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Article *in* Cell and Tissue Research · October 2005

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DOI 10.1007/s00441-004-1069-1

REGULAR ARTICLE

Jochen Lampel · Adriana D. Briscoe · Lutz T. Wasserthal

Expression of UV-, blue-, long-wavelength-sensitive opsins and melatonin in extraretinal photoreceptors of the optic lobes of hawkmoths

Received: 15 September 2004 / Accepted: 3 December 2004 / Published online: 21 July 2005

Abstract Lepidopterans display biological rhythms associated with egg laying, eclosion and flight activity but the photoreceptors that mediate these behavioural patterns are largely unknown. To further our progress in identifying candidate light-input channels for the lepidopteran circadian system, we have developed polyclonal antibodies against ultraviolet (UV)-, blue- and extraretinal long-wavelength (LW)-sensitive opsins and examined opsin immunoreactivity in the adult optic lobes of four hawkmoths, *Manduca sexta*, *Acherontia atropos*, *Agrius convolvuli* and *Hippotion celerio*. Outside the retina, UV and blue opsin protein expression is restricted to the adult stemmata, with no apparent expression elsewhere in the brain. Melatonin, which is known to have a seasonal influence on reproduction and behaviour, is expressed with opsins in adult stemmata together with visual arrestin and chaoptin. By contrast, the LW opsin protein is not expressed in the retina or stemmata but rather exhibits a distinct and widespread distribution in dorsal and ventral neurons of the optic lobes. The lamina, medulla, lobula and lobula plate, accessory medulla and adjacent neurons innervating this structure also exhibit strong LW opsin immunoreactivity. Together with the adult stemmata, these neurons appear to be functional photoreceptors, as visual arrestin, chaoptin and melatonin are also co-expressed with LW opsin. These findings are the first to suggest a role for three spectrally distinct classes of opsin in the extraretinal detection of changes in ambient light and to show melatonin-mediated neuroendocrine output in the entrainment of sphingid moth circadian and/or photoperiodic rhythms.

Keywords Adult stemmata · Brain opsins · Arrestin · Chaoptin · Photoperiodism · Hawkmoths (Insecta)

This work was partially supported by the Canadian Institute for Advanced Research (A.D.B.) and the National Science Foundation (grant nos. IBN-0082700 and IBN-0346765; A.D.B.).

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Introduction

All organisms show biological rhythms. They are expressed in daily circadian and seasonal photoperiodic rhythms associated with observed changes in organism physiology and behaviour and with changes in clock gene expression levels. In a series of pioneering experiments, egg hatching behaviour in silkmoths was shown to be controlled by circadian clocks (Riddiford and Johnson 1971) and extraretinal photoreceptors in the silkmoth brain were shown to control the timing of eclosion (Truman and Riddiford 1970; Truman 1972). Photoperiodic clocks have also been associated with eclosion in the hawkmoth, *Manduca sexta* (Shiga et al. 2003). Moreover, flight activity of migratory lepidopterans is known to be under circadian control (Mouritsen and Frost 2002; Froy et al. 2003).

The past few years have seen tremendous progress in our understanding of the molecular, biochemical, morphological and behavioural components of these clocks in insects (for a review, see Saunders 2002). One important *Zeitgeber* ("time giver") of these rhythms is the external light-dark cycle. Specialized photoreceptors are essential for the detection of this alteration in light stimuli. In *Drosophila melanogaster*, about which we know the most with regard to these clocks, a number of photoreceptor systems are needed for the entrainment of a circadian rhythm: the compound eyes, the dorsal ocelli, the extraretinal Hofbauer-Buchner eyelet and the blue-light flavoprotein cryptochrome located in some neurons of the brain (Hofbauer and Buchner 1989; Yasuyama and Meinertzhagen 1999; Helfrich-Förster et al. 2002; Malpel et al. 2002). Further-

more, a fifth unknown photopigment system has been reported to exist in the clock-gene-expressing dorsal neurons (Rieger et al. 2003). The photoreceptor basis of circadian and photoperiodic rhythms is less well understood for lepidopterans but, in addition to the compound eyes, extraretinal photoreceptors homologous to the *Drosophila* extraretinal Hofbauer-Buchner eyelet (Melzer and Paulus 1989) have been characterized anatomically and physiologically (Ichikawa 1991). In other insect orders, these adult stemmata have been found in hymenopterans (Felisberti and Ventura 1996), dipterans other than *Drosophila* (Seifert et al. 1987), coleopterans (Schulz et al. 1984; Felisberti et al. 1997; Hariyama 2000; Fleissner and Fleissner 2003) and trichopterans (Hagberg 1986), suggesting a similar role for this photoreceptive organ in the entrainment of circadian rhythms in insects.

Opsins have been shown to be the primary photopigment molecules that respond to stimuli from the light environment. Their pattern of spatial expression and mechanistic involvement in circadian clocks has been best resolved in *Drosophila* (e.g. Zordan et al. 2001; Rieger et al. 2003): five opsins are expressed in the compound eye (Cowman et al. 1986; Zuker et al. 1987; Montell et al. 1987; Chou et al. 1996; Huber et al. 1997; Papatsenko et al. 1997) and two of these are also expressed in the Hofbauer-Buchner eyelet (Yasuyama and Meinertzhagen 1999; Helfrich-Förster et al. 2002; Malpel et al. 2002). In sphingid moths, three visual pigments, UV-, blue- and long-wavelength (LW)-sensitive, have been found in the compound eye (Schwemer and Paulsen 1973; Bennett and Brown 1985; White et al. 2003). So far, little has been reported about the presence and distribution of extraretinal opsins in insects. Briscoe and White (2005) have found that two opsin mRNA transcripts (UV and green) are expressed in the adult stemmata of the butterfly *Vanessa cardui*. In the butterfly *Papilio glaucus*, immunohistochemical and in situ hybridization studies have demonstrated that one of six cloned opsins (Briscoe 1998, 2000), PglRh4 (exo-opsin), is not expressed in the retina but rather is found in some neurons of the brain (A.D. Briscoe, unpublished). An extraretinal opsin has been cloned and localized in the larval brain of *Bombyx mori* (Shimizu et al. 2001). Some neurosecretory neurons display anti-bovine opsin immunoreactivity in the protocerebrum of the moth *Antheraea pernyi* (Takeda et al. 1999). Finally, in the vetch aphid, the anterior ventral protocerebrum has been immunolabelled by several vertebrate and insect anti-opsin antibodies (Gao et al. 1999).

The photoperiodic timing of annual rhythms requires a functional circadian clock that compares the day length measured by extraretinal photoreceptors with an internal value (Saunders 2002). Such extraretinal opsins have been proposed to be the photoreceptors of the photoperiodic clock in insects (Numata et al. 1997; Shimizu et al. 2001).

Other than the above-mentioned photopigments, the neuroendocrine mediator of biological rhythms is also not well understood in any insect. The neurohormone melatonin, which is found in animals, plants, protozoans and bacteria, is presumably the major transmitter for the circadian and photoperiodic rhythm in metazoans (Vivien-Roels and Pévet 1993; Hardeland 1997). Vivien-Roels et al. (1984) first showed melatonin in the compound eyes of insects (locusts) by radioimmunoessays. Melatonin has also been detected in the *Drosophila* brain, together with a circadian variation in its synthesis (Callebert et al. 1991; Hintermann et al. 1996), and a higher peak of this compound at night has been described in the organs of other insects (for reviews, see Vivien-Roels and Pévet 1993; Saunders 2002). Lastly, L'Hélias et al. (1995) have shown a circadian rhythm of serotonin *N*-acetyltransferase (NAT), an enzyme essential for melatonin synthesis, in the brain and stemmata of the caterpillar *Pieris brassicae*.

Despite the existence of a number of physiological investigations of melatonin (see for example, Hodková 1989; Richter et al. 2000; Niva and Takeda 2003), few reports exist concerning its spatial distribution or the distribution of its synthesizing enzymes in the nervous system of insects. For instance, Nässel (1987) has described a melatonin-like immunoreactivity in the central body of the blowfly protocerebrum. The cockroach *Periplaneta americana* also exhibits melatonin-like labelling in several brain regions (Takeda et al. 1988), and NAT-like immunoreactivity has been demonstrated in some neurosecretory neurons of the brain of the silkmoth *Antheraea pernyi* (Takeda et al. 1999).

In the present study, we have examined four hawkmoth species in order to further our knowledge of the extraretinal photoreceptors present in the optic lobes of lepidopterans and to identify candidate neuroendocrine mediators of their responses to the photoperiod. Much is known about the neuroanatomy of hawkmoths such as *Manduca sexta* and their large brains represent an attractive system for physiological and behavioural manipulations. We have examined the structure of the adult stemmata by conventional anatomical and electron-microscopical techniques. In order to characterize these organs further, we have developed novel specific polyclonal antibodies against UV- and blue-sensitive opsins and examined their spatial expression patterns in the adult stemmata. We have also found immunocytochemical evidence for the existence of a second extraretinal photoreceptor system in some neurons of the optic lobes by using newly developed polyclonal antibodies against an extraretinal LW opsin. To test whether these neurons are indeed functional photoreceptors, we have also used antibodies against arrestin and chaoptin. Visual arrestin, which is important in the deactivation of rhodopsin (Dolph et al. 1993) has been found in the retina and some neurons of the brain of *Drosophila* (Lieb et al. 1991; Dolph et al. 1993; van Swinderen and Hall 1995). Other investigators have found arrestin in the optic lobes of beetles (Fleissner and Fleissner 2003) and caddisflies (Hagberg 1986), in the protocerebrum of aphids (Gao et al. 1999) and in the optic lobes and the central brain of blowflies (Nässel 1987; Cymborowski and Korf 1995). Finally, we have studied the expression of melatonin in the

retina, in the adult stemmata, and in the neurons containing the LW extraretinal opsin.

Materials and methods

Animals

Four hawkmoth species were used in this study: three from the Sphinginae subfamily, viz. *Acherontia atropos* (L.), *Agrius convolvuli* (L.) and *Manduca sexta* (L.), and one from the Macroglossinae subfamily, viz. *Hippotion celerio* (L.). The larvae were reared on their host plants (*Ligustrum* spp., *Acherontia*; *Convolvulus arvensis*, *Agrius*; *Cissus antarctica*, *Hippotion*) and *Manduca sexta* was provided with an artificial diet (Dr. J. Schachtner, Marburg, personal communication). The adults were kept in flight cages at 25°C and 80% relative humidity under natural environmental light conditions and allowed to feed on a 15% honey solution from artificial flowers. *Acherontia atropos* was fed by hand with a 50% honey solution every third day.

Morphology

Both males and females were used. The animals, which were dark-adapted in the evening for 1 h in the spring or summer, were anaesthetized with CO_2 and decapitated. The brains were dissected in phosphate-buffered saline (PBS, 0.1 M, pH 7.4) under red dim light from the posterior ventral side. For documentation, a Leica MZ8 photomacroscope with a MPS60 controlling unit was used.

Reduced silver staining

Brains were fixed in Carnoy's solution (60% ethanol, 30% chloroform, 10% glacial acetic acid) for 2–3 h at 4°C and, after dehydration in an ethanol series, embedded in paraffin. Sections were cut (10 μm thick), mounted on silane-coated slides, dewaxed in Rotihistol (Roth), rehydrated and stained according Tyrer et al. (2000). Briefly, slides were incubated in 20% silver nitrate for 3 h in the dark and then transferred into the impregnation solution (150 ml distilled water, 12 ml borax buffer pH 7.0, 12 ml 1% silver nitrate, 6 ml lutidine) for 24 h at 50°C. After being washed in distilled water, the sections were rinsed in 2% sodium sulphite for 2 min, washed again in distilled water and transferred into the developing solution (170 ml 9% sodium sulphite, 5 ml 5% silver nitrate, 11.25 ml 0.5% hydroquinone) for 6 min in the dark. The slides were washed in distilled water, tap water and distilled water for 5 min each. The 5-min immersions in a solution containing 0.2% gold chloride, a few drops of glacial acetic acid, 2% oxalic acid and 5% sodium thiosulphate were each interspersed with a 5-min wash in distilled water. The sections were dehydrated, transferred to Rotihistol, and mounted in DPX (BDH Laboratory Supplies).

Antibody generation

Polyclonal antibodies for the UV opsin, blue opsin and exo-opsin were generated commercially in rabbits (Quality Controlled Biochemicals) against C-terminal peptide sequences of the butterfly *Papilio glaucus* opsins PglRh4, PglRh5, and PglRh6 (Briscoe 1998, 2000; see Table 1 for the peptides used). The antiserum was immunoaffinity-purified on a $CNBr^-$-activated sepharose 4B column (Pharmacia Biotech) to which the peptides had been covalently attached. Briefly, 0.5 mg peptide was diluted in 5 ml sodium borate solution (0.5 M NaCl, 0.1 M sodium borate, pH 8.0), allowed to dissolve for 30 min and spun for 10 min at 4°C. The column was prepared by placing 300 mg sepharose-$CNBr^-$ beads on Whatman filter paper, saturating them with 200 ml 1 mM HCl and briefly applying a vacuum to dry the beads. The sepharose beads were added to the protein solution, mixed for 4 h at room temperature and spun for 5 min at 2000 rpm and the supernatant was removed. The beads were resuspended in 10 ml 0.5 M TRIS, pH 8.0, at room temperature, and spun again. The supernatant was again removed and the procedure repeated twice more, except that, on the last spin, 1 ml supernatant was left on the beads. The sepharose slurry was packed into a column, bubbles were eliminated with a Pasteur pipette and the column was washed with 6 ml 1.0 M NaCl, 0.1 M sodium borate, followed by 6 ml 1 M NaCl, 0.1 M sodium acetate, pH 4.0. These two washes were repeated and then 50 ml PBS/EDTA (PBS with 1 mM EDTA and 0.02% NaN_3) was applied to the column. The serum was spun briefly at 2000 rpm for 5 min to eliminate aggregates and diluted in PTX (0.15 M NaCl, 10 mM $NaHPO_4$ pH 7.5, 1 mM EDTA, 0.2% Triton X-100, 0.02% NaN_3). The PTX-diluted serum (20 ml) was loaded onto the column and fractions were collected and recycled through the column. The column was washed first with 20 ml 0.5 M NaCl-PTX, pH 7.5, to remove non-specific binding interactions and then with 50 ml PBS-EDTA. Antibodies were eluted with 8 ml 0.2 M glycine-HCl, pH 2.8, and 800-μl fractions were collected into tubes containing 200 μl 2 M TRIS, pH 8.0, to neutralize the glycine. The column itself was neutralized by washing with 5 ml 1 M K_2HPO_4, pH 9.4, followed by 50 ml PBS/A (PBS, 0.02% NaN_3). The antibodies were finally dialyzed against PBS/A for 4 h on ice and the antibody yield was quantified by using a spectrophotometer. Finally, the immunoaffinity purified antibodies were diluted 1:1 in glycerol and stored at −80°C.

Table 1 Peptides used for the preadsorption experiments

Antibody	Amino acid sequence of binding site
PglRh4	CQAASDEVDSVASNV
PglRh5	CISHPKYRQELQKRMP
PglRh6	CGVREQDPDSVSTS
F4C1 (arrestin)	YIDHVERVEPVDGVVLVDPEL

Western blot analysis

For immunoblotting, optic lobe and retina proteins were extracted in a lysis buffer consisting of 150 mM NaCl, 50 mM TRIS-HCl pH 7.4, 0.1% sodium dodecylsulphate (SDS), 0.5% Triton-X 100. Protein extracts (10–15 μg total protein in 10 μl) were mixed with 10 μl sample buffer (0.56 ml 1 M TRIS-HCl pH 6.8, 3.0 ml 10% SDS, 1.0 ml glycerol, 1.0 ml 2% bromphenolblue, 4.35 ml H_2O, 500 μl mercaptoethanol) and loaded into wells of a discontinuous (12% separating, 4% stacking) SDS-polyacrylamide gel. The proteins were transferred to nitrocellulose membranes by electroblotting. After incubation of the membranes in 5% non-fat milk powder for 1 h, immunolabelling was performed with anti-PglRh4 (exo-opsin) antibodies at a 1:200 dilution, anti-arrestin antibodies (MAb F4C1, kindly provided by Dr. L.A. Donoso, Philadelphia) at a 1:2000 dilution and anti-chaoptin antibodies (MAb 24B10, Developmental Studies Hybridoma Bank) at a 1:200 dilution for the optic lobe extracts and with anti-PglRh5 (UV) and anti-PglRh6 (blue) antibodies at a 1:200 dilution for the retina extracts for 1 h. Immunoreactivity was visualized by using the R.T.U. Vectastain Universal Elite ABC Kit and the 3,3′-diaminobenzidine substrate kit (DAB) for peroxidase with nickel intensification (Vector Laboratories).

Immunocytochemistry

Immunocytochemistry was performed on paraffin sections by means of the indirect peroxidase-antiperoxidase (PAP) method. The brains were fixed in Boer fixative (0.9% picric acid, 6% glutaraldehyde, 0.5% glacial acetic acid) for 2 h at 4°C and treated as mentioned above. After incubation in a 0.3% H_2O_2/100% methanol solution for 30 min, the slides were washed three times in TRIS-HCl buffer (0.05 M, 1.5% NaCl, pH 7.6) for 5 min. To reduce nonspecific background staining, the sections were incubated for 30 min with the blocking solution of The One Kit (Sternberger Monoclonals) and the primary antibodies diluted in PBT (PBS, 0.5% bovine serum albumin, 1% normal goat serum, 0.03% Triton X-100, 0.02% NaN_3) were applied for 16 h at 4°C in a humid chamber. The immunoaffinity-purified polyclonal rabbit anti-*Papilio*-extraretinal opsin antibody (PglRh4) was diluted 1:200, the monoclonal mouse anti-bovine-arrestin antiserum (MAbF4C1) was diluted 1:2,000 and the monoclonal mouse anti-*Drosophila*-chaoptin antiserum (MAb 24B10) was diluted 1:200. After several washes in TRIS-HCl buffer, sections were incubated at room temperature for 30 min with secondary antibodies of The One Kit followed by 30 min with The One Kit activity select PAP solution. Subsequently, staining was developed for 10 min with the DAB Kit. The sections were dehydrated and mounted in DPX.

Immunocytochemistry was also performed on cryostat sections by using the indirect immunofluorescence technique. After fixation in 4% formaldehyde in PBS for 2 h at 4°C, the tissue was infiltrated in a 16% sucrose solution overnight at 4°C and embedded in O.C.T. Tissue Tek (Sakura Finetek). Thick slices (5–10 μm) were cut, mounted on Superfrost Plus glass slides (Menzel), post-fixed in 100% ice-cold acetone for 5 min, permeabilized in a cytoskeletal buffer (10 mM HEPES pH 7.4, 200 mM sucrose, 3 mM $MgCl_2$, 50 mM NaCl, 0.5% Triton X-100, 0.02% NaN_3; Chou et al. 1996) for 5 min, treated for 5 min in a 0.5% SDS solution in PBS for antigen retrieval, washed three times in PBS containing 0.01% saponin and incubated in the primary antibodies for 16 h at 4°C in PBT with a 1:100 dilution for anti-PglRh5 (UV) and anti-PglRh6 (blue), a 1:1,000 dilution for anti-arrestin (MAb F4C1) and a 1:1,000 dilution for a polyclonal sheep anti-human-melatonin antiserum (AB/S/021, Stockgrand). Finally, Cy3-conjugated goat anti-rabbit, goat anti-mouse or donkey anti-sheep IgG (Jackson ImmunoResearch Laboratories) in PBT containing 1 μg/ml Hoechst 33258 (Sigma-Aldrich) was used as the secondary antibody at a 1:100 dilution and the slides were coverslipped in Fluoromount G (Southern Biotechnology Associates).

For double-immunofluorescence labelling, the optic lobes were treated as described above. The slides were incubated in a mixture of sheep anti-human-melatonin antiserum (1:1,000) and anti-PglRh4 antiserum (1:100). As a secondary IgG mixture, Cy2-conjugated goat anti-rabbit and Cy3-conjugated donkey anti-sheep IgG (Jackson ImmunoResearch Laboratories) were used at a 1:100 dilution.

Specificity controls included omitting the primary antibodies and preadsorbing the antibodies with specific opsin and arrestin peptides (Table 1) and with melatonin. The liquid-phase preadsorption of the diluted antisera was performed with 1 μM melatonin or peptide solution and eliminated all immunoreactivity in the optic lobes and retina.

All micrographs were taken with a Zeiss Axiophot microscope with differential interference contrast and epifluorescence. A F-View II-Camera (Soft Imaging System) was used to collect images. Selected images were digitally processed to improve contrast and brightness in Adobe Photoshop 7.0.

Transmission electron microscopy

The tissue was dissected in 0.08 M phosphate-buffered 2% glutaraldehyde/formaldehyde with 4% sucrose and fixed for 2 h at 4°C. After being washed in buffer and following postfixation in 2% OsO_4 at 4°C for 2 h, the specimens were dehydrated in ethanol and embedded in Epon 812. Ultrathin sections were cut, mounted on grids and, after being stained with uranyl acetate and lead citrate, examined in a Zeiss EM 10 electron microscope. Semithin sections were stained with 0.1% toluidine-blue/borax.

Results

Nomenclature for the neuroarchitecture of the optic lobes follows that of Homberg and Hildebrand (1989). Generally, the term “extraretinal photoreceptors” denotes opsin-

immunoreactive neurons in the optic lobes including the adult stemmata. In all four sphingid moth species investigated, almost no differences in the morphological and immunohistochemical results were found.

Morphology of the adult stemmata

In each optic lobe, dark pigmented structures were present along the posterior side. These retinular cells of the adult stemmata were distributed in clusters in *Hippotion celerio* (Fig. 1a) but were also dispersed all over the posterior region of the optic lobes as in *Manduca sexta* (Fig. 1b). In *Acherontia atropos* and *Agrius convolvuli*, the stemmata accumulated in clusters in the posterior median region of the optic lobes (data not shown). This cluster normally consisted of six stemmata innervating the accessory medulla, the former larval optic neuropil, by crossing the inner optic chiasma (Fig. 1c). Two nerve fibres seemed to branch into the medulla from the distal part of the accessory medulla (Fig. 1c). Additionally, a further nerve could be observed running to the accessory lamina (data not shown). The diameter of the stemmata varied between 50 μm and 150 μm within the investigated species. In Epon sections, we found that, in all species, the six adult stemmata were comprised of about 40 rhabdomere-containing retinular cells (or approximately 6–7 retinular cells per stemma). Six of these retinular cells can be seen in Fig. 1d. Additionally, some constituent parts of the dioptric apparatus of each stemma were still present, viz. the crystalline cones and their forming cells (Fig. 1d). Each stemma was also surrounded by a thick glial sheath (Fig. 1d). The location of the adult stemmata in the optic lobe relative to other neuropil is shown in Fig. 1e. At the ultrastructural level, we observed that the retinular cells were filled with globular electron-dense shielding pigments (Fig. 2a–c). Two to four retinular cells together formed a fused rhabdom by the individual contributions of their rhabdomeric microvilli (Figs. 1d, 2a, b_1). In some cases, the rhabdomeres appeared to be unfused (Fig. $2b_2$). The rhabdomeres themselves were oriented perpendicular to each other, perhaps indicating their polarized light sensitivity (Fig. $2b_{1,2}$). Rhabdom-turnover products were also present. Occasional multivesicular bodies, which presumably were involved in the maintenance and turnover of the microvilli (White 1968), were situated in the periphery of the rhabdoms and within the retinular cells (Fig. 2c). We also often found a rudimentary dioptric apparatus with the crystalline cones and their forming cells located at the rim of each stemma (Fig. 2d). The crystalline cones themselves appeared to be composed of orthogonally oriented, fibrous, tubular structures. A number of mitochondria were located around the periphery of the crystalline cones (Fig. 2d). Together, these morphological observations suggested that the stemmata present in the adult optic lobes contained functional photoreceptors.

Western blotting

In order to determine the specificity of the anti-UV and anti-blue opsin protein antibodies, an SDS-PAGE/blot analysis of retina homogenates of *Manduca sexta* was performed; two bands of proteins were seen (Fig. 3). An unknown protein with a molecular weight of about 40 kDa was detected by using the anti-PglRh6 (blue-sensitive opsin) antibody (Fig. 3, lane 1). A protein with a slightly higher molecular weight was detected by using the anti-PglRh5 (UV-sensitive opsin; Fig. 3, lane 2). Both lanes were accompanied by weak additional bands. Similarly sized bands (approximately 37 kDa) were detected for the P357, P450 and P520 opsins of *Manduca sexta* (White et al. 2003). Optic lobe homogenates without the retina were also investigated. A protein with an even larger molecular weight (>40 kDa) was detected with the anti-PglRh4 antibody (exo-opsin, Fig. 3, lane 3). A protein of about 50 kDa was detected by using the anti-arrestin (F4C1) antibody (Fig. 3, lane 4), which was similar in size (50 kDa) to a neuropteran in-vitro-translated arrestin originally cloned from UV photoreceptor cells of the compound eye (Bentrop et al. 2001). The anti-chaoptin (24B10) antibody detected a 160-kDa epitope (Fig. 3, lane 5). This was similar in size (160 kDa) to the *Drosophila melanogaster* chaoptin protein (Zipursky et al. 1984). Preadsorption with the corresponding peptides eliminated immunoreactivity completely (data not shown).

Immunocytochemistry of adult stemmata

Arrestin, chaoptin and two types of opsin We were interested in obtaining immunocytochemical evidence that the stemmata were functional in adult animals and so we used a set of antibodies to test for the presence of photoreceptor-cell-specific proteins. An antiserum against arrestin (F4C1) stained the whole retina of the compound eyes (Fig. 4b). This antiserum also labelled a subset of retinular cells of the adult stemmata, most intensely in the rhabdomeres (Fig. 4c). Separate immunolabelling of the retina of the compound eyes and the adult stemmata with an antiserum against chaoptin (24B10) revealed immunoreactivity within these photoreceptor structures (Fig. 4a, d). Antibodies against the butterfly UV opsin (PglRh5) and the blue opsin (PglRh6) exhibited immunostaining in a subset of retinular cells of the adult stemmata. The rhabdoms showed especially strong immunolabelling (Fig. 4e, f). However, we could not determine whether the UV and blue opsins were coexpressed in the same retinular cells. We observed no immunolabelling of either the retina or the stemmata by using the anti-LW (PglRh4) antibody. After preadsorption of the antibodies with specific peptides, the labelling was abolished (data not shown).

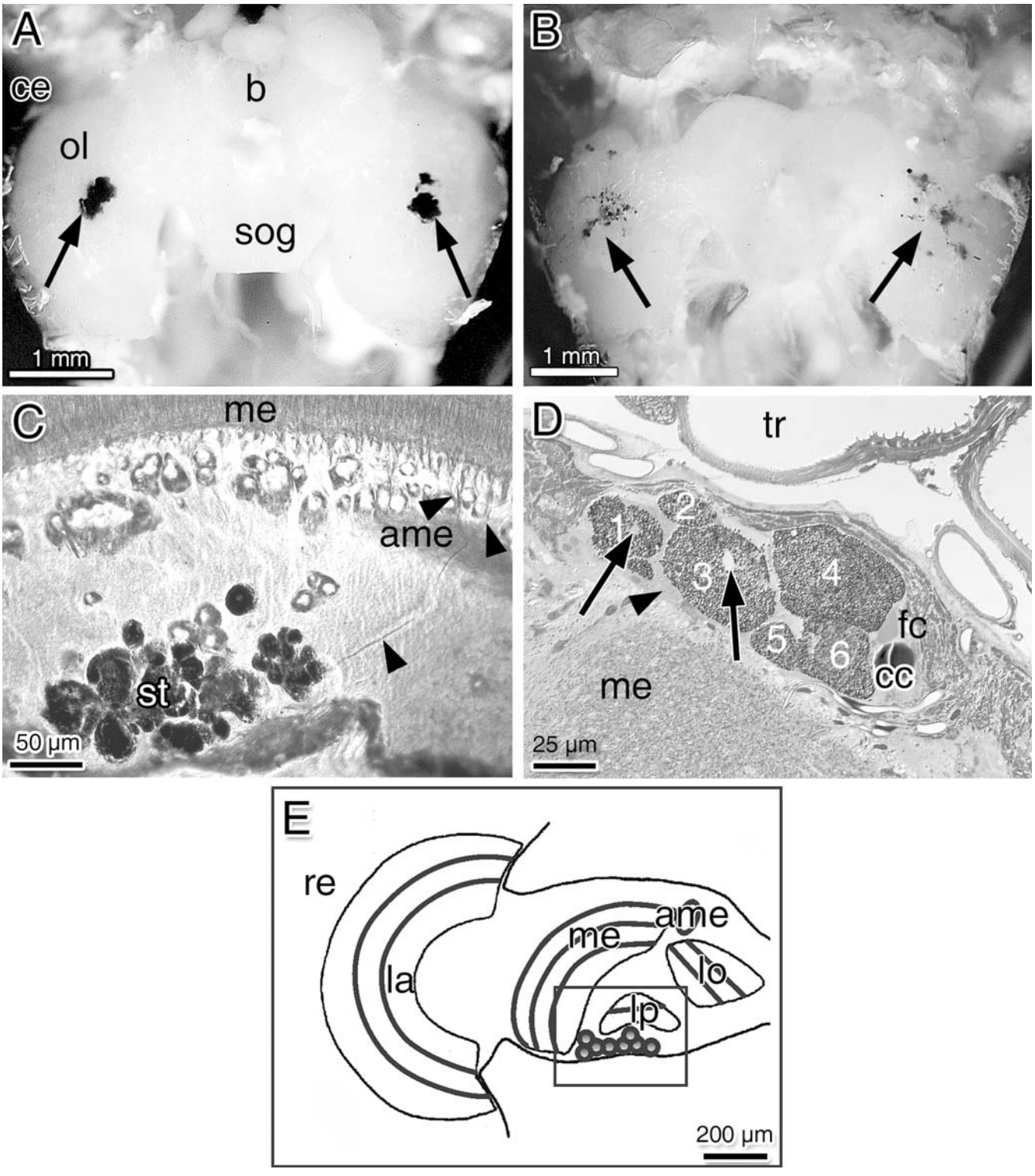

Fig. 1 **a** Posterior view of the brain (*b*), subesophageal ganglion (*sog*), compound eye (*ce*) and optic lobes (*ol*) of *Hippotion celerio* (*arrows* clusters of the stemma retinular cells at the posterior margin of the optic lobes). **b** Posterior view of the brain and the optic lobes of *Manduca sexta*. Note that the adult stemma retinular cells are dispersed all over the optic lobes (*arrows*). **c** Oblique sagittal paraffin section of the optic lobe of *Hippotion celerio*. Reduced silver staining. From the stemma (*st*), a nerve fibre projects to the accessory medulla (*ame*) and shows two arborizations to the medulla (*me*, *arrowheads*). **d** Frontal semithin section of the optic lobe of *Agrius convolvuli*. Six retinular cells (*1–6*) can be distinguished (*arrowhead* glial sheath, *arrows* fused rhabdom). A crystalline cone (*cc*) and its forming cell (*fc*) can still be seen (*tr* trachea, *me* medulla). **e** Diagrammatic dorsal view of the optic lobe (*box* position of the adult stemmata, *la* lamina, *lo* lobula, *lp* lobula plate, *re* retina, *me* medulla, *ame* accessory medulla)

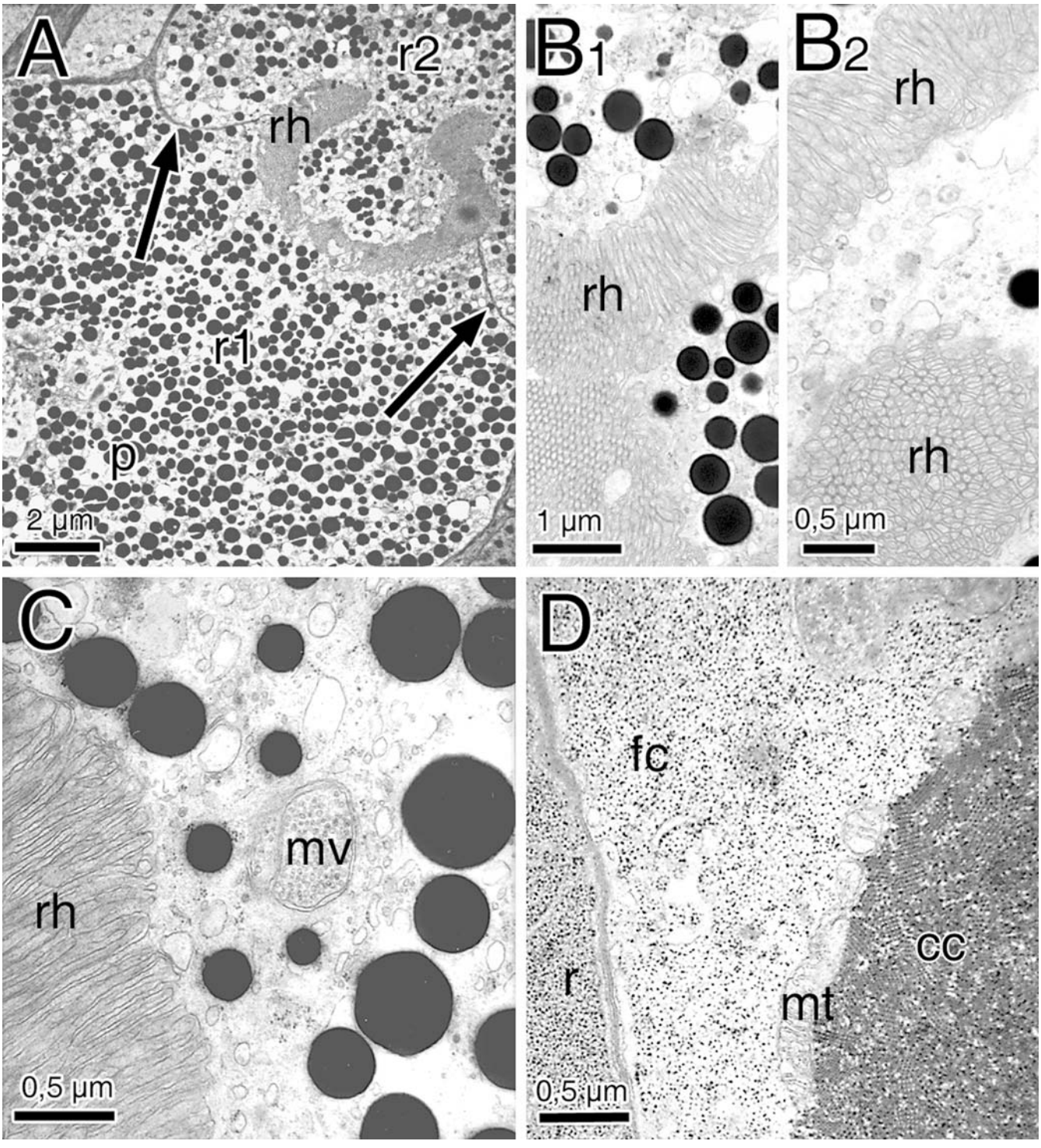

Fig. 2 Cross sections of an imaginal stemma of *Agrius convolvuli*. **a** Electron microscopy reveals two pigment (*p*) granule-containing retinular cells (*r1*, *r2*) forming a rhabdom (*rh*). Cell membranes are indicated (*arrows*). **b$_1$** Microvilli of the fused rhabdomeres (*rh*) are arranged orthogonally, indicating a possible polarization sensitivity. **b$_2$** Open rhabdoms (*rh*) can occasionally be found. **c** Multivesicular body (*mv*) at the periphery of the rhabdom (*rh*). **d** The crystalline cone (*cc*) is bordered by several mitochondria (*mt*) and consists of regularly arranged tubular material (*fc* lens-forming cell, *r* retinular cell)

Melatonin immunocytochemistry We were also interested in investigating the distribution of melatonin in the retina and optic lobe. The anti-melatonin antibody that we used gave strong melatonin-like immunoreactivity within the whole retina of the compound eyes. The basal region of the retina showed strong autofluorescence because of the presence of tracheoles in the tapetum (Fig. 5a). Weaker melatonin-like immunoreactivity was found in the former larval stemmata in which only a subset of the retinular cells was stained (Fig. 5b, arrowheads). Some surrounding

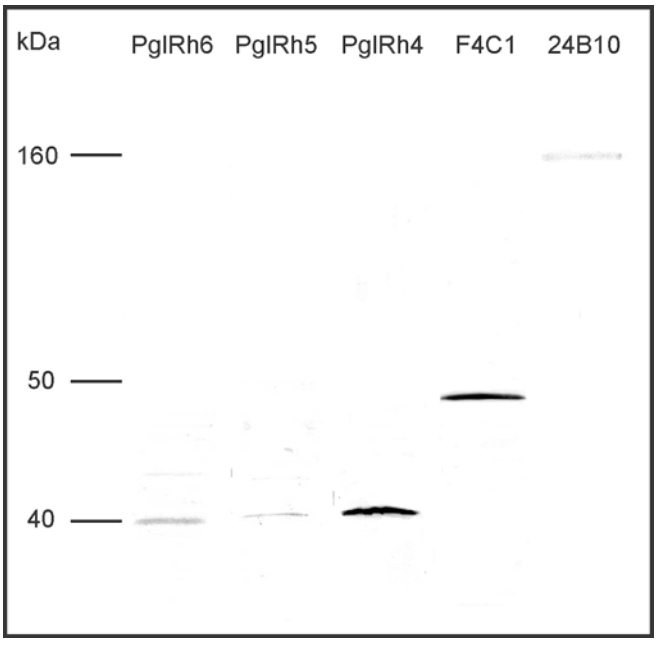

Fig. 3 Immunoblot detection of visual proteins. Detection of blue opsin (*PglRh6*) and UV opsin (*PglRh5*) in retina extracts and immunodetection of extraretinal opsin (*PglRh4*), arrestin (*F4C1*) and chaoptin (*24B10*) in optic lobe extracts of *Manduca sexta*. Positions of protein standards are indicated *left*

neurons also exhibited strong melatonin-like immunoreactivity. After liquid-phase preadsorption of the antibody with melatonin, the labelling was abolished (data not shown).

Extraretinal photoreceptors in neurons of the optic lobes

Arrestin and chaoptin immunocytochemistry Arrestin-like immunolabelling was found in the third layer of the second optic neuropil, in the medulla and in the first optic chiasma. In addition, some perikarya of the optic lobe group 1 and optic lobe group 2 (Homberg and Hildebrand 1989), located in the dorsomedial and the ventromedial edge of the medulla, showed strong to medium arrestin labelling (Fig. 6a). Weak chaoptin-like immunoreactivity was evident in perikarya surrounding the accessory medulla in which several cell bodies were stained (Fig. 6b).

Extraretinal opsin immunocytochemistry We found extraretinal opsin expressed in all neuropil structures and in a total number of about 300 perikarya of the optic lobe in all investigated species, by counting serial paraffin sections of six animals. We observed immunolabelling in the fibres of the lamina and accessory lamina and in some layers of the medulla (Fig. 7a, b). We also observed strong immunoreactivity in the accessory medulla located at the frontal rim of the medulla in which about ten immunolabeled somata were found with strong ramifications in this former larval neuropil (Fig. 7c). Additionally, the lobula and the lobula plate revealed weaker exo-opsin labelling (Fig. 7d, e). The surrounding neurons of these neuropils showed no immunoreactivity. Several immunoreactive perikarya were labelled at the dorsal and ventral edge of the medulla, in the periphery of the former larval stemmata and at the dorsal edge of the lobula (Fig. 7f–i).

Colocalization experiments Using double-immunofluorescence labelling, we found the co-labelling of exo-opsin and melatonin in identical neurons. Almost all perikarya that contained exo-opsin in the periphery of the accessory medulla (Fig. 7c) also expressed melatonin (Fig. 8a, b). Melatonin immunoreactivity was furthermore present in all other neurons that were stained with exo-opsin antibody in the various aforementioned optic lobe regions (data not shown). Interestingly, melatonin antibody only stained the cell bodies but not the neuropils. The expression of exo-opsin in the optic lobe is summarized in Fig. 9.

Discussion

Ablation experiments have demonstrated extraretinal photoreceptor involvement in photoperiodic rhythms in the brains of insects (for a review, see Numata et al. 1997). Several authors have postulated that extraretinal opsins are associated with a carotenoid as a receptor system for entrainment of photoperiodic periodicity (Numata et al. 1997; Shimizu et al. 2001). Lutein and β-carotene, for instance, have been found in the brain of caterpillars of *Pieris brassicae*. The importance of carotenoids for the photoperiodic induction of diapause has been shown in the silkworm and retinal and 3-hydroxyretinal have been found in the retina and the brain (for a review, see Numata et al. 1997; Saunders 2002). Thus, the overwhelming evidence that carotenoid-derivatives are present in the brains of lepidopterans suggests that extraretinal opsins are candidate functional photoreceptors, probably involved in the entrainment of photoperiodic rhythms.

Adult stemmata contain UV- and blue-sensitive opsins

Nearly all studies of lepidopteran stemmata have focused on the larval stages by using light and electron microscopy (Paulus and Schmidt 1978; Li and Chang 1991; Paulus 2000). One study has investigated adult stemmata by means of a combination of light microscopy and electrophysiology (Ichikawa 1991). Most recently, an in situ hybridization study of adult stemmata has reported expression of the same UV- and green-sensitive opsin mRNA transcripts as in the compound eye of the butterfly *Vanessa cardui* (Briscoe and White 2005). Here, by combining electron microscopy and immunocytochemistry, we present a new analysis of this adult structure in sphingid moths, specifically by employing antibodies against UV- and blue-sensitive rhodopsins, chaoptin and arrestin.

Adult stemmata are derived from larval precursors (Ichikawa 1991). Larval stemmata are typically composed of retinular cells arranged in a tiered fashion (Ichikawa and Tateda 1982; Li and Chang 1991). In the posterior margin

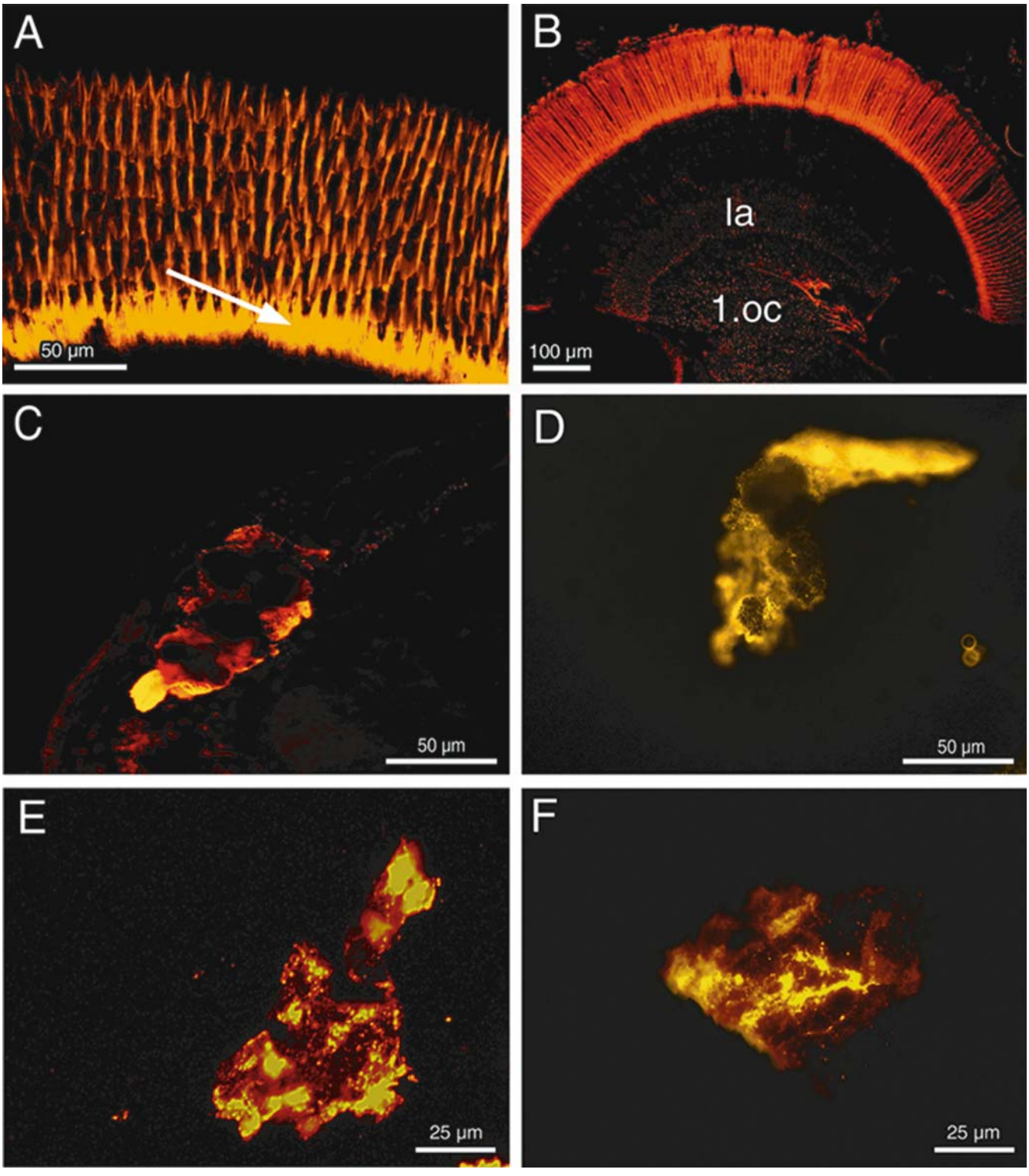

Fig. 4 Horizontal 10-µm-thick cryostat sections of the retina and the adult stemmata of *Agrius convolvuli*. **a** Chaoptin-like immunoreactivitiy can be observed in the retina of the compound eye (*arrow* tracheal tapetum showing autofluorescence). Arrestin-like (S-antigen) immunofluorescence is found in the retina of the compound eye (**b**) and in a subset of retinular cells of an adult stemma (**c**). Note the weak immunofluorescence in the lamina (*la*) and the first optic chiasma (*1. oc*) of the optic lobe (**b**). **d** A subset of retinular cells of the stemma exhibits chaoptin-like immunolabelling. Immunolabelling with anti-PglRh5 (**e**; UV opsin) and anti-PglRh6 (**f**; blue opsin) shows strong immunofluorescence within some regions of the rhabdoms

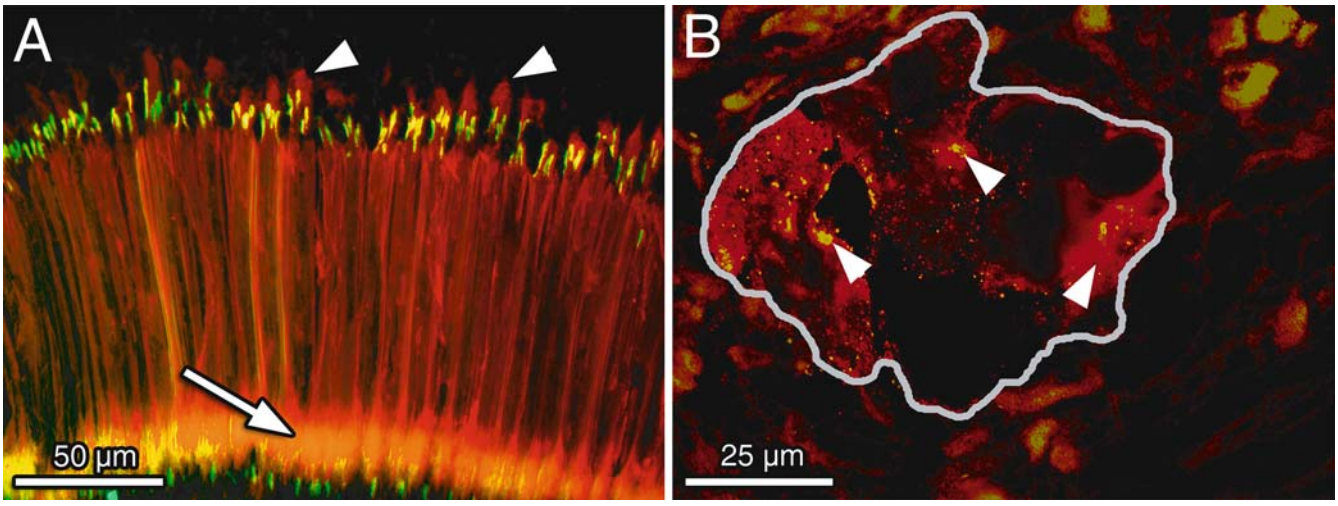

Fig. 5 Horizontal 10-µm-thick cryostat sections of the retina and the adult stemmata of *Acherontia atropos*. **a** Immunocytochemical detection of melatonin (*red*) in the retina. The whole retina is immunopositive (*arrowheads* cell bodies of the distal retinula cells, *arrow* tracheal tapetum with strong autofluorescence). The nuclei of the retinular and pigment cells are stained with Hoechst 33258 (*green*). **b** The adult stemmata (*white outline*) exhibit weaker melatonin immunoreactivity in a subset of retinular cells (*arrowheads*). Note melatonin-like labelling in some surrounding neurons

of the optic lobe of the adult moth, we have found approximately 40 photoreceptor cells organized into six pigmented stemmata (Fig. 1a,b). This number is consistent with larval stemma being composed of six to seven photoreceptor cells and with experimental studies that have found that only seven photoreceptor cells remain in the adult optic lobe following ablation of all but one larval stemma (D. Mischke and A. Ziegler, unpublished). In addition to retaining a similar number of cells in the adult stemma as observed in the larval eye, some of the retinular cell rhabdomeres are oriented perpendicular to each other, suggesting polarization sensitivity. This morphology is also consistent with the retention of a larval function in the adult stemmata, as larval lepidopterans have been shown to be responsive to polarized light (Wellington et al. 1957). The presence of intact rhabdomeres and multivesicular bodies (Fig. 2a–c), which are involved in membrane turnover (White 1968), also indicates that the photoreceptors in the adult stemmata are functional.

Electrophysiological investigations of lepidopteran larval stemmata have shown that they contain retinular cells that are maximally sensitive to UV, blue and green light (for a review, see Gilbert 1994) but the expression of UV-, blue- and green-sensitive opsin proteins has not previously been documented. In this study, we have developed novel polyclonal antibodies against lepidopteran UV- and blue-sensitive opsins; the antibodies recognize these proteins in the adult stemmata of all four of the investigated sphingid moth species (Fig. 4e,f). A third LW-sensitive opsin, exo-opsin, was found to be localized neither to this photore-

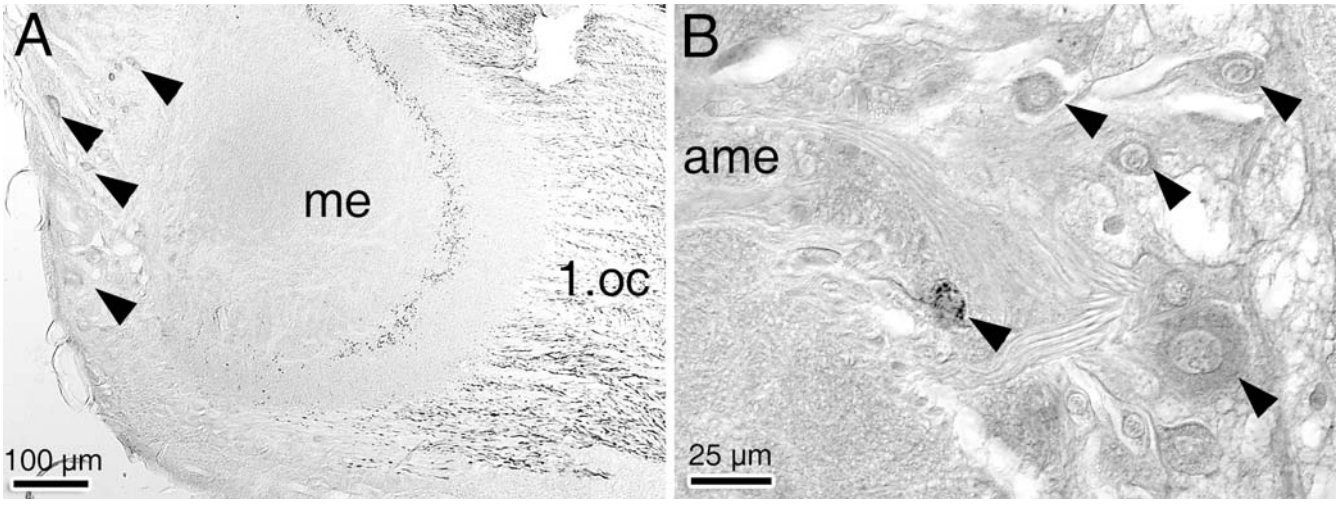

Fig. 6 Arrestin and chaoptin in the optic lobes. **a** Frontal paraffin section showing arrestin-like immunoreactivity in the third layer of the medulla (*me*) and massive labelling of the first optic chiasma (*1. oc*) in *Acherontia atropos* (*arrowheads* immunoreactive somata next to the medulla). **b** Horizontal paraffin section through the medulla region of *Agrius convolvuli*. Some neurons located next to the accessory medulla (*ame*) reveal chaoptin-like immunoreactivity (*arrowheads*)

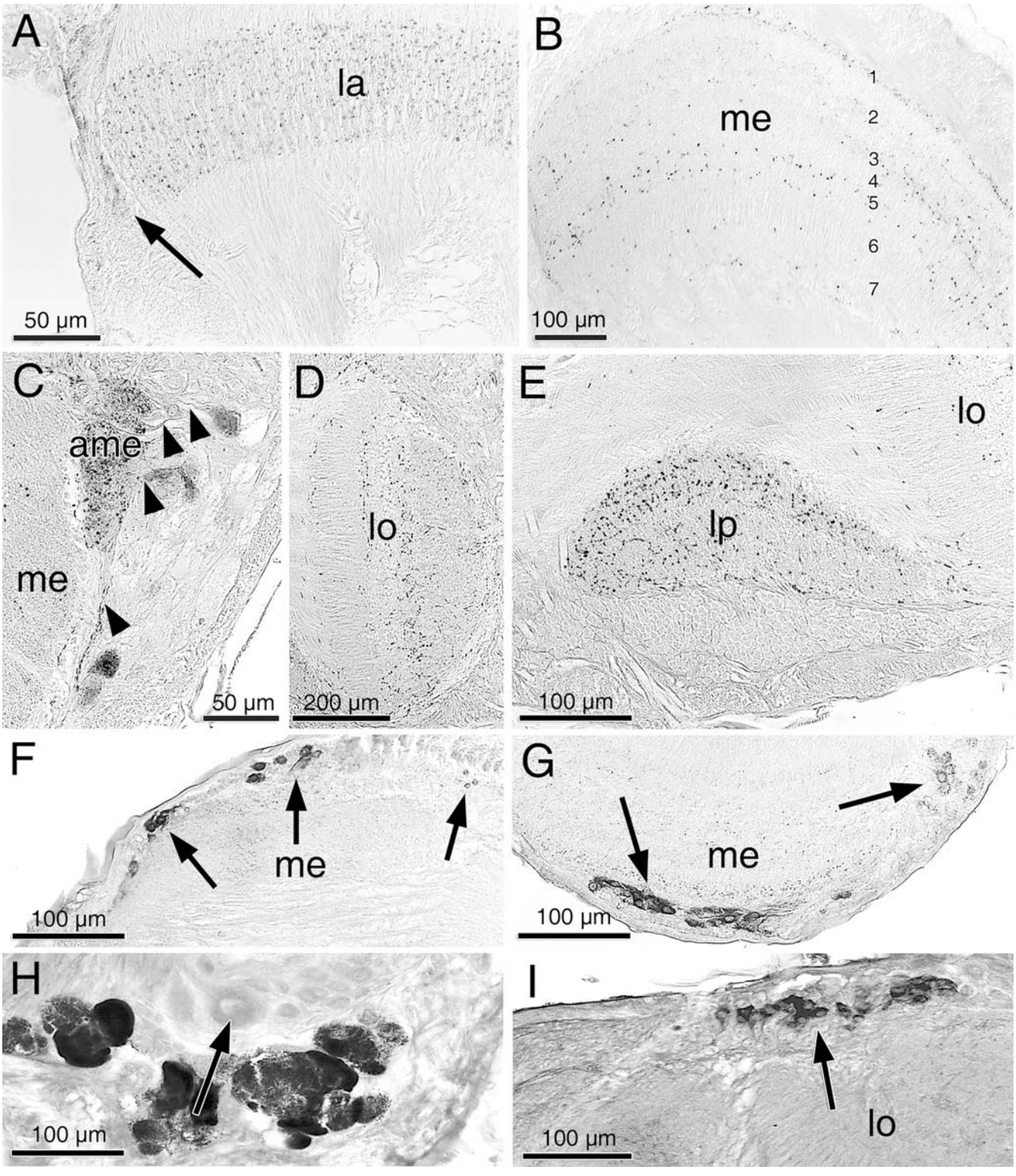

Fig. 7 Extraretinal opsin immunoreactivity (PglRh4) in the optic lobes of *Manduca sexta*. **a** Horizontal paraffin section showing strong immunoreactivity in the external plexiform layer of the lamina (*la*) and the accessory lamina (*arrow*). **b** Horizontal section through the medulla (*me*). Seven layers can be distinguished (*1–7*). Layers *1*, *3* and *5* show strong immunolabelling. **c** Horizontal section through the accessory medulla (*ame*). Five neurons exhibit opsin-like immunolabelling at the edge of the accessory medulla (*arrowheads*) and show axonal projections to this neuropil (*me* medulla). **d** Horizontal section through the lobula (*lo*) showing weaker immunoreactivity. **e** Exo-opsin immunoreactivity in the lobula plate (*lp*); horizontal section (*lo* lobula). Several immunoreactive fibres can be seen. **f** Frontal section of the dorsal medulla region (*me*); 15 perikarya show strong immunoreactivity (*arrows*). **g** Frontal section of the ventral medulla region (*me*). Several somata exhibit opsin-like immunolabelling (*arrows*). **h** Horizontal section showing the weak immunoreactivity of some neurons (*arrows*) next to the darkly pigmented stemmata. **i** Horizontal section through the lobula. Some perikarya located next to the lobula (*lo*) also express extraretinal opsin (*arrow*)

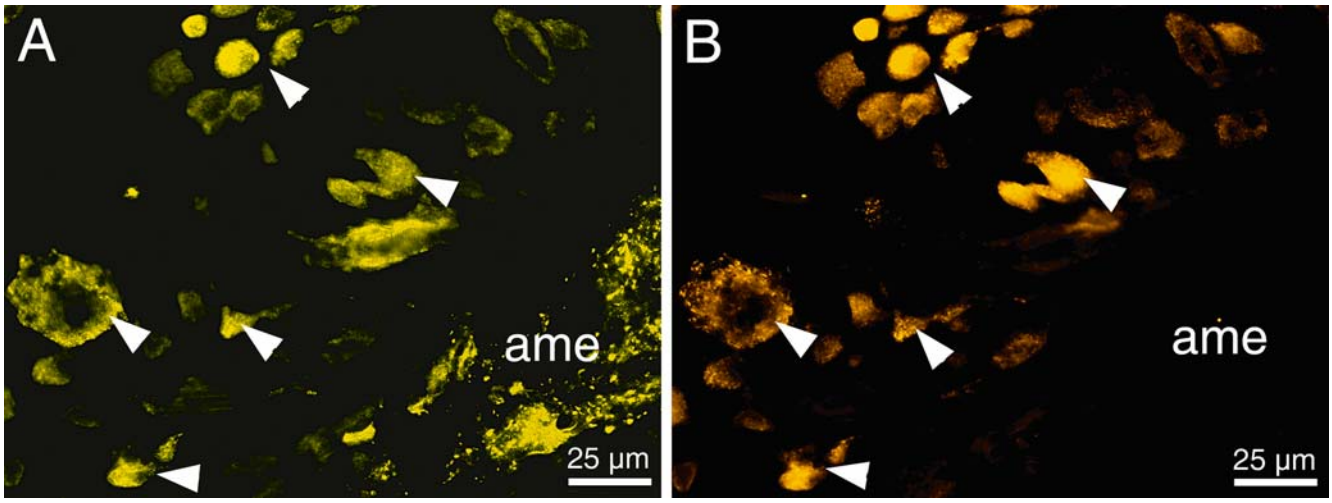

Fig. 8 Double-immunofluorescence labelling for exo-opsin (Cy2-conjugated secondary antiserum, *green*) and melatonin (Cy3-conjugated secondary antiserum, *red*) immunoreactivity in the accessory medulla region of *Acherontia atropos* (*arrowheads* the most prominent immunostained neurons). **a** Anti-exo-opsin labelling can be found in several perikarya and terminals of the accessory medulla (*ame*). **b** The same neurons exhibiting exo-opsin immunoreactivity also contain melatonin. Note the absence of melatonin in the accessory medulla fibres

ceptive organ nor to the retina of the compound eyes (see below). This is the first time the presence of UV- and blue-sensitive opsin proteins have been directly detected in the adult stemmata of moths.

With antibodies of any sort, polyspecificity or cross-reactivity with non-homologous peptide epitopes is always possible (Marchalonis et al. 2001). Indeed, in our anti-UV and blue opsin Western blot analysis, we have observed a few faint bands that might be attributable to non-specific binding. Nevertheless, several lines of evidence suggest that the strong immunolabeling of the adult stemmata with the anti-opsin antibodies reported here is specific. The first is that, as mentioned previously, UV opsin mRNAs have been localized to adult stemmata of butterflies (Briscoe and White 2005). Therefore, we expected to detect UV opsin protein in the adult stemmata of moths. Second, this UV opsin mRNA transcript has also been localized to the adult butterfly retina (Briscoe et al. 2003). This suggests that the same UV opsin protein is expressed in the retina as in the adult stemmata. Although the identity of the proteins being labelled in situ is difficult to confirm directly, the anti-UV opsin antibody that we have developed also cross-reacts with retinular cells of the compound eye (data not shown) and nowhere else in the optic lobe, a result that is at least consistent with the hypothesis that this antibody is specific. Moreover, although no blue opsin mRNA transcript has yet been found to be expressed in the butterfly adult stemmata (Briscoe and White 2005), both the adult stemmata and compound eyes of moths are labelled with the anti-blue opsin antibody, also suggesting its specificity. Confirmation of the antibody results by examining the spatial distribution of the sphingid moth UV and blue opsin mRNA transcripts will be examined in a future study.

Blue- and green-sensitive opsins (RH5 and RH6) and chaoptin have been detected in the equivalent "eyelet" structure in adult *Drosophila melanogaster* (Yasuyama and Meinertzhagen 1999; Helfrich-Förster et al. 2002; Malpel et al. 2002). The blue-sensitive opsins expressed in the moth stemma and eyelet are probably homologous, as only one blue opsin homologue has been reported in lepidopterans and dipterans so far (Briscoe 1998; Kitamoto et al. 2000; White et al. 2003). By contrast, a phylogenetic analysis suggests that lepidopteran LW (i.e. green) opsins were duplicated prior to the separation of moths and butterflies, but after the split between dipterans and lepidopterans (Briscoe 2000). This has given rise to a clade of lepidopteran "extraretinal" LW opsins, and a clade of LW opsins that are expressed in the retina. Consistent with this, we have not found expression of the LW-sensitive exo-opsin protein in the stemmata and retina but rather in other perikarya and neuropil regions (see below). The green-sensitive *Drosophila* RH6 opsin is expressed in both the compound eye and eyelet. It will be interesting to see whether a second LW opsin, probably the same as that expressed in the compound eye, is also expressed in the adult stemmata of hawkmoths. Taken together, the presence of blue-sensitive rhodopsin and chaoptin proteins in the adult stemmata of both moths and flies suggests that the expression of these proteins in these extraretinal photoreceptive organs is ancient.

As mentioned above, we have also observed the expression of a UV opsin in the stemmata. This is consistent with the finding of Briscoe and White (2005) of a UV opsin transcript in the adult stemmata of the butterfly *Vanessa cardui*. Is UV opsin expression found in the stemmata of other species? *Drosophila* has two duplicated UV opsins, RH3 and RH4. No ultraviolet-sensitive opsin expression has been reported in the eyelet (Helfrich-Förster et al. 2002; Malpel et al. 2002), nor are either of these genes apparently expressed in Bolwig's organ (F. Pichaud and C. Desplan, personal communication), the larval precursor to the eyelet, despite earlier reports to the contrary (Pollock and Benzer

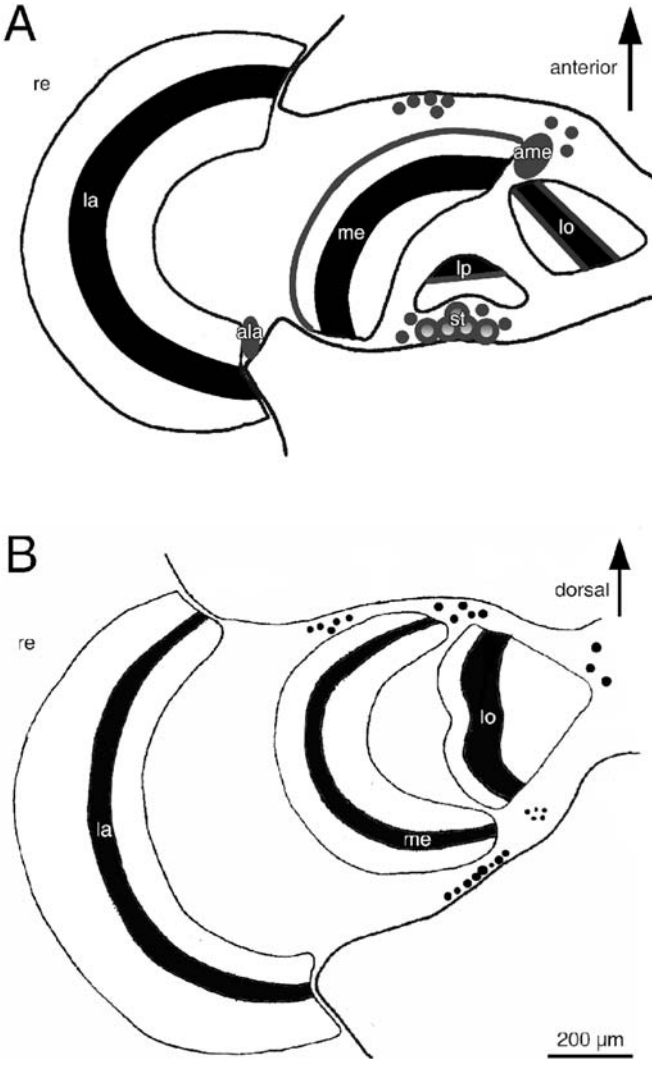

Fig. 9 Diagrammatic dorsal (**a**) and frontal (**b**) view of the optic lobe showing the location of cerebral photoreceptor cells (*dots*) and photosensitive neuropil areas. Exo-opsin was found in all labelled areas except the stemmata (*ala* accessory lamina, *ame* accessory medulla, *la* lamina, *lo* lobula, *lp* lobula plate, *me* medulla, *re* retina, *st* stemmata)

1988). The absence of UV opsins in the eyelet is therefore an interesting departure from the pattern observed in Lepidoptera.

In addition to finding morphological and immunohistochemical evidence that the stemmata photoreceptors are functional, we have obtained preliminary evidence that this structure may retain an input to the clock neurons in moths similar to that of the eyelet in *Drosophila*. In lepidopterans, it is well-known that the former larval optic neuropil still exists as the accessory medulla in the optic lobes of adults (Ichikawa 1994). We have found that the lepidopteran stemmatal photoreceptor cells have axons that project to a region adjacent to the accessory medulla of the adult brain (Fig. 1c).

In *Drosophila*, this same region contains the small ventral lateral neurons (sLN_vs), which express the products of the *period* (*per*) and *timeless* (*tim*) genes and are responsible for the control of locomotor activity and eclosion rhythms (for a review, see Malpel et al. 2002; see below for a discussion of *per* transcript distribution in *Manduca sexta*). These results are intriguing, particularly because the *per* transcript and protein of the giant silkmoth *Antheraea pernyi* have an extremely restricted expression pattern to four neurons in the dorsolateral protocerebrum (Sauman and Reppert 1996). Some differences between *Manduca* and *Drosophila* in the distribution of circadian neuropeptides (e.g. PDH distribution) in this region have previously been described (Wise et al. 2002). Higher-resolution studies are needed to clarify the exact inervation of the stemmatal nerves in this area. This might provide evidence that the light-dependent input to the clock neurons is indeed conserved between dipterans and lepidopterans.

LW-sensitive extraretinal opsin in neurons of the optic lobes

Several types of opsins have been found in various non-mammalian vertebrates in the pinealocytes of the pineal organ, a centre for the entrainment of circadian rhythms, and in other regions of the brain (for a review, see Bellingham and Foster 2002). By comparison, little is known about the presence and distribution of extraretinal opsins in the brain of insects. To further our understanding of extraretinal photoreceptors in insects, we have used antibodies that recognize an extraretinal opsin, arrestin, and chaoptin. We have found strong immunolabelling in a large number of dorsal and ventral neurons in the periphery of the medulla, lobula and the accessory medulla of the optic lobe (Figs. 6, 7, 8a). Additionally, all optic lobe neuropils show strong immunoreaction (Figs. 6, 7). Shimizu et al. (2001) have described an LW-sensitive opsin in some neurons of the brain of caterpillars of the silkmoth *Bombyx mori*, and Cymborowski and Korf (1995) have demonstrated arrestin immunoreactivity in some neurons of the optic lobes and the brain in the blowfly *Calliphora vicina*. Our study presents the first detailed investigation of the expression of an exclusively "extraretinal" opsin in neurons and other neuropil areas of an adult holometabolous insect. Once again, because of the possibility of polyspecificity mentioned above, the availability of independent markers, such as antibodies derived from other peptide epitopes of this protein, would be useful to verify the pattern of extraretinal LW opsin expression that we report here.

For animals in general, the possession of several photoreceptors with different spectral sensitivities to measure ambient light would be advantageous. Massive changes in the light environment take place at dusk and dawn. Thus, these two time points can effectively be used as *Zeitgeber* for adjusting endogenous clocks (Roenneberg and Foster 1997). Zordan et al. (2001) have ascertained, in behavioural studies with *Drosophila* mutants, that red and blue light entrains circadian locomotor activity. This is interesting as, at twilight, the irradiance spectrum shows high peaks in the blue and red. In hawkmoths, we have shown that former larval stemmata express two kinds of rho-

dopsins. Additionally, as a further photoreceptor, the LW-sensitive exo-opsin (probably having a maximum sensitivity in the green) is present in some neurons of the optic lobe. Thus, at least three different types of extraretinal rhodopsins exist with different spectral sensitivities interacting to recognize changes of the irradiance spectrum probably at twilight; this system might be just as important for the entrainment of lepidopteran locomotor rhythms as it is in *Drosophila*. Recently, Froy et al. (2003) have provided evidence for a clock-compass mechanism in the migration rhythms of the monarch butterfly *Danaus plexippus*. Many hawkmoth species are also known to migrate long distances. In particular, three of the four examined species in this study (*A. atropos*, *A. convolvuli*, *H. celerio*) show obvious migration behaviour, and thus extraretinal photoreceptors might play an important role in the control of this activity. Further physiological studies will be needed to demonstrate to what extent the optic lobes are involved in the entrainment of locomotor rhythms in sphingid moths.

An intriguing finding of our investigation is the strong exo-opsin immunoreactivity in some neurons lying adjacent to the accessory medulla and showing intense ramifications in this former larval neuropil (Figs. 6b, 7c, 8a). Fleissner (1982) has localized the circadian pacemaker to the lobula region of the optic lobe in beetles, a region next to the accessory medulla. More recently, the accessory medulla has been demsonstrated to be the centre for controlling the circadian rhythms in coleopterans (Fleissner et al. 1993). Ectopic transplantation of the adult accessory medulla into cockroaches without optic lobes restores the circadian locomotor activity rhythms (Reischig and Stengl 2003). Moreover, Wise et al. (2002) have found abundant antisense *per* transcripts in a cluster of 100–200 small neurons adjacent to the accessory medulla in the sphingid *Manduca sexta* but no labelling of this neuropil itself. Taken together, the accessory medulla is therefore known as the presumptive circadian pacemaker in several insect species (Helfrich-Förster et al. 1998). In addition to these findings, Homberg and Würden (1997) have recorded responses of accessory medulla neurons to light stimuli in locusts. Lastly, light-sensitive neurons with ramifications in the accessory medulla appear to be involved in the entrainment of the endogenous clock of the cockroach *Leucophaea maderae* (Loesel and Homberg 2001). Hence, the extraretinal LW-sensitive opsin in this specific brain region might play an important role in the entrainment of biological rhythms in hawkmoths.

Presence of melatonin in the optic lobes suggests a possible influence on photoperiodic rhythms

Melatonin is present in all organisms and is involved in the transmission of circadian and circannual clock information to the peripheral effector organs in higher metazoans (Hardeland 1997). A daily rhythm with a melatonin increase at night has also been demonstrated in insects (for a review, see Vivien-Roels and Pévet 1993). In the present study, we have found melatonin immunoreactivity in the whole retina of the compound eye, the former larval stemmata and, by colocalization experiments, the co-expression of melatonin in the same neurons as those containing the extraretinal LW-sensitive opsin in the accessory medulla (Figs. 5, 8).

The presence of melatonin in the hawkmoth retina is coincident with the findings of Vivien-Roels et al. (1984) who have described expression of this factor in the locust retina by radioimmunoessays and gas chromatography-mass spectrometry. L'Hélias et al. (1995) have shown NAT-like activity, a melatonin synthesizing enzyme, in the larval stemmata and the brain of pierid larvae. We have now demonstrated the expression and distribution of melatonin in the former larval stemmata and some optic lobe neurons in adult sphingid moths. Hence, the presence of melatonin in these photoreceptor structures argues for their possible involvement in conveying photoperiodic information.

As in vertebrates, melatonin affects several physiological and behavioural responses in insects and some of these effects might be also present in hawkmoths. Yamano et al. (2001) have reported that melatonin administered in drinking water affects the locomotor activity of crickets. Moreover, a seasonal polyphenism and gonadal development with the change of melatonin exposure has been described in the bug *Halyomorpha halys* (Niva and Takeda 2003). In another heteropteran species, this neuromodulator delays the oviposition rhythm after adult ecdysis by acting as a photoperiodic regulator and has an impact on diapause (Hodková 1989). One of the most fundamental influences of melatonin on photoperiodism in insects has been found in the cockroach *Periplaneta americana*; Richter et al. (2000) have described a neuroendrocrine-releasing effect of melatonin on prothoracicotropic hormone in the brain, a hormone that controls the production of ecdysone thereby initiating moulting in hexapods.

Concluding remarks

All of our results in hawkmoths suggest that the compound eyes, the former larval stemmata and some optic lobe neurons containing exo-opsin participate in the generation of circadian and/or photoperiodic rhythms, perhaps via the expression of melatonin. Our findings further indicate that these photoreceptors have a similar biochemical function as the pinealocytes of the pineal organ of non-mammalian vertebrates.

Acknowledgements We are grateful to Dr. Larry A. Donoso for the generous gift of a set of arrestin antibodies and peptides, to Dr. Joachim Schachtner for his gift of eggs of *Manduca sexta*, to Dr. Carol Gregorio for help with the antibody immunoaffinity purification and to Dr. Lisa Nagy for sponsoring us while the antibodies were being made. We also thank Angela Bruns and Claudia Obermeier for excellent rearing of our hawkmoth stocks. The monoclonal antibody 24B10 developed by Seymour Benzer was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the Department of Biological Sciences, University of Iowa, Iowa City, USA.

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