Molecular physiological investigation on a non-visual opsin melanopsin in

lower vertebrate cyclostomes

（下等脊椎動物円口類における非視覚型オプシン・メラノプシンに関する

分子生理学的解析）

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Contents

Abstract

Mammals contain one melanopsin (Opn4) gene, which is expressed in a subset of retinal ganglion cells to serve as a photopigment involved in non-image-forming vision such as photoentrainment of circadian rhythms.   In contrast, most nonmammalian vertebrates possess multiple melanopsins, which are distributed to various types of retinal cells, but their functions are still unclear.   Interestingly, the lamprey has only one type of mammalian-like melanopsin gene, showing a similarity to mammals in the melanopsin gene number.   Here I investigated the molecular properties and localization of melanopsin in the lamprey and other cyclostome hagfish retinas, which contribute to visual functions including image-forming vision and mainly to non-image-forming vision, respectively.   I isolated one type of mammalian-like melanopsin cDNA from the eyes of each species.   I showed that the recombinant lamprey melanopsin was a blue light-sensitive pigment, and that both the lamprey and hagfish melanopsins caused light-dependent increases of calcium ion concentration in cultured cells in a manner similar to that of the mammalian melanopsins.   I observed that melanopsin was distributed to several types of retinal cells, including horizontal cells and ganglion cells in

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the lamprey retina, despite only one melanopsin gene in lamprey.   In

contrast, melanopsin is almost specifically distributed to retinal ganglion cells

in the hagfish retina.   Taken together with my observation that the

melanopsin-expressing horizontal cells connected to the rhodopsin-containing

short photoreceptor cells in the lamprey, my findings suggest that the global

distribution of melanopsin in retinal cells might not relate to melanopsin gene

number but to extent of melanopsin contribution to visual function.

Introduction

1. Diversity of opsins

Most animals capture light signals through opsin-based pigments and utilize them for visual and non-visual functions such as photoentrainment of circadian rhythm.   Opsins, which comprise seven transmembrane domains, are typical for members of the G-protein coupled receptor (GPCR) family. Most opsins bind to the chromophore 11-*cis* retinal to form a light-sensitive pigment (opsin-based pigment, Fig. 1).   So far, thousands of opsins have been identified and divided into eight groups (Terakita 2005; Koyanagi and Terakita 2008; Nagata et al. 2010).   The accumulated evidence demonstrates that members of the opsin groups, except for peropsin and retinal photoisomerase (RGR/ retinochrome) groups, potentially serve as light-sensitive GPCRs; that is, opsin-based pigments absorb light and subsequently activate G-proteins (Fig. 2 and Fig. 3).   Basically, opsins belonging to different groups, particularly, Opn3 (encephalopsin/TMT-opsin), Gt-coupled opsin (vertebrate visual and related non-visual opsins), invertebrate Go-coupled opsin, Gs-coupled opsin, Gq-coupled opsin (invertebrate Gq-coupled visual opsin and vertebrate melanopsin), and
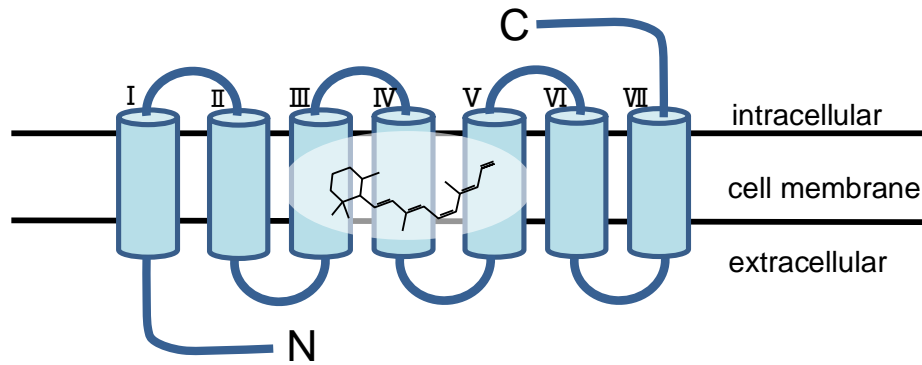
Fig. 1 Schematic drawing of secondary structure of opsin-based pigments.

Opsin-based pigments consist of a protein moiety opsin and a chromophore retinal.　The opsin has a seven-transmembrane structure, which is typical for members of the G-protein coupled receptors.
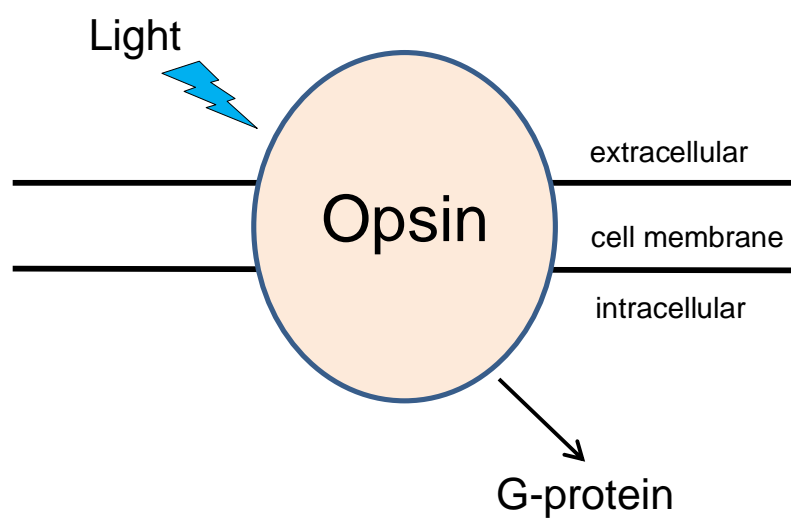
Fig. 2 Function of opsin-based pigments.

Opsin-based pigments absorb light and subsequently activate G-proteins.

Opn5 (neuropsin) groups, are coupled to different types of G proteins, Gi/Go (Koyanagi et al. 2013), Gt (Kuhn 1980), Go (Kojima et al. 1997), Gq (Terakita et al. 1993), Gs (Koyanagi et al. 2008), and Gi (Yamashita et al. 2010; Kojima et al. 2011), respectively (Fig. 3).   Humans have nine opsins: four visual opsins (one rhodopsin and three cone opsins) that serve as a foundation for image-forming and color visions and five non-visual opsins, the functions of which are not fully understood.   Therefore, questions regarding why and how animals utilize such diverse non-visual opsins remain unanswered.

Previous reports have described the basic spectroscopic characteristics of non-visual opsin-based pigments in a wide variety of animals.   For example, Opn3 homologues, one of the non-visual opsin-based pigments, convert to stable photoproducts upon light absorption, and the photoproducts revert to their original dark states by subsequent light-absorption (Koyanagi et al. 2013).   This photoregeneration ability or bistable nature has not been observed in vertebrate visual opsins, which release chromophores and are bleached after light absorption.   In addition, recent studies have suggested that another non-visual opsin-based pigment member, Opn5 (encephalopsin), also has bistable nature.   Members of the melanopsin group have also been characterized as bistable pigments in detailed spectroscopic studies on

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amphioxus and mouse melanopsins (Koyanagi et al. 2005; Matsuyama et al.

2012).   However, the physiological functions of these non-visual opsins,

except for mammalian melanopsin, are not fully understood.   In other words,

mammalian melanopsin is the best-studied non-visual opsin with respect to
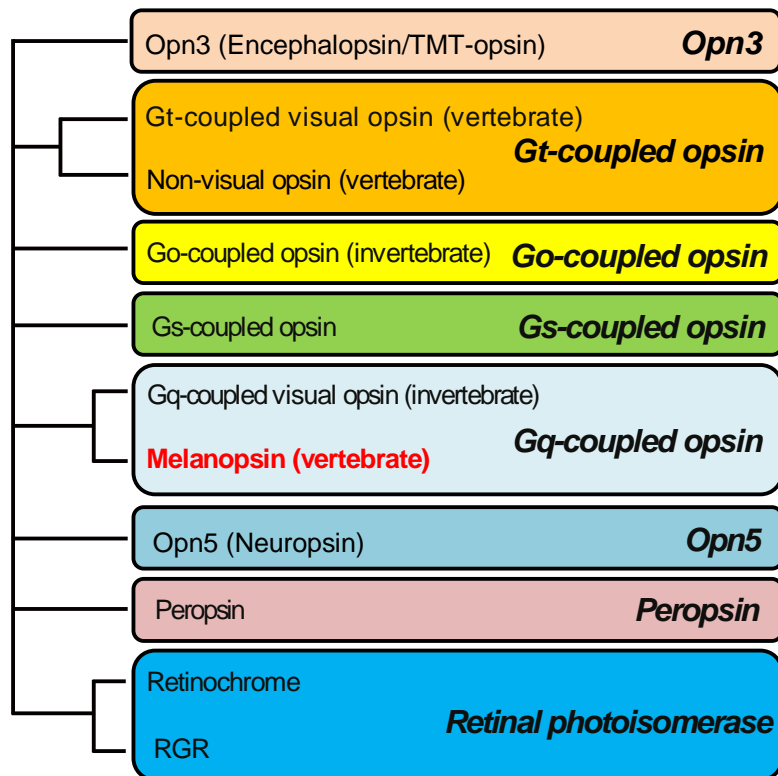
physiological function.

Fig. 3 Diversity of opsins.

Thousands of opsins have been identified thus far and they are divided into eight groups, Opn3 (encephalopsin/TMT-opsin) group, Gt-coupled opsin group, Go-coupled opsin group, Gs-coupled opsin group, Gq-coupled opsin group consisting of invertebrate Gq-coupled visual opsins and vertebrate melanopsins, Opn5 (neuropsin) group, peropsin group, and retinal photoisomerase (retinochrome/RGR) group.

2. Melanopsin-related physiological function in mammals

Melanopsin was first identified in frog melanophores (Provencio et al. 1998) and has since been identified in various deuterostomes, including echinoderms, cephalochordates, and vertebrates (Provencio et al. 2000; Raible et al. 2006; Koyanagi and Terakita 2008) . Melanopsin is similar to the invertebrate visual opsins that drive Gq-mediated signal transduction cascades. Several lines of evidence indicate that melanopsin activates Gq in a similar manner as the invertebrate visual opsins.

A mutant mouse model that lacked rod and cone visual cells still exhibited pupillary light reflexes and circadian rhythm photoentrainment, suggesting the existence of light sensor cells other than rod and cone visual cells (Hattar et al. 2002; Panda et al. 2002). It is now widely accepted that melanopsin is present in a subset of retinal ganglion cells, where it serves as a light-sensor for non-visual functions, light regulation of circadian rhythm and pupillary light reflex (Fig. 4) (Provencio et al. 2000; Panda et al. 2002; Ruby et al. 2002; Hattar et al. 2003; Lucas et al. 2003; Panda et al. 2003). Melanopsin-containing retinal ganglion cells are called intrinsically photosensitive retinal ganglion cells (ipRGCs). The ipRGCs transmit light signals to the suprachiasmatic nuclei (SCN) and olivary pretectal
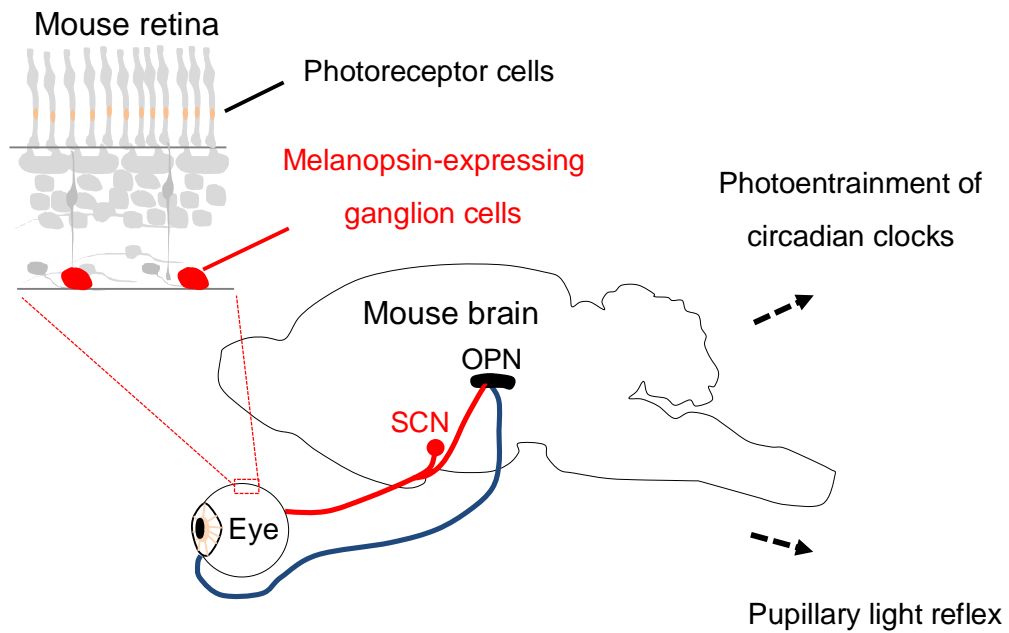
(Modified from Berson 2003)

Fig. 4 Melanopsin-related functions in mammals.

In mammals, melanopsin localizes to a small number of ganglion cells and underlies non-visual function.   These melanopsin-containing ganglion cells are intrinsically photosensitive and can response to light without photoreceptor cells.   The mammalian melanopsin-expressing ganglion cells project their axons to SCN (suprachiasmatic nucleus) and OPN (olivary pretectal nucleus) and transmit light signal to these regions, which are important for photoentrainment of circadian rhythm and pupillary light response in the mouse brain, respectively (Berson 2003).

nucleus (OPN) , which are important regions for circadian rhythm

photoentrainment and pupillary light reflexes, respectively, in the mouse brain

(Fig. 4).   Interestingly, a recent study also demonstrated melanopsin

localization in ON alpha RGCs, which constitute a conventional RGC type,

and suggested the possibility of melanopsin involvement in rough vision

(Schmidt et al. 2014).   A recent report suggested the presence of melanopsin

in blood vessels where it directly regulated the light-mediated relaxation of

blood vessels, indicating the possibility that melanopsin serves as an

extraocular light sensor (Sikka et al. 2014).

3. Melanopsins in nonmammalian vertebrates

3a Diversity of melanopsins in nonmammalian vertebrates

Multiple melanopsins have been isolated from various nonmammalian

vertebrates; for example, five from zebrafish and two from chicken.   The

vertebrate melanopsins can be divided phylogenetically into two types: the

Opn4m and the Opn4x (Bellingham et al. 2006) (Fig. 5).   Interestingly,

mammals possess only an *Opn4m* gene, indicating that the *Opn4x* gene was

secondarily lost during the evolutionary process that led to the mammalian

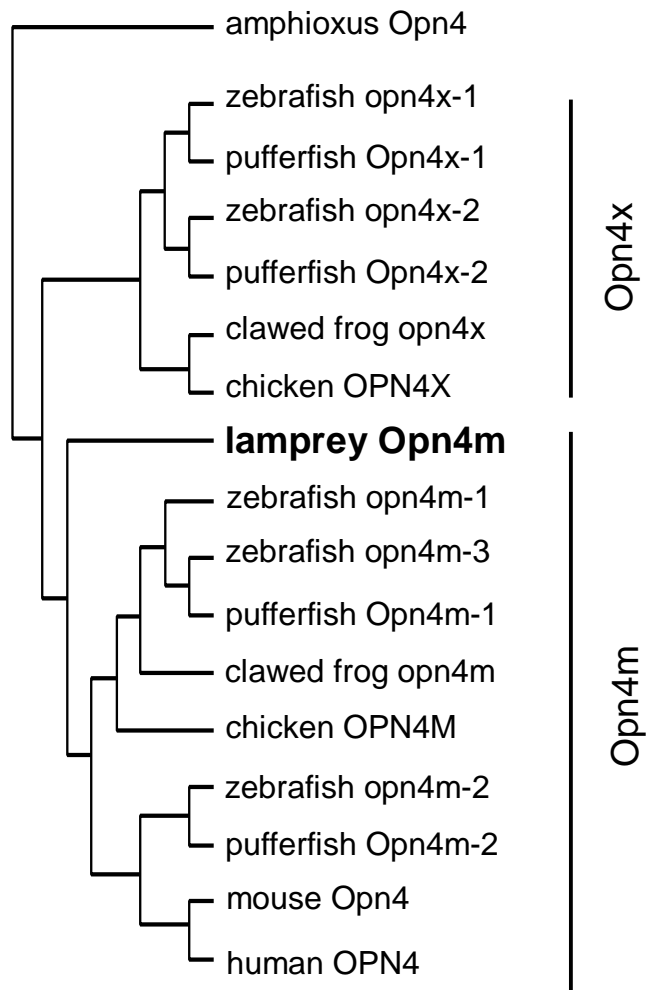lineage (Bellingham et al. 2006; Pires et al. 2007).   In contrast,

Fig. 5 Schematic presentation of phylogenic relationship among melanopsins.

Vertebrate melanopsins are divided into two types, the Opn4m and the Opn4x.   Mammals have only one *Opn4m* gene.   However, nonmammalian vertebrates, such as zebrafish (teleost), clawed frog (amphibian) and chicken (bird), have both types of melanopsin genes.

most nonmammalian vertebrates possess both melanopsin gene types (Fig. 5), suggesting that nonmammalian vertebrates exhibit more complicated melanopsin-related function than that in mammals.

3b Global distribution of melanopsin in nonmammalian vertebrates

In zebrafish, five melanopsin genes, two *Opn4x* and three *Opn4m* genes, are expressed in photoreceptor, horizontal, bipolar, amacrine, and ganglion cells (Matos-Cruz et al. 2011).   In the chicken retina, two melanopsin genes are widely expressed in various types of retinal cells, with the exception of the retinal pigment epithelium and the Müller cells (Chaurasia et al. 2005; Tomonari et al. 2005; Tomonari et al. 2007).   The distribution of multiple melanopsins in various types of retinal cells also suggests that melanopsins are related to more complicated biological functions in nonmammalian vertebrates.

3c Melanopsin in lamprey

Drs. Koyanagi and Terakita previously reported the identification of only one melanopsin (Opn4m) gene in the genome database of the cyclostome sea lamprey (*Petromyzon marinus*) (See Fig. 5) (Koyanagi and Terakita
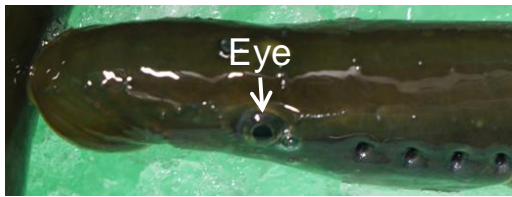
2008) , one of the most primitive nonmammalian vertebrates.   The number of

melanopsin genes (only one) in the lamprey differs considerably from that in

other nonmammalian vertebrates and instead is similar to that in mammals, as

mentioned above (Bellingham et al. 2006; Pires et al. 2007).   Therefore, as

only one *Opn4m* gene was identified in the lamprey genome database and

cyclostomes occupy phylogenetic positions at critical stages in vertebrate

evolution, the lamprey is a suitable animal for investigating melanopsin

functions in nonmammalian vertebrates.

4. Purpose of this study

In this study, I investigated the molecular properties and distribution of

melanopsin in the river lamprey (*Lethenteron camtschaticum*) and also

another cyclostome, the hagfish (*Eptatretus burgeri*) (Fig. 6).   The lamprey

possesses fully structured eyes which support visual (image-forming visual)

and non-visual (non-image-forming visual) functions, whereas the hagfish has

simply structured eyes that are buried beneath the skin, suggesting their

involvement in non-visual (non-image-forming visual) function rather than

visual (image-forming visual) function.   These functional differences in the

eyes/retinas will allow investigation of the relationship between the molecular

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properties and retinal distributions of melanopsin and the extent of the retinal

contribution to visual function.   In this thesis, I have therefore compared the

molecular properties and distributions of melanopsins in these organisms to

further understand melanopsin-related visual and non-visual functions in

nonmammalian vertebrates.

Lamprey
(*Lethenteron camtschaticum*)

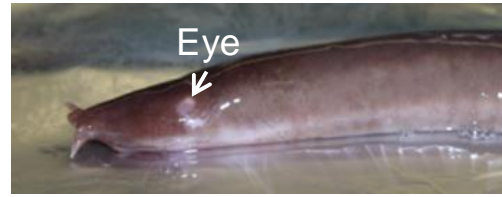Hagfish
(*Eptatretus burgeri*)

Eye

Eye

Fig. 6 The lamprey and hagfish eyes.

The lamprey possesses developed eyes that underlie visual (image-forming visual) and non-visual (non-image-forming visual) functions, whereas the hagfish eyes, which have no lens and are buried beneath the skin are primarily involved in non-visual function rather than visual function.

Materials and Methods

Ethics statement

This experiment was approved by the Osaka City University animal experiment committee (#S0032) and complied with the Regulations on Animal Experiments from Osaka City University.

Animals

River lampreys (*L. camtschaticum*) were kindly provided by Prof. Satoshi Tamotsu (Nara Women's University).   Hagfishes (*E. burgeri*) were commercially obtained.

Isolation of melanopsin cDNAs

Total RNA was isolated from the eyes of the lamprey and hagfish by using Sepasol-RNA I (Nacalai Tesque).   Total RNA was reverse transcribed to cDNA using oligo (dT) primers.   These cDNAs were subsequently used as templates for PCR amplification.   A partial cDNA of the lamprey melanopsin was obtained using gene-specific primers that were designed according to the genome sequence of the sea lamprey (*P. marinus*) melanopsin.   A partial

cDNA of the hagfish melanopsin was obtained using degenerate primers that were designed based on the conserved region of the seven transmembrane domains of vertebrate melanopsins, including the lamprey melanopsin.   The sense and antisense degenerate primers used to obtain the hagfish melanopsin cDNA fragments were as follows: sense, 5′ – TGGTCTGCITAYGTNCCNGARGG–3′, corresponding to the amino acid sequence WSAYVPEG; antisense, 5′ –TAYTTIGGRTGIGTDATNGC–3′, corresponding to the amino acid sequence AITHPKY; and 5′ – GCRTAIACDATIGGRTTRTGDAT–3′, corresponding to the amino acid sequence IHNPIVYA.   The full-length cDNAs of the lamprey and hagfish melanopsins were obtained using the 5′ RACE and 3′ RACE systems (Invitrogen).

Phylogenetic tree inference

Multiple alignment of the amino acid sequences of melanopsins, including the lamprey and hagfish melanopsins, was performed using the XCED software (Katoh et al. 2002).   The phylogenetic tree was inferred as described previously (Koyanagi et al. 2004).   In brief, the evolutionary distance was applied to the phylogenetic tree using the neighbor-joining

method (Saitou and Nei 1987). Bootstrap analysis was conducted using the

method of Felsenstein (Felsenstein 1985). The accession numbers of the

DDBJ/EMBL/GenBank or Ensembl databases regarding the sequences used

in the analyses are provided in the legend to Fig. 9.

Photopigment expression and spectrophotometry

The cDNAs of C-terminal-truncated melanopsin (387 amino acids in

lamprey melanopsin) were tagged with the monoclonal antibody rho 1D4

epitope sequence (ETSQVAPA). The tagged cDNAs were inserted into the

pMT2 vector obtained from Addgene (Addgene plasmid 15896). Pigment

expression in COS-1 cells and pigment purification were performed as

described previously (Koyanagi et al. 2002; Tsukamoto and Farrens 2013),

with some modifications. Briefly, to constitute the pigment, the expressed

proteins were incubated with 11-*cis* retinal overnight. The pigments were

then extracted with 1% (weight/vol) dodecyl β-D-maltoside in 20 mM HEPES

buffer (pH 7.0) containing 140 mM NaCl, 20 mM Tris, 0.2% cholesterol

hemisuccinate, and 10% glycerol. For purification, the pigments in the crude

extract were bound to 1D4-agarose, washed with 0.05% (weight/vol) dodecyl

β-D-maltoside in 20 mM HEPES buffer containing 140 mM NaCl, 1 mM Tris,

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0.2% cholesterol hemisuccinate, and 10% glycerol (buffer A), and eluted with

buffer A containing the 1D4 peptide.   The absorption spectra of the pigments

were recorded at 10°C using a Shimadzu UV-2450 spectrophotometer

(Shimadzu, Japan).

Calcium imaging assay

Full-length melanopsins of lamprey and hagfish were inserted into

pcDNA3.1, and the C-terminal-truncated melanopsins of amphioxus (Terakita

et al. 2008) and mouse (Matsuyama et al. 2012) were cloned into the

pcDNA3.1 and pMT2 vector, respectively.   All of the melanopsins were

tagged with the monoclonal antibody rho 1D4 epitope sequence.   The

melanopsin expression constructs were transfected into COS-1 cells with the

FuGENE HD Transfection Reagent (Promega).   After overnight incubation at

37°C with 11-*cis* retinal, the cells were loaded with 5 µM Fura 2-AM (Dojindo,

Japan) in Krebs–Ringer HEPES buffer (20 mM HEPES, 115 mM NaCl, 5.4

mM KCl, 0.8 mM $MgCl_2$, 1.8 mM $CaCl_2$ and 13.8 mM glucose, pH 7.4) for 1 h

at 37°C in the presence of 0.04% Pluronic F-127 and 1.25 mM Probenecid;

the cells were then rinsed with Krebs–Ringer HEPES buffer.   The ratios of

Fura-2 fluorescence at excitation wavelengths of 340 nm and 380 nm were

measured in Krebs–Ringer HEPES buffer with 1.25 mM Probenecid using a

fluorescence microscope (Olympus) and the MetaMorph software (Molecular

Devices).

Preparation of frozen sections

Lampreys and hagfish were quickly decapitated.   Their eyes were

immersion-fixed overnight in 4% paraformaldehyde in 100 mM sodium

phosphate buffer (PB, pH 7.4) at 4°C, and cryoprotected by immersion in 100

mM PB containing 15% sucrose, which was later replaced with 30% sucrose.

Finally, the eyes were embedded in OCT compound (Sakura, Japan) and 8–

12-μm frozen sections were prepared at −20°C using a cryostat (HM 520;

Microm International GmbH).

In situ **hybridization**

In situ hybridization analyses were performed as reported previously

(Koyanagi et al. 2004), with slight modifications.   In brief, digoxigenin (DIG)-

labeled antisense and sense RNA probes for the lamprey and hagfish

melanopsins were synthesized using a DIG RNA labeling kit (Roche Applied

Science).   The sections were treated with proteinase K (1 μg/mL) for 10 min,

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followed by hybridization with DIG-labeled RNA probes diluted in Ultrahyb-

Ultrasensitive Hybridization Buffer (Ambion) at 68°C overnight.   The probe

was detected on the sections by incubation with an alkaline phosphatase

(AP)-conjugated anti-DIG antibody (1:1000, Roche Applied Science), followed

by a blue 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium color

reaction.

For combined *in situ* hybridization/immunohistochemistry of the lamprey

retinas, the sections were treated without proteinase K.   After the

hybridization step, the sections were incubated with a mixture of the AP-

conjugated anti-DIG antibody and the anti-lamprey melanopsin antibody

(1:4500) (see below) overnight at 4°C.   The sections were then incubated

with Alexa Fluor 488 anti-rabbit IgG (1:500, Molecular Probes) for

immunohistochemical detection, followed by incubation with the fluorescent

substrate 2-hydroxy-3-naphthoic acid-2′-phenylanilide phosphate/4-chloro-2-

methylbenzene diazonium hemi-zinc chloride salt (HNPP/Fast Red TR, Roche

Applied Science), for visualization of *in situ* hybridization.

Preparation of an antibody specific to the lamprey melanopsin

A rabbit polyclonal antibody to the lamprey melanopsin was prepared

against the N-terminal peptide sequence MEEGSMLFGVHAEPGNYSL.   The

specific immunoreactivity of the antibody was examined using lamprey

melanopsin-expressing HEK293 cells using a method described previously

(Wada et al. 2012).   Lamprey melanopsins were detected in cultured cells

using the anti-melanopsin antibody (1:4500) and the rho 1D4 antibody
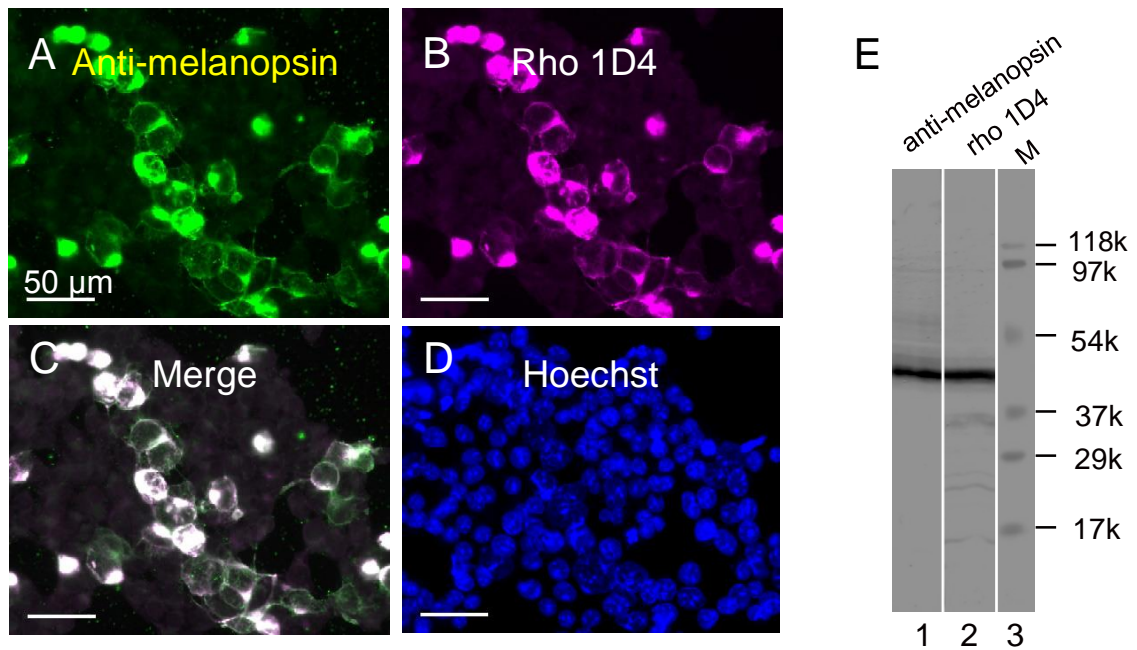
(hybridoma culture fluid) (Fig. 7).

Fig. 7 Immunoreactivity of the antibody against lamprey melanopsin.

HEK293 cells expressing lamprey melanopsin were immunostained with the
anti-lamprey melanopsin antibody (A) and rho 1D4, a monoclonal antibody to
bovine rhodopsin C-terminal sequence, which was tagged on the lamprey
melanopsin C-terminus (B).   (C) A merged image showing that the antibodies
labeled the same cells.   (D) Hoechst stains nuclei of the HEK293 cells.   (E)
Immunoblot profiles demonstrate that the anti-melanopsin and rho 1D4
antibodies specifically recognized a ~43 kDa peptide, melanopsin (lines 1 and
2).   M indicates the molecular weight standard marker (lane 3; Bio-Rad
Laboratories).   Scale bar, 50 µm.

Immunohistochemical analysis of lamprey melanopsin

The sections were incubated overnight at 4°C with the anti-lamprey melanopsin antibody (1:4500) and the anti-transducin antibody (1:500; TF15; CytoSignal). The sections were subsequently incubated with Alexa Fluor 488 anti-rabbit IgG and Alexa Fluor 594 anti-mouse IgG (1:500; Molecular Probes) for 5 h at room temperature.

Retrograde labeling

Retrograde labeling was performed as described previously (Kawano-Yamashita et al. 2007), with slight modifications. The neuronal tracer neurobiotin (Vector Laboratories) was applied to the optic nerves of the lamprey and hagfish eyes. These eyes were incubated in oxygenated Ringer solutions for lamprey (Kawano-Yamashita et al. 2007) and for hagfish (Holmgren and Fange 1981) overnight at 4°C, and fixed in 4% paraformaldehyde in 100 mM PB. To visualize neurobiotin, frozen sections of the lamprey and hagfish eyes were incubated with Alexa Fluor 594-conjugated and Alexa Fluor 488-conjugated streptavidin, respectively, for 5 h at room temperature. Additional immunohistochemistry and *in situ* hybridization were performed as described above for the visualization of

lamprey and hagfish melanopsin localization, respectively.　Nuclei were stained with Hoechst 33258 (1:3000; Dojindo, Japan) for 5 h.

For the quantification of melanopsin distribution in ganglion cells in the hagfish retina, the ratio of melanopsin-containing ganglion cells to total melanopsin-containing cells was investigated on 8 different slices of the hagfish retina.　The Wilcoxon signed-rank test was used for the analysis of the ratio.

Microscopy

Fluorescence microscopic was obtained using a Leica DM6000 B instrument (Leica Microsystems).

Results

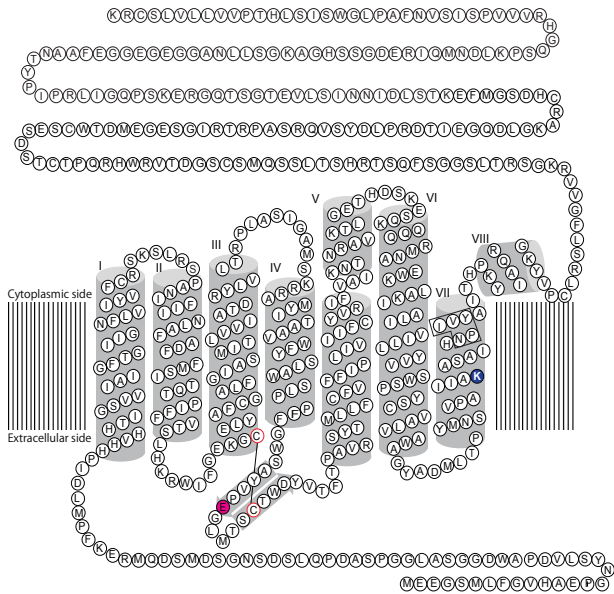**Isolation of lamprey and hagfish melanopsin cDNAs**

I isolated cDNAs encoding melanopsins from the eyes of the lamprey and the hagfish via PCR amplification (Fig. 8).   The molecular phylogenetic tree of melanopsins classified the hagfish melanopsin as well as the lamprey melanopsin into the Opn4m group (Fig. 9).   PCR-based screening against the hagfish eye cDNAs failed to identify the *Opn4x* gene.   These results suggest that the hagfish contains only one *Opn4m* gene, similar to the lamprey (Koyanagi and Terakita 2008).

Spectroscopic characterization and photosensitivity of the melanopsins

I first investigated the spectroscopic properties of the cyclostome melanopsins.   I successfully purified lamprey melanopsin from melanopsin-expressing COS-1 cells following reconstitution with 11-*cis* retinal chromophore, although I was unable to obtain a purified hagfish melanopsin. The absorption maximum of the lamprey melanopsin was approximately 480 nm (Fig. 10A), indicating that it is a blue light-sensitive pigment and has spectroscopic properties that are similar to the mammalian ones (Bailes and

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Lucas 2013; Matsuyama et al. 2012).   I subsequently investigated the light-

dependent functionalities of the lamprey and hagfish melanopsins by Ca^{2+}

imaging assay using Fura-2.   In cultured cells that expressed lamprey or

hagfish melanopsin, blue light stimulation induced a remarkable increase in

intracellular Ca^{2+}, which subsequently decreased to nearly baseline levels in a

manner that was similar to that observed in the mouse and amphioxus

melanopsin-expressing cells, in which light irradiation elevates Ca^{2+} levels via

the activation of the Gq-type G protein (Bailes and Lucas 2013; Qiu et al.

2005) (Fig. 10B).   These results indicate that, in the cultured cells, the

lamprey and hagfish melanopsins formed functional pigments with the retinal

chromophore.

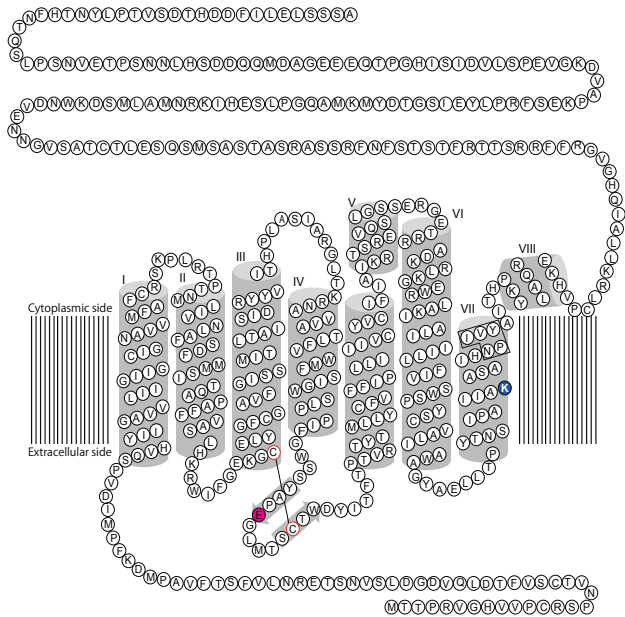**Lamprey melanopsin**　　　　**Hagfish melanopsin**

Fig. 8 Secondary structure model of the lamprey and hagfish melanopsin.

The seven membrane domains of lamprey and hagfish melanopsin are shown. Two conserved cysteine (C) residues at positions (red outlined circles) that are involved in disulfide bond formation. A highly conserved glutamic acid (E) (red circle) that serves as a counterion. The lysine (K) residue (blue circle) that binds to the retinal chromophore is highlighted. A conserved NPXXY motif (box), which assists in maintaining structural integrity upon activation of opsin-based pigments.
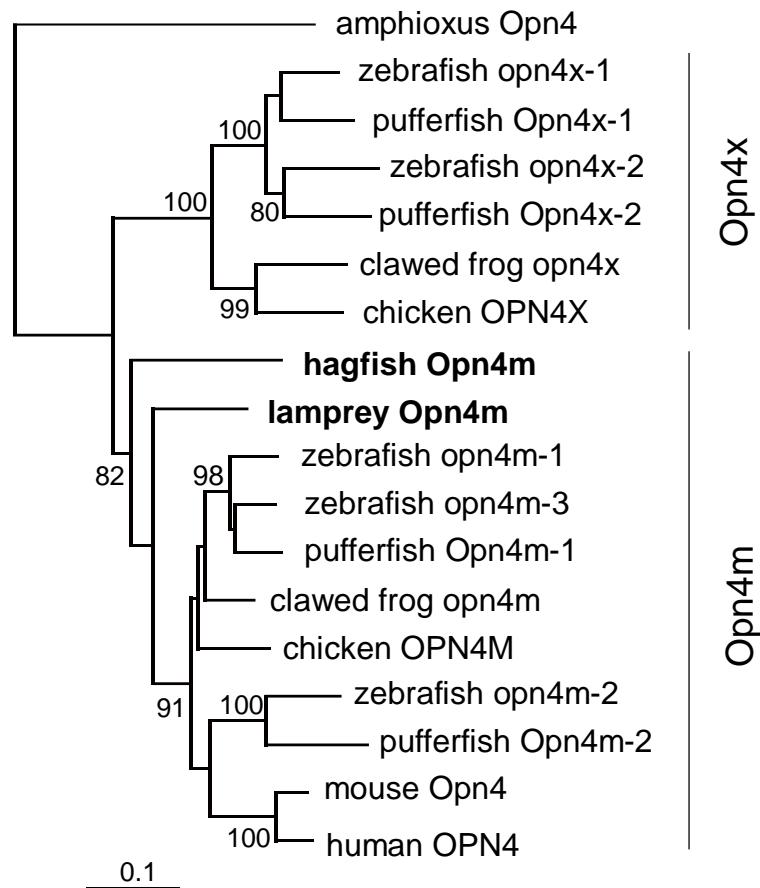
Fig. 9 Phylogenetic position of the lamprey and hagfish melanopsins.

Both the lamprey and hagfish melanopsins belong to the Opn4m group.

The bootstrap probabilities > 80% were indicated.　Scale bar, 0.1

substitutions per site.　The accession numbers of the sequences are as

follows: amphioxus Opn4, AB205400.　In Opn4x group: zebrafish opn4x-1,

GQ925718; pufferfish Opn4x-1, XP_003965597; zebrafish opn4x-2,

GQ925719; pufferfish Opn4x-2, XP_003974868; clawed frog opn4x,

AF014797; chicken OPN4X, AY036061.　In Opn4m group: hagfish Opn4m,

AB932627; lamprey Opn4m, AB932626; zebrafish opn4m-1, GQ925715;

zebrafish opn4m-3, GQ925717; pufferfish Opn4m-1, XP_003963814; clawed

frog opn4m, XP002937616; chicken OPN4M, AY882944; zebrafish opn4m-2, AY078161; pufferfish Opn4m-2, XP_003976773; mouse Opn4, AF147789; human OPN4, AF147788.
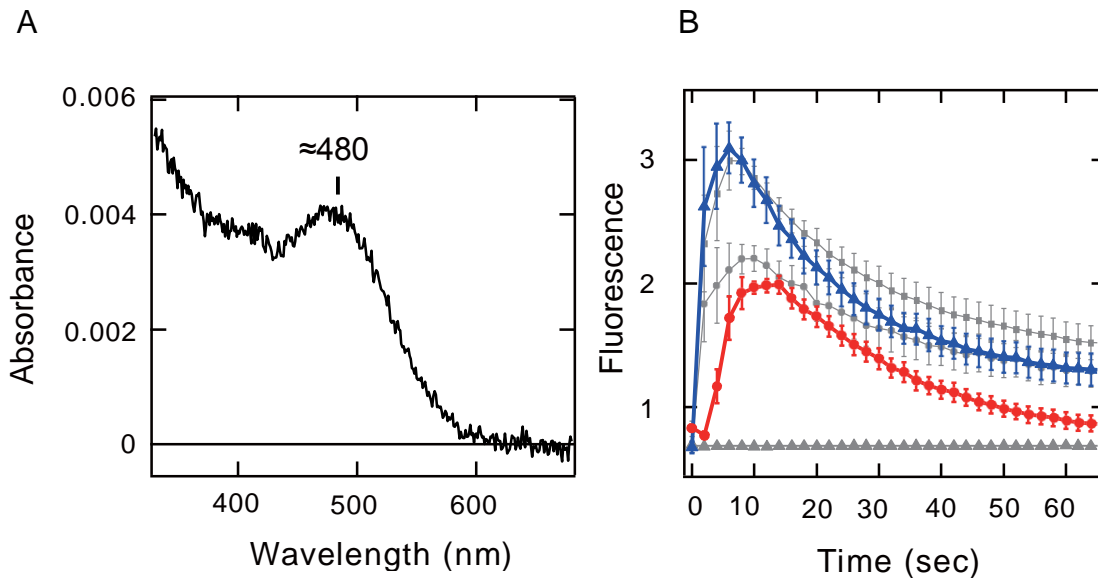
Fig. 10 Molecular properties of the lamprey and hagfish melanopsins.

A, An absorption spectrum of the dark-state lamprey melanopsin.   The absorption maximum is at approximately 480 nm.   B, Light-induced transient Ca^{2+} increases in lamprey (blue solid triangles), hagfish (red solid circles), amphioxus (gray solid circles) and mouse (gray solid squares) melanopsin-expressing cells were determined by using the Ca^{2+} indicator dye Fura-2. The cells were irradiated with blue light for 500 ms immediately after the first measurement point.   The mock-transfected cells exhibited no responses (gray solid triangles).   Full-length melanopsins of lamprey and hagfish and C-terminal-truncated melanopsins of amphioxus (Terakita et al. 2008) and mouse (Matsuyama et al. 2012) were used (see materials and methods section for details).   Each error bar represents the average value of 12 cells and indicates the standard deviation.

Distribution of melanopsin in the lamprey retina

Next, I investigated the localization of melanopsin in the lamprey retina.

Although only one *Opn4m* gene was found in the lamprey, *in situ* hybridization

revealed that melanopsin was expressed in horizontal cells and in other types

of cells in the inner nuclear layer (INL), as well as in the inner plexiform layer

(IPL), of the lamprey retina (Fig. 11A).

I also conducted immunohistochemical analyses to investigate

melanopsin distribution in the retina using an antibody against the N-terminal

region of the lamprey melanopsin, which reacted with the lamprey melanopsin

(Fig. 11B).   Immunostaining showed that melanopsin was widely distributed

in the inner horizontal cells and other types of cells in the proximal region of

the INL and IPL (Fig. 11B), which was consistent with the localization profile

revealed by *in situ* hybridization (Fig. 11A and Fig.12).

In mammals, a melanopsin gene is expressed in a subset of ganglion

cells (Hattar et al. 2002), and therefore I then investigated whether

melanopsin was expressed in the ganglion cells of the lamprey retina.

Double staining using the anti-melanopsin antibody and a retrograde tracer

applied to the optic nerve revealed that melanopsin was detected in the

retrograde tracer-stained ganglion cells located at the IPL (Fig. 13).   The

melanopsin-expressing cells in the INL did not overlap with the retrograde

tracer-stained ganglion cells (Fig. 13), suggesting that the melanopsin-

expressing cells located in the proximal region of the INL are not ganglion

cells; rather, they might be amacrine or bipolar cells, according to previous

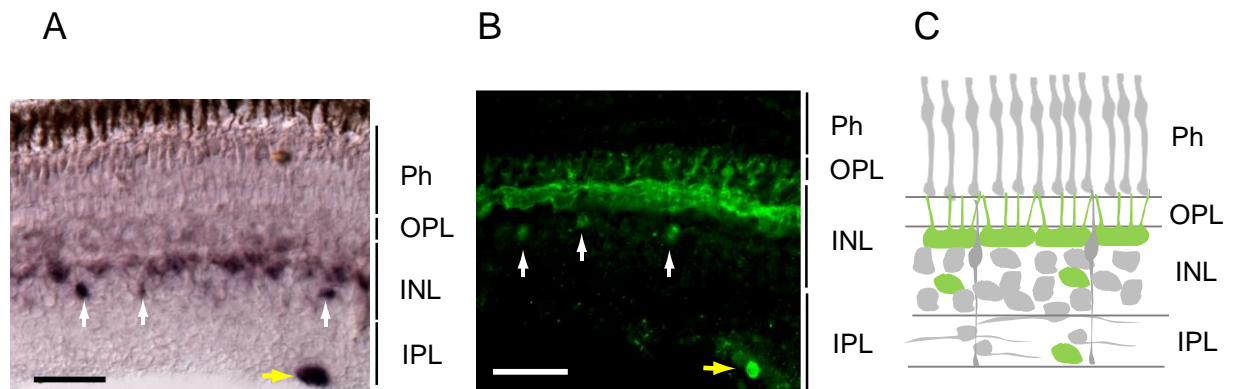morphological reports (Villar-Cheda et al. 2006).

Fig. 11 Localization of melanopsin in the lamprey retina.

Localization of melanopsin visualized by *in situ* hybridization with an antisense probe for melanopsin (A) and immunohistochemistry with an anti-melanopsin antibody (B) in the lamprey retina.   Melanopsin expression is observed in the inner horizontal cells of the INL and in other cells in the proximal region of the INL (white arrows) and IPL (yellow arrows).   A schematic drawing (C) shows melanopsin expression pattern (green).   Ph, photoreceptor cell; INL, inner nuclear layer; IPL, inner plexiform layer; OPL, outer plexiform layer.   Scale bar, 50 µm.
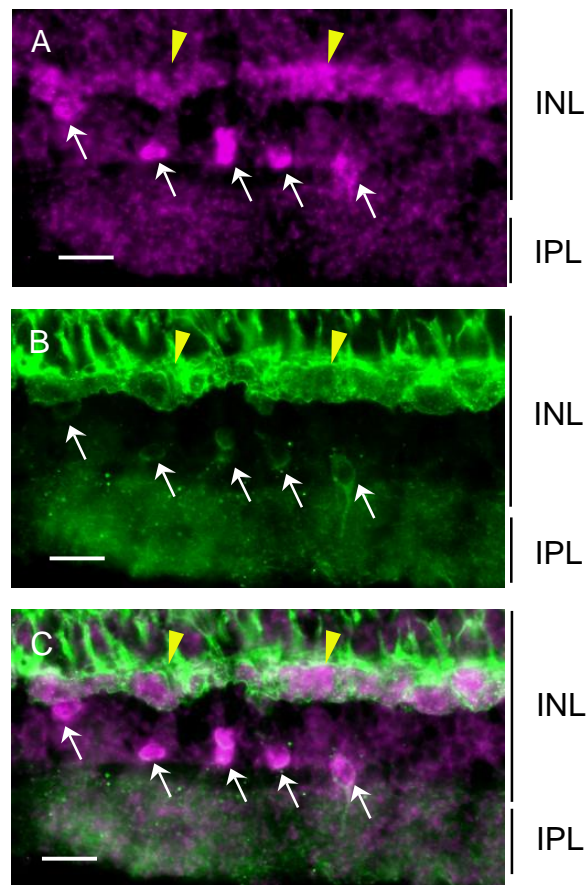
Fig. 12 Identification of melanopsin-expressing cells by combined *in situ* hybridization and immunohistochemistry.

Fluorescence *in situ* hybridization with HNPP/Fast Red staining (A) and immunohistochemistry with anti-melanopsin antibody (B) shows melanopsin expression in inner horizontal cells (arrowheads) of the INL and in the IPL (arrows) of the lamprey retina (see Materials and Methods section for details). A merged image (C) indicates that the anti-melanopsin antibody-labeled cells (green) overlap with the melanopsin probe-stained cells (magenta). INL, inner nuclear layer; IPL, inner plexiform layer. Scale bar, 25 µm.
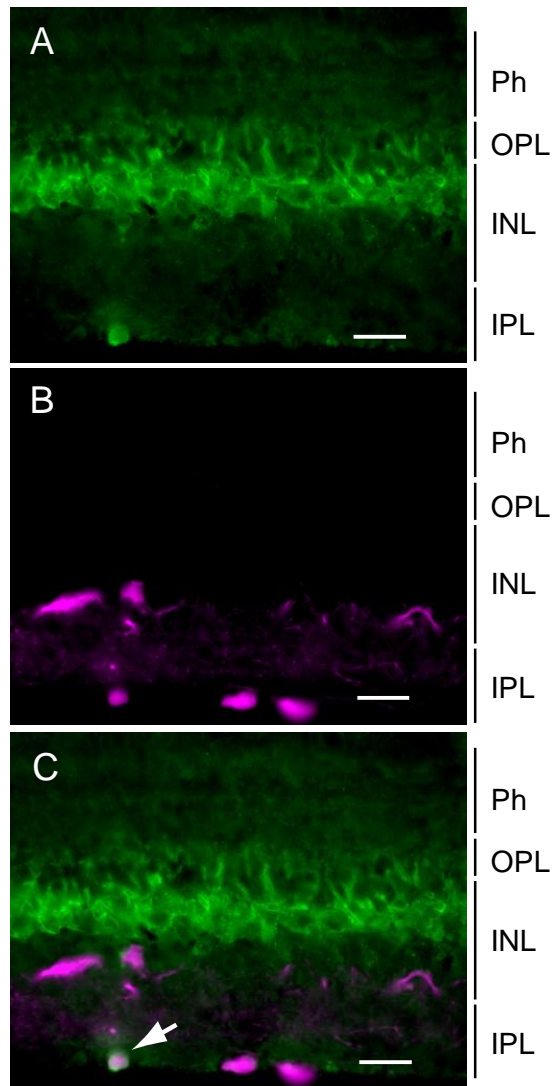
Fig. 13 Immunohistochemical localization of melanopsin to ganglion cells identified by retrograde tracing in the lamprey retina.

Melanopsin-expressing cells were immunohistochemically stained with the anti-melanopsin antibody (A, green) and ganglion cells were labeled by retrograde tracing with neurobiotin (B, magenta).   A merged image reveals that melanopsin is expressed in ganglion cells (C, white arrow).   Ph, photoreceptor cell; INL, inner nuclear layer; IPL, inner plexiform layer; OPL, outer plexiform layer.   Scale bar, 25 μm.

Distribution of melanopsin in the hagfish retina

I next investigated the distribution of melanopsin in the retina of another cyclostome, the hagfish, which is suggested to be primarily involved in non-visual (non-image-forming visual) function, rather than in visual (image-forming visual) function (Ooka-Souda et al. 1995; Kusunoki and Amemiya 1983; Fernholm and Holmberg 1975). Retrograde tracing clearly labeled cells in the proximal and distal regions of the inner layer, respectively (Fig. 14A and B). *In situ* hybridization revealed that melanopsin was localized to cells in both the proximal and distal regions of the inner layer (Fig. 14C), and most of the melanopsin-expressing cells were also stained by the retrograde tracing with neurobiotin (Fig. 14D). These findings suggest that the Opn4m-type melanopsin is primarily expressed in the ganglion cells of the hagfish retina, which mainly underlies non-visual (non-image-forming visual) function, rather than visual (image-forming visual) function.
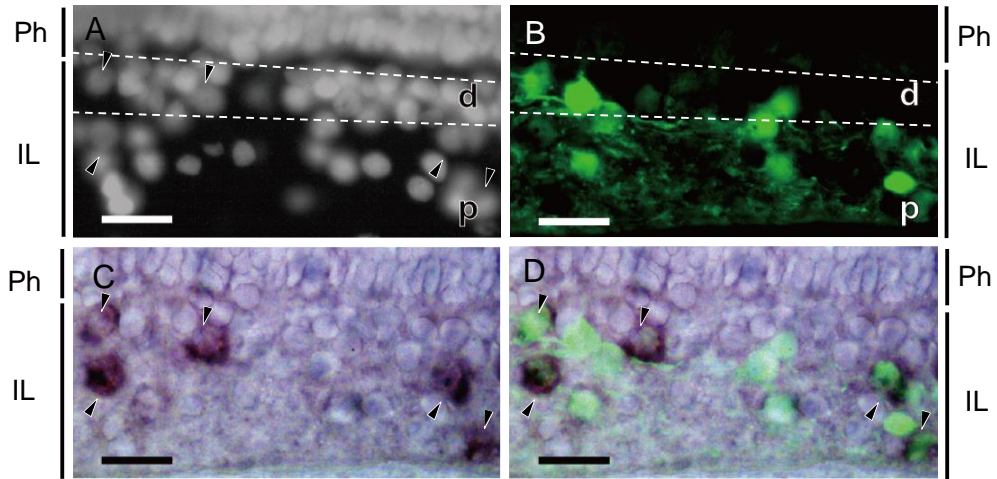
Fig. 14 Melanopsin-expressing ganglion cells in the hagfish retina.

(A) The central region of the hagfish retina stained with Hoechst consists of 2 layers, the photoreceptor (Ph) and inner layers (IL), which are further divided into two regions, the proximal region (p) and the distal region of the inner layer (d). The ganglion cells were stained by retrograde tracing (B, green), and melanopsin-expressing cells were stained by *in situ* hybridization (C, black arrowheads). Approximately 80% of melanopsin-expressing cells (75%, $p < 0.05$, Wilcoxon *t*-test) were stained by retrograde tracing in the merged image (D, black arrowheads). Scale bar, 25 µm.

**Histological connection between melanopsin-expressing horizontal cells
and photoreceptor cells in the lamprey retina**

It is well known that horizontal cells of teleost, chicken, and mammalian

retinas regulate the membrane potentials of photoreceptor cells via negative

feedback (Burkhardt 1977; Siminoff 1985; Verweij et al. 2003; Thoreson et al.

2008); therefore, I analyzed immunohistochemically the connection between

melanopsin-expressing horizontal cells and photoreceptor cells, to obtain

insights into the biological meaning of the global distribution of melanopsin in

the lamprey retina.   It has been reported that the lamprey has short and long

photoreceptor cells, which are distinguishable by the terminals that appear in

distinct layers (Dickson and Graves 1979).   Therefore, I analyzed to which

type of photoreceptor cells the melanopsin-expressing horizontal cells were

connected.   I performed double immunostaining using the anti-melanopsin

antibody and the anti-transducin antibody (TF15), which stained both types of

photoreceptor cells (Muradov et al. 2008) (Fig. 15B).   The dendrites of the

melanopsin-expressing horizontal cells that invaginated into the photoreceptor

cell terminals were stained with the anti-transducin antibody (Fig. 15A and

15C), and these connections were observed close to the scleral region, where

terminals of the short photoreceptor cells were located (Fig. 15D).   These

results suggest that the melanopsin-expressing horizontal cells were

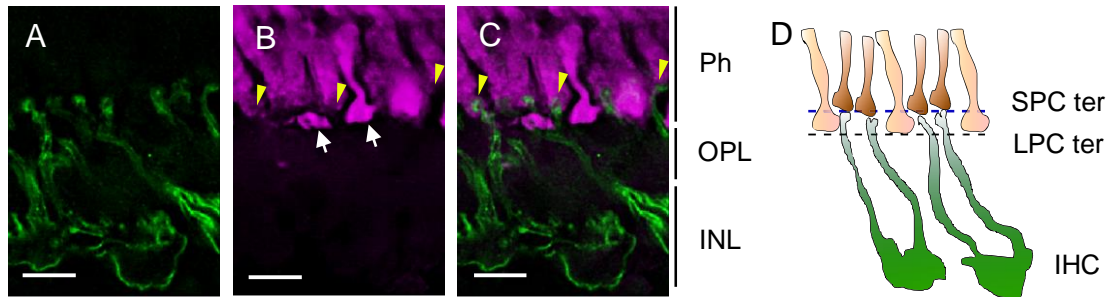connected to the short photoreceptor cells.

Fig. 15 Immunohistochemical characterization of the connection between melanopsin-expressing horizontal cells and photoreceptor cells in the lamprey retina.

Melanopsin-expressing horizontal cells (A, green) and two types of photoreceptor cells (B, magenta) are stained with the anti-melanopsin antibody and anti-transducin antibody (TF15), respectively.　The terminals of short photoreceptor cells (B, yellow arrowheads) and long photoreceptor cells (B, white arrows) are shown.　The connections between melanopsin-expressing horizontal cell dendrites and photoreceptor cell terminals are at the end of the short photoreceptor cell (C, yellow arrowheads).　A schematic drawing (D) shows that the terminals of short photoreceptor cells are closer to the scleral region than those of long photoreceptor cells.　Ph, photoreceptor cell; IHC, inner horizontal cell; INL, inner nuclear layer; LPC ter, long photoreceptor cell terminal; OPL, outer plexiform layer; SPC ter, short photoreceptor cell terminal.　Scale bar, 10 μm.

Discussion

In this study, I isolated mammalian-like melanopsins (Opn4m) from the lamprey and hagfish. The fact that the lamprey has only one *Opn4* gene in its genome has raised 2 possibilities. First, the common ancestor of cyclostomes had only one *Opn4* gene and *Opn4m* and *Opn4x* split after the cyclostome–gnathostome split in the gnathostome lineage. Second, it originally had *Opn4m* and *Opn4x* and lost *Opn4x*. However, the phylogenetic analysis revealed that the split of the Opn4m and Opn4x types occurred before the cyclostome–gnathostome split (Koyanagi and Terakita 2008; Raible et al. 2006), which supports the latter scenario. In addition, PCR-based screening against the hagfish eye cDNAs failed to identify the *Opn4x* gene, suggesting that the hagfish also contains only the *Opn4m* gene, similar to the lamprey. The result also supports the idea that the ancestral cyclostome had *Opn4m* and *Opn4x* genes and secondarily lost the *Opn4x* gene.

I reported the absorption spectrum of lamprey melanopsin, with its absorption maximum at approximately 480 nm, which was similar to those of other melanopsins. I also showed light-dependent Ca^{2+} increases in the lamprey and hagfish melanopsin-expressing cultured cells, similar to those

observed in mouse melanopsin-expressing cells (Bailes and Lucas 2013; Qiu

et al. 2005) (Fig. 10).　　Taken together with previous observations (Bailes and

Lucas 2013; Matsuyama et al. 2012; Matos-Cruz et al. 2011; Qiu et al. 2005),

these findings suggest that cyclostome melanopsins have similar biochemical

and absorption characteristics to mammalian ones.

　　My *in situ* hybridization and immunohistochemical analyses revealed that

strong melanopsin expression was observed in a large number of inner

horizontal cells of the lamprey retina.　　The dendrites of the melanopsin-

expressing horizontal cells formed contacts with the rhodopsin-containing

short photoreceptor cells (Fig. 15).　　It is widely accepted that a principal

function of retinal horizontal cells in various vertebrates is the provision of

negative feedback to photoreceptor cells (Burkhardt 1977; Siminoff 1985;

Verweij et al. 2003; Thoreson et al. 2008).　　Therefore, lamprey melanopsin in

horizontal cells may be involved in the regulation of negative feedback from

the horizontal cells to the rhodopsin-containing short photoreceptor cells in the

lamprey retina.　　In catfish and goldfish, it was reported that some cone

horizontal cells, which might contain melanopsin, exhibited inward calcium ion

currents as a response to light (Cheng et al. 2009).　　In addition, in a cyprinid

teleost, the roach (*Rutilus rutilus*), an electrophysiological study suggested

that a subset of horizontal cells are intrinsically photosensitive and light-dependently depolarized (Jenkins et al. 2001).   These observations suggest that the lamprey horizontal cells containing melanopsin might depolarize upon light absorption.   Therefore, one possibility for the function of melanopsin is the depolarization of the melanopsin-containing horizontal cells, which inhibits or cancels the hyperpolarization responses of horizontal cells that are triggered by light-dependent hyperpolarization of the photoreceptor cells, and, consequently, regulates the negative feedback to the photoreceptor cells. Accordingly, the possible depolarization of horizontal cells caused by melanopsin light absorption may contribute to membrane potential regulation in the rhodopsin-containing short photoreceptor cells, as a modulation of visual function.

Nonmammalian vertebrates, such as fish and chicken, possess multiple *Opn4* genes that are widely expressed in most retinal cell types, with the exception of retinal pigment epithelial and Müller cells (Tomonari et al. 2005; Tomonari et al. 2007; Davies et al. 2011; Matos-Cruz et al. 2011).   In contrast, mammals possess a single melanopsin gene that is limitedly expressed in ganglion cells.   In this study, I found that in the lamprey, melanopsin was distributed among various types of retinal cells, including

horizontal cells and ganglion cells, although the lamprey has only one

mammalian-like melanopsin gene.   Therefore, these findings indicate that

there is no significant relationship between the number of melanopsin genes

and the multiplicity of melanopsin-expressing cell types.   Rather, my results

highlighted the fact that, regardless of the number of melanopsin genes,

melanopsin(s) can be distributed in various retinal cells in nonmammalian

vertebrates, particularly in horizontal cells, which functionally contact with

photoreceptor cells.   In other words, melanopsin may contribute to the

regulation of various visual and non-visual functions, including both image-

forming and non-image-forming visions in nonmammalian vertebrates, such

as lampreys.   Conversely, in the hagfish retina, which is mainly involved in

non-visual (non-image-forming visual) function, melanopsin was primarily

distributed to the ganglion cells.   Although I compared the distribution of

melanopsin in only two cyclostome retinas, my findings allow us to speculate

that the global distribution of melanopsin to various kinds of retinal cells is not

correlated with the number of melanopsin genes, but might be related to the

extent of the retinal contribution to visual (image-forming visual) function in

cyclostomes.

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