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Molecular characterization and expression of the UV opsin in bumblebees: three ommatidial subtypes in the retina and a new photoreceptor organ in the lamina

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Summary

Ultraviolet-sensitive photoreceptors have been shown to be important for a variety of visual tasks performed by bees, such as orientation, color and polarization vision, yet little is known about their spatial distribution in the compound eye or optic lobe. We cloned and sequenced a UV opsin mRNA transcript from *Bombus impatiens* head-specific cDNA and, using western blot analysis, detected an eye protein band of ~41 kDa, corresponding to the predicted molecular mass of the encoded opsin. We then characterized UV opsin expression in the retina, ocelli and brain using immunocytochemistry. In the main retina, we found three different ommatidial types with respect to the number of UV opsin-expressing photoreceptor cells, namely ommatidia containing two, one or no UV opsin-immunoreactive cells. We also observed UV opsin expression in the ocelli. These results indicate that the cloned opsin probably encodes the P350 nm pigment, which was previously characterized by physiological recordings. Surprisingly, in addition to expression in the retina and ocelli, we found opsin expression in different parts of the brain. UV opsin immunoreactivity was detected in the proximal rim of the lamina adjacent to the first optic chiasm, which is where studies in other insects have found expression of proteins involved in the circadian clock, period and cryptochrome. We also found UV opsin immunoreactivity in the core region of the antennal lobe glomeruli and different clusters of perikarya within the protocerebrum, indicating a putative function of these brain regions, together with the lamina organ, in the entrainment of circadian rhythms. In order to test for a possible overlap of clock protein and UV opsin spatial expression, we also examined the expression of the period protein in these regions.

Supplementary material available online at
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Key words: circadian rhythm, visual pigment, photoreceptor, color vision, ultraviolet.

Introduction

The presence of ultraviolet (UV)-sensitive receptors is widespread and phylogenetically ancient in arthropods and vertebrates (Briscoe and Chittka, 2001; Shi et al., 2001). Such receptors enable the perception of light of wavelengths below 400 nm. UV vision is involved in all kinds of behaviors such as foraging, inter- and intraspecific communication, orientation and the entrainment of circadian rhythms (Goldsmith, 1994; Tovee, 1995). The UV-sensitive visual channel is employed by animals in two distinct contexts, the first of which is to extract information about the relative spectral composition of light (e.g. the color of an object). In this context, the information gathered by the UV receptor is processed together with the input of one or more different spectral receptor types in a postreceptor mechanism (Backhaus, 1998). Second, a UV receptor may be used to trigger wavelength-dependent behavior in which a behavioral response is exclusively based on information of only one photoreceptor type (Goldsmith, 1994).

One of the most intensively studied groups of arthropods with respect to UV vision are the bees, since they were first found to be sensitive to UV light more than 80 years ago (Kühn, 1924). In a bee's life, UV perception plays an essential role in flower detection and discrimination (Dyer and Chittka, 2004; Spaethe et al., 2001), polarization vision (Brines and Gould, 1979; Von Frisch, 1949) and orientation (Edrich et al., 1979; Moller, 2002). It has also been shown that UV information is utilized in both real color vision and wavelength-specific behavior.

UV perception in general is mediated by visual pigments, which are composed of a chromophore and an opsin protein. Opsins belong to the large group of G-protein-coupled receptors and are subdivided into distinct classes according to the part of the light to which they tune the absorbance properties of the chromophore. Physiological and behavioral studies have shown that all bees investigated so far have one

receptor that is most sensitive in UV light (Briscoe and Chittka, 2001; Peitsch et al., 1992). However, despite the huge amount of data showing the significance of UV perception in Apidae, molecular data for the UV opsin are rare. Among the estimated 20 000 bee species (Michener, 2000), the UV opsin from only a single species, the honeybee *Apis mellifera*, is molecularly characterized (Townson et al., 1998), and opsin spatial expression data are completely missing. The goal of the present study, therefore, was to characterize molecularly the UV opsin-encoding gene and determine its protein expression pattern in order to obtain a better understanding of possible functions of the UV visual pigment in bees. We used the bumblebee *Bombus impatiens* as our study system. This species is one of the most common North American bumblebee species and is intensively used in agriculture for pollination of cucumbers, peppers, tomatoes, strawberries, melons and squash (Meisels and Chiasson, 1997; Stubbs and Drummond, 2001; van Ravestijn and van der Sande, 1991). The spectral sensitivity of the *B. impatiens* retina for short-wavelength light has been characterized and shows a single peak most sensitive to 350 nm (Bernard and Stavenga, 1978).

Materials and methods

cDNA library construction, PCR, cloning and sequencing

Colonies of *Bombus impatiens* Cresson were bought from a commercial breeder (Koppert, distributed by Plant Sciences, Inc., Watsonville, CA, USA). We extracted total RNA from a single worker head according to the Trizol protocol (Gibco BRL, Gaithersburg, MD, USA). cDNA was synthesized from total RNA using the Marathon cDNA amplification kit (BD Biosciences, Clontech, Mountain View, CA, USA). To detect the UV opsin transcript, we ran a PCR (2 min at 94°C and then 35 cycles of 30 s at 94°C, 1 min at 50°C, and 1 min at 68°C) with the cDNA as a template and used an opsin-specific degenerate forward primer (5′-GAA CAR GCW AAR AAR ATG A-3′) and a reverse primer that is complementary to the adapter sequence. After amplification, the PCR product was cloned into a pGEM-T Easy plasmid (Promega, Madison, WI, USA). Clones were prepared using a FastPlasmid Miniprep kit (Eppendorf, Westbury, NY, USA), screened for inserts by *Eco*RI digestion and subsequently sequenced using the BigDye Terminator v. 3.1 Cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). We conducted a BLAST search to identify clones encoding the UV opsin. We identified a partial sequence of the UV opsin cDNA and used this fragment to design a gene-specific reverse primer (5′-GCC ATC ACG CCA TAC GGA GTC-3′) for 5′ RACE to obtain the complete cDNA sequence. The sequenced 5′ and 3′ RACE products were imported into SeqMan (DNASTAR, Inc., Madison, WI, USA), assembled and manually edited.

Phylogenetic analysis

Compared to the insect long-wavelength (LW)-sensitive opsin gene family, for which more than 240 complete or partial sequence data are published (Ascher et al., 2001; Briscoe, 2001; Kawakita et al., 2003; Ortiz-Rivas et al., 2004; Spaethe and Briscoe, 2004), only a few UV-sensitive opsin gene sequences are available in GenBank (Briscoe, 2000; Chase et al., 1997; Gao et al., 2000; Kitamoto et al., 2000; Smith et al., 1997; Vanhoutte et al., 2002). We downloaded 13 full-length UV opsin cDNA sequences from four insect orders and the blue-sensitive opsin sequence from *Apis mellifera* as an outgroup into the Alignment Explorer in MEGA 3.0 (Kumar et al., 2004). We aligned the translated amino acid sequences using the ClustalW algorithm (Thompson et al., 1994).

Heterogeneous patterns of nucleotide or amino acid substitution between sequences can produce erroneous branching patterns (see literature cited in Kumar and Gadagkar, 2001). We therefore tested 1st + 2nd nucleotide positions, 3rd nucleotide positions and amino acid sequences for composition homogeneity among lineages using the disparity index test (Kumar and Gadagkar, 2001) as implemented in MEGA 3.0 (Kumar et al., 2004). Our goal was to only use molecular characters in our phylogenetic reconstruction that appear to be evolving with a similar pattern of substitution and/or to remove any sequences that violated the homogeneity assumption. Third nucleotide positions appeared to have evolved in a significantly non-homogeneous fashion (in 71% of all comparisons after Bonferroni correction). First + 2nd nucleotide positions and the protein sequences were found to be homogeneous except for 1st + 2nd nucleotide positions in *Camponotus abdominalis* and for amino acids in *Drosophila* Rh4, which were significantly different from one or more of the other species sequences ($P<0.002$; all P-values were adjusted for multiple comparisons using Bonferroni correction; data not shown). We therefore decided to use 1st + 2nd nucleotide positions and amino acid sequences in the phylogenetic analysis. In addition, we looked for possible effects of the non-homogeneously evolving sequences on the tree topology by running phylogenetic analyses both with and without them. We applied the neighbor-joining (NJ) algorithm under the Tajima-Nei model for 1st + 2nd nucleotide positions with complete deletion of gaps, and Poisson correction for the amino acid sequences to construct phylogenetic trees, including a total of 1176 aligned nucleotide and 392 aligned amino acid sites. Robustness of the NJ trees was tested using the bootstrap method with 500 replicates. All phylogenetic analyses were conducted using MEGA 3.0 (Kumar et al., 2004).

Tissue preparation

Bumblebee workers were immobilized with CO_2, decapitated and the heads cut sagittally in two halves and fixed in phosphate-buffered paraformaldehyde (4%) for 1–3 h at room temperature or at 4°C overnight. In some bees, for a better overview of the entire brain, we cut a small window in the head capsule and pre-fixed the brain for 30 min. The brain with the entire retina and cornea was then removed from the head capsule and fixed for a total of 1–3 h at room temperature. After fixation, the tissue was stepped through a 10% and 20%

phosphate-buffered sucrose solution and finally infiltrated for at least 12 h in a 30% sucrose solution. It was then embedded and frozen in Tissue Tek O.C.T. freezing compound (Sakura Finetek, Inc., Torrance, CA, USA) and 12–14 μm sections were cut on a cryostat (MICROM HM 500 OM, Walldorf, Germany).

Recent investigations have shown that, in the honeybee, *period* mRNA expression level is highest during the first hours of the dark phase when bees were entrained to a 12 h:12 h L:D cycle (Bloch et al., 2003; Toma et al., 2000). Based on this finding, we expected the highest level of period protein (PER) during the first hours of the dark phase. To obtain the strongest immunoreactivity when applying anti-PER antibody, we kept groups of bumblebees for at least three days in small boxes (15×15×20 cm) entrained to a 12 h:12 h L:D cycle before dissecting the retina and brain two hours after the commencement of the dark phase under red light conditions as described above.

UV opsin western blot analysis

The immunoaffinity-purified polyclonal rabbit anti-PglRh5 (UV) antibody we used was made against a 15 amino acid peptide domain within the C-terminus of the butterfly *Papilio glaucus* Rh5 opsin (Briscoe and Nagy, 1999; Lampel et al., in press). Twelve of the 15 amino acid sites were conserved between the butterfly opsin and the predicted *B. impatiens* UV opsin sequence (Fig. 1), so we assumed that cross-reactivity of the *Papilio* Rh5 antibody with the *B. impatiens* UV opsin was very likely. To test for the likelihood that the antibody might also cross-react with other peptides, we performed a tBLASTn search of GenBank, including against the whole translated honeybee genome, using both the butterfly peptide sequence and the bee sequence, which varies by three amino acids. We then tested the antibody on a western blot of eye extract subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Eight retinas of four worker bees were dissected and homogenized for 10 min in 25 μl ice-cold homogenization buffer, pH 7.5 (50 mmol l^{-1} Mops, 1 mmol l^{-1} EDTA, 1 mmol l^{-1} EGTA, 120 mmol l^{-1} KCl, 5 mmol l^{-1} $MgCl_2$, 250 mmol l^{-1} sucrose and 1× Halt protease inhibitor cocktail mix; Pierce, Rockford, IL, USA). After centrifuging for 5 min at 3000 ***g*** and 4°C, the supernatant was transferred into a new tube and centrifuged for 15 min at 18 000 ***g*** and 4°C (Jouan BR4i; ThermoElectron Corporation, Milford, MA, USA). The supernatant was diluted in 2× NuPAGE LDS sample buffer (Invitrogen, Carlsbad, CA, USA) supplemented with 1× NuPAGE Reducing agent and 1× Halt protease inhibitor, to a final concentration of 0.15 retina eq. μl^{-1}. Proteins from 3.75 retina supernatant equivalents per lane were separated on NuPAGE 4–12% gradient Bis-Tris SDS-PAGE minigel and transferred onto a PVDF membrane. The blot was blocked for 1 h in 2.5% normal horse serum (Vectastain ABC kit; Vector Laboratories, Burlingame, MA, USA) and then either probed with the primary antibody (0.00453 nmol ml^{-1}) diluted in 1× TTBS (25 mmol l^{-1} Tris, pH 7.5, 150 mmol l^{-1} NaCl, 0.1% Tween 20) overnight at 4°C in the presence or absence of 100× molar excess of peptide (0.453 nmol ml^{-1}) mixed with antibody (0.00453 nmol ml^{-1}) for 1 h at room temperature prior to being applied to the blot. Then the membrane was incubated for 30 min with a secondary antibody conjugated with biotinylated horseradish peroxidase (Vectastain ABC kit) and visualized with DAB (Pierce). As a control, each membrane was subsequently stained with Coomassie blue to ensure that comparable amounts of protein were loaded in each lane.

Immunocytochemistry

The anti-PER serum was kindly provided by J. C. Hall (Brandeis University; Liu et al., 1992). It is a polyclonal rabbit

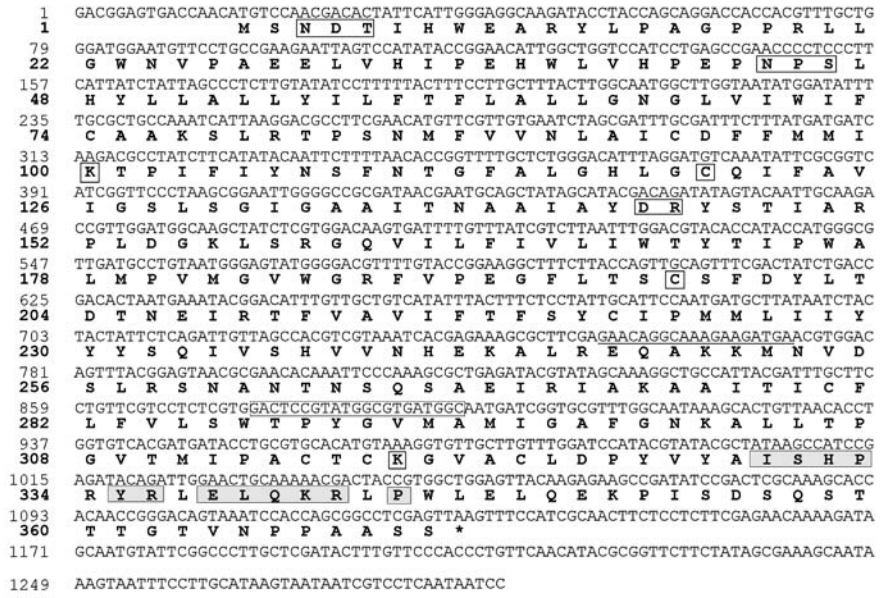

Fig. 1. The nucleotide sequence, including the untranslated regions, and the deduced amino acids of the UV opsin cDNA. Unbolded numbers are for nucleotides, and bolded numbers are for amino acids. The nucleotide sequence to which the gene-specific reverse primer binds is boxed. The location of the degenerate primer used in 3′ RACE is underlined. Grey boxes indicate the amino acid residues that are conserved between *B. impatiens* and *P. glaucus* within the peptide domain used to generate the anti-UV antibody (Lampel et al., in press). The first two boxes indicate candidate glycosylation sites (NXS/T) (O'Tousa, 1992), and the third box indicates the lysine (K) that confers UV sensitivity (Salcedo, 2001; Salcedo et al., 2003). A pair of conserved cysteines (C) that form an essential disulfide bridge in bovine rhodopsin (Karnik et al., 1988) are located at amino acid positions 120 and 197 (boxed). The site of chromophore attachment (amino acid 318; Bownds, 1967) is also boxed. A highly conserved motif (E/DR) involved in G-protein binding/activation (Baldwin, 1997) is present at amino acid positions 144 and 145. GenBank accession number: AY655163.

anti-PER serum raised against the full-length *Drosophila melanogaster* period protein. Bloch et al. (2003) previously showed its cross-reactivity and specificity in the honeybee, *Apis mellifera*, a species belonging to the same subfamily (Apinae) as *B. impatiens*.

Sections were first post-fixed in 100% ice-cold acetone, washed three times for 10 min in 1× PBS, once for 5 min in 0.5% SDS in 1× PBS, and then blocked for 30 min with 4% normal goat or normal donkey serum in PBST (1× PBS, 0.3% Triton X-100). Either affinity-purified rabbit anti-UV antibody (1:200 to 1:1000) or anti-PER serum (1:100 to 1:500) was then added to sections and incubated for 2–3 h at room temperature or overnight at 4°C. After washing for 30 min in 1× PBS, sections were incubated with a secondary antibody [Cy3-conjugated goat anti-rabbit (Jackson Immuno Research Laboratories, Inc., West Corore, PA, USA) or Alexa Fluor 488-conjugated donkey anti-rabbit (Molecular Probes, Eugene, OR, USA); 1:1000 in PBS] for 1 h, washed for 30 min and counterstained for 15 min with 0.1% 4,5-diamidino-2-phenylidone (DAPI; Molecular Probes). Slides were then washed in PBS again and mounted with Aqua Poly Mount (Polysciences, Inc., Warrington, PA, USA). As a control for specificity of the antibody, we also performed a peptide competition experiment in which a 0.023 nmol ml^{-1} dilution of the anti-UV opsin antibody in blocking solution was mixed for 1 h with 2.3 nmol ml^{-1} peptide (Biosource, Hopkinton, MA, USA), and then applied to slides. The slides were then processed as described above in parallel with adjacent sections to which a 0.023 nmol ml^{-1} dilution of the anti-UV opsin antibody had been applied (peptide omitted). Sections were examined and photographed using an Axioskop 2 plus microscope connected to an AxioCam HRc (Zeiss, Goettingen, Germany), and images were processed using Adobe Photoshop (version 7.0).

In situ *hybridization*

As an additional control for the specificity of our anti-UV opsin antibody, we also performed *in situ* hybridizations to examine the distribution of the UV opsin mRNA transcript in the compound eye and optic lobe. Sense and antisense RNA probes (riboprobes) of the UV opsin cDNA were synthesized with digoxigenin-labeled UTPs using a DIG RNA labeling kit (Roche Diagnostics, Indianapolis, IN, USA). Sections were incubated in hybridization buffer in a humid chamber for 30 min at 60°C as previously described (Briscoe et al., 2003). The labeled probe was diluted in the hybridization buffer, corresponding to approximately 45 ng of probe per slide. The sections were incubated overnight at 55–60°C in a humid chamber and then washed for 10 min with, in turn, 2×, 1× and 0.1× standard saline citrate (SSC) and 0.1% Tween to increase accessibility of the probe. The probes were localized by incubation with an anti-digoxigenin alkaline phosphatase-conjugated antibody (1:1000), diluted in 1× PBS plus 0.1% Tween for two hours. After three 10 min washes with 1× PBS, the slides were washed in alkaline phosphatase developing solution with levamisol for 5 min before detection. The probes were detected by a colorimetric reaction produced by nitro blue tetrazolium, 5-bromo-4-chloro-3-indolylphosphate and 10% Tween in alkaline phosphatase developing solution.

Results

Isolated UV opsin gene from cDNA

We obtained a cDNA sequence with a total length of 1290 nucleotides (GenBank Accession number: AY655163) (Fig. 1). From the deduced amino acids, we identified a putative start as well as stop codon, which indicates that we probably obtained the full length. The aligned amino acid sequences of the UV opsin from *B. impatiens* and the honeybee *Apis mellifera* (370 amino acids) were almost identical in length except for an additional Ser at the C-terminus in *B. impatiens*. Altogether, we obtained 1113 nucleotides of coding region, 15 nucleotides of the 5′ UTR and 162 nucleotides of the 3′ UTR.

UV opsin gene phylogeny

The phylogenetic analysis using 1st and 2nd nucleotide positions showed that the *B. impatiens* sequence is most closely related to the *A. mellifera* opsin (100% bootstrap support) and that both sequences form, together with the other two hymenopteran sequences, a well-supported group (100% bootstrap support) (Fig. 2). Removing the *Camponotus* sequence from the analysis did not affect the tree structure, thus we retained the sequence in our subsequent analyses. All lepidopteran and dipteran sequences formed two strongly supported groups (100% and 84%, respectively). Using amino acid sequences instead of 1st and 2nd nucleotide positions revealed a very similar tree structure except that *Anopheles* forms, with the hymenopteran sequences, a sister group to all *Drosophila* opsins. However, when we removed the *Drosophila* Rh4 amino acid sequences, which have evolved under a significantly different rate from Rh3 (Carulli and Hartl, 1992), the identical tree structure to that obtained with 1st and 2nd nucleotide positions was recovered (data not shown).

Western blotting

Our BLAST search of both butterfly and bee peptides (against which the polyclonal antibody is likely to bind) revealed over 30 hits to only insect UV opsin homologues, including those from the honeybee, *Apis mellifera*, the mosquito *Anopheles gambiae* and several species of *Drosophila* and butterflies. On the western blot from the retina tissue, the anti-UV polyclonal antibody labeled a band with an apparent molecular mass of approximately 41 kDa (Fig. 3A, lane 2). This size matches closely the predicted UV opsin molecular mass of 41.4 kDa that was obtained from our predicted UV opsin amino acid sequence using the ExPASy analysis tool (Gasteiger et al., 2003). Two smaller bands were also present at 30 and 29 kDa. In the peptide competition assay where the UV opsin-antibody was incubated with the peptide before application, none of the three bands could be detected

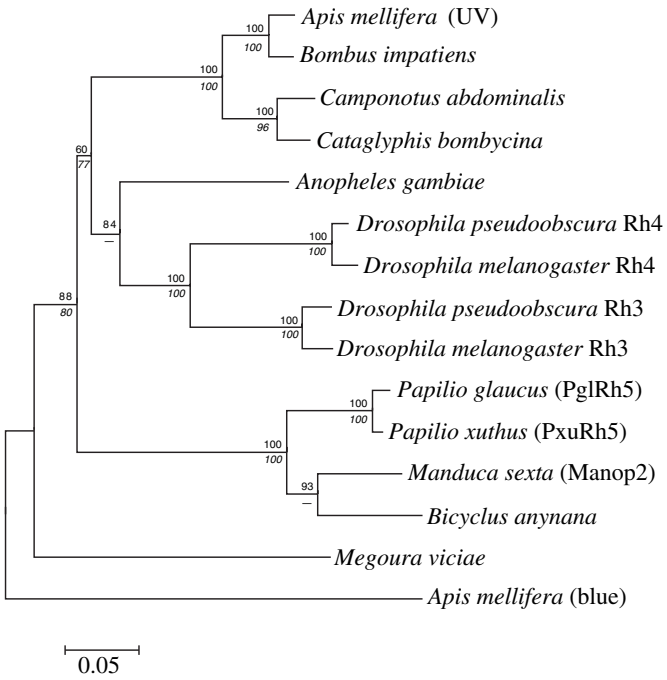

Fig. 2. Phylogeny of insect UV opsins based on 1st and 2nd nucleotide positions and the Tajima-Nei model of evolution with complete deletion of gaps. The *A. mellifera* blue opsin sequence was used as an outgroup. Bootstrap values based on 500 replications are shown above nodes for 1st and 2nd positions and below nodes for amino acids (italics). Branch length indicates nucleotide substitutions per site. GenBank accession numbers for sequences used in our analysis are as follows: *Anopheles gambiae* GPRop8 (AAAB01008960 gi|19612143: 3070327-13075141), *Apis mellifera* (UV, AF004169; blue, AF004168), *Bicyclus anynana* (AF484248), *Camponotus abdominalis* (U32502), *Cataglyphis bombycina* (U32501), *Drosophila melanogaster* (Rh3, M17718; Rh4, M17719), *Drosophila pseudoobscura* (Rh3, X65879; Rh4, X65880), *Manduca sexta* (L78081), *Megoura viciae* (AF189715), *Papilio glaucus* (AF077191), *Papilio xuthus* (AB028218).

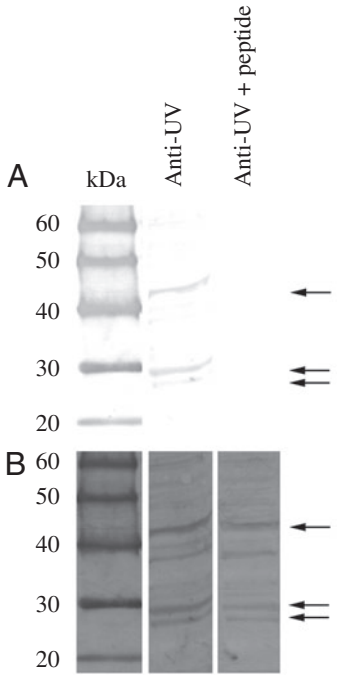

Fig. 3. UV opsin immunoreactivity on a western blot of crude protein extracted from retinas of four *B. impatiens* female workers with and without peptide treatment. (A) Lane 1, MagicMark protein standard (Invitrogen). Lane 2, anti-UV opsin antibody. The primary antibody concentration was 1:1000 (0.00453 nmol ml^{-1}) and the membrane was incubated at 4°C overnight. The specific opsin immunoreactive band with an approximate molecular mass of 41 kDa is indicated by an arrow. Two smaller bands, which might represent alternatively glycosylated states of the UV opsin protein and/or proteolytic breakdown products, are present at approximately 30 and 29 kDa (arrows). Lane 3, anti-UV opsin antibody + UV opsin peptide. The antibody (0.00453 nmol ml^{-1}) was mixed with 0.453 nmol ml^{-1} peptide for 1 h before incubation with the membrane at 4°C overnight. (B) Same membranes as above except stained with Coomassie blue to visualize total protein present on blots.

(Fig. 3A, lane 3), even though the bands were present on the membrane, as shown by a subsequent Coomassie staining (Fig. 3B, lanes 2 and 3).

Retina and ocelli

Bee ommatidia are composed of one small proximal and eight elongate distal photoreceptor cells. UV opsin immunoreactivity (-ir) was found in most but not all ommatidia across the retina (Fig. 4A–C,F). The strongest staining was found in the rhabdoms of the ommatidia, where most of the opsin protein is localized at high concentration in the microvillous membranes. In some cases, vesicles in the cytoplasm of some photoreceptor cells also showed UV opsin-ir (Fig. 4C). We found three different ommatidial types with respect to UV opsin-ir: those containing two, one and no UV opsin-ir photoreceptor cells (Fig. 4B,C). A diagram of a single ommatidium in longitudinal view and of the three ommatidial types in cross section found in the main retina is shown in Fig. 4E. Because of its small size, we did not investigate the presence or absence of the UV opsin in the tiny ninth cell. In the median-central part of the retina of a worker bee (N=3), we estimate that 25% of ommatidia (63 out of 252) contain two UV opsin-ir photoreceptor cells, 48% (121 out of 252) contain one UV-ir photoreceptor cell, and 27% (68 out of 252) contain no UV opsin-ir photoreceptor cells. UV opsin-ir receptor cells were most densely packed in the dorsal rim area (Dra) of the eye compared with the rest of the retina (Fig. 4F). We could not tell whether there were more than two photoreceptor cells per ommatidium in the Dra that had UV opsin expression, as has been suggested by some investigations (Meyer, 1984).

At the top of the bee head, three additional photoreceptor organs are located: the median and two lateral ocelli. We detected UV opsin expression in some of the retinula cell rhabdoms in all three ocelli of the worker bee (Fig. 5A). Incubation of adjacent sections with the anti-UV opsin antibody and peptide for 1 h abolished the specific staining in the rhabdoms of both the retina (Fig. S2A,B in supplementary material) and ocelli (Fig. 5B).

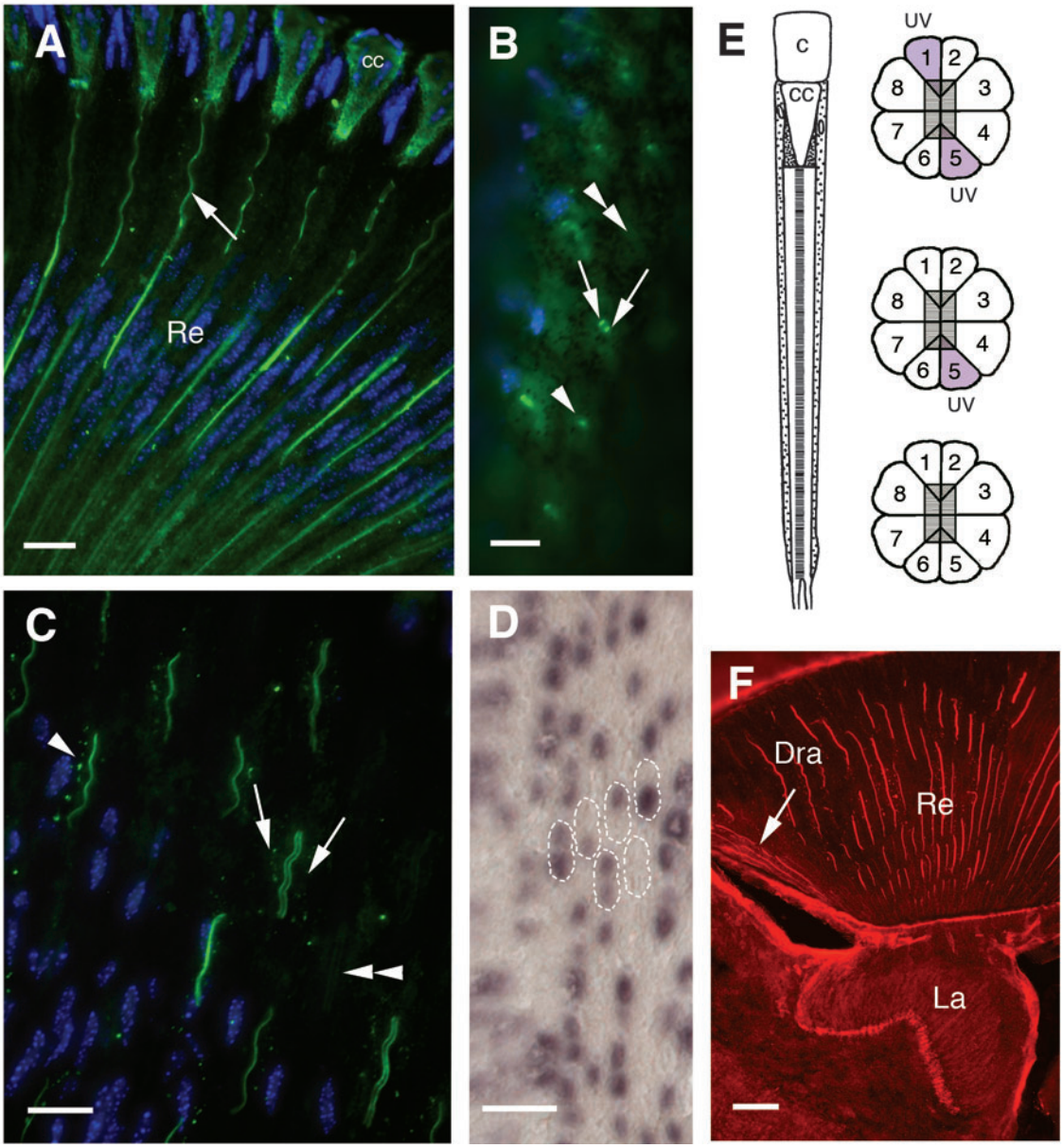

Fig. 4. UV opsin immunoreactivity (-ir) in the retina, visualized with a secondary antibody attached to Alexa Fluor 488 (green) or Cy3 (red). Cell nuclei were stained with DAPI (blue). (A) Frontal section showing the distally twisted rhabdomeres with UV opsin-ir (arrow). Note the strong autofluorescence of the crystalline cones (cc). (B) Tangential section of the central part of the eye showing three types of ommatidia, those with zero (double arrowhead), one (single arrowhead) and two (arrows) UV opsin-ir rhabdomeres. An electron microscopy study of the bumblebee fused rhabdom indicates that the rhabdomeres of R1, 2 and 5,6 are physically separated by the rhabdomeres of the other photoreceptor cells (Meyer-Rochow, 1981). (C) Semi-tangential section showing the heterogeneous UV opsin-ir in the rhabdomeres of different ommatidia. Ommatidium with one (arrowhead), two (arrows) and no (double arrowhead) UV opsin-ir retinula cells. Note the UV opsin-ir vesicles (flecks) in the cell bodies adjacent to the UV opsin-ir rhabdoms. (D) Tangential section showing UV opsin mRNA expression in the cell bodies of R1 and R5 photoreceptor cells, visualized with antisense digoxigenin-labeled riboprobes and an alkaline phosphatase-conjugated anti-digoxigenin antibody. Dotted lines indicate the boundaries of individual ommatidia. Three ommatidial types are shown, those with zero, one or two UV opsin mRNA-expressing photoreceptor cells. (E) Diagram of a longitudinal view of a single bee ommatidium showing the cornea (c), crystalline cone (cc), primary pigment cells (darkly speckled), secondary pigment cells (lightly speckled) and retinular cells (white) surrounding the fused rhabdom (hatched). Tangential views of the three ommatidial types observed in the *Bombus* retina with respect to UV opsin-ir (purple) are shown. Numbers indicate retinula cells R1–8. The R1 and R5 cells are UV opsin-ir, following the convention of Menzel and Blakers (1976). Note that in ommatidia in which only one rhabdomere is UV opsin-ir, that cell can be either the R1 or R5 cell (only one shown). (F) Longitudinal section of the retina showing the more densely packed UV opsin-ir dorsal rim area (Dra) (arrow). La, lamina; Re, retina. Scale bars, 20 μm (A,C); 10 μm (B); 25 μm (D); 50 μm (F).

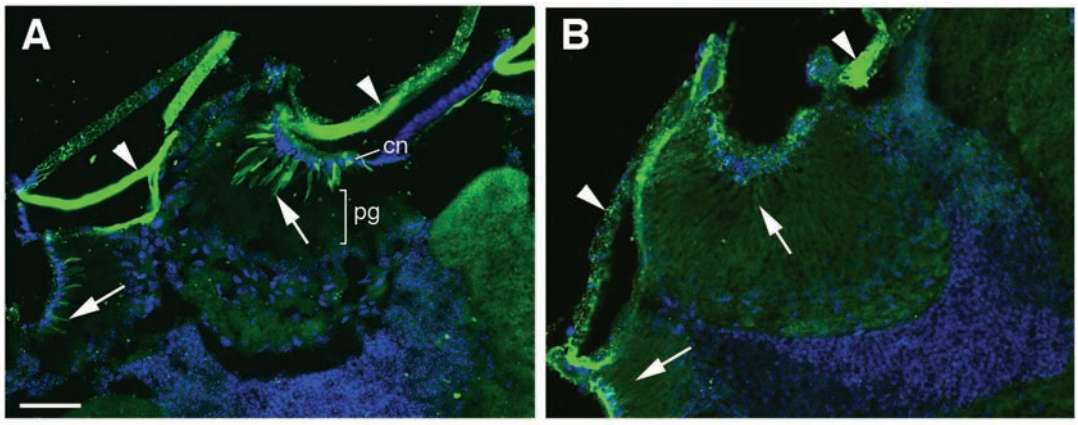

Fig. 5. UV opsin-ir in the dorsal ocelli of a worker bee (Alexa Fluor 488; green). Nuclei were stained with DAPI (blue). (A) Tissue was fixed for 45 min at 4°C and then 20 min at room temperature in 4% paraformaldehyde before being sectioned on a cryostat. Sections (12 µm) were incubated with a 0.023 nmol ml^{-1} dilution of the anti-UV opsin antibody. Arrows indicate UV opsin-ir rhabdomeres in some but not all photoreceptor cells in both ocelli. The median ocellus is on the left and one of the lateral ocelli is on the right. Electron microscopy studies of the dorsal ocelli in the worker bee, *Apis mellifera*, have previously identified both planar and non-planar rhabdoms (Toh and Kuwabara, 1974). The arrow on the right points to a planar-like rhabdom, and on the left to a non-planar rhabdom. The rhabdoms lie directly beneath a layer of corneagenous cell nuclei (cn). The cell bodies of the retinular cells subtend the rhabdomeres and are filled with pigment granules (pg) (not shown). Arrowheads indicate strongly autofluorescing cuticle. (B) Adjacent section incubated with 0.023 nmol ml^{-1} dilution of the anti-UV opsin antibody mixed for 30 min with 2.3 nmol ml^{-1} peptide prior to application to the slides and parallel processing with the section shown in A. Arrows indicate the location of the rhabdomere layer, where the UV opsin protein immunoreactivity has been abolished. Arrowheads indicate the strongly autofluorescing cuticle. The corneagenous cells of the ocelli, like that of the compound eye, are themselves also autofluorescing. cn, nucleus of the corneagenous cell; pg, pigment granule layer of retinular cells. Scale bar, 50 µm.

Optic lobe

UV opsin-ir was found in the first optical layer, the lamina, as well as in at least 10 perikarya located ventrally of the lamina (Fig. 6). Immunoreactivity within the lamina was restricted to the 3rd layer (C-layer; Mobbs, 1985), which is formed by the lamina cartridges (Ribi, 1979) and is embedded between two layers of cell bodies (Fig. 6A–E). Immunoreactivity in this layer was found throughout the entire lamina and formed a disc-like shape with an increasing thickness towards the laminar margin (Fig. 6C). Sections incubated with the secondary antibody but without the primary anti-UV antibody showed no labeling (Fig. 6B), and sections labeled with the primary antibody mixed with the peptide also showed no labeling (compare Fig. S2C,D in supplementary material). In most immunolabelings, we also observed a weak UV opsin-ir in the medulla [where the long fibers of the UV opsin-expressing retinula cells terminate (Menzel and Blakers, 1976)], which was not found in any of the controls (Figs S1, S2C,D in supplementary material). *In situ* hybridization using a DIG-labeled UV opsin antisense riboprobe revealed mRNA expression in the retina (Fig. 4D), in the cell bodies that form part of the 3rd layer of the lamina and in the cell bodies along the distal margin of the medulla (Fig. 6F).

Protocerebrum and antennal lobes

As opsins, together with their chromophore, form the light-sensitive part of the visual system in insects, we expected UV opsin protein expression in the retina and, perhaps, in the optic lobe to which the axons of the receptor neurons project (Menzel and Blakers, 1976; Mobbs, 1985). Opsin expression has been reported below the basement membrane of the retina in the region of the retinula cell axons in *in situ* hybridization studies in both crayfish (Hafner et al., 2003) and butterflies (Briscoe and White, 2005) and in autoradiographic studies of opsin synthesis in crayfish (Hafner and Bok, 1977). Since the short-wavelength photoreceptor axons are known to terminate in the medulla (Ribi, 1975), we did not expect the strong immunostaining in the 3rd layer of the lamina, the 'lamina organ'. In addition to the immunoreactivity observed in the lamina, we also found strong immunoreactivity in the neuropils and perikarya of the protocerebrum and deuterocerebrum, respectively, as well as on the ventral edge of the medulla (Fig. 7A,C). In the protocerebrum, a cluster of UV opsin-ir perikarya was found between the lobula and the anterior lateral protocerebrum (Fig. 7D,E). The cell cluster had a ball-like shape and comprised approximately 10–15 perikarya. The first olfactory neuropil in insects, the antennal lobe, is composed of small spherical subunits, called glomeruli, in which the synaptic connections between the invading antennal sensory neurons and antennal lobe interneurons take place. We found that probably all glomeruli exhibited a strong UV opsin-ir (data not shown). The UV opsin expression was mostly restricted to the core region of the glomeruli (Fig. 7G). The core region mainly comprises processes of local interneurons and dendrites of projection neurons whereas in the cap region the connections between those neurons and the invading receptor neurons take place (Arnold et al., 1985; Mobbs, 1985), Thus, we speculate that our anti-UV opsin antibody labeled dendrites of secondary olfactory neurons rather than primary sensory afferent neurons. Also, the fibers of the upper division of the central body, which is part of the central complex, showed UV opsin-ir (Fig. 7H). In all control experiments, the UV opsin-ir was completely eliminated when either the primary antibody

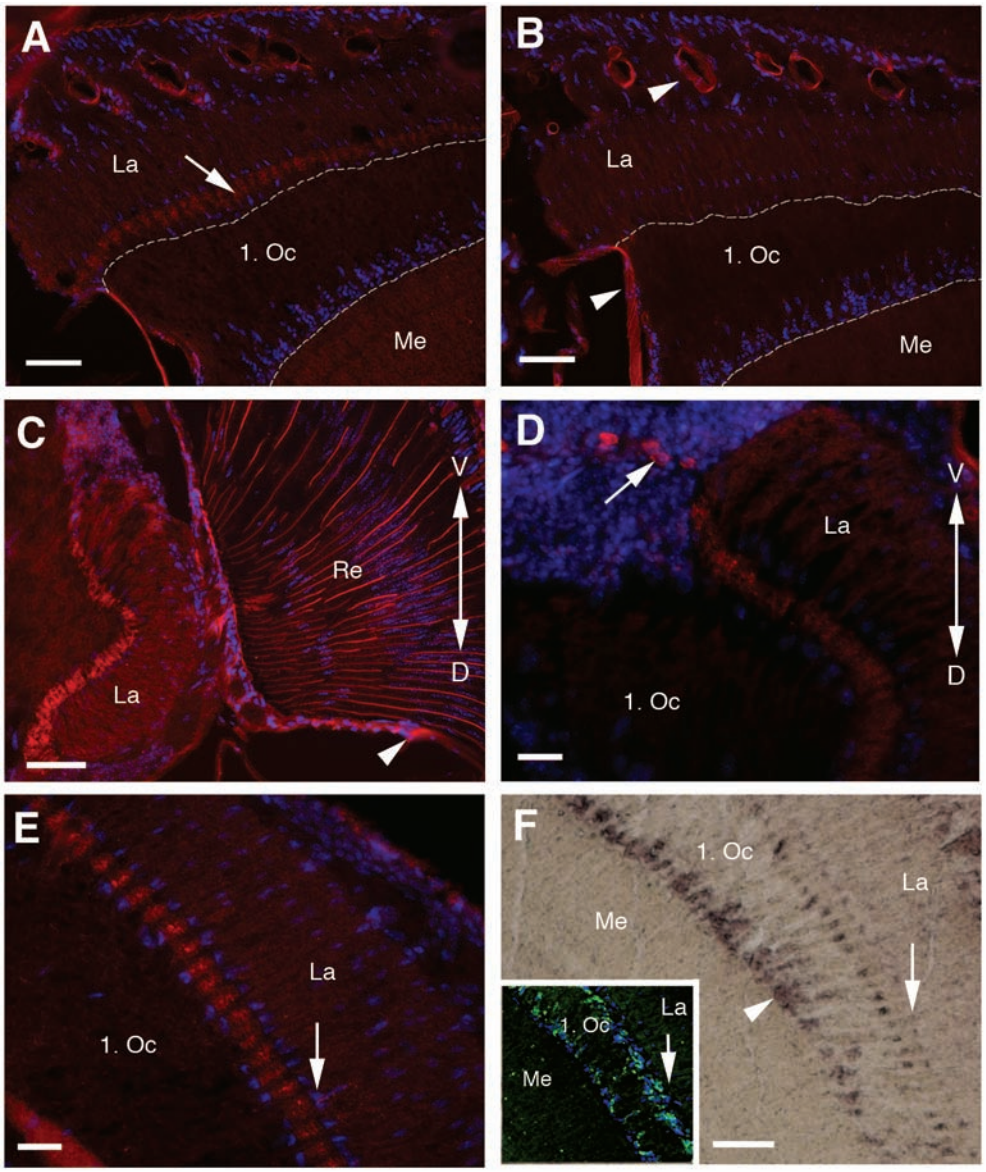

Fig. 6. (A–E) UV opsin-ir in the retina and optic lobe (Cy3, red). Nuclei were stained with DAPI (blue). (A) UV opsin-ir of the most proximal part of the lamina (C-layer), adjacent to the first optic chiasm (arrow). (B) Adjacent section to A processed with the primary antibody omitted. Strong autofluorescence of the cuticle and trachea are indicated here and in C by arrowheads. (C) UV opsin-ir in the retina and the lamina. The width of the labeled neuropil increases towards the lateral edges of the lamina (bottom left). (D) Perikarya cluster at the ventral edge of the lamina (arrow). The maximum number of labeled cells we could detect in a single section was 15. (E) The column-like structure of the UV opsin-ir neuropil in the proximal part of the lamina. Two layers of nuclei border the labeled neuropil (blue). The structure of UV opsin-ir columns found in our study shows high similarity with the optical cartridges described at the same location in the honeybee by Sinakevitch et al. (2003) using reduced silver staining. Arrow indicates cell layer comprising glial nuclei or nuclei of the monopolar cells that communicate with the long photoreceptor cell fibers. (F) UV opsin mRNA detected by *in situ* hybridization using an antisense riboprobe. Hybridization signal was found in the perikarya of the two layers bordering the lamina columns (see also arrow in E) in between which the UV opsin-ir was detected and in perikarya at the distal rim of the medulla (arrowhead), adjacent to the first optic chiasm. (Inset) PER-like-ir (green) was found in the perikarya layer that is located adjacent to the distal rim of the medulla in the first optic chiasm and in the 3rd layer of the lamina (arrow). Nuclei are stained with DAPI (blue). Arrows in F and in the inset indicate similar location. D, dorsal; 1. Oc, first optic chiasm; La, lamina; Me, medulla; Re, retina; V, ventral. Scale bars, 50 µm (A,B,C,F); 20 µm (D,E).

was omitted (e.g. Fig. 7I) or the antibody was preabsorbed with the peptide against which it was raised (e.g. Fig. 7B; Fig. S2,E,F in supplementary material).

Spatial characterization of the period protein

The period protein (PER) plays a central role in the maintenance of the circadian rhythms and in photoperiodic timing of annual rhythms that are entrained by light (Hall, 1995), and thus we were interested if partial overlap of PER and visual pigment expression occurs. The anti-PER serum we applied was raised against the full length of the *Drosophila melanogaster* PER protein (Liu et al., 1992) and was shown to be cross-reactive in the honeybee (Bloch et al., 2003). PER-ir was obtained in the 1st and 2nd layer of the optic lobe and in the deuterocerebrum. We found PER-ir in the perikarya layer, which is located adjacent to the distal rim of the medulla in the outer chiasm (Fig. 6F, inset). A second nuclear staining was found in the 3rd layer of the lamina. A similar pattern of PER expression in the optic lobe was previously reported in the honeybee (Bloch et al., 2003). Double staining of the sections with DAPI confirmed that PER-ir perikarya in the lamina form the double layer of cell bodies that borders the lamina cartridges, i.e. the lamina region in which we detected UV opsin-ir (Fig. 6F, inset) as well as UV opsin mRNA expression (Fig. 6F). None of the other perikarya within the lamina exhibit PER-ir (data not shown).

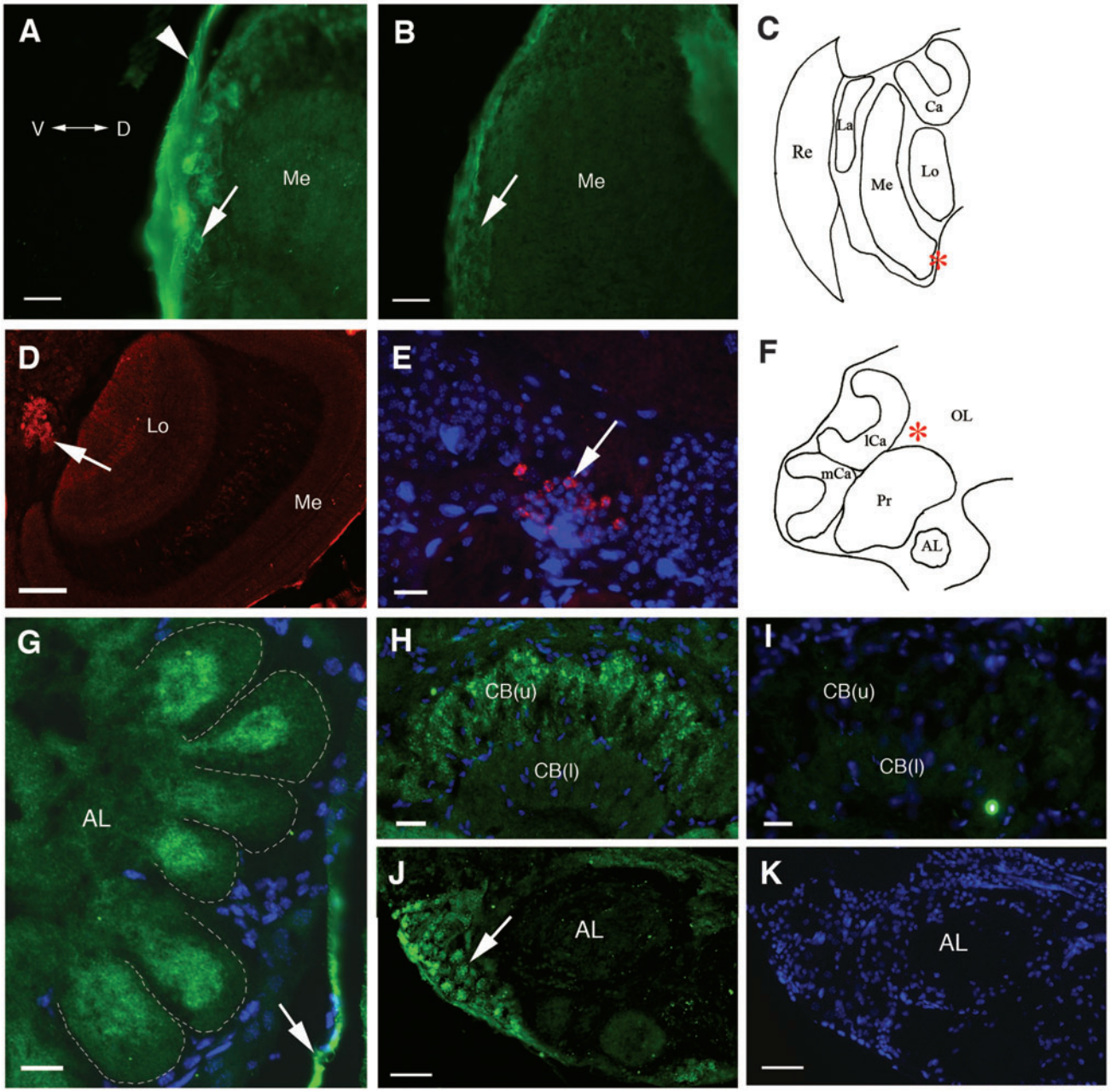

Fig. 7. UV opsin-ir (Alexa Fluor 488, green or Cy3, red) in perikarya along the edge of the medulla (Me) and the protocerebrum (Pr), within the central body (CB) and antennal lobes (AL), and PER-ir in the AL. Nuclei are stained with DAPI (blue). (A) UV opsin-like ir was found in a cluster of approximately 10 perikarya located at the ventral edge of the medulla (arrow). Arrowhead indicates autofluorescing cuticle. (B) An adjacent section to A except that the antibody was incubated prior to application with the peptide against which it was raised for 1 h at room temperature (see Materials and methods). Arrow indicates the location of perikarya in which the UV opsin-ir is undetectable. (C) Sketch of a posterior section through the optic lobe of a worker bee showing the location of the UV opsin-ir perikarya (red asterisk) shown in A. (D,E) UV opsin-ir (Cy3, red) of a cell cluster between the dorsal-median rim of the lobula (Lo) and the lateral calyces of the mushroom bodies. These cells are in a similar location to pigment dispersing hormone (PDH)-ir cells in the honeybee (Bloch et al., 2003). The arrow in D is pointing to the cell cluster and in E to an individual cell. (F) Sketch of an anterior section of a worker bee brain showing the location of the UV opsin-ir cell cluster (red asterisk) shown in D and E, which is located between the anterior lateral protocerebrum and the optic lobe. (G) Frontal section of the antennal lobe. Strongest UV opsin-ir was found in the core region of each glomeruli. (H) UV opsin-ir (Alexa Fluor 488, green) was found in the upper part of the central body. (I) Adjacent section to H, processed with the primary antibody omitted. (J) PER-ir in the deuterocerebrum. Strongest labeling was found in the lateral cell cluster (arrow). These perikarya belong to local interneurons and projection neurons (Rybak and Menzel, 1993). Dorsal and ventral clusters exhibit weaker labeling. (K) Same section as in J, showing nuclei of all antennal lobe cell bodies labeled by DAPI (blue). CB(u), central body, upper region; CB(l), central body, lower region; Lo, lobula; Me, medulla; Pr, protocerebrum; OL, optic lobe; lCa, lateral calyx; mCa, median calyx; D, dorsal; V, ventral. Scale bars, 20 μm (A,B,E, G,H,I); 50 μm (D,J,K).

The lateral cell cluster of the ALs, which comprises predominantly perikarya from local AL and projection neurons (Rybak and Menzel, 1993; Witthoeft, 1967), also exhibited strong PER-ir (Fig. 7J). In addition, many cell populations across the entire brain were found to display a weak PER-ir that was not present in the controls (data not shown). The high frequency of perikarya PER-ir found in our study is in accordance with PER expression data from the honeybee and other insects (Bloch et al., 2003; Wise et al., 2002). A schematic representation of UV opsin and PER protein distribution is shown in Fig. 8.

Discussion

A number of conserved features of the UV opsin amino acid sequence suggest that, together with the chromophore, it encodes a functional visual pigment. For instance, a lysine in transmembrane domain seven, to which the chromophore is bound in bovine rhodopsin (Bownds, 1967), is present at amino acid position 318 (Fig. 1). A highly conserved motif (E/DR) involved in G-protein binding/activation (Baldwin, 1997) is present at amino acid positions 144 and 145. An essential disulfide bridge in bovine rhodopsin (Karnik et al., 1988), connecting transmembrane domain three to the second intracellular loop, is conserved in the *Bombus* UV opsin (amino acids 120 and 197). Finally, a total of nine serine and threonine residues at the C-terminus are candidate sites of phosphorylation in photoactivated rhodopsin by rhodopsin kinase (Ohguro, 1996).

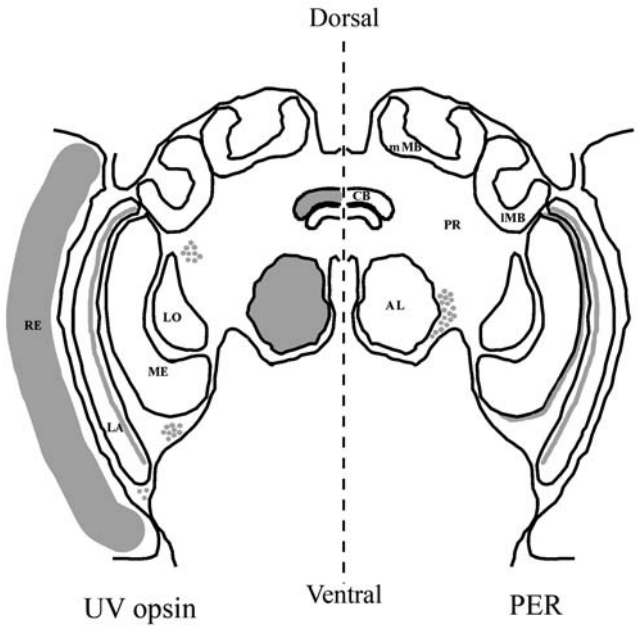

Fig. 8. Schematic frontal view of a bumblebee brain showing the location of UV opsin-ir (left) and PER-ir (right), in grey, found in this study. AL, antennal lobe; CB, central body; LA, lamina; lMB, mushroom body – lateral calyx; LO, lobula; mMB, mushroom body – median calyx; ME, medulla; PER, period protein; PR, protocerebrum; RE, retina.

The molecular basis of invertebrate ultraviolet vision has recently been identified, through a series of site-directed mutagenesis experiments (Salcedo et al., 2003), to be due primarily to the presence of a lysine residue at a site that is homologous to glycine 90 (G90) in bovine rhodopsin. We therefore inspected the translated *B. impatiens* amino acid sequence for the presence of this residue, which is important for conferring the short-wavelength spectral sensitivity of the putative rhodopsin. We indeed found a lysine at this site in the *B. impatiens* sequence and, on the basis of the presence of this amino acid, and the robust results of our phylogenetic analysis, we conclude that the encoded opsin protein is likely to produce the 350 nm UV-sensitive photopigment previously characterized by Bernard and Stavenga (1978).

In addition to these inferences regarding the putative function of this protein from the sequence analysis, three major conclusions can be drawn from our anatomical results. (1) The UV-sensitive photoreceptor cells are heterogeneously distributed among the ommatidia in the retina. We found three different types: ommatidia with two, one and no UV opsin-ir receptor cells. (2) UV opsin-ir cells are not only present in the retina but were also found in the ocelli and the optic lobe, as well as in the protocerebrum and the ALs. (3) Both UV opsin-ir and PER-ir were found in the most proximal (internal plexiform) layer of the lamina and the AL. Collectively, these results (especially the latter) suggest that both the UV opsin and PER might be part of a light-sensitive extra-retinal input channel of the circadian system.

Retina

The honeybee, *Apis mellifera*, is the only hymenopteran species where the distribution of different photoreceptor types within an ommatidium has been investigated. Combined intracellular recordings with morphological characterizations suggested the presence of only one ommatidial type within the entire retina of the worker honeybee (Menzel and Blakers, 1976). Each ommatidium was thought to contain two UV-, two blue- and four green-sensitive photoreceptor cells plus a small proximal ninth cell, which was thought to be UV-sensitive. [Intracellular recordings from photoreceptor cells in the *Bombus hortorum* compound eye confirm the presence of three types of photoreceptor with a peak sensitivity in the UV (353 nm), the blue (430 nm) and the green (548 nm); Meyer-Rochow, 1981.] However, reliable morphological characterization of the different bee receptor types is difficult due to the twist of the ommatidium around its long axis and possible (but, in most cases, neglected) morphological receptor variation in different parts of the eye (see discussion in Menzel and Blakers, 1976). Also, it has not been clear whether the correlation between spectral sensitivity and morphology found in the honeybee and two ant species (Menzel, 1972; Menzel and Blakers, 1975) is extendible to all hymenopterans.

In contrast to these early studies, our results have revealed three different ommatidial types in the worker bumblebee retina with respect to both UV opsin protein and mRNA expression (Fig. 4). Opsin immunocytochemistry and *in situ*

hybridization studies in dipterans (Chou et al., 1996; Huber et al., 1997; Montell et al., 1987; Papatsenko et al., 1997; Zuker et al., 1987) and lepidopterans (Briscoe et al., 2003; Kitamoto et al., 2000; Wakakuwa et al., 2004; White et al., 2003) have shown that the presence of different ommatidial types within the retina is a common feature of all investigated insects. For instance, the major ommatidial types in the butterfly *Vanessa cardui* are similar to those in the bumblebee and comprise seven green-sensitive cells and either two UV-, one UV- and one blue-, or two blue-sensitive cells (in *Vanessa*, the small ninth cells express a green-sensitive opsin in contrast to the supposed UV-sensitive opsin in honeybees) (Briscoe et al., 2003). Our data, together with results from other insects, suggest that the honeybee retina is presumably also comprised of different ommatidial types, which were not detected with the more classical techniques. Opsin-expression studies on the honeybee retina are, of course, necessary to verify this hypothesis.

In many insects, it has been shown that the dorsal-most rows of ommatidia (Dra) contain an increased number of UV-sensitive receptor cells, which are also sensitive to polarized light (Sauman et al., 2005; Labhart and Meyer, 1999). In *Drosophila*, for example, it has been shown that both the R7 and R8 cells of the Dra express the UV-sensitive RH3 opsin whereas, in the main retina, RH3 is expressed in only a subset of R7 cells and never in R8 (Wernet et al., 2003; Zuker et al., 1987). In this region in the bumblebee retina, we found the highest density of UV opsin-ir ommatidia (Fig. 4E). We were, however, unable to determine the number of UV opsin-labeled cells per ommatidium in this area.

Ocelli

Bumblebees have been shown to use their ocelli alone or in conjunction with the dorsal portion of the compound eye to navigate by polarized light (Wellington, 1974). While the compound eye seems particularly critical for form and color perception and works well during the day, when the environment is brightly illuminated, ocelli are especially important at dusk, when the surroundings are too dim for the compound eye to distinguish landmarks. By making use of polarized light cues, bumblebees are thereby able to prolong their foraging. Physiological investigations indicated that honeybee and bumblebee ocelli have a peak sensitivity in the blue-green as well as in the UV part of the light (Meyer-Rochow, 1981; Ruck and Goldsmith, 1958). Meyer-Rochow (1981) estimated the UV peak sensitivity of the bumblebee median ocellus to be 353 nm, which is indistinguishable from the peak sensitivity of the UV visual pigment of the compound eye (Bernard and Stavenga, 1978). It is therefore not entirely surprising that we found UV opsin-ir rhabdomeres in all three ocelli. The physiological similarity between the UV visual pigment in the compound eye and ocelli, in combination with the finding of only one UV opsin-encoding gene in the recently released *Apis mellifera* genome and our immunohistochemical results, suggests that the same opsin is expressed in both the compound eye and ocelli.

The honeybee ocelli show an additional peak sensitivity at around 490 nm (Ruck and Goldsmith, 1958), which is ~50 nm shifted towards shorter wavelengths compared with the long-wavelength photoreceptor cells found in the retina (Menzel and Blakers, 1976), and in *Bombus hortorum*, the ocelli have a secondary peak around 519 nm (as estimated by ERG) (Meyer-Rochow, 1980). In Drosophila, the Rh2 opsin is exclusively expressed in the ocelli (Pollock and Benzer, 1988) and shows a 60 nm blue-shift compared to the paralogous main retinular opsin Rh1 (Britt et al., 1993; Zuker et al., 1988). We recently found a novel LW-sensitive opsin gene (*LW Rh2*) that is paralogous to the already known LW opsin gene (*LW Rh1*) in hymenopterans (Spaethe and Briscoe, 2004). The mRNA expression level of *LW Rh2* is much lower compared with the already known *LW Rh1* opsin gene (J.S. and A.D.B., unpublished observation) and, although its cellular location is currently unclear, it is possible that it is expressed only in the ocelli, like Rh2 in *Drosophila*.

Extra-retinal opsin expression

So far, extra-retinal opsin expression has been studied in only a few insects and crustaceans (Sandeman et al., 1990). As one example, the expression of green-, blue- and UV-sensitive opsins in lepidopteran adult stemmata, which correspond to the former larval photoreceptors, has only recently been examined (Briscoe and White, 2005; Lampel et al., in press). In addition, even within the same insect order there appears to be heterogeneity in terms of which opsins are expressed. In the butterfly *Vanessa*, for instance, only UV and green opsin transcripts were detected in the stemmata, while in hawkmoths, all three were found. In the *Drosophila* Hofbauer–Buchner eyelet, the homologue of the adult lepidopteran stemmata, the green-sensitive Rh5 and the blue-sensitive Rh6 rhodopsins were found to be expressed but, in contrast to lepidopterans, no UV-sensitive (Rh3 or Rh4) rhodopsin was detected (Malpel et al., 2002; Yasuyama and Meinertzhagen, 1999). In both instances, all opsins that are found in the stemmata are also expressed in the retina. Whether the UV opsin-ir perikarya at the ventral rim of the lamina that we found in the bumblebee (Fig. 6D) are homologous to the butterfly stemmata is unknown; earlier studies suggested that stemmata are probably absent in aculeate hymenopterans (Gilbert, 1994; but see Felisberti and Ventura, 1996). Also, we found no associated crystalline cones with these perikarya, as were found in lepidopterans (Briscoe and White, 2005).

Lepidopterans and aphids are the only insect groups so far in which opsins have also been reported in other parts of the brain. Shimizu et al. (2001) cloned a long-wavelength sensitive opsin (*boceropsin*) from a larval brain cDNA of the silkworm, *Bombyx mori*, and showed its protein expression in various neuronal clusters in the larval brain. Gao et al. (1999) tested several antibodies directed against invertebrate (*Drosophila* Rh1) and vertebrate (chicken cone, mammalian blue cone, and rod) opsins in the aphid *Megoura viciae*, and an anterior ventral neuropil region in the brain was labeled. However, *in situ* hybridization experiments with two *M. viciae* opsin-encoding

antisense riboprobes found expression only in the retina but not in other parts of the brain, indicating that an additional opsin might be expressed in the aphid brain (Gao et al., 2000). In four different hawkmoth species, Lampel et al. (in press) found widespread expression of a new LW-sensitive opsin ('brain opsin') in various parts of the optic lobe, namely the lamina, lobula, lobula plate, medulla, accessory medulla and adjacent neurons innervating the accessory medulla. In contrast to the opsins found in both the retina and stemmata, the brain opsin in the hawkmoths was exclusively expressed outside the retina, including in perikarya along the ventral rim of the medulla, as we found in bees.

Our data show that in bumblebees, UV opsin that is expressed in the retina and supposed to be used primarily for vision is broadly expressed in different parts of the brain as well. In contrast to previous studies in lepidopterans and dipterans, where expression of retinal opsins outside the retina was shown to be restricted to stemmata, opsin-ir in *Bombus* was also found in larger neuropils like the proximal rim of the lamina (Fig. 6), the AL and central body (Fig. 7). This situation in *Bombus* is not unlike that of the crayfish *Cherax destructor*, where the major rhodopsin of the retinula cells of the compound eye was also found to be expressed in cell sommata along the anterior margin of the cerebral ganglion (Sandeman et al., 1990), and highlights the flexibility of opsin expression patterns in different invertebrate lineages. It will be interesting to see whether the blue and LW eye opsins of bumblebees are also expressed extraretinally.

With all antibody studies, however, it is important to note that there is always the possibility of cross-reactivity with multiple epitopes (Marchalonis et al., 2001). The results of our western blot indicate the presence of a major band at ~41 kDa, which is the predicted size for the UV opsin protein. We also note the presence of two smaller bands in the same blot, at 30 and 29 kDa. Multiple bands have been observed in opsin western blots in *Drosophila* (Bentrop et al., 1997), in hawkmoths (White et al., 2003) and in *Limulus* (Battelle et al., 2001), and therefore the observation of multiple bands in our western blot is not entirely surprising. In *Drosophila*, this is due to the fact that nascent Rh1 opsin protein is glycosylated and then deglycosylated as it matures. On a western blot, this is observed as multiple bands, at 40 and 35 kDa (Ozaki et al., 1993). We inspected the *Bombus* UV opsin amino acid sequence for the presence of putative glycosylation sites, indicated by the consensus sequence NXS/T (O'Tousa, 1992), and found two in the N-terminal domain (see Fig. 1), one more than is present in the *Drosophila* Rh1 opsin (Katanosaka et al., 1998). Therefore, one possible explanation for these additional bands is that the UV opsin protein in bees exists in several different states of glycosylation. Another possibility is that these bands also represent proteolytic breakdown products of the opsin protein. Lastly, the results of our tBLASTn search for peptide sequences similar to that part of the protein to which we directed our antibodies, including against the whole *Apis mellifera* genome, yielded only UV-sensitive insect opsin proteins, so it would seem that this peptide motif is, at least, not common among the known genomes in GenBank.

Several lines of evidence in fact suggest that the opsin expression patterns we observed in the retina and brain may be specific. First, staining in the ocelli (Fig. 5B), perikarya at the edge of the medulla (Fig. 7B), in the retina, lamina organ, cluster of cells between the lobula and the mushroom bodies (Fig. S2 in supplementary material), within the antennal lobes and central body (not shown) were all abolished when the antibody was first incubated with the peptide. Second, we confirmed the pattern of opsin protein expression in the compound eye by performing *in situ* hybridizations. Third, the cluster of UV opsin-ir cells that we observed between the lobula plate and the calyces of the mushroom bodies is similar in position to a cluster of pigment-dispersing hormone (PDH)-ir cells in *Apis mellifera* (Bloch et al., 2003). Neurons that are immunoreactive for PDH appear to be significant components of the optic lobe pacemakers in a variety of insects (Helfrich-Forster et al., 1998). Finally, in intracellular recordings from neurons in the central body of the locust, several were found to be light sensitive (Vitzthum et al., 2002). Our finding of UV opsin-like staining in the central body is consistent with this and may in part explain some of these findings. Clearly, much additional work needs to be performed to corroborate our results. As one example, it would be interesting to examine the distribution of visual arrestin and PDH in relation to the UV opsin expression described above.

Putative non-visual extra-retinal photoreceptors

Besides vision, a primary function of photoreceptors in insects is the entrainment of circadian and seasonal photoperiodic rhythms. Biological rhythms are associated with organismal changes in physiology and behavior and are controlled by clock genes at the cellular level (Devlin and Kay, 2001; Jackson et al., 2001). The 'circa'-dian changes of clock gene expression levels are entrained by external photic stimuli, for instance by changes in the surrounding light intensity or spectral composition (Zordan et al., 2001). However, the contribution of the various non-visual photic input channels is complex and, even in *Drosophila*, not fully understood. Behavioral experiments with different fly mutants showed that, besides the compound eyes and the ocelli, at least three different non-visual channels are additionally involved in synchronizing the circadian clock: the Hofbauer–Buchner eyelet (discussed above), the blue-light photopigment cryptochrome, and unknown photopigments in clock-gene-expressing dorsal neurons (Helfrich-Forster et al., 2001; Rieger et al., 2003). Other putative extra-retinal photoreceptors described in different hemi- and holometabolous insect orders are the lamina and lobula organs, located in the optic lobe (Fleissner and Fleissner, 2003). The laminar organs have been found on the proximal dorso-frontal rim of the lamina, the part of the optic lobe where we found strong UV opsin- and PER-immunoreactivity in the bumblebee (Fig. 6). This photoreceptive organ was detected by means of

immunocytochemistry using antibodies against proteins of the phototransduction cascade and cryptochrome in combination with ultrastructural investigations. However, neither the presence of opsins nor PER was tested. We speculate that the proximal part of the lamina in bumblebees serves a similar function to the laminar organ in other insects. However, to demonstrate that the proximal part of the lamina in bumblebees is homologous to the previously described lamina organ in other insects, we need to check for the presence of cryptochrome in the bumblebee lamina, for opsins in the laminar organ of other insect orders, and for similar morphological and developmental origins.

We also found UV opsin and PER expression in AL neurons. No photoreceptive system has been described in the bee AL so far. However, the extraretinal LW brain opsin in adult hawkmoths, mentioned above, is also expressed in the core region of hawkmoth antennal lobe glomeruli (Lampel et al., 2002; J. Lampel, A.D.B. and L. T. Wasserthal, unpublished). Therefore, these neurons might form independent photoreceptive clock neurons in the bee AL, as in *Drosophila*, where autonomous circadian oscillators are found in different organs and tissues all over the body (Plautz et al., 1997). Also, electroantennograms in *Drosophila* have shown a robust circadian rhythm of electrophysiological responses of the antenna that was abolished in a mutant fly where PER expression was restricted to the optic lobe but was not expressed in peripheral circadian oscillators (Krishnan et al., 1999). An alternative explanation is that the UV opsin serves a different, yet unknown, function in the AL than photoreception. For instance, arrestins play an important role in visual signaling processes by interacting with the rhodopsin and terminating visual stimulation. In recent studies in *Anopheles* and *Drosophila*, it has been shown that visual arrestins are also expressed in olfactory neurons and are required for normal olfactory physiology, demonstrating a function in both visual and olfactory signal transduction systems (Merrill et al., 2002).

To summarize, UV opsin was found in different parts of the bumblebee brain, the proximal rim of the lamina, antennal lobe, central complex and different cell clusters in the protocerebrum. Also, PER, an important component of the circadian clock, was found in the proximal rim of the lamina and the antennal lobe, indicating a putative function of these brain regions as putative extra-retinal photoreceptors in the entrainment of circadian rhythms. Further studies are needed, however, to clarify whether UV opsin and PER are co-expressed in the same cells and whether PER protein levels in the lamina and in other UV-expressing areas described here oscillate over a 24 h light:dark cycle.

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