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Are the eyespots of *Microstomum lineare* light-sensitive?

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Introduction

The predominant photoreceptors in Platyhelminthes are the cerebral pigment-cup eyes. The argumentation for their photoreceptive function is based on observations of light-induced ultrastructural changes as well as the phototactic behaviour of intact and eyeless planarians (see TALIAFERRO, 1920; HYMAN, 1950; FOURNIER, 1984). Furthermore, their spectral sensivity suggests the presence of a single rhodopsin-like pigment (BROWN et al., 1968). In addition to the pigment-cup eyes, two completely different structures have been associated with photoreception in flatworms: 1) non-pigmented rhabdomeric «retinal clubs», and 2) ciliary «lamellate bodies» (FOURNIER, 1984).

Ciliary lamellate bodies have been described from a number of free-living and parasitic platyhelminths (for references see XYLANDER, 1984). They are presumed to be photoreceptors mainly by morphological similarities with known and probable photoreceptive structures in other invertebrates. Phototactic behaviour has also been reported in the miracidia and cercariae of *Schistosoma mansoni*, although they lack cerebral pigment-cups (MASON and FRIPP, 1977; SALADIN, 1982). However, neither electrophysiological studies nor biochemical analyses have been made on the lamellate bodies in platyhelminths.

A recent investigation of the eyespots in *M. lineare* (PALMBERG et al., 1980) showed four different cell types: 1) ciliary lamellate body (ciliary photoreceptor cell in the 1980 paper), 2) pigment cell, 3) sensory cell with long cilia and 4) sensory cell with balloon-like cilia (Fig. 1). Of these, the lamellate body, if any, was presumed to be photoreceptive by its morphological similarities with other presumed photoreceptors. The aim of the present study is to further test the photoreceptive function of the eyespots (with special reference to the lamellate bodies) by using four different methods: (1) behavioural tests, (2) (^{3}H)vitamin-A autoradiography, (3) micro-spectrophotometry and (4) electrophysiology.

Material and methods

Specimens of *Microstomum lineare* were collected from shallow brackish water at Stortervo, Pargas (SW Finland). Worms used for behavioural tests were collected the day before the experiment and kept in the dark at about 18 °C.

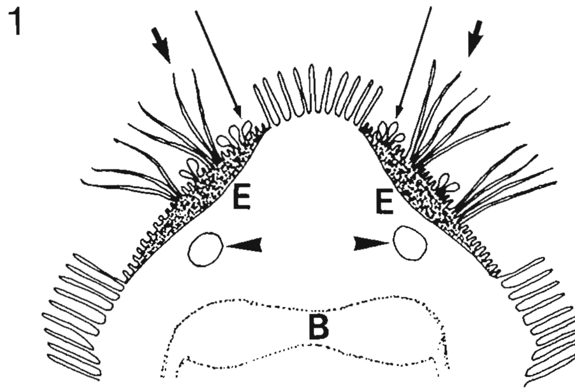

Fig. 1: Diagram of head of *Microstomum lineare* showing eyespot area (E), ciliary lamellate body (arrowhead), balloon-like cilia (long arrow), long cilia (short arrow), brain (B).

Light-response experiments

During the light-response experiments the worms were kept in glass troughs, 5 cm long, 2 cm broad and 1 cm deep, made from glass tubes and divided into three compartments, which could be separated from each other with rubber walls. The troughs which were black-painted on the outside were exposed to a light gradient: One end compartment was covered with matt black paper, the mid-compartment with a neutral filter (Wratten No 96) passing 10% of the incoming light, while the other end was left uncovered.

The troughs with animals were kept in a dark box for 1 h between light exposures. During illumination a rectangular lid in the box was removed for 2 min or 5 min. Following exposure the compartments were immediately separated by rubber walls and the number of worms in each of them registered. The distance from the 100 W tungsten lamp light source to the experimental animals was 20 cm. A heat-absorbing glass filter (Schott, KG 1) and a 2 cm deep heat-absorbing fluid filter were placed 3 cm above the trough.

A ground-glass inserted in the optical system made the field of illumination almost uniform. The irradiance level during the experiment was measured with a Lunasix 3 Gossen-photometer at the surface of the troughs. The recorded white light was 1500 lux in the uncovered compartment (within the wavelength band 400–800 nm, this corresponds to about 2000 $\mu W/cm^2$). The control worms were held in identical troughs and boxes, but not exposed to light. Their distribution in the trough-compartments was recorded at one-hour intervals and the distribution compared to those of animals exposed to light. Students t-test was used to detect possible significant differences between the numbers of worms in the differently illuminated compartments, and between the numbers observed after 2 min and 5 min exposures respectively.

Autoradiography

An equimolar amount of bovine serum albumin (BSA) was added to a tritium labelled vitamin-A solution (Concentration: 1.0 mCi, 20 μg vitamin-A in 1 ml; specific activity 14,3 Ci/mmol; New England Nuclear). Aliquots of this stock solution were then added to filtered brackish water to give the final concentrations of 10, 4 and 0.4 μCi/ml. All solutions were kept in the dark until specimens of light- and dark-adapted *M. lineare* were

transferred to labelled vitamin-A and exposed in that for 2–4 h. Subsequent steps in fixing processes and light microscopic autoradiography followed the method described in PALMBERG and REUTER (1983). Ultrathin sections were mounted on carbon-coated nickel grids, covered with a thin film of Ilford L-4 emulsion and exposed for 2–3 months at 4 °C. After developing in Kodak D-19 the gelatin was removed by 0.5 N acetic acid for 30 min in 37 °C. The sections were post-stained with uranyl acetate and lead citrate.

Microspectrophotometry

The animals were mounted on the microspectrophotometer stage between two coverslips in a drop of water containing 1% $MgSO_4$ and 15% dextran (to prevent movements). The instrument was a «single-beam, double-position, microspectrophotometer», constructed by one of us (V.G.). During the measurement its stage jumped several tens of micrometres 15 times per second, shifting the measuring beam between the *M. lineare* eyespot and a transparent pigment-free part of the animal's body. An analog electronic circuit calculated the logarithm of the ratio of the intensities of the lights transmitted through the two positions, thus recording the optical density (= absorbance) of the sample.

The dark-adapted specimens were prepared in weak room illumination or under red light (> 630 nm). The red eyespot pigment could not, however, be seen under red light. Thus, we used a weak blue light for placing the circular measuring beam (diam. 5 μm) in proper position within the eyespot. This blue light would have bleached about 10% of the rhodopsin in a vertrebrate rod.

Electrophysiology

The electrophysiological experiments were carried out with DC amplification, and with conventional light stimulation and signal display systems (for details, see Lindström and Nilsson, 1983). The dark-adapted animals were kept on moist blotting paper in a brackish water film containing 10% dextran. Pilot studies with two recording techniques were performed: (1) the eyespots were penetrated with 10–20 MΩ glass micropipettes (tip diam. about 0.5 μm) filled with 1 M NaCl; (2) the whole front part of the animal, including eyespots, was sucked into a glass pipette (inner diam. 50–100 μm) filled with brackish water (0.6% NaCl). The DC resistance of this electrode in brackish water typically increased from 170 to 400 kΩ when the animal was sucked into it. The indifferent electrode was an Ag-AgCl wire in contact with the brackish water film bathing the caudal part of the worm.

Results

Light response experiments

The first indications of phototactic behaviour (i. e. movements away from bright light) were received from preliminary observations in ordinary microscopes without any heat absorbing filters. However, we would like to point out that in an inverted Nikon Diaphot TMD microscope (low voltage, brightness value 4 = 350 lux) the worms seemed to be indifferent. When the swimming movements were followed from below in a stereomicroscope, no special reactions were seen when the worms crossed borders between dark and bright fields or between fields of different colour (yellow, green, violet).

The distribution of worms was recorded in 72 light response experiments, where 8–12 worms/trough were exposed to a light gradient. In 48 of these experiments the worms were exposed to light for 2 min and in 24 experiments for 5 min. As controls 92 countings were made on 8–12 worms/trough kept in darkness. No significant difference, at the 0.05 level of significance, was observed with Students t-test between experimental and control worms (Table 1). There was no significant difference between worms exposed for 2 min and 5 min respectively, either.

Table 1: Average proportions of worms distributed in light gradient in 72 experiments and 92 controls. No difference at 0.05 level of significance between experimental and control worms. t and p: values received from Student's t-test.

Average proportions of worms in:	Experiment	Control	t	p
uncovered compartment	0.30 ± 0.17	0.32 ± 0.19	0.48	0.63
middle compartment	0.35 ± 0.18	0.31 ± 0.17	1.43	0.15
covered compartment	0.34 ± 0.19	0.37 ± 0.18	0.88	0.34

Autoradiography

Observations on light microscopic sections revealed a selective incorporation of (^{3}H)vitamin-A in the eyespot area (Fig. 2). Furthermore, more silver grains than background labelling were observed over the whole body, especially over the epidermis. The electron microscopic analysis showed an abundance of (^{3}H)vitamin-A incorporation in the pigment cells. In these cells, the labelling was restricted to cytoplasmic vacuoles, while the nuclei appeared unlabelled (Fig. 3). Labelling of the same type of vacuoles was observed beneath the epidermal cell web (Fig. 4). Only sparsely distributed silver grains were observed over the lamellated bodies (Fig. 5). The density of silver grains over the sensory processes at the body surface did not exceed that of the surrounding cells. No special distribution pattern was revealed between the processes bearing long or balloon-like cilia.

Microspectrophotometry

A light-microscopic examination at high magnification reveals that the eyespots of living or fresh *M. lineare* specimens contain a large number of yellow, orange or orange-red granules (diam. 0.5–1 μm). Similar granules can also be found sparsely distributed in other epidermal regions. In addition, the whole animal, including the eyespots, occassionally contains brown particles the abundance of which seems to increase when the animal is under physiological stress.

Stable microspectrophotometric recordings were obtained from a total of 22 eyespots. Spectra were measured from up to six positions within, or at the border of, an eyespot. Absorbance peaks or shoulders were observed at 400–410, 460–475 and 490–510 nm (Fig. 6A, B and C). In some specimens, the 460–475 nm peak exceeded the 500 nm peak (Fig. 6C), while the opposite was true in other animals (Fig. 6A). This variation suggets that at least two pigments build up the absorbance in the 450–500 nm region. As some of the orange granules have very high optical densities, while some light passes between granules, the absorbance spectra are somewhat distorted by «fractional saturation».

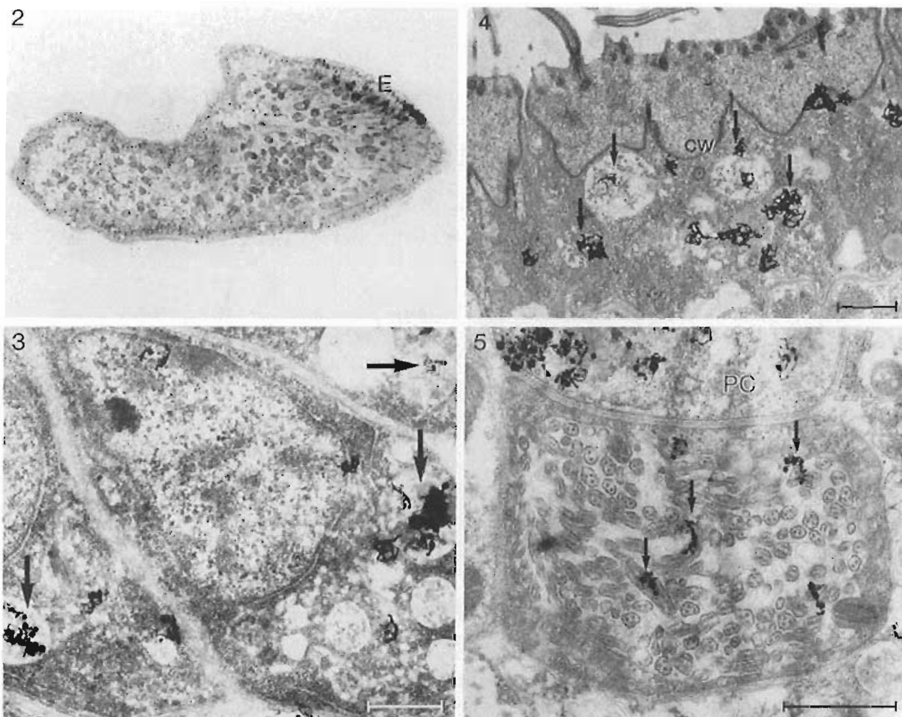

Fig. 2: *Microstomum lineare*. Light microscopic sagittal section of frontal part showing (^{3}H) vitamin-A labelling of eyespots (E) and epidermis.

Fig. 3: Details of pigment cells with (^{3}H) vitamin-A labelled vacuoles (arrows). Bar = 1 μm

Fig. 4: Detail of epidermis showing (^{3}H) vitamin-A labelled vacuoles (arrows) beneath cell web (cw). Bar = 1 μm

Fig. 5: *Microstomum lineare*. Ciliary lamellate body with sparsely (^{3}H) vitamin-A labelling over cilia (arrow). Note also labelling of pigment cell (PC). Bar = 1 μm

When recordings were made from an eyespot containing many brown particles, the 400 nm shoulder grew to a dominating peak. Similar 400 nm peaks were obtained from other regions in the body containing brown particles but no orange granules. Thus it is possible that the shoulder around 400 nm is always affected by this brown pigment.

The recordings marked «1» in Fig. 6 were obtained from dark-adapted specimens. They were followed by 1–2 min exposures to a white light intense enough to bleach over 90% of the rhodopsin in a vertebrate retina in less than 10 s. The recordings marked «2» were made after those exposures. No light-induced changes were observed. In other experiments the eyespots were exposed to intense and prolonged violet, blue and green irradiations. However, no significant and consistent spectral changes were noted.

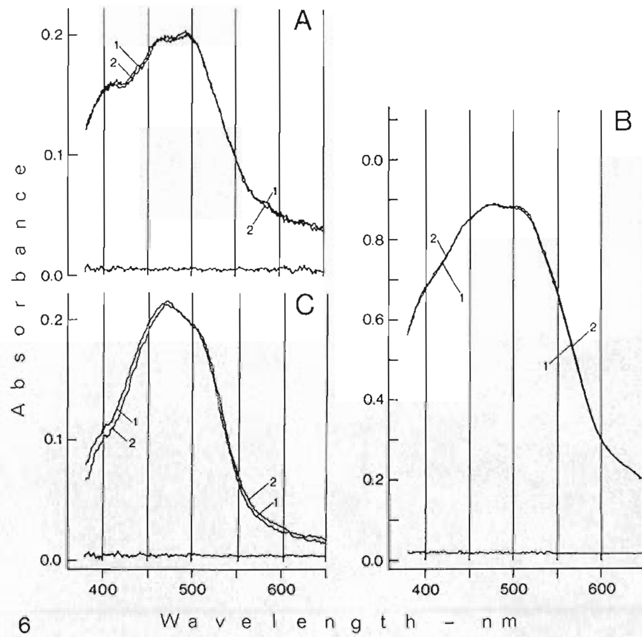

Fig. 6: *Microstomum lineare*. Absorbance spectra measured through three eyespots (A, B, C). No significant effects of light exposures are observed. Recordings marked «1» are measured before; recordings marked «2» after 1–2 min strong white light exposures. Lowermost horisontal tracings mark reference level. Note different ordinate scales.

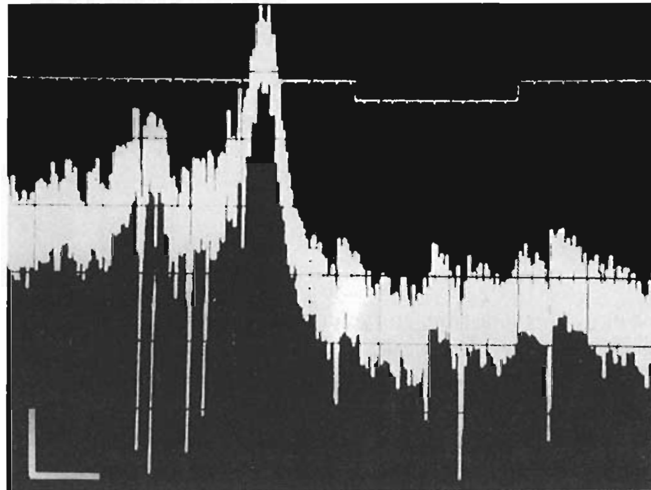

Fig. 7: Suction pipette recording from frontal part of initially dark-adapted animal. No voltage changes correlated with light exposures are observed. Upper tracing shows timing of strong white 1.2 s stimulus. Calibration bars at lower left corner indicate 0.5 s and 20 μV.

Electrophysiology

Neither microelectrode nor sucking electrode recordings revealed light-dependent responses exceeding the 5–20 μV noise fluctuations. The sucking electrode recordings showed slow potential changes and 10–15 ms «spikes» probably reflecting movements of the animal. This activity was not, however, related to light stimulation (Fig. 7).

Discussion

In the previous paper (Palmberg et al., 1980) the argumentation for the photoreceptory function of the eyespots in *M. lineare* was based only on structural similarities with other *presumed* photoreceptors. Especially, the lamellate body-type was presumed to be photoreceptive. Similar lamellate bodies have been observed in cercariae and miracidia of *Schistosoma mansoni* (Brooker, 1972; Short and Gagne', 1975). These larval stages which have no other presumed photoreceptors are known to show phototactic behaviour by reacting on light differences with typical shadow response (Saladin 1979, 1982) and by aggregating in light (Mason and Fripp, 1977). In the preliminary tests on *M. lineare*, however, neither aggregation at any irradiance level nor any response to shadow was observed. Furthermore, no light responses of worms were observed in the electrophysiological recordings from the eyespot area or in the behavioural observations in a light gradient.

Our light microscopical results of selective incorporation of (^{3}H)vitamin-A in the eyespot area, together with studies on well-developed visual systems of *Helix aspersa* (see Eakin and Brandenburg, 1968; Brandenburg and Eakin, 1970) and *Octopus* (see Robles et al., 1984) showing that vitamin-A uptake, intracellular transport and rhodopsin synthesis can be followed in invertebrate photoreceptor organelles, led to further investigations of (^{3}H)vitamin-A uptake in *M. lineare*. No clear indication of a vitamin-A based photopigment could, however, be demonstrated in the presumed photoreceptors in *M. lineare*. The sparse labelling over these cells could also be a result of radiation spread or translocation of the label from the abundantly labelled pigment cells. In addition the omission of BDMA (Borane-dimethylamine), to ensure stabilization of protein linkage, may have lead to translocation of the label during tissue processing (Hall and Bok, 1982).

No irradiation-induced spectral changes indicating rhodopsin bleaching or classical photoreceptor function were observed. However, our microspectrophotometric recordings cannot exclude the possibility that the eyespots contain small amounts of a real photosensitive (retinal- or flavinbased) receptor pigment. Even if the cilia membranes in the lamellate body (which has a diameter of only 10–15 μm) contained a normal rhodopsin-type pigment in a concentration comparable to that in vertebrate photoreceptor membranes, the amount of pigment might not be detectable by microspectrophotometry.

The photostable pigment seen in Fig. 6 has its main absorption in the 400–500 nm band. Since many biological pigments (i.e. carotenoids, flavins, ommochromes, pteridines) absorb in this same region, our microspectrophotometric recordings cannot be used for a reliable chemical identification, especially as the spectra were obtained from non-homogenous clusters of orange-red granules and occasional brown particles. However, a paper chromatographic analysis of an extract of 100 *M. lineare* heads did not support the pteridine hypothesis (M. Reuter, unpubl. results). The general shape of the curves in Fig. 6, and the red fluorescence of the pigment cell vacuoles observed with the Falck-Hillarp method (Palmberg et al., 1980), are suggestive of a carotenoid nature of the

pigment. The accumulation of vitamin-A in these vacuoles agrees with the idea that they are lipophilic.

What could be the function of this presumed carotenoid? It is well known that many biological pigments, including carotenoids, serve a protective function by quenching photosensitizers and «exited oxygen» and thereby conferring protection against oxidative damage to organisms living in the presence of light and oxygen (Krinsky 1978; Presti, 1983; Ostrovsky et al., 1987). We therefore suggest that the eyespots of *M. lineare* contain some photo-oxidation-sensitive structures (whic of course may be photoreceptors) and that the role of the orange granules may be to protect this structure (or these structures) from photo-damage.

We want to emphasize that neither the microspectrophotometric, nor the electrophysiological methods employed were very sensitive in relation to the task of detecting one or a few small photoreceptors, and that an autoradiographic labelling of an invertebrate photoreceptor may be difficult, since a typical invertebrate visual pigment is not split into opsin and vitamin-A by light. Synthesis of new photo-pigment may therefore be a prerequisite for a vitamin-A uptake.

Thus, at present, although we have no positive evidence for a photoreceptive function of the eyespots, we have no very strong evidence against that hypothesis, either.

Acknowledgements

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