

RESEARCH ARTICLE

Expression and light-dependent translocation of β -arrestin in the visual system of the terrestrial slug *Limax valentianus*

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ABSTRACT

Vertebrates, cephalopods and arthropods are equipped with eyes that have the highest spatiotemporal resolution among the animal phyla. In parallel, only animals in these three phyla have visual arrestin specialized for the termination of visual signaling triggered by opsin, in addition to ubiquitously expressed β -arrestin that serves in terminating general G protein-coupled receptor signaling. Indeed, visual arrestin in *Drosophila* and rodents translocates to the opsin-rich subcellular region in response to light to reduce the overall sensitivity of photoreceptors in an illuminated environment (i.e. light adaptation). We thus hypothesized that, during evolution, visual arrestin has taken over the role of β -arrestin in those animals with eyes of high spatiotemporal resolution. If this is true, it is expected that β -arrestin plays a role similar to visual arrestin in those animals with low-resolution eyes. In the present study, we focused on the terrestrial mollusk *Limax valentianus*, a species related to cephalopods but that has only β -arrestin, and generated antibodies against β -arrestin. We found that β -arrestin is highly expressed in photosensory neurons, and translocates into the microvilli of the rhabdomere within 30 min in response to short wavelength light (400 nm), to which the *Limax* eye exhibits a robust response. These observations suggest that β -arrestin functions in the visual system of those animals that do not have visual arrestin. We also exploited anti- β -arrestin antibody to visualize the optic nerve projecting to the brain, and demonstrated its usefulness for tracing a visual ascending pathway.

KEY WORDS: Spatiotemporal resolution, *Lehmannia*, Optic nerve, Rhodopsin, Retina, Gastropod

INTRODUCTION

Most metazoan animal species have eyes as a sensory organ specialized for the detection of ambient light. The evolution of eyes has been discussed in terms of their structural and functional characteristics, such as the directionality of photoreception, membrane stacking, integration time, functionality of the lens, and so on (Land and Nilsson, 2012; Nilsson, 2013). Acquisition and refinement of these elements resulted in the development of eyes with high performance.

Among animal phyla, vertebrates, cephalopods and arthropods have acquired the most exquisite eyes with high temporal and spatial

resolution (classified as ‘class IV’ by Nilsson, 2013). Are there any molecular bases for the exquisiteness of the eyes of the animals in these three phyla? Regulation of the lifetime of rhodopsin signaling is one of the determinants of the temporal resolution of eyes. Arrestin is thought to play an important role in this process because it takes part in the termination of rhodopsin signaling (Arshavsky, 2003; Calvert et al., 2006). Here, we have noticed that the photoreceptors of the animals belonging to these three phyla possess arrestin specialized for vision (visual arrestin) in addition to β -arrestin as a signaling component of phototransduction (Gurevich and Gurevich, 2006; Yoshida et al., 2015). Arrestin family proteins function in terminating G protein-coupled receptor (GPCR) signaling by binding the intracellular domain of the GPCR, thereby sterically blocking further G protein activation. Of these, β -arrestin has a clathrin-binding domain in its C-terminus, and is ubiquitously expressed, serving as a terminator for all GPCR signaling. In contrast, visual arrestin is devoid of a clathrin-binding domain, and is specialized for the termination of opsin signaling in photoreceptors (Gurevich and Gurevich, 2006). If the presence of visual arrestin is a key factor for high performance of class IV eyes, it must be subserving the high temporal resolution by promoting rapid turnover of activation/inactivation of rhodopsin signaling.

If the above-mentioned parallelism is actually meaningful, it is expected that animals with less-developed eyes should cope with living tasks using only β -arrestin. In the present study, we focused on the gastropod *Limax* because it is evolutionarily related to cephalopods but considered to have less elaborate eyes, and seems to have a lifestyle that is less dependent on speed. According to Nilsson (2013), the gastropod eye is categorized as class III (having a medium spatiotemporal resolution), as a result of the small number of photoreceptors and under-focused lenses.

Visual arrestin is known to translocate to the rhabdomeric region/outer segment of the photoreceptors in response to light in *Drosophila* and vertebrates (Broekhuysen et al., 1985; Philp et al., 1987; Kiselev et al., 2000; Nair et al., 2002; Lee et al., 2003). Such translocation serves to down-regulate the overall signaling from rhodopsins. The light-dependent translocation of arrestin, therefore, is thought to play a pivotal role in the light adaptation of the photoreceptor (Calvert et al., 2006). However, it is uncertain whether β -arrestin exhibits similar light-dependent behavior in the photoreceptors involved in vision of those animals that lack visual arrestin.

Immunohistochemical visualization of β -arrestin is also important for tracing the flow of visual information in the slug brain. It has been suggested in previous studies that some of the visual information entering the brain from the eye crosses the commissure of the cerebral ganglia, and inputs into the contralateral side of the brain (Tuchina et al., 2011; Matsuo et al., 2014b). In these studies, the visual tract was marked with neurobiotin incorporated from the cut end of the optic nerve. Such bilateral crosstalk probably enables the slug’s negative phototaxis behavior through comparison of the light intensities between the bilateral eyes, thereby allowing efficient

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List of abbreviations

CNS	central nervous system
ERG	electroretinogram
FMRamide	Phe-Met-Arg-Phe-NH ₂
GPCR	G protein-coupled receptor
GST	glutathione S-transferase
NHS	N-hydroxysuccinimide
PBS	phosphate-buffered saline

escape from a light place (Matsuo et al., 2014b). However, incorporation of neurobiotin does not necessarily mean that the optic nerve directly projects to the other side of the cerebral ganglion, because neurobiotin easily passes gap junctions (Hampson et al., 1992). Hence, the commissural nerve may be distinct from those neurons projecting to the brain from the eye. Therefore, a different molecular tool is necessary for this analysis. β -Arrestin is a good candidate to serve this role. Indeed, the optic nerves have previously been visualized by immunohistochemical staining of this protein in planaria because β -arrestin protein is enriched in the photoreceptor cells and optic nerves in this species (Cross et al., 2015).

In the present study, we first confirmed that the *Limax* eye has a simple structure, and that *Limax* has β -arrestin but is devoid of visual arrestin. We then cloned β -arrestin cDNA from *Limax valentianus* to analyze its expression in the eye and in other parts of the central nervous system (CNS). We also raised antibodies against β -arrestin and rhodopsin to analyze the anatomical features of the eye. We then used immunohistochemical staining to investigate whether β -arrestin exhibits light-dependent translocation in the photosensory neurons, and analyzed its wavelength dependency. Finally, anti- β -arrestin antibody was applied to visualize the optic nerve to trace the flow of visual information in the brain, as well as to monitor the re-innervation of optic nerves during tentacle regeneration after tentacle amputation.

MATERIALS AND METHODS**Animals**

Terrestrial slugs *Limax valentianus* Férussac 1822 have been maintained in our laboratory at 19°C for at least 29 generations as a closed colony. They were fed a diet of humidified powder mixture consisting of 500 g of potato starch, 521 g of rat chow (Oriental Yeast, Tokyo, Japan) and 21 g of mixed vitamins (Oriental Yeast). The age of the slugs was 3–4 months after hatching at the start of all experiments.

Toluidine Blue staining and electron microscopy

Slugs were deeply anesthetized with an injection of ice-cold Mg²⁺ buffer (57.6 mmol l⁻¹ MgCl₂, 5.0 mmol l⁻¹ glucose, 5.0 mmol l⁻¹ Hepes, pH 7.0) into the body cavity, and the superior tentacle was isolated. The tentacle was fixed by immersion in 2% paraformaldehyde and 2% glutaraldehyde in 0.02 mol l⁻¹ phosphate buffer (pH 7.3) at 4°C overnight. The tissues were rinsed, osmified, dehydrated and embedded in epoxy resin. Semi-thin (1 μ m-thick) sections were cut on an ultramicrotome (Leica EM UC7, Leica Microsystems, Wetzlar, Germany) and stained with 0.5% Toluidine Blue dissolved in 1% borate buffer. Images were obtained with an Eclipse E800 microscope (Nikon, Tokyo, Japan) equipped with a DXM1200F digital CCD camera (Nikon) and a $\times 40$ (NA 0.95) objective lens. Ultrathin sections were cut on the ultramicrotome, stained with uranyl acetate and lead citrate, and examined with an electron microscope (JEM-1400plus, JEOL, Tokyo, Japan).

Molecular cloning of arrestin cDNA

Arrestin cDNA was cloned by 5'-SMART rapid amplification of cDNA ends (5'-SMART-RACE, Takara, Kusatsu, Japan) and reverse transcription-PCR (RT-PCR) using template cDNAs synthesized from RNAs derived from the brain and the tentacle. The PCR primers were designed based on the nucleotide sequence of putative arrestin partial cDNA found in RNA-seq data of *Limax* (also called *Ambigolimax*, NCBI BioProject accession number PRJDB3972). The nucleotide sequences of the PCR primers were 5'-CGCCACATGGCTTGCCAGTGTCTCCAGGTG-3' and 5'-T-ACAGAAAGCTGGGGAATTGGTGGCAGCTC-3' for the first and second gene-specific primers, respectively, in 5'-SMART-RACE, and 5'-CACCTGGAGACACTGGCAAG-3' and 5'-TGGC-AGACTCACTTGTTTAGAAAC-3' for molecular cloning of the C-terminal region of arrestin cDNA. The obtained PCR fragments were purified using the Wizard SV Gel and PCR clean-up system (Promega, Fitchburg, WI, USA) according to the manufacturer's instructions, and then precipitated with ethanol. The termini of the concentrated PCR product were phosphorylated by T7 polynucleotide kinase, and fragments were ligated into a cloning vector (pBluescriptII, Agilent Technologies, Santa Clara, CA, USA) that had been digested with *EcoRV* then dephosphorylated with shrimp alkaline phosphatase (Takara). These plasmids were transformed into competent *Escherichia coli* (Dh5 α) for amplification, and subsequently extracted using a GenElute plasmid miniprep kit (Sigma-Aldrich, St Louis, MO, USA). The nucleotide sequences of the plasmid inserts were confirmed by DNA sequencing (Genetic Analyzer 3500, Applied Biosystems, Waltham, MA, USA). The nucleotide sequence information of *L. valentianus* arrestin (β -arrestin) was deposited in GenBank (accession number: LC218443).

Phylogenetic analysis

Phylogenetic tree inference was performed as described previously (Koyanagi et al., 2004). Briefly, multiple alignment of the amino acid sequences of arrestins, including *L. valentianus* β -arrestin, was carried out with the aid of XCED software (Kato et al., 2002). A molecular phylogenetic tree was inferred by the neighbor-joining method (Saitou and Nei, 1987). Bootstrap analysis was performed based on 1000 sets of resampled sequences using Felsenstein's method (Felsenstein, 1985). The accession numbers of the sequences used for analysis were as follows: human (*Homo*) rod arrestin, NM_000541; medaka (*Oryzias*) rod arrestin-1, AB002554; medaka rod arrestin-2, AB029392; human cone arrestin, NM_004312; medaka cone arrestin, AB002555; lamprey (*Lethenteron*) visual arrestin, AB495339; human β -arrestin-1, NM_004041; zebrafish (*Danio*) β -arrestin-1, NM_001159822; human β -arrestin-2, NM_004313; rainbow trout (*Oncorhynchus*) red blood cell arrestin, U48410; lamprey β -arrestin, AB495338; ascidian (*Ciona*) arrestin, AB052669; fruitfly (*Drosophila*) arrestin-1, NM_057333; fruitfly arrestin-2, NM_079252; octopus (*Octopus*) visual arrestin, XM_014928485; splendid squid (*Loligo*) visual arrestin, AF393635; pygmy squid (*Idiosepius*) visual arrestin, LC021441; bobtail squid (*Euprymna*) eye arrestin, EU344779; fruitfly kurtz, AF221066; nematode (*Caenorhabditis*) β -arrestin, NM_075782; oyster (*Crassostrea*) β -arrestin, XM_011435491; limpet (*Lottia*) β -arrestin, XM_009058330; scallop (*Argopecten*) Arr12, HQ695998; scallop Arr23, HQ695999; octopus β -arrestin-1-like, XM_014914803; pygmy squid β -arrestin, LC021440; nautilus (*Nautilus*) β -arrestin, LC021439; sea hare (*Aplysia*) β -arrestin-1-like, XM_013090298; and slug (*Limax*) β -arrestin, LC218443.

Expression analysis of β -arrestin by RT-PCR

Molecular cloning of β -arrestin was performed by RT-PCR as described previously (Fukunaga et al., 2006) using template cDNAs synthesized from RNAs derived from the cerebral ganglia, subesophageal ganglia or superior tentacle. The nucleotide sequences of the PCR primers were: 5'-CATGACTCCACTGCTGTCA-AAC-3' and 5'-GTGGCTGCTCTCAAGATCCAC-3' for β -arrestin, and 5'-GCTTACCAAGCTCCGACCCTCGTGG-3' and 5'-CGTCACTACCTCCCCGTGCCGGG-3' for 18S ribosomal RNA (rRNA).

In situ hybridization of β -arrestin and rhodopsin

cDNA fragments of β -arrestin and rhodopsin (GenBank accession number: LC223120) were amplified by RT-PCR using the template cDNAs synthesized from RNAs derived from the whole CNS and the superior tentacle of the slug, respectively. The nucleotide sequences of the PCR primers were 5'-CACCTGGAGACACTG-GCAAG-3' and 5'-TGCGAGACTCACTTGTTTAGAAAC-3' for β -arrestin, and 5'-GCCGCAATGAGCAGGATGGAC-3' and 5'-GACTGCGTGGAGGCTGCTGC-3' for rhodopsin. The PCR fragments were ligated into a cloning vector (pCRII-TOPO, Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The obtained plasmid clones were used as templates for *in vitro* transcription of digoxigenin-labeled cRNA probes as described previously (Fukunaga et al., 2006). The final concentrations of antisense and sense cRNA probes were determined so that their titers were equivalent. Fresh-frozen sections (14 μ m thick) of superior tentacle and brain were mounted onto a glass slide coated with Vectabond (Vector Laboratories, Burlingame, CA, USA). Hybridization (at 52°C) and washing were performed as described in Fukunaga et al. (2006). The hybridized cRNA probes were detected using anti-digoxigenin antibody conjugated with alkaline phosphatase (Roche Diagnostics, Basel, Switzerland). The fluorescence substrates of alkaline phosphatase (HNPP fluorescence detection set, Roche Diagnostics; used according to the manufacturer's instructions) were used because the tissues in the superior tentacle are intrinsically brown colored, which made it difficult to distinguish the positive signals using a color development method. The reaction was stopped by incubation of the sections with TE buffer for 10 min (10 mmol l⁻¹ Tris pH 8.0, 1 mmol l⁻¹ EDTA). The nuclei were then stained with 0.1 μ g ml⁻¹ 4'-diamidino-2-phenylindole (DAPI) in TE for 10 min, followed by a wash with TE buffer. The images were obtained with an Eclipse E600 microscope (Nikon) equipped with a DP70 digital CCD camera (Olympus, Tokyo, Japan) and a \times 20 (NA 0.50) objective lens.

Preparation of β -arrestin protein and generation of anti- β -arrestin antibody

β -Arrestin protein was bacterially expressed as a glutathione *S*-transferase (GST) fusion protein using the pGEX-6P-2 expression vector (GE Healthcare, Little Chalfont, UK), and was purified essentially as described previously (Matsuo et al., 2014a). Briefly, a full open reading frame of β -arrestin was ligated into pGEX-6P-2 using *Bam*HI and *Xho*I restriction sites, and transformed into competent *E. coli* (Dh5 α). The expression of the β -arrestin fused to the C-terminus of GST was induced by 0.2 mmol l⁻¹ isopropyl β -D-thiogalactopyranoside (IPTG) at 22°C overnight. The recombinant protein was purified using Glutathione Sepharose 4B beads (GE Healthcare) as described previously (Yamagishi et al., 2012). The fusion protein was then digested with PreScission Protease (GE Healthcare), and the GST moiety and

PreScission Protease were removed using Glutathione Sepharose 4B beads. Antiserum was obtained by immunizing a rabbit with the purified recombinant β -arrestin protein, and the antibody was affinity purified from the antiserum using the recombinant β -arrestin protein covalently attached to *N*-hydroxysuccinimide (NHS)-activated Sepharose 4 Fast Flow beads (GE Healthcare) as described previously (Matsuo et al., 2001). The concentration of the purified antibody was determined using a Pierce BCA protein assay kit (Thermo Scientific) according to the manufacturer's instructions.

Generation of anti-rhodopsin antibody

A rabbit was immunized with the C-terminal 19 amino acids (VITQADVGSYSNKAYQID) of *Limax* rhodopsin, conjugated to Keyhole limpet hemocyanin via a cysteine residue added to the peptide's N-terminus. The antibody was purified from the antiserum using the same peptide conjugated to NHS-activated Sepharose 4 Fast Flow beads. The concentration of the purified antibody was determined using a Pierce BCA protein assay kit.

Cell culture and transfection

COS-7 cells were cultured in 6-well dishes in Dulbecco's modified Eagle's medium (Thermo Scientific) supplemented with 10% fetal bovine serum (Sigma-Aldrich) at 37°C in 5% CO₂, 95% air. Mammalian expression vector pcDNA3.1 (Thermo Scientific) harboring a full open reading frame of β -arrestin was transfected using Lipofectamine 3000 reagent (Thermo Scientific) according to the manufacturer's instructions. After 24 h, the cells were washed with cold phosphate-buffered saline (PBS), and harvested with ice-cold TNE buffer [10 mmol l⁻¹ Tris pH 7.5, 1 mmol l⁻¹ EDTA, 1% (v/v) Nonidet P-40, protease inhibitor cocktail (Wako, Osaka, Japan)]. The protein concentration was determined using a Pierce BCA protein assay kit.

Preparation of protein samples and western blotting

To obtain samples for western blotting of β -arrestin, the brain (without the buccal ganglia) and the superior tentacles were isolated under anesthesia; the brain was further dissected into cerebral and subesophageal ganglia. The tissues were homogenized in ice-cold TNE buffer. To obtain samples for western blotting of rhodopsin, a crude membrane fraction was prepared as follows: the brain or the superior tentacle was homogenized in ice-cold buffer consisting of 10 mmol l⁻¹ Tris pH 8.0, 0.25 mol l⁻¹ sucrose, 1 mmol l⁻¹ EDTA and 10 mmol l⁻¹ β -mercaptoethanol, supplemented with protease inhibitor cocktail, followed by centrifugation at 1400 *g* for 10 min at 4°C. The supernatant was then ultracentrifuged at 100,000 *g* for 60 min at 4°C, and the pellet was dissolved in TNE buffer supplemented with protease inhibitor cocktail. The protein concentrations of the homogenates were determined using a Pierce BCA protein assay kit. An equivalent volume of SDS sample buffer (50 mmol l⁻¹ Tris pH 6.8, 4% SDS, 10% glycerol, 10% mercaptoethanol, 0.1% Bromophenol Blue) was added to the homogenates, and the mixture was boiled at 100°C. Lysates containing 3 or 7.5 μ g protein were electrophoresed on a 12% SDS-polyacrylamide gel for the β -arrestin western blot, and those containing 5 μ g protein were electrophoresed on a 10% SDS-polyacrylamide gel for the rhodopsin western blot. The electrophoresed protein was electrically transferred to nitrocellulose membrane (GE Healthcare). The membrane was blocked with 5% skimmed milk in TTBS (20 mmol l⁻¹ Tris pH 7.5, 137 mmol l⁻¹ NaCl, 0.2% Tween-20) at 4°C overnight then incubated with 0.3 μ g ml⁻¹ anti- β -arrestin antibody or anti- α -tubulin mouse monoclonal antibody (1:5000, clone B-5-1-2, Sigma-Aldrich) in

TTBS at room temperature for 1 h, washed 3 times in TTBS, and then incubated with horseradish peroxidase-conjugated anti-rabbit IgG (for β -arrestin) or anti-mouse IgG antibody (for α -tubulin; both 1:10,000, GE Healthcare) in TTBS for 50 min at room temperature. The blots were washed 3 times in TTBS, and the signals were detected using ImmunoStar LD (Wako) and LuminoGraph I (ATTO, Tokyo, Japan).

Tentacle amputation

To monitor the process of tentacle regeneration using anti- β -arrestin antibody, we surgically amputated the left superior tentacle from the slugs under anesthesia, essentially as described previously (Yamagishi et al., 2008) with slight modification. Briefly, a small cut was made with microscissors on the left side of the head, and the superior tentacle (including the optic nerve) was cut at the base near the procerebrum. The left superior tentacle was then pulled out. After surgery, 300–400 μ l of physiological saline (70 mmol l⁻¹ NaCl, 2.0 mmol l⁻¹ KCl, 4.7 mmol l⁻¹ MgCl₂, 4.9 mmol l⁻¹ CaCl₂, 5.0 mmol l⁻¹ glucose and 5.0 mmol l⁻¹ Hepes adjusted to pH 7.0) was injected into the body cavity to facilitate recovery from the anesthesia.

Immunohistochemistry

The brain or superior tentacle was isolated as described above, and frozen in Surgipath FSC 22 Clear Frozen Section Compound (Leica Microsystems) using liquid nitrogen. Cryostat sections (14 or 6 μ m thick) were cut and mounted onto glass slides coated with Vectabond. They were fixed in 4% paraformaldehyde/0.1% glutaraldehyde in PBS for 30 min at room temperature. To immunostain an optic nerve entering the brain, the isolated brain was fixed by immersion in 4% paraformaldehyde in PBS for 1 h followed by cryoprotection in 20% sucrose in PBS at 4°C overnight before being frozen in Surgipath FSC 22 Clear Frozen Section Compound using liquid nitrogen. Sections (14 μ m thick) were post-fixed with 5% neutralized formalin for 20 min at room temperature. They were then treated with 0.1% Triton X-100 dissolved in PBS (PBST) for 10 min. After a brief wash in PBS, the sections were blocked in blocking buffer (PBST supplemented with 2.5% goat serum and 2.5% BSA) at room temperature for 1–8 h, and then incubated with anti- β -arrestin antibody (0.3 μ g ml⁻¹ in blocking buffer) or anti-rhodopsin antibody (0.5 μ g ml⁻¹ in blocking buffer) at 4°C overnight. In some experiments, the primary antibody was omitted from the blocking buffer to show the absence of non-specific binding of the secondary antibody. The sections were washed three times in PBS, followed by incubation for 50 min with a secondary antibody against rabbit IgG labeled with Alexa Fluor 488 (1:500 in blocking buffer; Life Technologies, Carlsbad, CA, USA) at room temperature. After a wash in PBS, the sections were incubated with 0.1 μ g ml⁻¹ DAPI in PBS for 10 min, then washed again in PBS. The sections were mounted under a coverslip in Fluoromount G (SouthernBiotech, Birmingham, AL, USA). To confirm the specificity of the primary antibody, anti- β -arrestin antibody (900 μ l, 0.3 μ g ml⁻¹ in blocking buffer) was incubated with the recombinant β -arrestin protein covalently attached to NHS-activated Sepharose 4 Fast Flow beads (100 μ l volume, see above) at 4°C overnight, and the supernatant was used for the primary antibody reaction. For dual staining of β -arrestin and α -tubulin, 6 μ m-thick sections were cut, and the primary antibody was a mixture of anti- β -arrestin antibody (0.3 μ g ml⁻¹) and anti- α -tubulin mouse monoclonal antibody (1:2000, clone B-5-1-2, Sigma-Aldrich) in blocking buffer, and the secondary antibody was a mixture of anti-rabbit IgG (Alexa 488-labeled, 1:500) and anti-

mouse IgG (Alexa 594-labeled, 1:500, Life Technologies) in blocking buffer. The fluorescence images were obtained with a microscope (Eclipse E600) equipped with a CCD camera (DP70), and $\times 20$ (NA 0.50) and $\times 40$ (NA 0.75) objective lenses. The brightness and contrast were adjusted for some images using the software Canvas X (Deneba, Victoria, BC, Canada), if necessary. To calculate the ratio of the mean pixel intensities between the cell body and the rhabdomeric regions, the images of two serial sections (14 μ m thick) almost intersecting the center of the eye were chosen. Mean pixel intensity was measured using the software Photoshop CS2 (Adobe, San Jose, CA, USA), and the ratios were calculated and then averaged between the two sections. The measurements were performed in 6–8 independent superior tentacles.

Electroretinogram (ERG) recording

The ERG was recorded from the surface of an isolated eye in physiological saline. A glass electrode (tip diameter ~ 50 μ m) filled with physiological saline was attached to the outer surface of the isolated eye. The signals were amplified using a differential amplifier (Model 3000, A-M Systems, Sequim, WA, USA), and were recorded on a computer via an A/D converter (PowerLab/2/26, AD Instruments, Dunedin, New Zealand). The ERG signals were low-pass filtered at 3 Hz, and the difference between the highest and lowest peaks was calculated as the ERG amplitude. Monochromatic light was delivered by a 500 W xenon arc light system (model XW-500Q, Sanso, Tokyo, Japan). The light intensity was measured by a photopower meter (model TQ8210, Advantest, Tokyo, Japan). The eye was illuminated with six different wavelengths (400, 440, 480, 520, 560 and 600 nm) of monochromatic light for 1 s, with 30 min intervals in between. Light of equivalent photon flux density (2.25×10^{13} photons cm⁻² s⁻¹) was delivered for different wavelengths. In some experiments, 600 nm monochromatic light (3.77×10^{14} photons cm⁻² s⁻¹) was delivered for 2 h. Each eye sample underwent a single series of illuminations either from 400 to 600 nm or from 600 to 400 nm, with the direction counterbalanced among all preparations. The ERG amplitude was expressed as a ratio to the maximum ERG amplitude in each preparation, and was averaged across all preparations ($n=8$). All recordings were performed at 16–24°C.

Incorporation and staining of neurobiotin

Neurobiotin (Vector Laboratories) was incorporated anterogradely or retrogradely from the cut end of the optic nerve of an isolated brain as described previously (Matsuo et al., 2014b). For the dual staining of β -arrestin and neurobiotin anterogradely incorporated into the brain, the brain was fixed with 4% paraformaldehyde in PBS for 1 h followed by cryoprotection in 20% sucrose in PBS at 4°C overnight. For the dual staining of FMRFamide (Phe-Met-Arg-Phe-NH₂) and neurobiotin, the brain was fixed in Bouin fixative (2.9% picric acid, 8.8% formaldehyde, 4.8% acetic acid) for 1 h and was immersed in 70% ethanol for 6 h followed by cryoprotection in 20% sucrose in PBS at 4°C overnight. For retrograde incorporation into the eye, the superior tentacle, including the eye, was fixed in 4% paraformaldehyde in PBS for 1 h at room temperature, followed by cryoprotection in 20% sucrose in PBS at 4°C overnight. The fixed tissues were frozen in Surgipath FSC 22 Clear Frozen Section Compound using liquid nitrogen. Cryostat sections (14 μ m thick) were mounted onto glass slides coated with Vectabond. The sections were stained with Alexa 594-labeled streptavidin (1:1000, Thermo Scientific) and with either anti- β -arrestin antibody (0.3 μ g ml⁻¹) or anti-FMRFamide antibody (1:3000, Neuromics, Northfield, MN, USA), as described previously (Matsuo et al.,

2014b). The secondary antibody was anti-rabbit IgG labeled with Alexa Fluor 488 (1:500).

Statistical analysis

The data were statistically analyzed using a one-way ANOVA with a *post hoc* Scheffé test, or by a Student's *t*-test, with a significance level of $P < 0.05$. The data are expressed as means \pm s.e.m.

RESULTS

Structure of the *Limax* eye

We first analyzed the architecture of the *Limax* eye. Toluidine Blue staining of a semi-thin section revealed the elements of the eye, including the lens, rhabdome, pigment layer and optic nerve (Fig. 1A). The number of neurons having an apical projection was not large. To further analyze the structure of the rhabdomic region where the proteins involved in photoreception are thought to exist, an electron microscope image was acquired (Fig. 1B). As reported previously in other pulmonates (Kataoka, 1975; Katagiri et al., 2001), numerous microvilli were evident around the apical projection, which extrudes from the pigment layer and contains a lot of mitochondria. To examine whether all of the principal elements of the eye are the subcellular structures of a photoreceptor, neurobiotin was incorporated efferently (retrogradely) from the cut end of an optic nerve. Neurobiotin spread into the rhabdomic region as well as the cell body region (Fig. 1C–E), supporting the view that a single photoreceptor is composed of a rhabdomic region, pigment layer, cell body layer and optic nerve, as revealed by previous electron microscopy observations (Kataoka, 1975; Katagiri et al., 2001).

Molecular phylogenetic analysis of arrestin

We first searched for arrestin-like transcripts in *Limax*, and found a single arrestin-like transcript in our RNA-seq data of *Limax* (NCBI BioProject accession number PRJDB3972). Based on the partial

cDNA sequence of this arrestin-like transcript, we obtained the nucleotide sequence information of the cDNA encompassing a putative full open reading frame using a PCR-based method.

To confirm that there is only one arrestin gene in *Limax*, we searched for arrestin-like transcripts in the NCBI nucleotide sequence database. We detected six transcripts with a deduced amino acid sequence highly similar (>86% identity) to that of *Limax* β -arrestin in the 'Gastropoda' subcategory of the non-redundant nucleotide collection using a TBLASTN program (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>; Altschul et al., 1997; GenBank accession numbers XM_013090298.1, XM_013212299.1, XM_013212298.1, XM_009058330.1, XM_013212300.1, XM_013090835.1). The deduced amino acid sequences of four of the six transcripts contained a clathrin-binding domain, whereas the remaining two lacked this domain probably because they were only partial sequences (XM_013212300.1 and XM_013090835.1). Therefore, *Limax* (and probably other gastropods) seem to have only one arrestin gene.

A molecular phylogenetic tree was inferred using the amino acid sequences of various arrestin proteins, including both visual arrestin and β -arrestin from vertebrates and invertebrates (Fig. 2A). The tree was grouped into four clusters: vertebrate visual arrestin, vertebrate β -arrestin, invertebrate visual arrestin and invertebrate β -arrestin. *Limax* arrestin was clearly grouped with invertebrate β -arrestin, which is consistent with the presence of a clathrin-binding motif in its C-terminus (Fig. 2B). Yoshida et al. (2015) previously reported that several cephalopod mollusks (such as octopus and squid) possess visual arrestin lacking a clathrin-binding motif in its C-terminus, and these arrestin genes were grouped with a distinct cluster from that which includes *Limax* arrestin (Fig. 2A).

Expression of β -arrestin in the CNS of *Limax*

We analyzed the expression of β -arrestin protein in the CNS of *L. valentianus* using an antibody raised against the recombinant

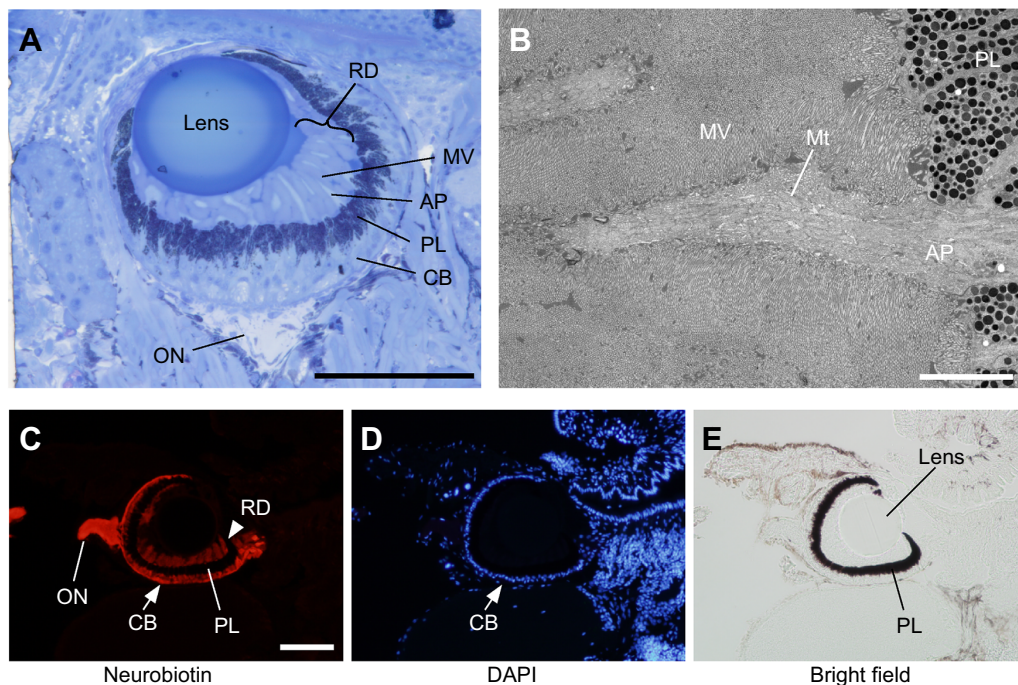


Fig. 1. Structure of the *Limax* eye. (A) A Toluidine Blue-stained semi-thin section of an eye. (B) A photograph of the rhabdomic region viewed by electron microscopy. Numerous microvilli emerge from the apical projection of a photoreceptor. Many mitochondria are visible in the apical projection. (C) Retrograde incorporation of neurobiotin from the cut end of an optic nerve. (D) DAPI staining of the optic nerve in C. (E) A bright field image of the optic nerve in C. Scale bars: A, C, 100 μ m; B, 5 μ m. AP, apical projection; CB, cell body layer; Mt, mitochondria; MV, microvilli; ON, optic nerve; PL, pigment layer; RD, rhabdomic layer.

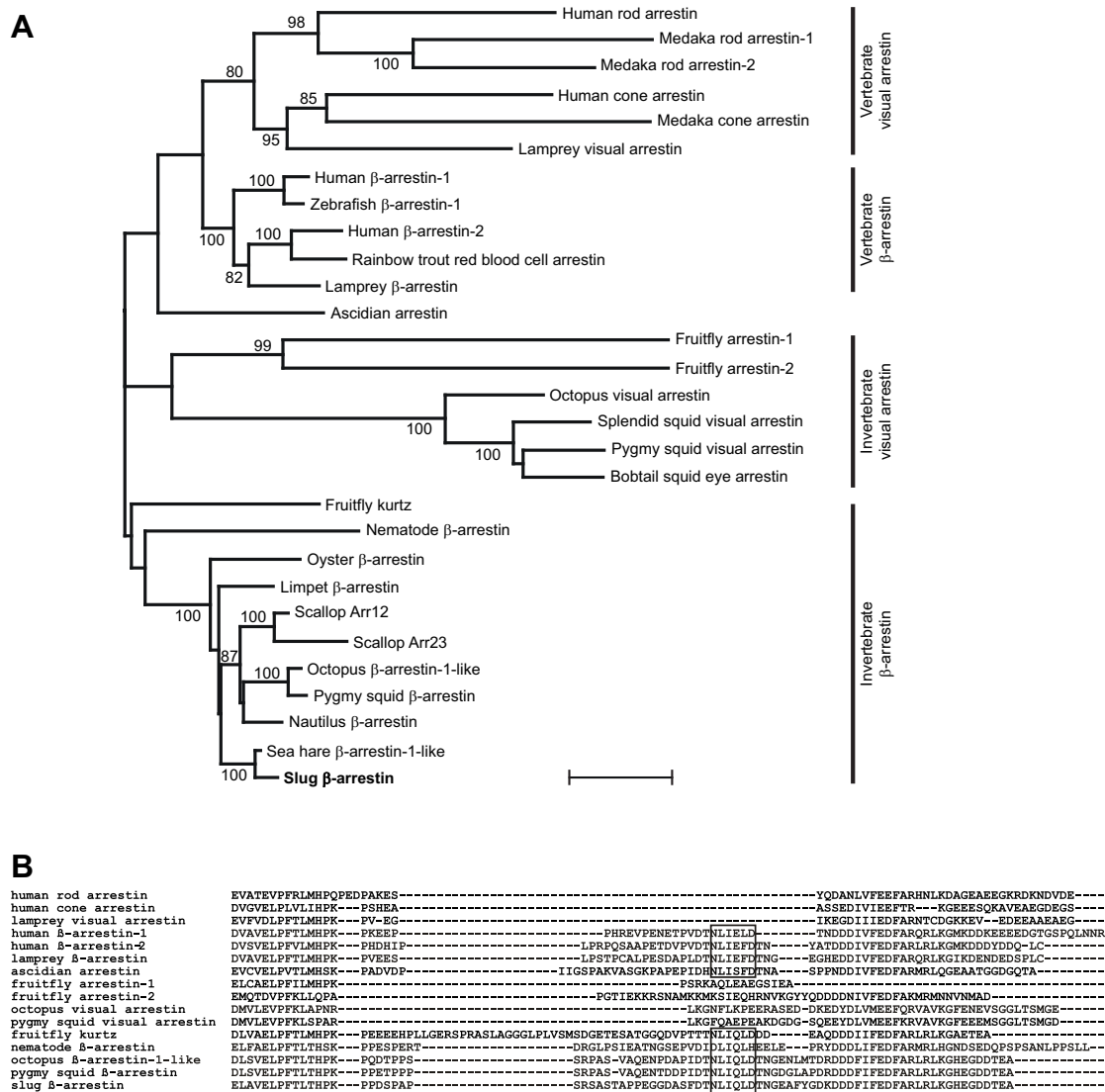


Fig. 2. Phylogenetic position of *Limax* β-arrestin. (A) An unrooted molecular phylogenetic tree of the arrestin family. Invertebrate arrestins are divided into two groups: β-arrestin and visual arrestin. *Limax* (slug) β-arrestin is grouped with the invertebrate β-arrestins. The bootstrap probabilities ($\geq 80\%$) are indicated at each branch node. Scale bar, 0.1 substitutions per site. (B) Comparison of the amino acid sequences of the C-terminal region of vertebrate and invertebrate arrestins. A clathrin-binding motif (boxed) is conserved in both vertebrate and invertebrate β-arrestin, including *Limax* β-arrestin.

β-arrestin protein. The antibody gave rise to a strong immuno-reactive signal in the rhabdomeric region of the eye, and less-intense signals in the remaining parts of the superior tentacle (Fig. 3A). The signals almost disappeared if the primary antibody was pre-incubated with recombinant β-arrestin protein (Fig. 3A) or if the primary antibody was omitted (Fig. S1). In the brain, there were no conspicuous signals. However, the weak immuno-reactive signals detected throughout the brain almost completely disappeared following pre-incubation of the primary antibody with the recombinant β-arrestin protein (Fig. 3B).

The antibody was also suitable for western blotting because it gave rise to bands with electrophoretic mobility identical to that of the full-length β-arrestin protein expressed in COS-7 cells, although the detected bands exhibited a slightly larger molecular mass than that predicted from the amino acid sequence of β-arrestin (48.4 kDa; Fig. 3C).

Expression of β-arrestin mRNA was also examined by RT-PCR and fluorescence *in situ* hybridization. In RT-PCR, the band intensities had a trend similar to those in western blotting, being

lower in the superior tentacle than in the brain ganglia (Fig. 3C,D). Expression of β-arrestin mRNA was prominent in the cell body layer of the eye when an antisense probe was used in *in situ* hybridization, whereas no discernible signal was detected with the sense probe (Fig. 3E).

Co-localization of β-arrestin and rhodopsin in the rhabdomere of the eye

To see whether β-arrestin protein is localized to the rhabdomeric region of the retina, we generated a polyclonal antibody against the C-terminal peptide of rhodopsin (also called G_q -coupled opsin or rhabdomeric opsin), and visualized the rhabdomeric region by immunohistochemical staining of rhodopsin. The specificity of the anti-rhodopsin antibody was confirmed by western blotting, where single bands slightly larger than the predicted molecular size of 60 kDa were detected in the total lysate and crude membrane fraction of the superior tentacle, but not in the brain (Fig. 4A). The expression of rhodopsin mRNA was also confirmed in the cell body layer of the retina by *in situ* hybridization (Fig. 4B). The prominent

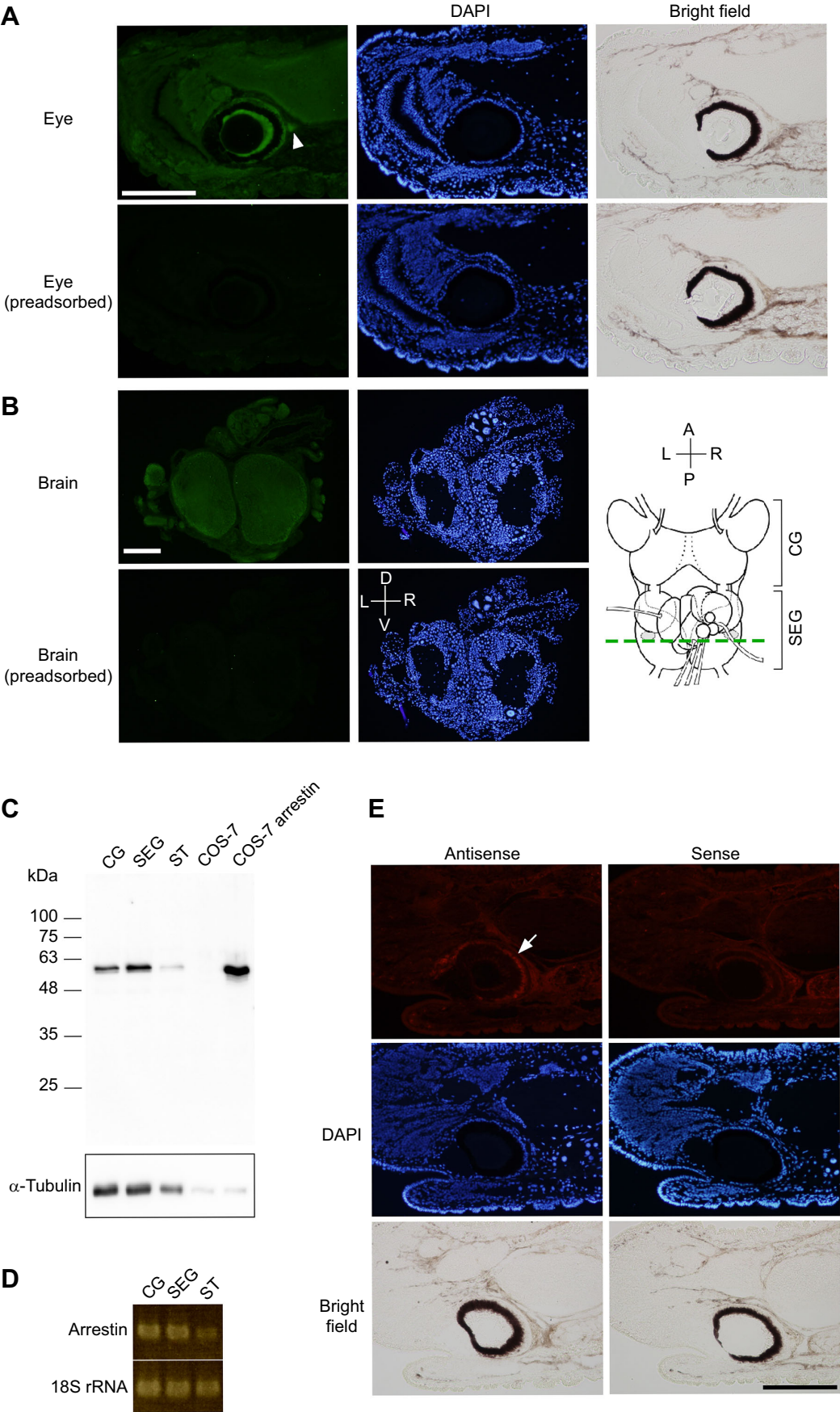


Fig. 3. See next page for legend.

Fig. 3. Expression of β -arrestin in *Limax valentianus* eye and brain.

(A) Immunohistochemical staining of β -arrestin in the superior tentacle (left). Immunoreactivity almost disappeared following preadsorption of the primary antibody with recombinant β -arrestin protein. DAPI staining (middle) and bright field images (right) show the cell nuclei and the pigment layer, respectively. The white arrowhead indicates the proximal end of the optic nerve. (B) Immunohistochemical staining of β -arrestin in the brain. Immunoreactivity almost disappeared following preadsorption of the primary antibody with recombinant β -arrestin protein. DAPI staining shows the distribution of cell nuclei. The cartoon on the right shows the cutting plane (dashed green line) of the brain section (dorsal view). (C) Western blot with β -arrestin antibody. The blot was also probed with α -tubulin antibody to demonstrate the equivalence of the amount of protein loaded (3 μ g; bottom). Note the band intensities of α -tubulin differ between the brain homogenates (left 3 lanes) and COS-7 cell lysates (right 2 lanes), possibly because of the difference in the animal species (slug versus African green monkey) and the tissue type. (D) RT-PCR of β -arrestin in different parts of the CNS. Amplicons of 18S rRNA served as an internal control for the equivalence of the cDNA templates used. (E) Fluorescence *in situ* hybridization of β -arrestin in the eye (top). The white arrow indicates positive signals in the cell body layer of the retina. DAPI staining (middle) and bright field images (bottom) show the location of the cell bodies and the pigment granule layer, respectively. Scale bars: 200 μ m. A, anterior; CG, cerebral ganglia; D, dorsal; L, left; P, posterior; R, right; SEG, subesophageal ganglia; ST, superior tentacle; V, ventral.

immuno-reactive signals of β -arrestin matched those of rhodopsin (Fig. 4C–H), and these corresponded to the rhabdomeric region of the retina (Fig. 1A,C).

Light-dependent translocation of β -arrestin in the photosensory neuron

Thus far, most of the studies on the light-dependent translocation of arrestin have been carried out with visual arrestin in the eyes of vertebrates and fruitflies (Broekhuysse et al., 1985; Philp et al., 1987; Kiselev et al., 2000; Nair et al., 2002; Lee et al., 2003). As an exception, however, it has been demonstrated that β -arrestin translocates in a light-dependent manner in the pineal photosensory cell of the lamprey (Kawano-Yamashita et al., 2011). To examine whether β -arrestin in the slug's eye exhibits light-dependent subcellular translocation in photoreceptors, we analyzed the distribution of β -arrestin in the retina immunohistochemically. Illumination with 400 nm monochromatic light (2.25×10^{13} photons $\text{cm}^{-2} \text{s}^{-1}$) for 3 h resulted in a change in the distribution of β -arrestin in the retina, with a reduction in the cell body layer and an increase in the rhabdomeric region (Fig. 5A). The ratio of fluorescence intensity of the rhabdomeric region to that of the cell body layer increased over 3 h (Fig. 5B, $F_{4,33}=6.505$, $P=0.0006$), and began to increase as early as 30 min when compared with the dark-adapted state ($P=0.0066$, two-tailed Student's *t*-test).

We found that the *L. valentianus* retina exhibits a more robust ERG response to short wavelength light than to long wavelength light (Fig. 5C). The distribution of fluorescence was thus compared among the groups that were dark adapted (2 h), or delivered short wavelength (400 nm) or long wavelength (600 nm) light. Light

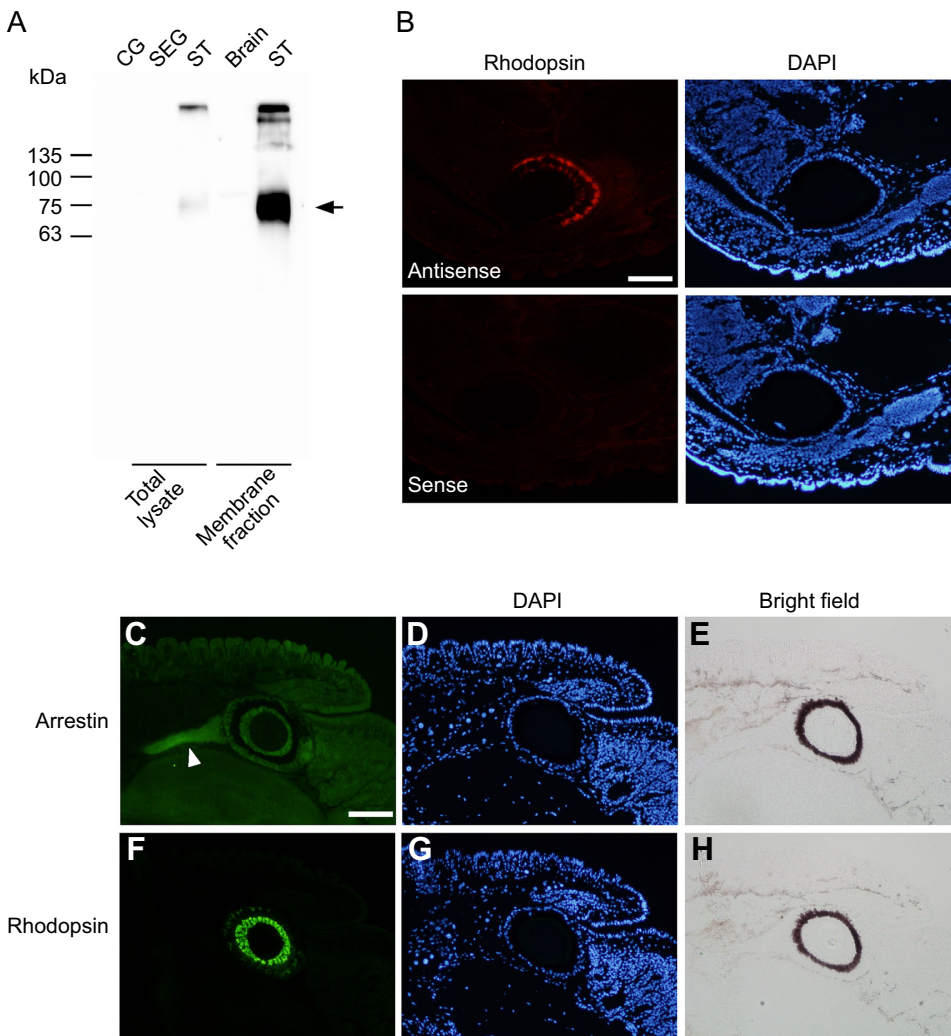


Fig. 4. Expression of β -arrestin and rhodopsin in the rhabdomere. (A) Western blotting of rhodopsin. The arrow indicates bands with slightly reduced mobility than the predicted size of 60 kDa. (B) Fluorescence *in situ* hybridization of rhodopsin in the eye.

DAPI staining (right) shows the location of the cell body layer. (C,F) Serial sections (6 μ m thick) of the superior tentacle stained with anti- β -arrestin (C) and anti-rhodopsin (F) antibodies. The white arrowhead indicates the optic nerve. (D,G) DAPI staining of the sections in C and F, respectively. (E,H) Bright field images of the sections in C and F, respectively. Scale bars: 100 μ m. CG, cerebral ganglia; SEG, subesophageal ganglia; ST, superior tentacle.

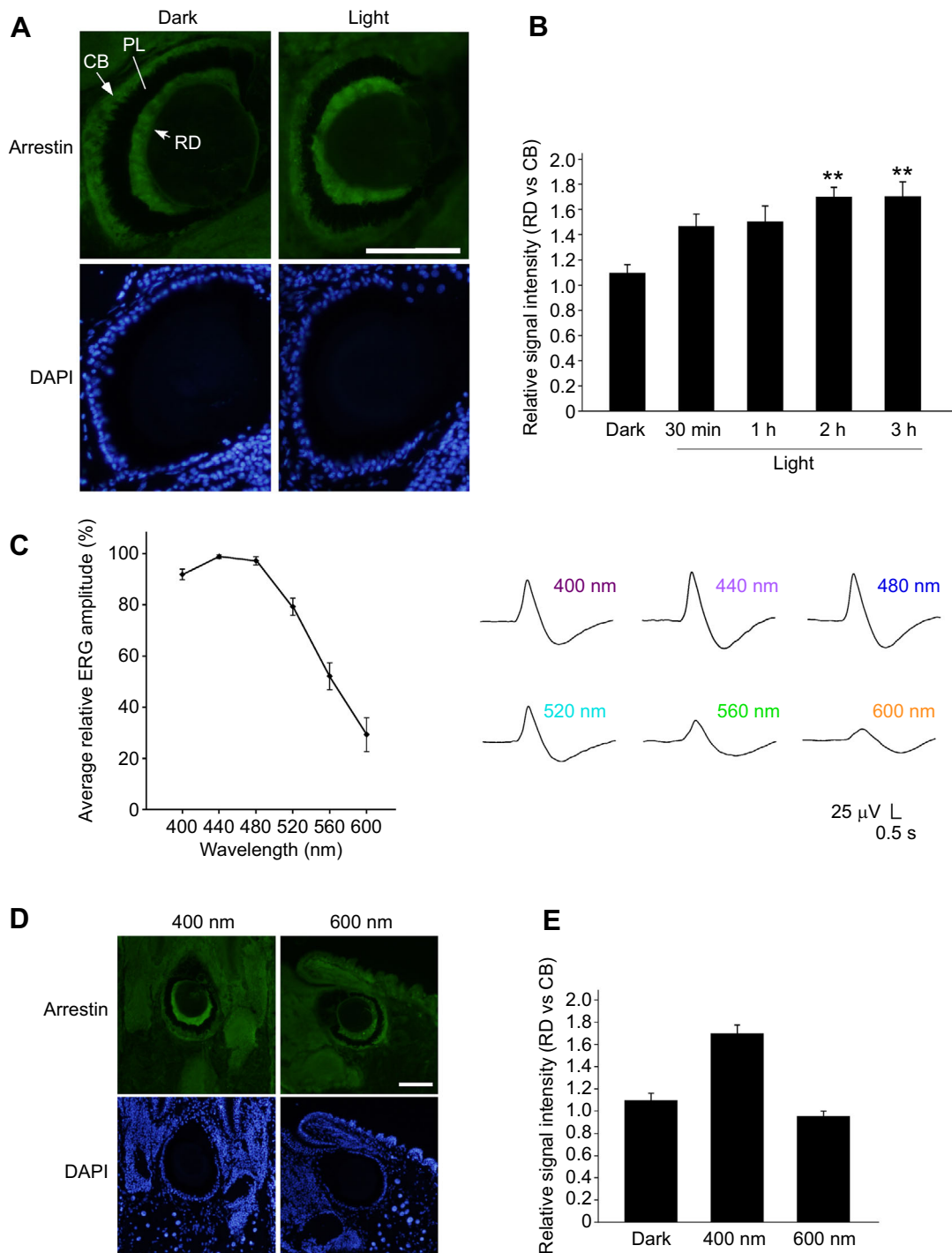


Fig. 5. Light-dependent translocation of β -arrestin in the retina. (A) Immunohistochemical staining of β -arrestin in the superior tentacle kept in the dark for 2 h (left) and subsequently illuminated (400 nm light) for 3 h (right). (B) Change in the relative intensity of β -arrestin immunoreactivity over time. The superior tentacles were kept in the dark for 2 h, followed by illumination (400 nm) for 30 min, 1 h, 2 h or 3 h. Means \pm s.e.m. among 6–8 independent samples. $^{**}P<0.01$ versus dark, *post hoc* Scheffé tests. (C) The wavelength dependence of electroretinogram (ERG) amplitude. Means \pm s.e.m. ($n=8$). Representative ERG responses are shown on the right. (D) Immunohistochemical staining of β -arrestin in samples kept in the dark for 2 h, followed by illumination at 400 or 600 nm. Illumination with 600 nm light did not elicit translocation of β -arrestin. (E) Mean \pm s.e.m. of the relative signal intensity ($n=8$). $^{**}P<0.01$ versus dark and 600 nm light group, *post hoc* Scheffé tests. Scale bars: 100 μ m. CB, cell body layer; PL, pigment layer; RD, rhabdomeric layer.

intensity was adjusted so that equivalent photon flux densities were delivered between 400 and 600 nm (2.25×10^{13} photons $\text{cm}^{-2} \text{s}^{-1}$). As shown in Fig. 5D, no apparent increase of the fluorescence signals in the rhabdome was evident in the long wavelength (600 nm) group. The ratio of fluorescence intensity of the

rhabdomeric region to that of the cell body layer was larger in the short wavelength (400 nm) group than in the long wavelength (600 nm) group or in the dark-adapted group (Fig. 5E; $F_{2,21}=38.40$, $P<0.0001$), whereas there was no difference between the dark-adapted and long wavelength groups ($P>0.05$, *post hoc* Scheffé

test). Moreover, the ratio increased moderately when more intense 600 nm light (3.77×10^{14} photons $\text{cm}^{-2} \text{s}^{-1}$) was delivered for 2 h (Fig. S2; $F_{2,22}=24.65$, $P<0.0001$ among 400 nm at 2.25×10^{13} photons $\text{cm}^{-2} \text{s}^{-1}$, 600 nm at 2.25×10^{13} photons $\text{cm}^{-2} \text{s}^{-1}$, 600 nm at 3.77×10^{14} photons $\text{cm}^{-2} \text{s}^{-1}$).

Closer inspection of the thinner (6 μm -thick) sections revealed more detailed light-dependent subcellular re-distribution of β -arrestin. In the dark-adapted eye, the immunoreactivity of β -arrestin exhibited a stripe-like pattern in a section near the center of the eye (Fig. 6A). The stripe-like pattern largely corresponded with that of α -tubulin (Fig. 6A–E,S), which is reportedly enriched in the apical projection of the rhabdomere (Fig. 1B; Kataoka, 1975; Martinez et al., 2000). Similarly, the dot-like distribution of β -arrestin was almost equivalent to that of α -tubulin in a section crossing the apical projection of the rhabdomere (Fig. 6F–I,S). In contrast, the stripe-like pattern and dot-like distribution of β -arrestin became less

evident in eyes illuminated with 400 nm monochromatic light for 2 h (Fig. 6J–Q), suggesting the re-distribution of β -arrestin into the microvilli of the rhabdomere in response to light (Fig. 6T).

Because visual arrestin is known to be phosphorylated in a light-dependent manner in *Drosophila* and squid (Kahn and Matsumoto, 1997; Alloway and Dolph, 1999; Robinson et al., 2015), there is a possibility that the change in the immunoreactivity of β -arrestin is caused by the change in the binding affinity to the primary antibody resulting from phosphorylation. To test this possibility, the protein samples of the dark-adapted and light-adapted superior tentacles were western blotted with anti- β -arrestin antibody. There was no apparent mobility shift or change in band intensity between the two samples (Fig. 6R), suggesting that the light-dependent phosphorylation of β -arrestin, if any, does not affect the binding affinity to the polyclonal primary antibody used in the present study.

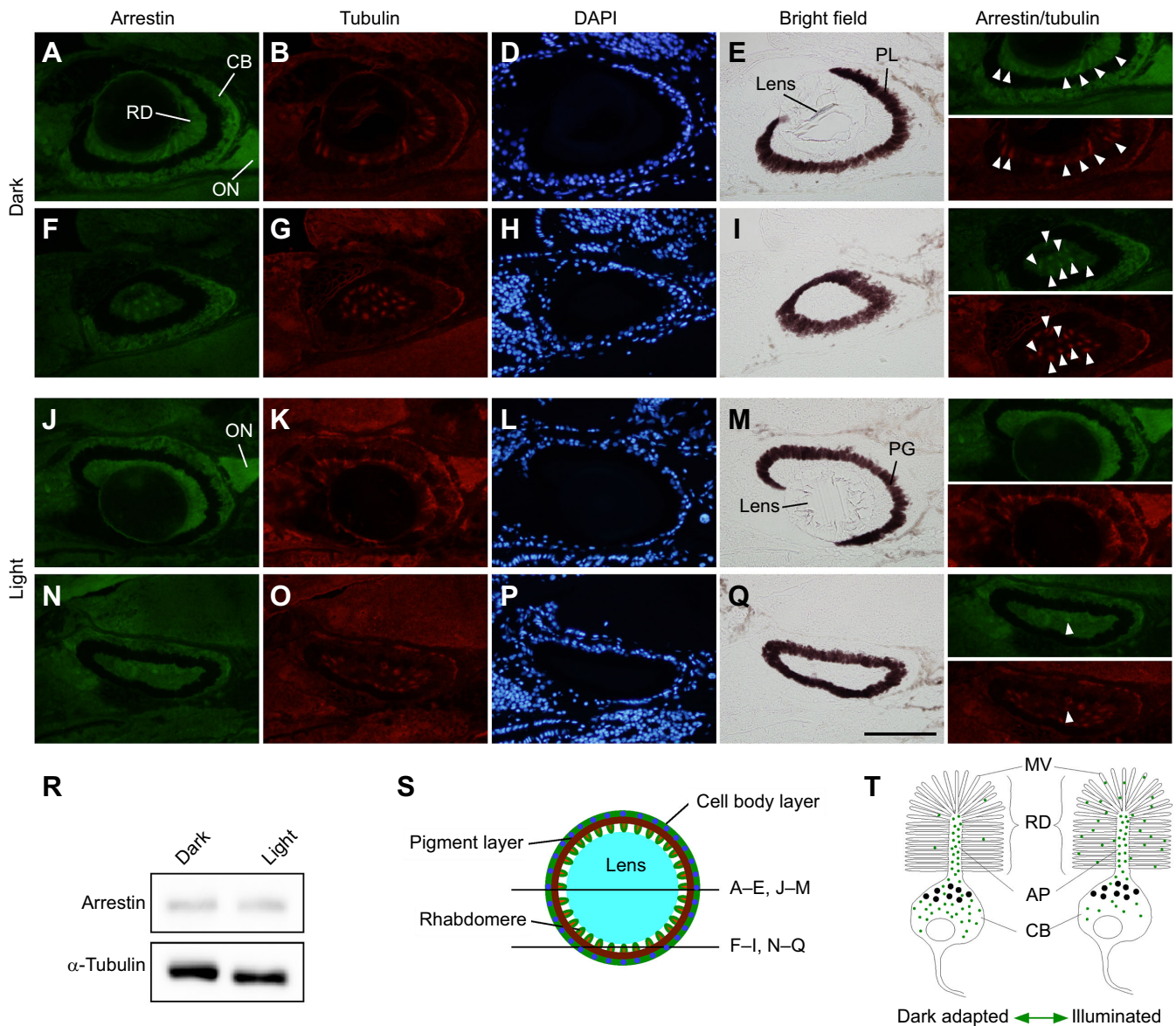


Fig. 6. Dispersal of β -arrestin immunoreactive signals from the apical projection of the rhabdomere during illumination with 400 nm light. (A–I) Dark-adapted (2 h) eyes. (J–Q) Eyes illuminated with 400 nm light (2 h) following 2 h dark adaptation. White arrowheads in the right panels indicate the co-localization of β -arrestin and α -tubulin. (R) Western blotting of β -arrestin showing the absence of apparent changes in molecular mass or immunoreactivity of β -arrestin after illumination. (S) Schematic diagram showing the cutting planes of the sections. (T) Schematic diagram of the light-dependent redistribution of β -arrestin. Scale bar: 100 μm . AP, apical projection; CB, cell body layer; MV, microvilli; ON, optic nerve; PL, pigment layer; RD, rhabdomeric layer.

β -Arrestin as a marker of the optic nerve

Because β -arrestin is enriched in the optic nerve as well as in the retina (Figs 3A, 4C; Cross et al., 2015), we exploited the immunoreactivity of β -arrestin to visualize and trace the visual input pathway in *L. valentianus*. Neurobiotin, a tracer molecule, was incorporated afferently from the cut end of the optic nerve, and the brain sections were stained with anti- β -arrestin antibody to see whether the commissural fiber that incorporated neurobiotin

(Matsuo et al., 2014b) is a branch of the optic nerve. The neurobiotin signal co-localized with that of β -arrestin at the input region into the cerebral ganglia (Fig. 7A–C), whereas at the cerebral commissure, no prominent β -arrestin signal was evident, and the neurobiotin signal did not overlap that of β -arrestin (Fig. 7D–F). Similarly, the immunoreactivity of FMRamide, which has been demonstrated in the optic nerve of the pulmonate *Lymnaea* (Tuchina et al., 2012), also overlapped with the neurobiotin signal beneath the

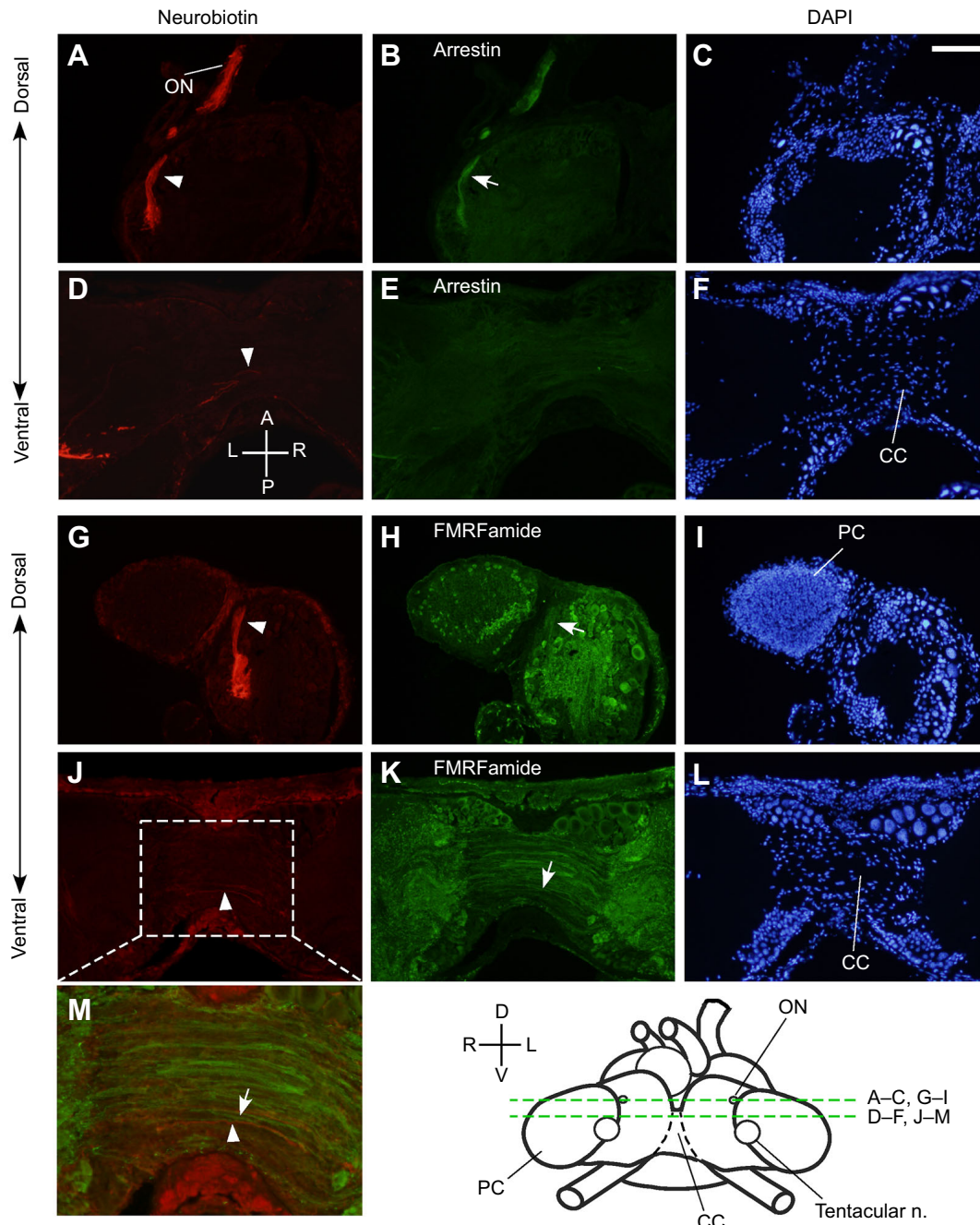


Fig. 7. Visualization of the optic nerve entering the brain by immunohistochemical staining of β -arrestin. (A,D) The visual input pathway traced by neurobiotin (arrowhead) incorporated from the cut end of the left optic nerve. (B,E) Immunostaining of β -arrestin (arrow) in the same sections as in A and D, respectively. (C,F) DAPI staining of the sections in A and D, respectively. (G,J) The visual input pathway traced by neurobiotin incorporated from the cut end of the left optic nerve. (H,K) Immunostaining of FMRamide in the same sections as in G and J, respectively. (I,L) DAPI staining of the sections in G and J, respectively. (M) A merged image of J and K, corresponding to the region circumscribed with a dashed line in J. The arrowhead and arrow indicate the signals of neurobiotin and FMRamide, respectively. The schematic diagram on the right shows the cutting planes of the sections (anterior view of the brain). Note that the background red fluorescence is greater in the sections of the brain fixed with Bouin fixative (G,J,M) than in those fixed with paraformaldehyde (A,D). Scale bar: 100 μ m. A, anterior; CC, cerebral commissure; D, dorsal; L, left; ON, optic nerve; P, posterior; PC, procerebrum; R, right; V, ventral.

entry point into the brain (Fig. 7G–I), although this is less evident. However, these signals did not overlap each other at the cerebral commissure (Fig. 7J–M). Therefore, it is unlikely that the neurobiotin-stained commissural nerve is a branch of the optic nerve, but rather these commissural nerves may be part of a neuron distinct from the photoreceptors.

Monitoring of optic nerve regeneration following amputation

As an optic nerve can be visualized by immunostaining of β -arrestin, the spontaneous regeneration of an optic nerve was examined by immunohistochemistry in the coronal section of the brain, 14 or 49 days following the amputation of the left superior tentacle. We have previously demonstrated that the olfactory nerves degenerate and retract 15 days following tentacle amputation (Matsuo et al., 2010; Kobayashi et al., 2010), whereas olfactory function is restored at 31 days (Koga et al., 2016). We therefore expected that the β -arrestin signal would be absent in the brain at 14 days, whereas it would re-appear at 49 days. Indeed, we found that optic nerves could not be observed on the left side of the brain as a signal of β -arrestin immunoreactivity at 14 days (Fig. 8A–H), whereas they were detected on both sides of the brain at 49 days (Fig. 8I–P).

DISCUSSION

In the present study, we found that β -arrestin is highly expressed in the retina of the *Limax* eye, and localized in the rhabdomeric region where photoreception takes place. Moreover, we found that β -arrestin translocates in response to light. Translocation into the microvilli of the rhabdomere would promote the rapid turnover of rhodopsin in a light environment, preventing saturation of the photosensory neuron response (Arshavsky, 2003; Calvert et al., 2006). As far as we know, this is the first report on the light-dependent translocation of β -arrestin in invertebrate retina, suggesting a functional role of this molecule as a terminator of opsin signaling in the retina of those animals that lack visual arrestin. To demonstrate the role of β -arrestin more decisively, further investigation will be needed, such as an analysis of light-dependent binding of β -arrestin to the intracellular region of rhodopsin.

We demonstrated that β -arrestin translocates to the microvilli of the rhabdomere in the photoreceptor by illuminating it with short wavelength light (400 nm) to which the eye exhibits a robust ERG response. Translocation was not evident when long wavelength light (600 nm) was delivered at an equivalent photon flux density, whereas moderate translocation occurred when 600 nm light

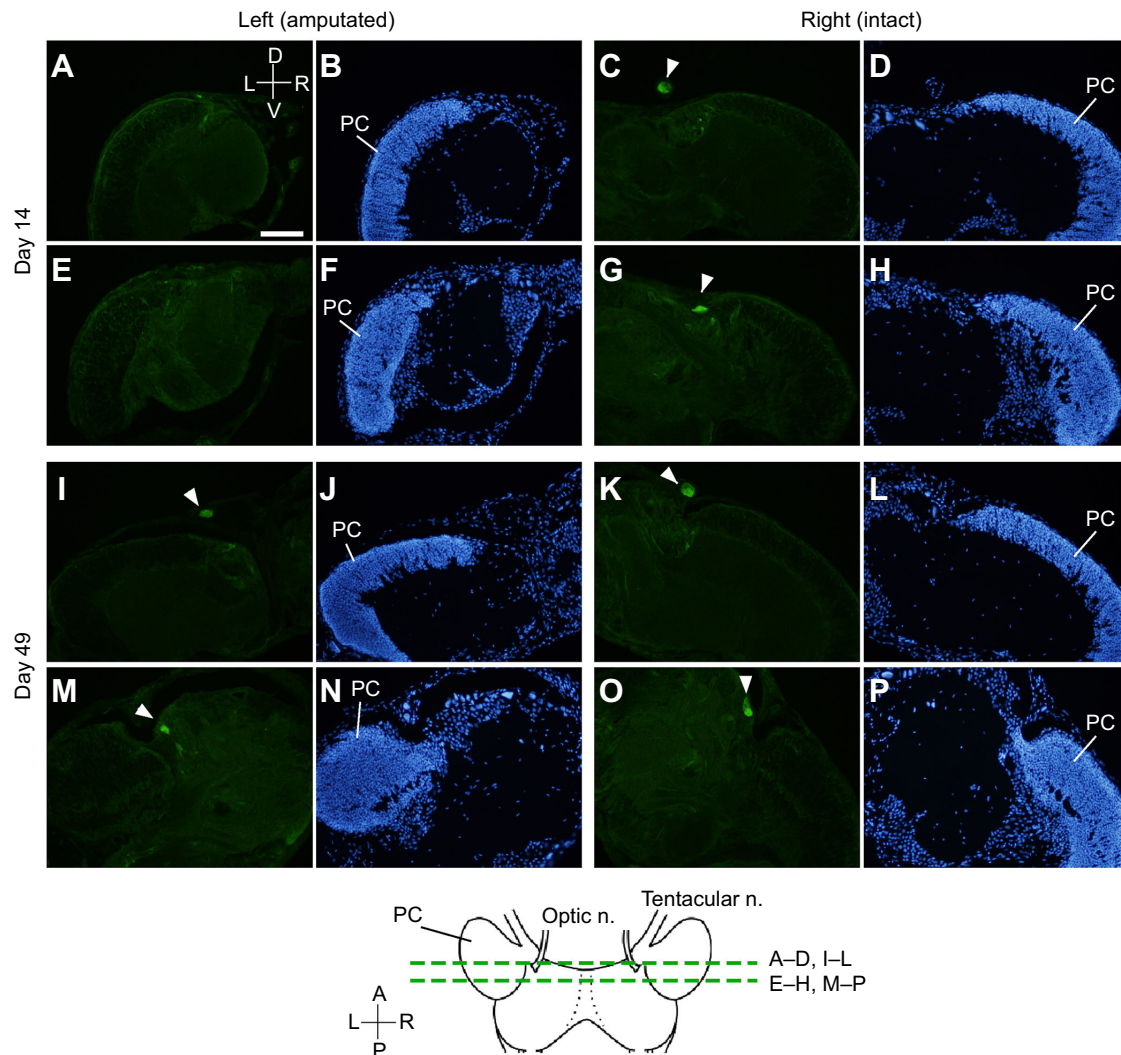


Fig. 8. Regeneration of the optic nerve visualized by immunostaining of β -arrestin. Coronal sections of the cerebral ganglia were immunostained with β -arrestin 14 days (A–H) or 49 days (I–P) after the surgical amputation of the left superior tentacle. Right panels (B,D,F,H,J,L,N,P) show DAPI staining of corresponding images to the left. White arrowheads indicate β -arrestin signals in the optic nerve. The schematic diagram at the bottom shows the cutting planes of the sections. Scale bar: 200 μ m. A, anterior; D, dorsal; L, left; P, posterior; PC, procerebrum; R, right; V, ventral.

with higher photon flux density was delivered. Therefore, the translocation of β -arrestin is dependent on the activation of rhodopsin, consistent with a previous report that light-induced translocation of visual arrestin is absent in mice deficient in rhodopsin activation (Mendez et al., 2003).

We investigated the localization and light-dependent translocation of β -arrestin using a specific antibody raised against bacterially expressed β -arrestin protein. The specificity of the antibody was confirmed not only by preadsorption in immunohistochemistry experiments but also by the observation that the expression pattern of β -arrestin mRNA (by RT-PCR and *in situ* hybridization) and β -arrestin protein (by immunohistochemistry and western blotting) corresponded (Fig. 3). The presence of β -arrestin throughout the brain tissue reflects the fact that a single β -arrestin functions in terminating all G-protein signaling in gastropods. Neural tissues generally express higher levels of β -arrestin proteins than non-neural tissues (Gainetdinov et al., 2004), and therefore lower expression levels of β -arrestin in the superior tentacle than in other brain areas (Fig. 3C,D) can be explained by the fact that the superior tentacle consists of both neural and non-neural tissues such as retractor muscles and epidermis.

We have previously observed that the fine commissural nerves between the bilateral cerebral ganglia incorporate neurobiotin delivered from the cut end of an optic nerve (Matsuo et al., 2014b). Similar results have been described in the marine gastropod *Aplysia* and the freshwater snail *Planorbarius* (Olson and Jacklet, 1985; Tuchina et al., 2011). Olson and Jacklet (1985) reported that the cell bodies of some neurons in the circumesophageal ganglia were labeled by horseradish peroxidase incorporated from the cut end of an optic nerve in *Aplysia*. These neurons were expected to be efferent neurons projecting to the eye. In contrast, some of the labeled neuronal cell bodies in the study by Tuchina et al. (2011) may be secondary neurons receiving visual input from a photosensory neuron via gap junctions, because neurobiotin, but not horseradish peroxidase, passes through gap junctions, thereby potentially labeling all the neurons connected with optic nerves via electrical synapses. Because immunostaining of β -arrestin specifically labels optic nerves, it is possible to distinguish optic nerves entering the brain from the efferent neurons or the secondary neurons connected via gap junctions.

The versatility of β -arrestin immunostaining could also be utilized to monitor the regeneration of an optic nerve during recovery from tentacle amputation or injury. Spontaneous recovery from tentacle amputation, such as cell proliferation and re-innervation, is a very complex and chaotic process in terrestrial pulmonates (Flores et al., 1992; Matsuo et al., 2010). β -arrestin antibodies, therefore, will be a useful tool to trace the process of regeneration of optic nerves and re-innervation to the brain. Future studies should determine the precise order of re-innervation of the visual pathway during spontaneous regeneration of the superior tentacle.

In most of the invertebrate rhabdomere as well as lamprey pineal organ, the photoreceptor cells express bi-stable opsin, which photo-converts to a stable photoproduct that reverts to its original dark state by subsequent light absorption. In the lamprey pineal organ, these bi-stable opsins use β -arrestin instead of visual arrestin (Kawano-Yamashita et al., 2011; Koyanagi and Terakita, 2014). β -Arrestin can vigorously internalize activated opsin molecules by endocytosis mediated by a direct interaction with clathrin (Kawano-Yamashita et al., 2011). Internalization would be a useful strategy for a bi-stable opsin to retrieve its original dark state (Terakita et al., 2012; Koyanagi et al., 2017). In contrast, the photoreceptor cells expressing bleaching opsins, whose photoproduct releases its retinal chromophore over time and decays (bleaches), such as

vertebrate rhodopsin, have a visual arrestin lacking a clathrin-binding motif in its C-terminus. Therefore, it would be reasonable that gastropods have only β -arrestin (Fig. 2A), because they seem to use a bi-stable rhabdomeric opsin like other mollusks (Koyanagi and Terakita, 2014).

However, insects and many of the cephalopods, whose visual pigments are also bi-stable, possess both visual arrestin and β -arrestin (Fig. 2A), and the expression levels of these mRNAs are comparable in the eye of the pygmy squid *Idiosepius* (Yoshida et al., 2015). What are the roles for these two arrestin proteins in the eye? Visual arrestin seems to play a major role in phototransduction signaling in the photosensory neurons of cephalopods: visual arrestin of the splendid squid *Loligo* binds activated rhodopsin and functions in shutting off rhodopsin signaling (Swardfager and Mitchell, 2007). Moreover, visual arrestin exhibits light-dependent translocation to the microvilli of the rhabdomere in *Drosophila* (Kiselev et al., 2000; Lee et al., 2003; Satoh et al., 2010). Therefore, visual arrestin might have taken over the role of β -arrestin in phototransduction during the course of the evolution of vertebrates and insects/cephalopods independently. β -Arrestin, in contrast, seems to still function in the internalization of GPCRs other than opsins independently or in collaboration with visual arrestin in the photoreceptors of these animals (Deming et al., 2015). It is currently uncertain whether the emergence of visual arrestin precedes the divergence of the Lophotrochozoa (including cephalopods) and Ecdysozoa (including insects), although our molecular phylogenetic analysis supports the emergence of visual arrestin in the common ancestor of the two taxa (Fig. 2A).

Why did insects and cephalopods gain visual arrestin during evolution despite their eyes expressing bi-stable opsin? The correlation between the possession of visual arrestin and the temporal/spatial resolution of the eye is of note. The eyes of animals in the vertebrate, cephalopod and arthropod phyla have the highest temporal/spatial resolution and sensitivity of the metazoan animals. Nilsson (2013) has argued that the eyes of animals in these phyla have a well-developed light-gathering system (lens), densely arrayed photoreceptors and numerous membrane stacking of a photoreceptor, all of which enable the animal to perform daily tasks that require a visual system with high temporal/spatial resolution. Concomitantly, the performance of arrestin must be high enough in the photoreceptors of these animals to satisfy the high temporal resolution required. Indeed, the difference between the binding affinities for the active and inactive phosphorylated GPCRs is greater in visual arrestin than in β -arrestin in vertebrates (Clever et al., 2002). This suggests a more efficient dissociation of visual arrestin from phosphorylated inactive opsins, probably allowing visual arrestin to do the next round of opsin signaling termination as well as rapid dephosphorylation of opsins by phosphatases for their reuse (Gurevich and Gurevich, 2015). It would be intriguing to see whether a similar kinetic difference exists between visual and β -arrestin in arthropods and cephalopods.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: R.M.; Methodology: R.M., S.H.; Investigation: R.M., Y.T., S.H., M.K., Y.M.; Writing - original draft: R.M.; Writing - review & editing: R.M., Y.T., S.H., M.K., Y.M.; Supervision: R.M.; Project administration: R.M.; Funding acquisition: R.M.

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Data availability

Limax β -arrestin and rhodopsin nucleotide sequences have been deposited in GenBank (accession numbers: LC218443 and LC223120, respectively).

Supplementary information

Supplementary information available online at <http://jeb.biologists.org/lookup/doi/10.1242/jeb.162701.supplemental>

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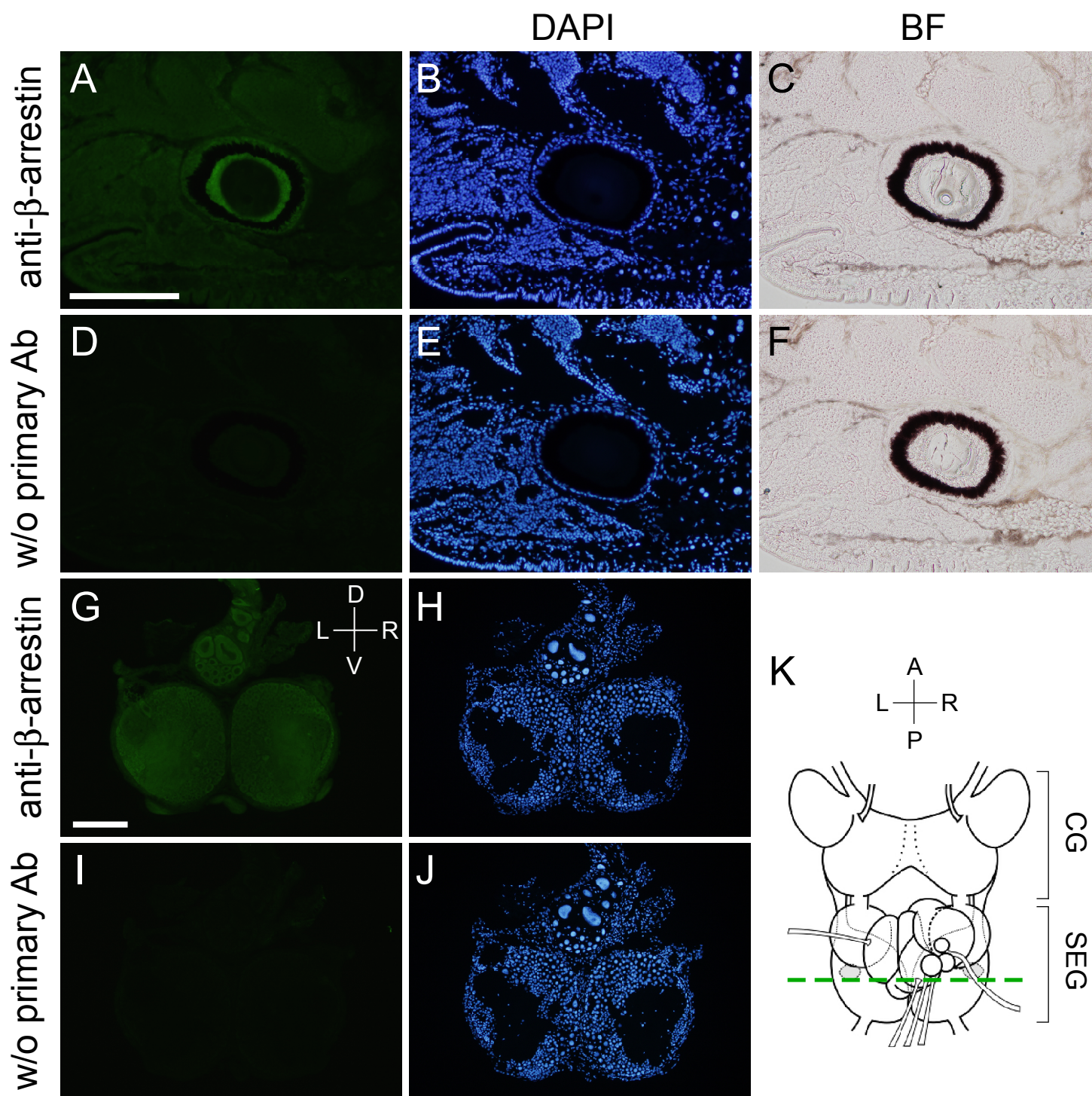


Figure S1 Specificity of anti β -arrestin antibody.

Immuno-positive signals of β -arrestin (A, G) were absent if the primary antibody was omitted (D, I), suggesting that the secondary antibody does not bind to the section in a non-specific manner. (B, E, H, I) are the fluorescence images of DAPI of (A, D, G, I). (C, F) are the bright field images of (A, D). (K) A schema showing the cutting plane of the brain section of (G-J). Scale bars: 200 μ m. CG, cerebral ganglia; SEG, subesophageal ganglia; A, anterior; P, posterior; R, right; L, left; D, dorsal; V, ventral.

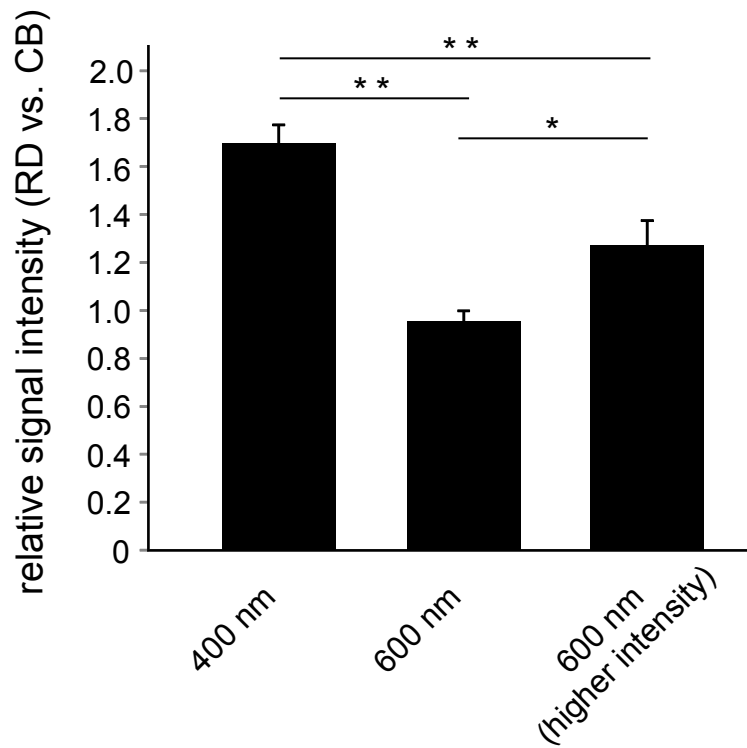


Figure S2 Illumination with 600 nm light with higher intensity (3.77×10^{14} photons $\text{cm}^{-2}\text{sec}^{-1}$) moderately elicited the translocation of β -arrestin.

The superior tentacles were kept in the dark for 2 h, followed by illumination (400 nm or 600 nm) for 2 h. The data for 400 nm (2.25×10^{13} photons $\text{cm}^{-2}\text{sec}^{-1}$) and 600 nm (2.25×10^{13} photons $\text{cm}^{-2}\text{sec}^{-1}$) are reproduced from Fig. 5E. Error bars indicate \pm SE of the mean among 7 - 8 independent samples.

** $P < 0.01$, * $P < 0.05$ by post hoc Scheffe tests.