 [AB052889]

  GTNFYVPFSNKTGVVRNPFEYPQYYLAEPWKFSALAAYMF   45
  TTRSSVFVYTNSNNTRGPFEGPNYHIAPRWVYNLTTLWMF   60
  MVGDDDFYLFKNISKVGPWDGPQYHIAPAWTFYFQTAFMG   40

  LLILLGFPVNFLTLYVTIQHKKLRTPLNYILLNLAFANHF   85
  FVVAASVFTNGLVLVATMKFKKLRHPLNWILVNLAIADIA  100
  FVFFVGTPLNAIVLIVTVKYKKLRQPLNYILVNVSLAGFT   80
                     VVP-F5 ——►
  MVFGGFPVTMYSSMNGYFVFGVTGCNIEGFFATLGGEIGL  125
  ETLIASTISVINQIFGYFILGHPLCVIEGYTVSVCGITGL  140
  FCIFSVFSVFVASSQGYFIFGKFMCEMEAFLGSVSGLVTG  120

  WSLVVLAIERYVVVCKPMSNFRFGENHAIMGVMFTWIMAL  165
  WSLTIIAWERWFVVCKPFGNIKFDGKLAAAGIIFSWAWAA  180
  WSLAFLAIERYIVICKPMGNFRFASKHALMIVLTTWVIGF  160

  ACAAPPLFGWSRYIPEGMQCSCGVDYYTLKPEVNNESFVI  205
  FWCAPPIFGWSRYWPHGLKTSCGPDVFSGNSDPGVQSFMI  220
  SVSIGPLVGWSRYIPEGLQCSCGPDWYTVGTKYNSETYTW  200

  YMFVVHFLIPISIISFCYGRLVCTVKEAAAQQQESATTQK  245
  TLMSTCCILPLSIIILCYVQVWWAIRQVAMQQKESESTQK  260
  FLFIFCFIIPLSLICFCYSQLLGALRAVAAQQQESATTQK  240
                                  VVP-F1 —
  AEREVTRMVIIMVVAFLICWVPYASVAFYIFCNQGSDFGP  285
  AEREVSRMVVVMIMAYIFCWGPYTFFVCFAAANPGYSFHP  300
  AEREVTRMVIVMVASFCVCYVPYAAMAMYMVNNRNHGLDL  280
  ——►
  VFMTVPAFFAKSSSIYNPVIYIVLNRQFRNCMITTLCCGK  325
  LAASLPAYFAKSATIYNPIIYVFMNRQFRNCIYQLF--GK  338
  RLVTIPAFFSKSACVYNPIIYSFMNKQFRACIMETV-CGT  318
              ◄——VVP-R2
  NPFGEDETTSAATSKTEASSVSSSQVSPA  354
  KVDDGSELS--SSSRTEVSSVSNSSVSPA  365
  PITDESDVS-TSSNKTEVSSVSSSQVSPN  347
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Fig. 1. Deduced amino acid sequences of newt opsins. Each arrow shows the amino acid sequence corresponding to the degenerate primers, VVP-F5′, VVP-F1′ and VVP-R2′. The nucleotide sequence data have been deposited to the EMBL nucleotide sequence databases with accession nos. AB043890 (Cp-Rh), AB043891 (Cp-LWS) and AB052889 (Cp-SWS1).

3.2. In situ hybridization and immunohistochemistry of normal retina

The sequence analysis indicated that Cp-Rh was quite similar to rhodopsins of other vertebrates. Our in situ hybridization study showed Cp-Rh mRNA localized in rod inner segments, but not in cone inner segments (Fig. 3a). We therefore concluded that Cp-Rh was newt rhodopsin. Our previous observation that Rh29 monoclonal antibody raised against bovine rhodopsin selectively recognizes rod outer segments of newt [11] strongly suggests that Rh29 would recognize Cp-Rh (newt rhodopsin) in newt retina (Fig. 3b).

Fig. 3c and d shows the localization of Cp-LWS and Cp-SWS1 mRNAs, respectively. Each opsin mRNA was found in the myoid regions and inner segments of a subpopulation of cone cells. Each cone cell, which we investigated by two-color fluorescent in situ hybridization, expressed a single opsin (data not shown). This suggested that each of these opsins was fundamentally expressed in a certain subclass of cones.

3.3. In situ hybridization of regenerated retina

About 3 months after the surgical removal of the original retinas, newts began to show behavioral reactions against objects moving in front of their eyes, that was

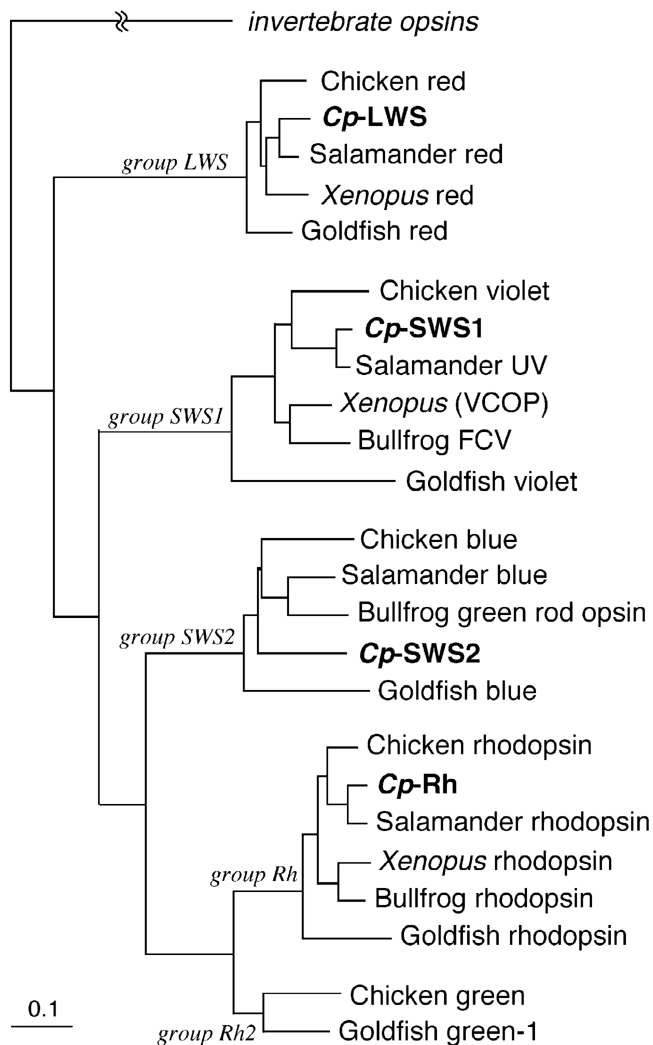

Fig. 2. Phylogenetic analysis of newt opsin sequences. Cp-LWS, -SWS1, -SWS2 and -Rh (rhodopsin) each belongs to group LWS, SWS1, SWS2 and Rh, respectively. As seen in this analysis, each of the newt opsins shows high homology with other amphibian opsins. The sequence data used in the present analyses were taken from EMBL and SWISS-PROT databases (accession nos. in parentheses): chicken rhodopsin (P22328), and red (P22329), green (P28683), blue (P28682) and violet (P28684) cone opsins; newt MS opsin (Cp-MS) (AB040148); bullfrog rhodopsin (P51470), and green rod (Rc-MS) (AB010085) and FCV (Rc-S) (AB001983) opsins; tiger salamander rhodopsin (U36574), and red (AF038947), blue (AF038946) and ultraviolet (AF038948) cone opsins; Xenopus rhodopsin (U23808), and red cone opsin (U90895) and violet cone opsin (VCOP) (U23463); and goldfish rhodopsin (P32309), and red (P32313), green-1 (P32311), blue (P32310) and ultraviolet (Q90309) cone opsins.

similar to that of unoperated (normal) animals. It was likely that they had recovered their vision [7]. Localization of opsin mRNAs in these regenerated retinas was investigated by in situ hybridization. Similar to in normal retina, rhodopsin mRNA was expressed only in rods (Fig. 4a), and Cp-LWS (Fig. 4b), Cp-SWS2 (Fig. 4c) and Cp-SWS1 (Fig. 4d) opsin mRNAs was selectively expressed in

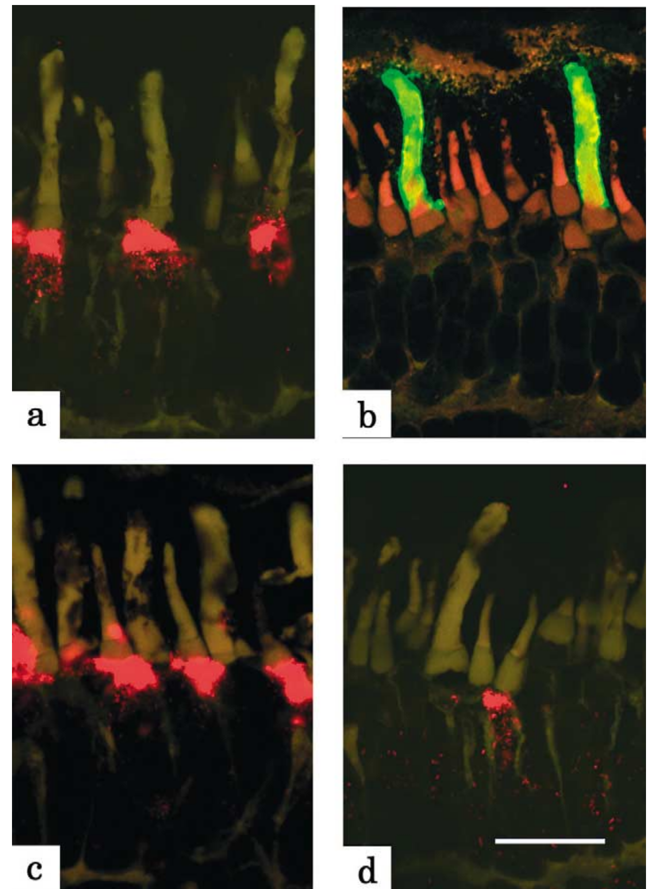

Fig. 3. Localization of opsin mRNAs in normal newt retina. (a) Rhodopsin (Cp-Rh) mRNA is expressed in rods, and (b) anti-bovine rhodopsin antibody (Rh29) recognizes the outer segments of rods (green fluorescence). (c) Cp-LWS and (d) Cp-SWS1 mRNAs are expressed in cones. Hybridization signals are seen as red fluorescence in the myoid regions of photoreceptor cells. Scale bar, 20 μm.

cones. This indicated that opsins were similarly expressed in the regenerated and normal retina.

3.4. In situ hybridization of regenerating and developing retinas

The expression of opsin mRNAs was examined in retinas at several regenerating stages (Fig. 5). Fig. 5a shows a section of an intermediate II-regenerating retina. The retina is characterized by multilayered cells with only an innermost row of rounded cells providing evidence of ganglion cell differentiation [5]. At this stage of regeneration, no apparent rhodopsin mRNA was detectable. Fig. 5b shows the weak expression of rhodopsin mRNA in a few cells of an intermediate III-regenerating retina (arrow heads) which corresponds to a stage at the beginning of, or just after, the formation of the synaptic layers. Fig. 5c shows a retina early in the late-regenerating stage where the inner plexiform layer (IPL) separates the inner nuclear layer (INL) and the ganglion cell layer (GCL) (arrow). The

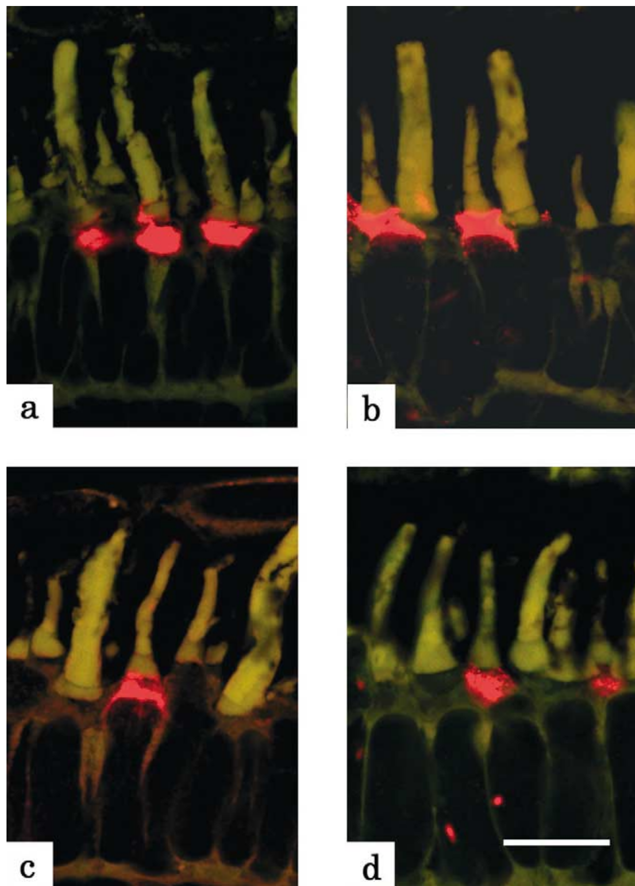

Fig. 4. Localization of opsin mRNAs in regenerated newt retina. (a) Rhodopsin (Cp-Rh) mRNA is expressed in rods, and (b) Cp-LWS, (c) Cp-SWS2 and (d) Cp-SWS1 mRNAs are expressed in cones. Signals are seen as red fluorescence. Scale bar, 20 μm.

expression of rhodopsin mRNA was observed at this stage in some cells abutting the retinal pigment epithelium (RPE); cells expressing rhodopsin mRNA also increased in number (Fig. 5c, arrowheads).

The expression of rhodopsin mRNA during development was examined on retinal sections from embryonic stages 32–49 (Fig. 6). Rhodopsin mRNA was not detectable at developmental stage 32 well before the formation of the IPL (Fig. 6a). Fig. 6b shows a section at stage 39 where the IPL was apparent in the central part of the retina (arrow) Rhodopsin mRNA-positive cells were detected at the central part of the retina (arrowheads). Fig. 6c shows rhodopsin mRNA expression in the retina at stage 44 while rhodopsin mRNA-positive cells become apparent even at the peripheral part of the retina. At stage 49, distribution of rhodopsin mRNA is similar to that of adult normal retina (Fig. 6d). Notably, hybridization signals are observed all around the cell body at the early stage (Fig. 6b), and are restricted in the upper side of the cells during subsequent development (Fig. 6c and d).

3.5. Immunohistochemistry of regenerating retina

Corresponding to the appearance of rhodopsin mRNA, our immunohistochemical study using Rh29 antirhodopsin monoclonal antibody shows that newt rhodopsin is first observed at the beginning of the formation of the IPL in regenerating retina (Fig. 7a). In the magnified images, we find three distinguishable patterns of signals in photo-receptor precursor cells (Fig. 7b–d). In Fig. 7b, rhodopsin exists all around the plasma membrane of immature

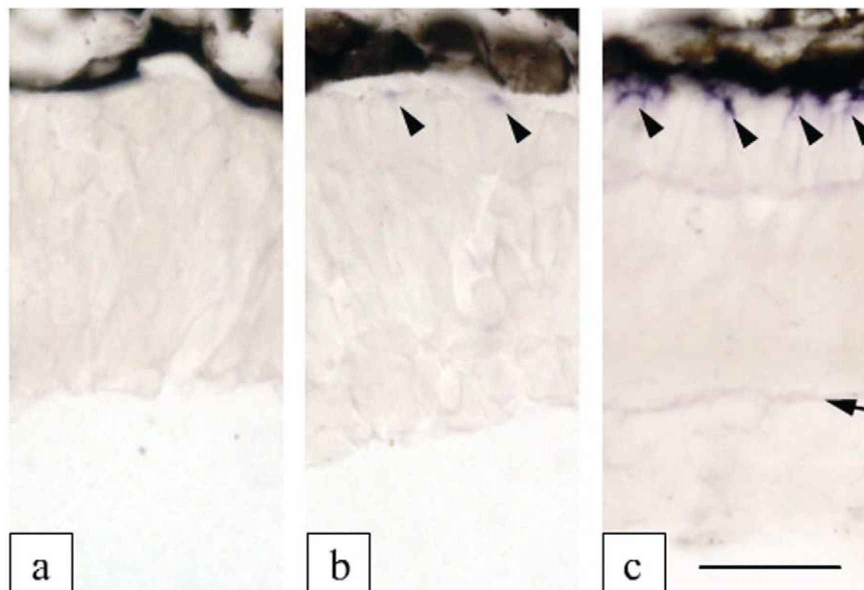

Fig. 5. Expression of rhodopsin (Cp-Rh) mRNA in regenerating newt retina. (a) Rhodopsin mRNA was not detected at Intermediate 2, (b) but was first seen at the regeneration stage between Intermediate 3 and Late when the inner plexiform layer appeared. (c) At a late stage of regeneration, the number of rhodopsin-expressing cells increased. Arrowheads indicate the hybridization signals, and an arrow indicates the newly formed inner plexiform layer. Scale bar, 50 μm.

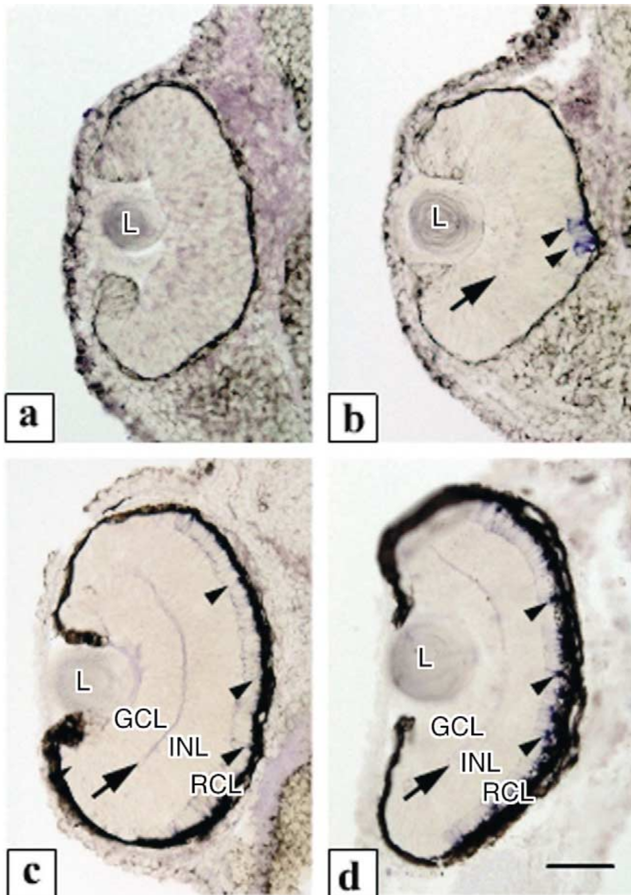

Fig. 6. Expression of rhodopsin (Cp-Rh) mRNA in developing newt retina. (a) Rhodopsin mRNA was not detected at stage 32 (b) but was first observed at the central zone of developing retina (arrowheads) when the newly formed inner plexiform layer first appeared (arrow). At stages 44 (c) and 49 (d), the expression of rhodopsin mRNA became clearer all around the retina. Abbreviations: inner plexiform layer, IPL; inner nuclear layer, INL; ganglion cell layer, GCL; receptor cell layer, RCL. Scale bar, 100 μm.

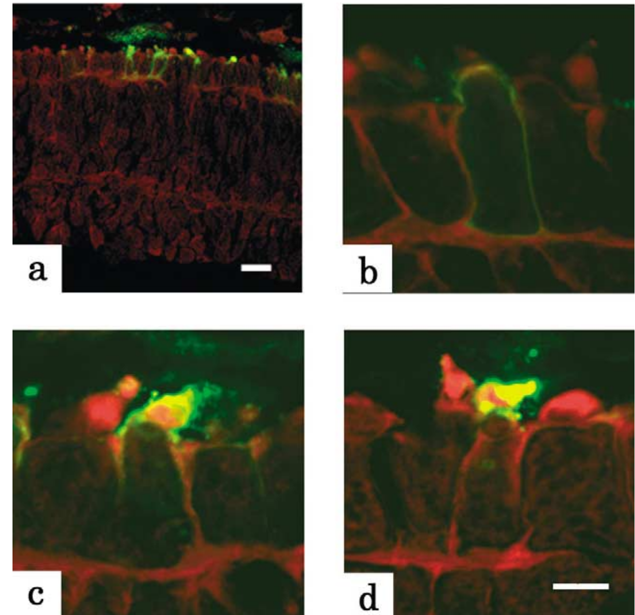

Fig. 7. Reactivity of an antirhodopsin antibody (Rh29) in regenerating newt retina. (a) Signals, seen as green fluorescence, first appeared at Intermediate 3 stage in regenerating newt retina. (b) In the regenerating process, rhodopsin exists isotropically in the plasma membrane of the photoreceptor cell whose pre-outer segment cilium has not yet been formed. (c) The pre-outer segment cilium begins to form and signals are seen both at the cilium and at the cell body. (d) Rhodopsin localizes only at the cilium. Scale bars, 20 μm (a) and 10 μm (b)–(d).

photoreceptors without outer segments. Fig. 7c shows that rhodopsin is observed both in the plasma membrane and in the apical projection (the so-called 'pre-outer segment cilium') which is thought to form the rod outer segment. It is likely that Fig. 7c shows an intermediate stage between the Fig. 7b and d. In Fig. 7d, signals are found only at the apical region. Since rhodopsin is localized in rod outer segments in mature photoreceptor cells, our result suggests that rhodopsin exists all around the plasma membrane of immature photoreceptors before the formation of pre-outer segment cilium, and undergoes translocalization from plasma membrane to the apical region in regenerating newt retinas.

4. Discussion

In this study, we showed that four kinds of opsins are expressed in *C. pyrrhogaster*; Cp-Rh (rhodopsin) in rods and the other three opsins in cones. In newt retina, it is difficult to discriminate each cone subtype morphologically, but we clearly showed that there are at least three subtypes. The distributions of opsin mRNAs are quite similar between normal and regenerated newt retinas. It is also likely that regenerated retina restores spectral sensitivity, as supported by ERG [19,29] and behavioral studies [7].

As the primary structures of group Rh2 opsins share high homologies with those of group Rh opsins (rhodopsins), it was expected that a cDNA encoding the group Rh2 opsin could be obtained by low-stringency screening using a newt rhodopsin cDNA fragment as a probe. Moreover, we performed plaque hybridization using the cDNA fragment of dg2, a group Rh2 opsin of *P.m. longinsulae*, as a probe. In both cases, only Cp-Rh clones were obtained in our screening. Group Rh2 opsin has not been identified in any amphibian retinas, and there is some negative opinion, based on electrophysiology, regarding the existence of this opsin type in amphibians [26]. It has been suggested that the Rh29 monoclonal antibody recognizes the N-terminal region of the rhodopsin sequence [34] and those of similar sequences in group Rh2 opsins, such as medaka Rh2 opsin [24]. No cones in newt retina were recognized by Rh29 anti-rhodopsin antibody, suggesting that group Rh2 opsin is not expressed in newt retina, as also found in other amphibian species.

Previously, opsin expression has been investigated in developing and regenerating retinas of another newt species, *Notophthalmus viridescens*. It has been reported that the opsin expression is first detected, but in a very few cells, a little earlier than the formation of plexiform layers during embryonic retinal development of newt [21]. In retinal regeneration, opsin expression was observed at the beginning or shortly after the IPL could be detected [3], but the direct correlation of opsin expression between developing and regenerating retinas was not clear. Here, we showed that the expression of newt rhodopsin (Cp-Rh) in developing and regenerating Japanese common newt (*C. pyrrhogaster*) retina begins at almost the same time as the formation of plexiform layers. This timing is somewhat later than that of the appearance of cone photoreceptor cells [5,22]. Recently, Kaneko et al. briefly described the expression of rhodopsin mRNA in regenerating *C. pyrrhogaster* retina, their data showing a correspondence to ours [13]. Both sets of data indicate that on a molecular level the reformation of photoreceptor cells is almost identical to the formation during embryonic generation.

During the maturation of photoreceptor cells in regenerating newt retina, the rod outer segments appeared after the synaptic separation [3]. In developing macaca retina, rhodopsin was first found in the inner segment and cilia [23]. Our results suggest that in regenerating newt retina, isotropical expression of rhodopsin in plasma membrane precedes formation of the cilium, and that rhodopsin is then gradually localized to pre-outer segment cilium. It has been suggested that translocalization of rhodopsin is closely related to the cytoskeleton [4], and that possibly, the reorganization of cytoskeleton takes place in rods that locate rhodopsin into the cilium.

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