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RHODOPSIN-LIKE PROTEINS IN PLANARIAN EYE AND AURICLE: DETECTION AND FUNCTIONAL ANALYSIS

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Summary

The presence of rhodopsin-like proteins in the eyes and auricles of the freshwater planarian *Dugesia japonica* was confirmed using anti-frog-rhodopsin rabbit IgG. The apparent relative molecular masses of these proteins were 65×10^3 and 62×10^3, and positive reactions to IgG were localized to the microvilli of the photoreceptor cells in the eyes and to the sensory cilia, rootlets and microvilli in the auricles. Eye- or head-excised planarians showed no negative phototaxis, whereas intact or auricle-excised planarians did. During regeneration in head-excised planarians, the appearance of rhodopsin-like proteins in the regenerating eyes corresponded to the recovery of negative phototaxis behaviour. Head or auricle excision enhanced asexual fission under continuous illumination. However, eye excision had no such effect. These results suggest that the rhodopsin-like proteins in the eyes work as photoreceptors for negative phototaxis behaviour and that, in the auricles, they are involved in asexual fission originating from the circadian rhythm.

Key words: rhodopsin-like protein, eye, auricle, fission, phototaxis, chemotaxis, *Dugesia japonica*, planarian.

Introduction

Sensory systems exist widely in animals ranging from unicellular organisms to humans. Planarians have two sensory organs, a visual system, consisting of the pigment-cup ocular eyes (Taliaferro, 1920; Milne and Milne, 1959; Röhlich and Török, 1961; MacRae, 1964; Carpenter *et al.* 1974; Kuchiiwa *et al.* 1991; Eakin, 1972), and the auricles (Koehler, 1931; MacRae, 1967). On the basis of excision and structural analysis, the auricles are thought to be important for the tactile system and in chemoreception (Koehler, 1931; Lorenzo, 1957; Bronstein and Ivanov, 1965; Ivanov, 1966; MacRae, 1967; Bronstein and Pyatkina, 1969; Pigon *et al.* 1974; Ferrero *et al.* 1980; Ferrero and Bedini, 1989). Very little is known about the ultrastructure and detailed functions of the auricles. It has been reported that rhodopsin-like proteins (RLPs) exist in the planarian head (Fujita *et al.* 1991). In the present study, immunoreactivity to anti-frog-rhodopsin rabbit IgG was detected in the auricles as well as the eyes. This study provides immunocytochemical, behavioural and physiological evidence for the presence of RLPs in the eyes and auricles of a planarian.

Materials and methods

Planarians

Dugesia japonica Ichikawa and Kawakatsu is a common and polymorphic species of freshwater planarian distributed widely in the Far East (the Japanese Islands, Taiwan, the Korean Penisula, China and Primorskiy, Northeastern Siberia in Russia: Ichikawa and Kawakatsu, 1964; Kawakatsu *et al.* 1995). Specimens of *Dugesia japonica* were collected in glass bottles from a stream in the suburbs of Kiryu using chemotaxis to chicken liver. The worms were kept in dechlorinated water (boiled city water) at 16 °C and fed raw beef liver. Prior to experimentation, the worms were starved for 2 weeks.

Reagents

Foetal calf serum was obtained from Gibco Laboratories or Life Technologies, Inc. Protein A-gold (5 nm), LR white acrylic resin and FITC-conjugated goat anti-rabbit IgG were obtained from Bio Cell Research Laboratories, Polyscience, Inc. (Warrinton, PA, USA) or E. Y. Laboratories, Inc., USA. All other reagents were of the highest analytical grade available.

Whole-mount immunocytochemistry

The localization of RLPs in intact worms was visualized using anti-frog-rhodopsin rabbit IgG and a whole-mount technique. The worms were fixed for 1 day at 4 °C in 0.1 mol l^{-1} sodium phosphate buffer (PBS; pH 7.4) containing 4 % paraformaldehyde (PFA). After washing with PBS, worms were permeabilized by incubation in methanol (–20 °C,

20 min). Non-specific binding was prevented by incubation in PBS containing 0.1 % Triton X-100 and 10 % foetal calf serum. The fixed worms were incubated with anti-frog-rhodopsin IgG (primary antibody, prepared as described by Shinozawa *et al.* 1987) solution (1:50) overnight at 4 °C. To detect binding of IgG, the worms were incubated with FITC-conjugated goat anti-rabbit IgG (1:100, secondary antibody) for 1 h. Washing steps (in PBS containing 0.1 % Triton X-100 for 3 h) were performed between incubations with each antibody. The worms were mounted on glass slides in glycerol/PBS (1:3). Control incubations were performed without the addition of the primary antibody. After all the above steps, the sections were washed three times with PBS, mounted in glycerol/PBS (1:3) solution and examined with a Nikon Fluophot microscope system.

Electron microscopy and immunogold labelling

For morphological studies, planarian sections containing eyes and auricles were fixed with 2 % glutaraldehyde in 50 mmol l^{-1} cacodylate–HCl buffer at pH 7.2, and then further fixed in 2 % osmium tetroxide in the same buffer, dehydrated through a graded ethanol series and finally embedded in epoxy resin (Epok 812, Okenshoji Co. Ltd, Tokyo).

For immunogold labelling, samples were fixed in a mixture of 0.2 % glutaraldehyde, 2 % PFA and 1 % picric acid in 50 mmol l^{-1} cacodylate buffer (pH 7.4) for 2 h at 4 °C. Cryo-ultramicrotomy (preparation of ultrathin sections) was performed according to the method of Tokuyasu (1986). Small pieces of tissue were infiltrated with 2.3 mol l^{-1} sucrose in 0.1 mol l^{-1} PBS (pH 7.4) overnight.

A tissue block mounted on a holder was quickly frozen in liquid nitrogen, and ultrathin frozen sections were cut with a diamond knife at approximately –90 °C on a Reichert ultracut microtome equipped with an FC4E cryocut attachment. The sections were picked up in a drop of 2.0 mol l^{-1} sucrose and 0.5 % gelatin in 0.1 mol l^{-1} PBS and placed on Formvar-carbon-coated nickel grids. The samples were rinsed well with PBS containing 0.02 mol l^{-1} glycine (glycine–PBS), and the sections were preincubated in 1 % PBS containing 1 % bovine serum albumin (BSA) for 30 min. Sections were then incubated overnight, without rinsing, in the primary antibody, rinsed with glycine–PBS, and labelled with protein A-gold (particle diameter 5 nm) for 90 min. The sections were then rinsed with PBS, fixed in 1 % glutaraldehyde in 0.1 mol l^{-1} PBS (pH 7.4) for 5 min, post-fixed in 2 % osmium tetroxide in the same buffer for 15 min, and stained with 0.5 % uranyl acetate in barbital/acetate buffer at pH 5.2. The sections were then dehydrated through a graded ethanol series and immediately infused with absolute LR white acrylic resin, which was then polymerized at 60 °C according to the method of Keller *et al.* (1984). Specimens were observed using a JEM 1200EX electron microscope operating at 80 kV.

Western blotting

Thirty specimens were decapitated and their eyes and auricles were removed. A sharp blade was used for decapitation (head excision). To excise the eyes or auricles, a glass capillary tube (0.9 mm in diameter, Corning) was used to punch out the tissue. The eyes and auricles were homogenized in a solution of 10 mmol l^{-1} Tris–HCl (pH 7.5), 1 mmol l^{-1} dithiothreitol (DTT), 4 mol l^{-1} urea and 2 mol l^{-1} LiCl in a Teflon–glass homogenizer and then sonicated at 100 W for 15 s (three times). The homogenate was centrifuged at 38 000 ***g*** for 20 min, and the precipitates were washed twice by centrifugation in the same solution and once in a solution of 10 mmol l^{-1} Tris–HCl (pH 7.5) and 1 mmol l^{-1} DTT. The precipitated protein fractions were treated with 2 % digitonin containing 10 mmol l^{-1} Hepes–KOH (pH 7.5) and 100 mmol l^{-1} NaCl to solubilize RLPs. Solubilized proteins were then precipitated using 10 % trichloroacetic acid (TCA) and 0.13 % sodium cholate. The precipitates were washed with 10 % TCA and then with ice-cold acetone. The membrane proteins thus obtained were solubilized in sodium dodecylsulphate (SDS) sample solution (7 % SDS, 70 mmol l^{-1} DTT and 20 mmol l^{-1} EDTA). Protein composition was analyzed by SDS–PAGE.

After SDS–PAGE, the gel was either stained with Coomassie Brilliant Blue R-250 (CBB: Nakarai Chemicals Ltd, Japan) to visualize proteins or used for blotting. The relative molecular masses of the electrophoresed proteins were determined using molecular mass standards (Pharmacia). For blotting, the proteins in the gel were transfered electrophoretically to a polyvinylidenefluoride (PVDF) membrane (Atto Co., Japan) for 90 min at 300 mA according to the method of Burnette (1981). The proteins on the membrane were incubated with anti-frog-rhodopsin rabbit IgG and then with horseradish-peroxidase-labelled goat anti-rabbit IgG as described previously (Nakaoka *et al.* 1991). The binding of the secondary antibody was detected by the peroxidase reaction using 4-chloro-1-naphthol and H_2O_2 as substrates. Protein concentrations were determined using the method of Lowry *et al.* (1951) using BSA as a standard.

Extraction and detection of retinoids

Extraction and HPLC analysis of retinoids from pieces of head, eye and auricle obtained from 300 planarian worms were carried out as described by Azuma *et al.* (1992). The planarian samples were homogenized in a solution of 100 mmol l^{-1} NH_2OH (pH 7.2) and methanol (final concentration 60–70 %). The homogenates were mixed with dichloromethane and *n*-hexane (1:2, v/v), shaken vigorously and centrifuged at 2500 ***g*** for 15 min, and the upper (dichloromethane/hexane) layers were collected. This extraction procedure was repeated three times.

An HPLC system equipped with a column of YMC-Pack A-003-3 SIL (Yamamura Chemical La. Co. Ltd, Japan) was used. The eluent was a mixture of *n*-hexane, diethylether and ethanol (90:10:0.1, v/v) and was used at a flow rate of 1.3 ml min^{-1} for 50 min. All procedures were carried out under dim red light. The absorbances of each fraction at 350 nm and 280–350 nm were measured using a detector (UVDEC-100, Jasco, Japan) and with a multi-wavelength detector (MULTI-340, Jasco), respectively. Measurements with the MULTI-340 were carried

out to obtain absorption spectra over a wide range of wavelengths. The amounts of several retinoids were estimated from their absorption coefficients and the peak areas of known amounts of standard retinoids.

Photosensitivity

Thirty samples each of intact, head-, eye- and auricle-excised planarians were illuminated for 10 min with a halogen lamp (20 lx, wavelength 400–800 nm, fibre-optic light source, Olympus Optical Co. Ltd, Japan). The light source was at a distance of 5 cm from the surface of the water to one side of a dish (10 cm diameter). The head, eye and auricle excisions were carried out as described above. To obtain quantitative data, the numbers of worms farthest from the light source and in the centre area of the dish were counted after illumination. Quantitative analysis of negative phototaxis during regeneration was carried out in a plastic tray 10 cm in width, 15 cm in length and 5 cm in height. The tray, containing planarians in 3 cm of dechlorinated water, was placed in a box open on one side (10 cm width) with the other five sides shaded. The non-shaded side was illuminated by a 30 W fluorescent lamp. Twenty specimens were placed on the non-shaded side in the tray. After illumination for 10 min, the numbers of worms in areas 0–5 cm, 5–10 cm and 10–15 cm from the open side were counted and were defined as +, ± and − for negative phototaxis, respectively (see Table 1).

Light microscopic immunocytochemistry

Worms were decapitated behind the auricles, and the heads were fixed for 1 day in 4% paraformaldehyde (PFA) in 0.1 mol l^{-1} PBS at pH 7.4. After washing with PBS, the heads were infiltrated with graded 0.6 mol l^{-1}, 1.2 mol l^{-1} and 2.3 mol l^{-1} sucrose in 0.1 mol l^{-1} PBS and frozen in dry-ice/hexane. The frozen sections (4 μm) were mounted on gelatin-coated glass slides. The sections were then incubated in normal horse serum for 30 min to inhibit non-specific binding. After washing with PBS, the sections were incubated overnight with anti-frog-rhodopsin rabbit IgG (1:30). Incubation with horseradish-peroxidase-labelled goat anti-rabbit IgG (1:100) was then carried out for 30 min. Peroxidase activity was detected using diaminobenzidine (DAB) as substrate.

Asexual fission of excised planarians

Forty specimens each of intact, head-, eye- and auricle-excised planarians were segregated into two groups of 20 samples each. Each group was kept in light conditions under a halogen lamp, as described above, or in dark conditions for 7 days in 100 ml of dechlorinated water at 17 °C. Asexual fission was measured each day at the same time of day.

Chemotaxis assay

Chemotaxis assays were carried out by observing the responses of planarians to chicken liver (Miyamoto and Shimozawa, 1985). Ten samples each of intact, auricle- or head-excised planarians were kept separately in glass Petri dishes (diameter 9.0 cm) containing dechlorinated water (1.0 cm depth) and a piece of chicken liver (5.0 g). Five dishes of each were covered with aluminium foil and kept at room temperature for 10 min. After incubation, worms attached to the chicken liver were removed, and the numbers of attached worms were counted as scoring positively for chemotaxis.

Results

Detection of rhodopsin-like proteins (RLPs) and retinoids in planarian eye and auricle

In western blotting analyses of proteins in the planarian head, anti-frog-rhodopsin rabbit IgG detected RLPs with apparent relative molecular masses of 65×10^3 and 62×10^3 (Fujita *et al.* 1991). Fig. 1A is a light micrograph of the planarian head area. In whole-mount immunocytochemistry using anti-frog-rhodopsin rabbit IgG, a fluorescent immunoreaction was detected in the region of the eyes and

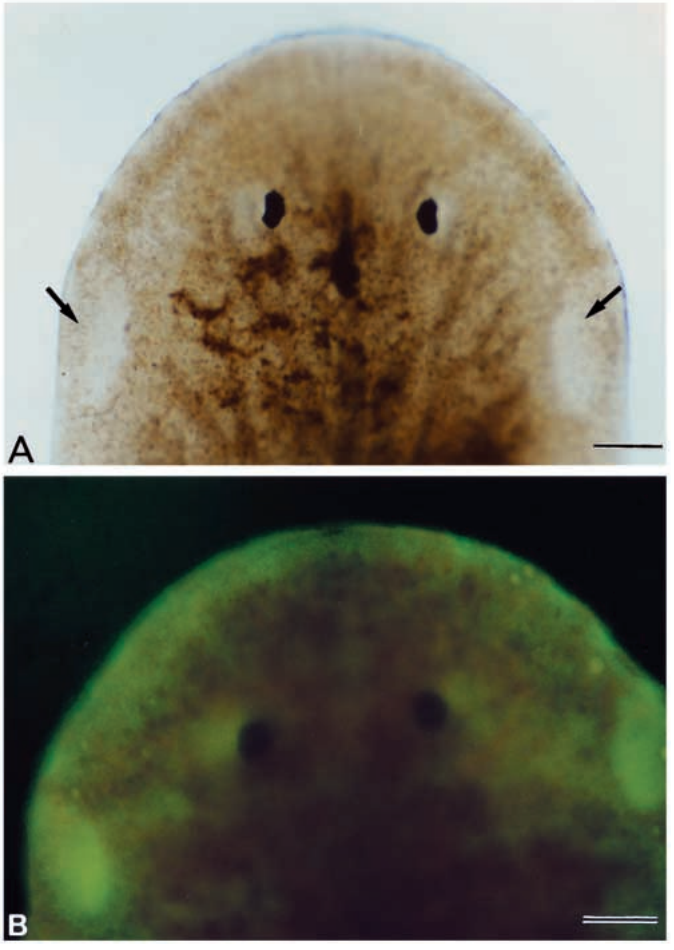

Fig. 1. Indirect immunofluorescent staining of whole-mount worms using anti-frog-rhodopsin rabbit IgG. (A) Light micrograph of the planarian head area. The arrows show the auricles. (B) Indirect immunofluorescent staining with anti-frog-rhodopsin rabbit IgG. Scale bars, 20 μm.

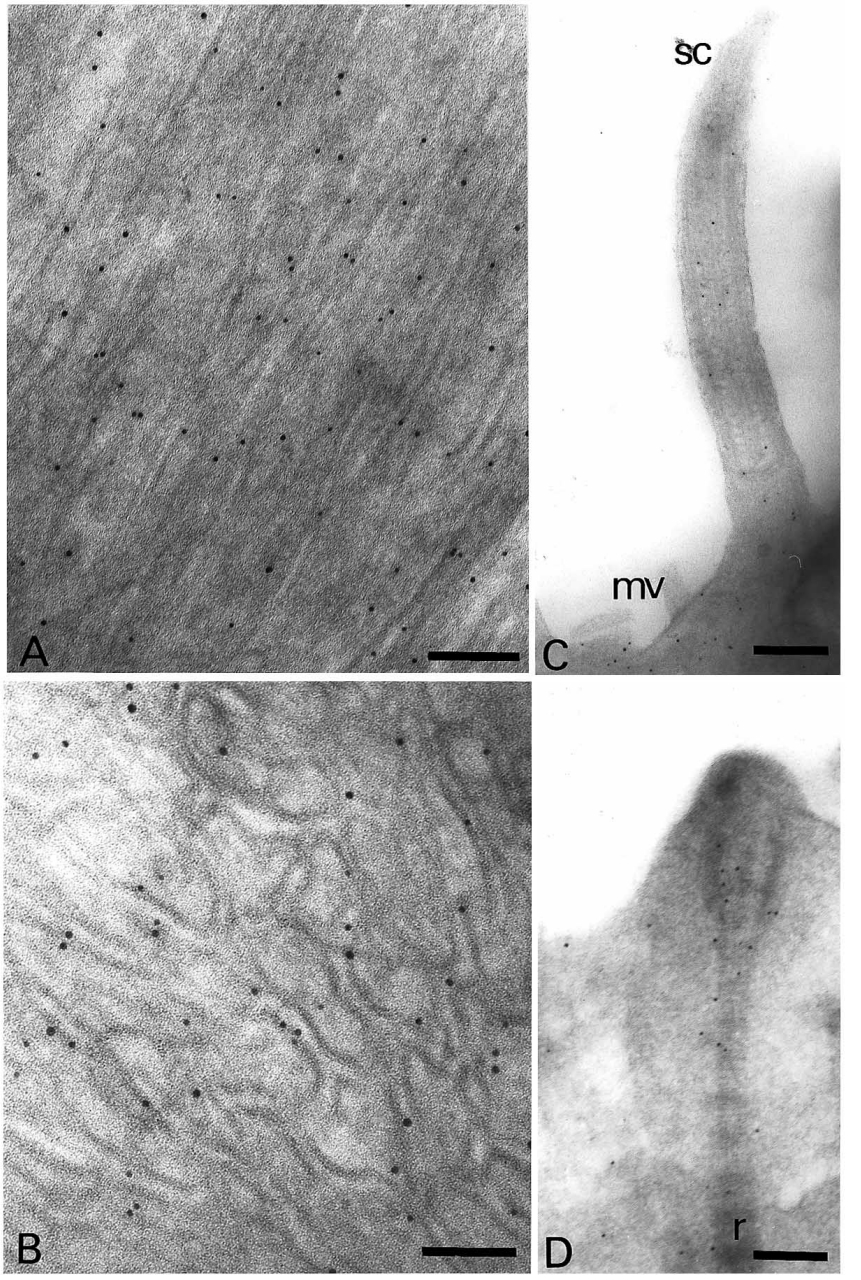

Fig. 2. Electron immunomicroscopy using anti-frog-rhodopsin rabbit IgG and gold-labelled protein A. Sagittal section (A) and transverse section (B) of photoreceptor cells in the eye. Scale bars, 100 nm. (C,D) Sagittal sections through the auricles. sc, sensory cilium; mv, microvilli; r, rootlet. Scale bars, 100 nm.

auricles (Fig. 1B). The reaction seen at the front edge of the head is autofluorescence and was always detected irrespective of the primary antibody used. To analyze the localization of RLPs more precisely, electron microscopic observations of ultra-thin sections from the area of the eyes (Fig. 2A,B) and auricles (Fig. 2C,D) were carried out using anti-frog-rhodopsin rabbit IgG (primary antibody) and gold particle (5 nm diameter)-labelled protein A. RLPs detected as gold particles were localized on the membranes of the microvilli of photoreceptor cells in the eyes (Fig. 2A,B). In the auricles, RLPs were localized in the sensory cilia, microvilli and rootlets (Fig. 2C,D).

The specificity of antibody binding to membrane proteins prepared from the area of the eyes and auricles was examined by immunoblotting after SDS–PAGE. As shown in Fig. 3, proteins with apparent relative molecular masses of 65×10^3 and 62×10^3 were detected in samples of auricles (Fig. 3, lane 2B) and eyes (Fig. 3, lane 2A). To detect the RLPs chromophore, HPLC analysis of the retinoids was carried out. Fig. 4 shows HPLC profiles of retinoids extracted from the eyes and auricles obtained from 300 planarian worms. Peaks for all-*trans* retinol (ROL) and *syn* all-*trans* retinal (*syn* all-*trans* RALOX) were detected in both samples, but 11-*cis* retinal (*syn* 11-RALOX) was detected only in the eye samples. The mean amounts of 11-*cis* retinal and all-*trans* retinal in a single eye were 0.23 pmol and 0.45 pmol, respectively. The amount of all-*trans* retinal in one auricle fragment was 0.41 pmol.

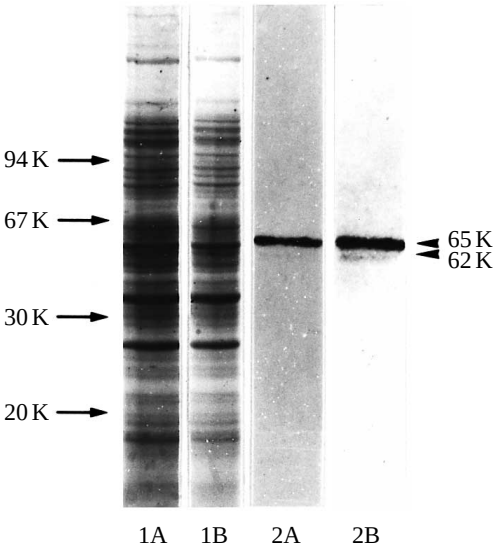

Fig. 3. Western blot of planarian membrane proteins using anti-frog-rhodopsin rabbit IgG. Planarian membrane proteins from the area of the eyes (2.4 µg, lane 1A; 3.2 µg, lane 2A) and auricles (2.4 µg, lane 1B; 3.2 µg, lane 2B) were subjected to SDS–PAGE. Electrophoresed proteins were stained with Coomassie Brilliant Blue R-250 (lanes 1A, 1B) or transferred electrophoretically to a polyvinylidenefluoride membrane. The transferred proteins were treated with anti-frog-rhodopsin rabbit IgG and horseradish peroxidase (lanes 2A, 2B). Relative molecular masses of standard proteins are shown on the left.

Photoresponse of planarians: effect of excision on negative phototaxis and correlation between the appearance of negative phototaxis and RLPs during regeneration

It is well known that planarians exhibit negative phototaxis (Taliaferro, 1920). Using this characteristic, the function of the eye and auricle in photo-response behaviour was investigated (Fig. 5). Intact (Fig. 5A) or auricle-excised (Fig. 5C) planarians showed negative phototaxis, whereas eye-excised (Fig. 5B) or head-excised (Fig. 5D) planarians did not. The results of a quantitative analysis are shown in Table 1. In this study, at least 90 % of both intact and auricle-excised specimens exhibited negative phototaxis. Statistical analysis using Student's *t*-test indicated that data to be statistically significant ($P<0.05$).

Table 1. *Quantitative analysis of the effects of excision on negative phototaxis in planarians*

		Negative phototaxis			
Excised area	*N*	+	–	ND	Total (%)
None (intact)	300	286	8	6	95.3±4.2
Head	300	23	249	28	7.7±5.6
Eye	300	24	254	22	8.0±6.9
Auricle	300	270	18	12	90.1±5.3

See Materials and methods for details.
ND, not determined.
Total values are means ± S.D.
+ indicates clear negative phototaxis; ± indicates that the responses were indeterminate; – indicates the absence of phototaxis.

These results suggest that the auricles are not involved in phototaxis and that RLPs in the eyes participate in planarian photosensitive behaviour (negative phototaxis). During regeneration in decapitated planarians, negative phototaxis was investigated over a period of 7 days (Fig. 6). Between the first and fourth day of regeneration, there was no significant change in the number of specimens showing negative phototaxis (+, ± and –). From the fifth day onwards, the number of specimens showing negative phototaxis (+) increased significantly. Correspondingly, the number of specimens not showing negative phototaxis decreased. By the seventh day, the numbers of specimens displaying negative phototaxis ratings of +, ± and – were close to the values for intact planarians. The

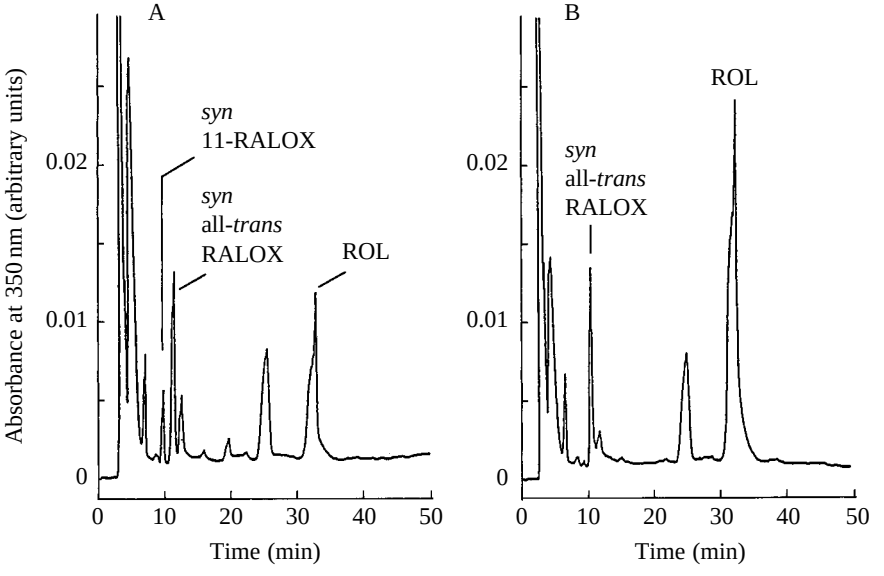

Fig. 4. HPLC chromatogram of retinoids extracted from planarian eyes and auricles. (A) Extract of 600 eyes; (B) extract of 600 auricles. *syn* 11-RALOX, *syn* 11-*cis* retinaloxime; *syn* all-*trans* RALOX, *syn* all-*trans* retinaloxime; ROL, all-*trans* retinol.

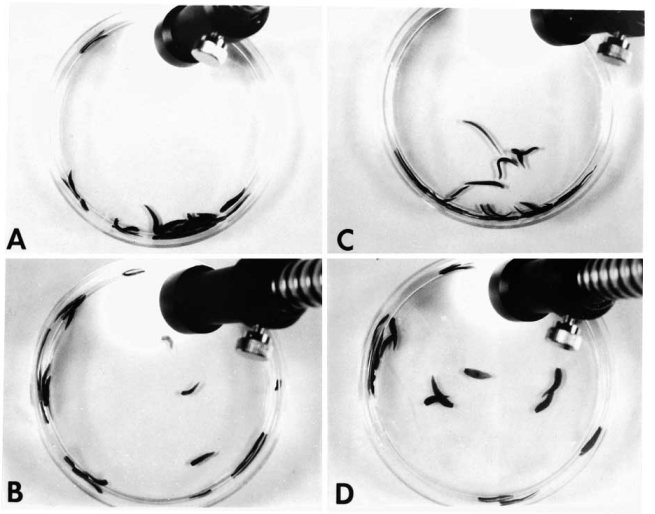

Fig. 5. Effect of excision on negative phototaxis of planarians. Photographs were obtained after illumination for 10 min of intact (A), eye-excised (B), auricle-excised (C) and head-excised (D) planarians.

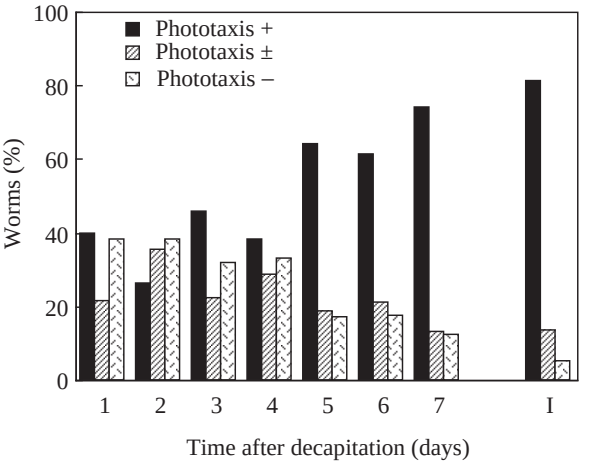

Fig. 6. Photosensitivity of planarians during regeneration. Negative phototaxis in 20 decapitated (head-excised) planarians was analyzed during regeneration for 7 days. Analyses were conducted ten times per day and mean values are shown. Experiments using intact planarians (I) were also carried out on the first day. +, ± and – indicate clear negative phototaxis, indeterminate behaviour and the absence of phototactic behaviour, respectively.

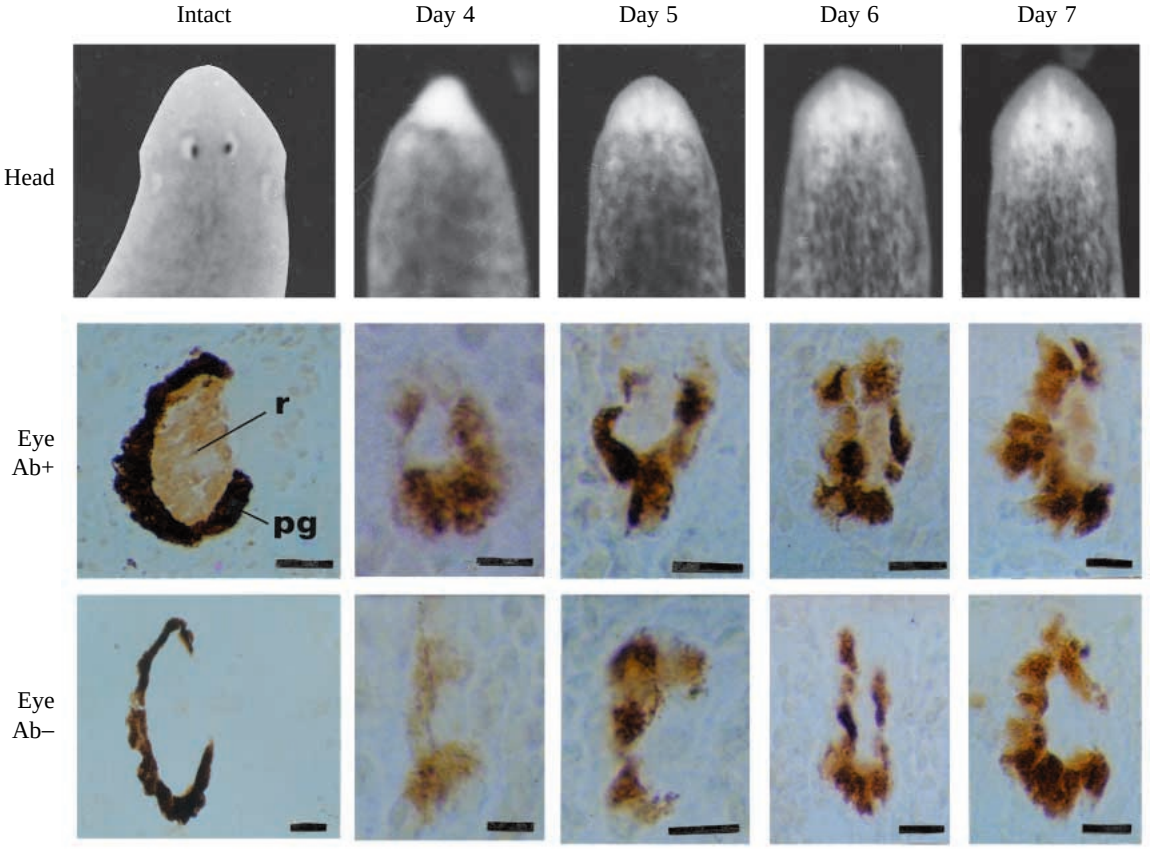

Fig. 7. Photomicrographs and light microscopic immunocytochemistry of intact and regenerating planarian heads. Top row (Head): photomicrographs showing the morphology of intact and regenerating planarian heads. Middle and bottom rows: horizontal sections in the area of the eye. Middle row (Eye Ab+): eye samples treated with anti-frog-rhodopsin rabbit IgG (primary antibody) and horseradish-peroxidase-labelled goat anti-rabbit IgG (secondary antibody). r, rhabdomere-type photoreceptor cells; pg, pigment cells. Bottom row (Eye Ab–): negative control for each reaction. Sections were treated with phosphate-buffered saline containing 0.1 % bovine serum albumin instead of the primary antibody. Scale bars represent 10 μm for intact and 5 μm for other samples.

appearance of RLPs was investigated using anti-frog-rhodopsin rabbit IgG. Fig. 7 shows photomicrographs to indicate morphology and the detection of RLPs in intact and regenerating planarians. The detection of RLPs was carried out by light microscopic immunocytochemistry using anti-frog-rhodopsin rabbit IgG (primary antibody) and horseradish-peroxidase-labelled goat anti-rabbit IgG (secondary antibody). Photomicrographs of the regenerating head (top row in Fig. 7) show regenerating eyes to be lightly labelled on the fifth day and clearly labelled on the sixth day. In the planarian eye, the rhabdomere-type photoreceptor cells were surrounded by pigment cells. Immunocytochemistry of intact eyes showed a clear reaction to horseradish peroxidase, which detects RLPs, in the area of the photoreceptor cells. In the absence of this primary antibody, this reaction was not detected. In the case of regenerating eyes, RLPs were detected faintly on the fifth day. The reaction was much clearer on the sixth day. These experiments were repeated each day from the first to the tenth day of regeneration. No RLPs were detected until the fifth day. Thus, during regeneration, the appearance of RLPs correlates with the appearance of negative phototaxis.

Photoresponse of planarians: effect of excision on asexual fission

The asexual fission of planarians is affected by the photoperiod and by melatonin (Morita and Best, 1984). In order to investigate the physiological function of RLPs in the auricle, experiments on asexual fission were carried out.

Fig. 8 shows the effects of excision (head, eyes or auricles) and illumination on asexual fission. Under continuous light conditions (Fig. 8A), both head- and auricle-excised specimens showed a high incidence of fission. Among head-excised planarians, 12 of the 20 planarians underwent fission. However, intact and eye-excised planarians did not undergo fission. In contrast, under continuous dark conditions (Fig. 8B), fission occurred in all groups (11–13 of 20 specimens).

Because the function of the planarian auricle is considered to be chemotaxis, the effects of auricle or head excision on chemotaxis were analyzed (Fig. 9). Most (6–9 out of 10) intact planarians attached to the chicken liver, showing chemotaxis, within 10 min on days 1, 3 and 6. Some (3–6) auricle-excised planarians showed chemotaxis on day 1 after excision, and the number of worms showing chemotaxis increased in parallel with the regeneration of auricles, as observed using an inverted microscope. In the case of head-excised (decapitated) planarians, the chemotactic ability was completely abolished; it partially recovered 6 days after decapitation. These data suggest that the auricle contributes only partially to chemotaxis.

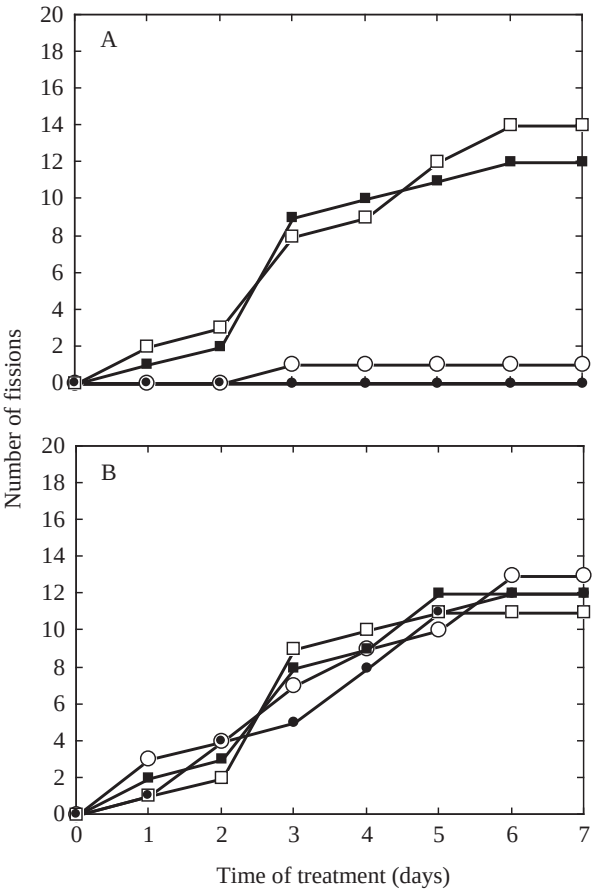

Fig. 8. Effect of excision on asexual fission. The number of asexual fissions was measured under continuous light conditions (A) and under continuous dark conditions (B) in intact (●), head-excised (■), eye-excised (○) and auricle-excised (□) planarians. The experiment was carried out three times and averaged data (±10 %) are shown.

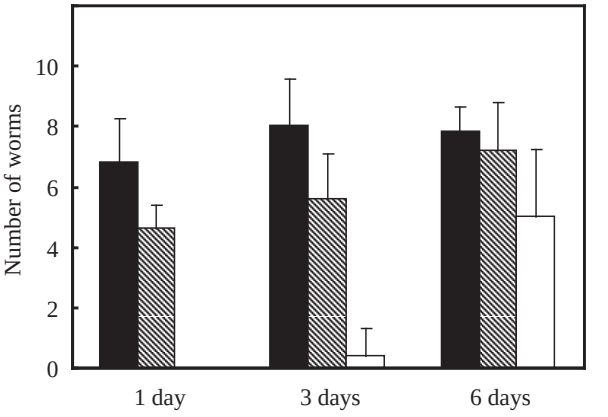

Fig. 9. Effect of excision on planarian chemotaxis. Chemotaxis of 10 worms in a dish containing intact (filled columns), auricle-excised (hatched columns) or head-excised (open columns) planarians was assayed 1, 3 and 6 days after (excision) treatment. Mean values + S.D. for five experiments of each treatment are shown.

Discussion

We report the detection and analysis of rhodopsin-like proteins (RLPs) in the eyes and auricles of planaria. Using anti-frog-rhodopsin rabbit IgG, the presence of RLPs in the eyes

and the auricles was confirmed. Using whole-mount immunocytochemistry, we detected fluorescent reactions due to the binding of IgG to RLPs in the area of the eyes and auricles. Electron microscopic observations showed RLPs to be localized on the membranes of microvilli in the eye and on the sensory cilia, microvilli and rootlets in the auricles. As previously reported (Röhlich and Török, 1961; Kishida, 1965; Eakin, 1965, 1972; Carpenter *et al.* 1974; Kuchiiwa *et al.* 1991), the planarian eye is of the rhabdom type, consisting of microvilli connected to each other *via* their membranes. In the case of the auricles, comparable structures occur in the chemoreceptors of catfish (*Parasilurus asotus*), lamprey (*Lampetra fluviatilis*) and beetle (*Acilius salcatus*) as well as in the olfactory epithelium of mammals (Lorenzo, 1957; Bronstein and Ivanov, 1965; Ivanov, 1966; MacRae, 1967; Bronstein and Pyatkina, 1969; Pigon *et al.* 1974; Ferrero and Bedini, 1989). Our observations that photoreceptor cells in the eye have a microvillous-type rhabdom and that the sensory cells in the auricle are ciliated olfactory-like cells are similar to those of previous reports. RLPs in the auricles and eyes were shown by western blotting to have apparent relative molecular masses of 65×10^3 and 62×10^3. These relative molecular masses are the same as those previously reported (Fujita *et al.* 1991). HPLC analysis detected the presence of all-*trans* retinal, all-*trans* retinol and all-*trans* retinyl ester in extracts from both head and tail pieces, while 11-*cis* retinal was detected only in head pieces (Azuma *et al.* 1992). In our study, all-*trans* retinol and *syn* all-*trans* retinal were detected in both eye and auricle samples; however, 11-*cis* retinal was detected only in eye samples. One reason that 11-*cis* retinal was not detected in the auricle area might be that it is present in only low levels. Another reason might be that the RLP chromophore in the auricles is not 11-*cis* retinal but another retinal derivative, e.g. 3- or 4-hydroxy 11-*cis* retinal. The latter has been found as the visual chromophore in several invertebrate species (Matsui *et al.* 1988; Gärtner and Towner, 1995) and is not detectable by the present HPLC methods. By detecting RLPs in the eye and auricle, the co-existence of microvillar and ciliated photoreceptors in planarians was confirmed. This observation shows similarities to the investigations of Eakin (1965).

To elucidate the function of RLPs in the eye and auricle, analyses of both behaviour (negative phototaxis) and physiology (asexual fission) were carried out. Although excision of the eyes or decapitation abolished negative phototaxis, excision of the auricles did not. Also, the reappearance of negative phototaxis was closely correlated with the reappearance of RLPs during regeneration. The period of 5–6 days required for the reappearance of RLPs and negative phototaxis is close to that required for neuronal repair in a flatworm (Faisst *et al.* 1980). In addition, the results indicate that RLPs in the eyes participate in the behavioural photoresponse. Asexual fission in planarians is influenced by the photoperiod and occurs only at night. Decapitation of planarians enhances fission (Best *et al.* 1969), and the addition of melatonin inhibits it (Morita and Best, 1984). Melatonin also inhibits head and tail regeneration (Yoshizawa *et al.* 1991). The concentration of melatonin in the head is higher in the dark than in the light (Morita *et al.* 1987) and also higher than in other body parts (Yoshizawa *et al.* 1991). Pigon *et al.* (1974) analyzed the influence of grouping (number of worms in a tray) and surgical ablation on asexual fission. Although grouping was found to inhibit fission, ablation of the auricles and/or snout enhanced it. They suggested that the neural cilia, located in the ablated area, act as sensors for social stimuli. In our experiments, ablation of the auricles and decapitation enhanced asexual fission even in the light. From these observations, it appears possible that light reception by the auricle controls the synthesis of melatonin and that the diffusion of melatonin controls asexual fission. Measurements of melatonin concentrations in various planarian parts under light and dark conditions will confirm this possibility. The structural similarity between the cells in the planarian auricle and the chemoreceptor cells in other animals has led several authors to suggest that the auricles function as chemoreceptors (Koehler, 1931; MacRae, 1967; Pigon *et al.* 1974; Ferrero *et al.* 1980; Ferrero and Bedini, 1989). Our results show that the auricles contribute only slightly to chemoreception and that the head area is also involved in chemoreception.

In conclusion, RLPs were detected in the auricles and eyes. RLPs in the eyes act as photoreceptors influencing behaviour, while RLPs in the auricles influence the circadian rhythm (and therefore asexual fission). The possibility that the auricles have some pineal-organ-like function, such as that seen in lower vertebrates (Quay, 1965; Kappers, 1967; Hafeez and Quay, 1969; Falcón and Collin, 1989), is suggested.

The possibility that ciliated photoreceptor cells evolved from chemoreceptor cells has been suggested (Eakin, 1965; Burr and Burr, 1975). Because of the ciliated structure of the auricular cells, it is of great interest to determine whether a bimodal (photoreception and chemoreception) stage has occurred during evolution.

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