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RESEARCH ARTICLE

Visual phototransduction components in cephalopod chromatophores suggest dermal photoreception

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ABSTRACT

Cephalopod mollusks are renowned for their colorful and dynamic body patterns, produced by an assemblage of skin components that interact with light. These may include iridophores, leucophores, chromatophores and (in some species) photophores. Here, we present molecular evidence suggesting that cephalopod chromatophores – small dermal pigmentary organs that reflect various colors of light – are photosensitive. RT-PCR revealed the presence of transcripts encoding rhodopsin and retinochrome within the retinas and skin of the squid *Doryteuthis pealeii*, and the cuttlefish *Sepia officinalis* and *Sepia latimanus*. In *D. pealeii*, $G_{q\alpha}$ and squid TRP channel transcripts were present in the retina and in all dermal samples. Rhodopsin, retinochrome and $G_{q\alpha}$ transcripts were also found in RNA extracts from dissociated chromatophores isolated from *D. pealeii* dermal tissues. Immunohistochemical staining labeled rhodopsin, retinochrome and $G_{q\alpha}$ proteins in several chromatophore components, including pigment cell membranes, radial muscle fibers, and sheath cells. This is the first evidence that cephalopod dermal tissues, and specifically chromatophores, may possess the requisite combination of molecules required to respond to light.

KEY WORDS: Rhodopsin, Retinochrome, Extraocular photoreceptor, Skin

INTRODUCTION

Many animals have complex image-forming eyes. Photoreceptor cells within these eyes are organized into a retina, which is responsible for detecting light and initiating neuronal signals. While eyes and their retinal photoreceptors are the most familiar light detectors, extraocular photoreceptors (i.e. those located outside the eye) that detect light for non-visual functions are common. Such photoreceptors do not form images and have been identified in many species and various tissues, most commonly within the central nervous system (CNS). Many vertebrates, annelids and arthropods have photoreceptors in the CNS that are involved in diverse physiological responses, including circadian timing, orientation, concealment and photoperiodism (Foster and Soni, 1998; Bertolucci and Foà, 2004; Arendt et al., 2004; Shintani et al., 2009; Hanna et al., 1988; Prosser, 1934; Welsh, 1934). However, extraocular photoreceptors are also frequently located outside of the CNS. For example, photoreceptors in light organs of bobtail squid are thought to function within a feedback system that controls the emittance of light from the bioluminescent organ (Tong et al., 2009). Opsin proteins, which are components of all visual pigments in animals, are used in almost all known extraocular photoreceptors, including bobtail squid light organs.

When bound to a vitamin-A-derived chromophore and stimulated by a photon of light, opsins activate a heterotrimeric G-protein, which initiates a signal cascade, resulting in the opening or closing of ion channels. While opsin proteins are obviously involved in phototransduction in eyes, a great diversity of opsins can also be found in extraocular tissues (Porter et al., 2011). The term 'non-visual opsins' refers to opsins involved in photoreception that does not lead to the perception of images (Peirson et al., 2009). They can exist in eyes (e.g. melanopsin in the vertebrate retina) or outside them, and are commonly associated with diverse types of photoreceptors. Many studies have focused on the locations and functions of non-visual opsins, such as melanopsin, parapinopsin, pinopsin, encephalopsin, peropsin and neuropsin (Provencio et al., 1998; Kawano-Yamashita, et al., 2007; Okano et al., 1994; Blackshaw and Snyder, 1999; Eriksson et al., 2013; Tarttelin et al., 2003; for review, see Terakita, 2005). These particular examples typically couple to phototransductive pathways that are distinct from those used in visual phototransduction in the same animal. However, extraocular photoreceptors can also express opsins identical to those in the retina and may potentially use visual phototransductive pathways. Examples of these include rhodopsin in the light organ and parolfactory vesicles of squids and cone opsins in the dermis of fish (Hara and Hara, 1980; Tong et al., 2009; Ban et al., 2005; Kasai and Oshima, 2006; Chen et al., 2013). Nile tilapia (*Oreochromis niloticus*) and neon tetra (*Paracheirodon innesi*) are particularly notable, because opsins identical to those in the retina play a role in initiating signals that result in expansion and contraction of pigment cells (chromatophores) or modulation of the color reflected from iridophores. Thus, while visual opsins are by definition involved in retinal image detection, they can also contribute to other kinds of photoreception.

Coleoid cephalopods generally have only a single photoreceptor class in the retina, which expresses a single type of rhodopsin (Bellingham et al., 1998). When illuminated, the visual pigment (which consists of a rhodopsin protein bound to a retinal chromophore) activates a heterotrimeric G-protein, thought to be of the G_q class (Davies et al., 1996). Dissociation of the heterotrimeric G-protein signals a downstream cascade, which involves phospholipase C (PLC) and the second messengers inositol triphosphate (IP3) and diacylglycerol (DAG) (Arendt, 2003). This cascade ultimately leads to the opening of ion channels, which are thought to be a type of TRP channel called squid transient receptor potential channel (sTRP) (Monk et al., 1996), thereby initiating a cellular signal. The presence of these signaling molecules as well as retinal opsin, indicates that a particular cell type may function in photoreception.

In addition to retinal photoreceptors, there are a few well-studied extraocular photoreceptors known in cephalopods. The Japanese flying squid, *Todarodes pacificus,* has a photoreceptive system in the

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parolfactory vesicles (also called parolfactory bodies) located near the optic tract, which apparently involves a rhodopsin protein that is identical to that expressed in the retina (Messenger, 1967; Hara and Hara, 1976, 1980). The parolfactory vesicles also express retinochrome, a retinal photoisomerase thought to regenerate the chromophore in inner segments of cephalopod retinal photoreceptors (Hara and Hara, 1980). Parolfactory vesicles have been implicated in long-term monitoring of ambient light and in diel vertical migration (Cobb and Williamson, 1998). More recently, the bioluminescent light organ of the Hawaiian bobtail squid *Euprymna scolopes* has been found to possess photoreceptors expressing visual rhodopsin and the retinal phototransduction proteins arrestin and rhodopsin kinase (Tong et al., 2009). These photoreceptors detect light emitted from the bioluminescent light organ and potentially regulate the light output of the organ. Finally, opsin transcripts have been identified in skin from the fin and ventral mantle of the cuttlefish *Sepia officinalis* (Mathger et al., 2010), suggesting the presence of dermal photoreceptors. The presence of rhodopsin in the skin of cuttlefish and the involvement of cone opsins in the modulation of fish chromatophores provides some of the impetus for the current study.

Here, we show that visual opsins and other components of visual phototransduction exist in the skin of three coleoid cephalopod species, and specifically in chromatophore organs, indicating the potential for dermal light sensing.

RESULTS

RT-PCR

Rhodopsin

A single full-length rhodopsin transcript was identified in the retina and throughout all skin regions tested in the squid *Doryteuthis pealeii* (supplementary material Table S1; accession number KR107044). Full-length rhodopsin gene transcripts were identified in the retinas and partial rhodopsin gene transcripts (>200 amino acids in the transmembrane region) were identified throughout all skin regions tested in the cuttlefishes *S. officinalis* and *S. latimanus* (accession numbers KR107053, KR107052, KR107049, KR107048). The predicted amino acid sequence for each respective species is the same for all sequences amplified from that species, regardless of tissue region (supplementary material Fig. S1). Thus, only a single opsin mRNA sequence was found throughout all tissues in each species. Rhodopsin sequences from *D. pealeii* and *S. officinalis* were identical to previously published sequences in these species (Go and Mitchell, 2003; accession no. AY450853; Bellingham et al., 1998; accession no. L47533), while that of *S. latimanus* rhodopsin was previously unreported (accession number KR107049). Rhodopsin transcripts were not located in RNA extracts from the fin nerve or stellate ganglion in *D. pealeii*, which served as negative controls.

Retinochrome

A single full-length retinochrome transcript was identified in the retina and throughout the skin of *D. pealeii* (accession number KR107043). Full-length retinochrome gene transcripts were identified in the retinas and partial transcripts (>170 amino acids in the transmembrane region) were identified throughout the skin of *S. officinalis* and *S. latimanus* (supplementary material Table S1; accession numbers KR107051, KR107050, KR107047, KR107046). Retinochrome transcripts were identified by comparison to the published retinochrome transcript from *Todarodes pacificus* (Hara et al., 1990; accession no. X57143). A single transcript was found in each species investigated. The predicted amino acid sequences for retinochrome were the same for all recovered sequences in each respective species and identical to retinal retinochrome (supplementary material Fig. S2). Retinochrome sequences from *D. pealeii*, *S. officinalis* and *S. latimanus* were previously unknown. As with rhodopsin, retinochrome transcripts were not found in RNA extracts from the fin nerve or stellate ganglion in *D. pealeii*.

$G_{q\alpha}$

A full-length G_q α-subunit transcript was identified in the retina of *D. pealeii* (supplementary material Table S1; accession number KR107041) and was identical to the *D. pealeii* $G_{q\alpha}$ previously reported by Go and Mitchell (2003; accession no. AF521583). A second full-length $G_{q\alpha}$ transcript was also found in all dermal tissue regions of this species (accession number KR107042). However, the dermal sequence differed in amino acid composition from the retinal sequence by 16 amino acids near the C-terminus (supplementary material Fig. S3). The presence of a $G_{q\alpha}$ transcript was not investigated in the fin nerve or stellate ganglion of *D. pealeii*.

sTRP

A partial squid transient receptor potential (sTRP) channel transcript was identified in the retina and skin of *D. pealeii* (supplementary material Table S1). sTRP channel transcripts located in skin RNA extracts had a predicted amino acid sequence identical to that of transcripts from retinal tissue (supplementary material Fig. S4). sTRP from *D. pealeii* had not been sequenced prior to this search (accession number KR107045). Primers used to identify sTRP in *D. pealeii* were designed using the sequence from *Loligo forbesi* (Monk et al., 1996). The partial sTRP sequence (216 amino acids) identified in *D. pealeii* differs by one amino acid from the sequence identified in *L. forbesi* (Monk et al., 1996). While sTRP is thought to function as the ion channel involved in retinal phototransduction in cephalopods, this has not been empirically confirmed (Monk et al., 1996). The presence of a sTRP transcript was not examined in the fin nerve or stellate ganglion of *D. pealeii*.

Dissociated chromatophores

Full-length rhodopsin, retinochrome and $G_{q\alpha}$ mRNA transcripts were identified in chromatophores dissociated from the ventral mantle, dorsal mantle, lateral mantle and fin of *D. pealeii* (supplementary material Table S2). The predicted amino acid sequence for each transcript was the same as the corresponding amino acid sequence from the retina, except for $G_{q\alpha}$ (supplementary material Figs S1-S3), which differed by 16 amino acids near the C-terminus and was identical to the sequence from skin samples reported earlier. The presence of an sTRP transcript was not examined in dissociated chromatophore RNA.

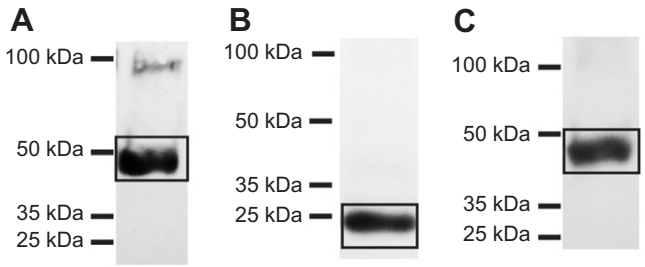

Fig. 1. Western blots of retinal protein tissue extracts from the squid *Doryteuthis pealeii*. (A) Rhodopsin (47 kDa), (B) retinochrome (24 kDa) and (C) $G_{q\alpha}$ (48 kDa). Boxes indicate expected molecular mass of bands representing each protein. The band at 100 kDa on the rhodopsin blot is a rhodopsin dimer.

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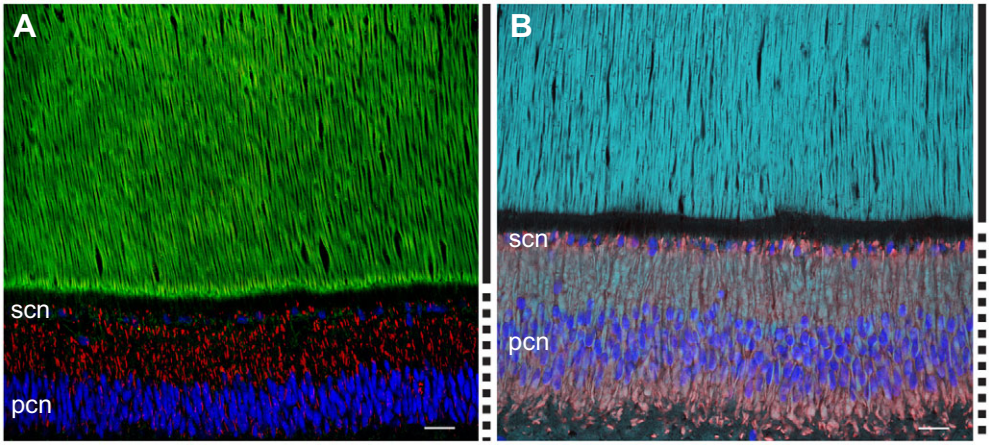

Fig. 2. Immunohistochemical labeling of the retina of *D. pealeii*. (A) Rhodopsin (green) and retinochrome (red). (B) $G_{q\alpha}$ (cyan) and retinochrome (red). Labeled rhodopsin is present in outer segments. Retinochrome is present in inner segments. Retinochrome label appears pink in the inner segments when colabeled with $G_{q\alpha}$, suggesting that these two proteins are coexpressed in the same cells of the inner segments. $G_{q\alpha}$ label is also present in inner and outer segments. The location of the outer segments is represented by the vertical solid black lines; that occupied by inner segments is represented by the vertical dotted lines. DAPI labeling of nuclei in the photoreceptor cells (pcn) and in supporting cells (scn) where the inner (dashed lines) and outer (solid lines) segments meet is blue. Scale bars: 25 μm.

Antibody studies

Western blot

Anti-rhodopsin labeled a retinal protein of ~47 kDa molecular mass in *D. pealeii* (Fig. 1A). This finding agrees with values reported for several squid rhodopsins identified through amino acid analysis and SDS-polyacrylamide gel electrophoresis using anti-rhodopsin antibodies (Nashima et al., 1979). The secondary-only control for anti-rabbit HRP conjugate shows no labeling (supplementary material Fig. S5A). Similarly, an absorption control using rhodopsin antibody and peptide shows no labeling (supplementary material Fig. S5B). Anti-retinochrome labeled a retinal protein of ~24 kDa molecular mass in *D. pealeii* (Fig. 1B). This finding also agrees with previous reports of antibody labeling against *T. pacificus* retinochrome (Hara and Hara, 1984). The secondary-only control for anti-chicken HRP conjugate shows no labeling (supplementary material Fig. S6A). Similarly, an absorption control using retinochrome antibody and peptide shows no labeling (supplementary material Fig. S6B). Anti-$G_{q/11\alpha}$ labeled a retinal protein with a molecular mass of ~48 kDa in *D. pealeii* (Fig. 1C). This protein is similar in size to a protein identified in the retina of the firefly squid *Watasenia scintillans* (Narita et al., 1999), where it is thought to be the alpha subunit of the G_q protein.

Immunolabeling of rhodopsin and retinochrome

Rhodopsin and retinochrome were simultaneously immunolabeled in some preparations. Rhodopsin antibody labels the outer segments of the retina, where opsin protein is known to be present (Fig. 2A). Retinochrome antibody labels the inner segments of retinal photoreceptors, where retinochrome is thought to function as a photoisomerase to regenerate *cis*-retinal for use by the visual pigment rhodopsin (Fig. 2A; Hara and Hara, 1972). DAPI, included in the mountant, labels a large band of nuclei in the inner segments, belonging to the photoreceptor cells. A single layer of supporting cell nuclei in the inner segments distal to the photoreceptor nuclei is also labeled (Fig. 2A). Immunolabeling in the retina is used as a positive control, since protein expression in this tissue is known (Hara and Hara, 1972). Negative controls containing only secondary antibodies in the retina show no non-specific binding or cross-reactivity of secondary antibodies (supplementary material Fig. S7).

Rhodopsin and retinochrome antibody labeling is localized to components of many dermal tissues (Fig. 3), including ventral mantle, dorsal mantle, fin, each of the four arm pairs (only arm 1 shown), and tentacle. Specifically, both labels are consistently seen in pigment cell membranes of chromatophores, as well as in radial muscle fibers and sheath cells (see Cloney and Florey, 1968 for chromatophore ultrastructure). Rhodopsin and retinochrome immunolabeling is apparent in these organs in cross-sections of the ventral mantle and tentacle, and in orthogonal sections of the dorsal mantle, fin, and arm 1 (see Fig. 4 and Materials and methods for orientation descriptions). Depending on the angle of sectioning, there are sections where the outer membrane of the pigment sac is labeled (Fig. 3C). This is the case when the outer pigment membrane is present in the section, and other sections where outer pigment membrane is not present do not show this staining (Fig. 3D,E). Negative controls containing only secondary antibodies or antibodies absorbed by antigenic peptide show no non-specific binding or cross-reactivity of secondary antibodies in dermal tissues (supplementary material Figs S7, S8, S9, and S10).

Immunolabeling of $G_{q\alpha}$ and retinochrome

$G_{q\alpha}$ and retinochrome were also colabeled in some preparations. $G_{q\alpha}$ antibody binds to the inner and outer segments of the retina, where $G_{q\alpha}$ is thought to function in phototransduction (Fig. 2B; Narita et al., 1999). $G_{q\alpha}$ and retinochrome labeling overlap, and appear pink in the inner segments of photoreceptor cells (Fig. 2B). Controls show no non-specific binding or cross-reactivity in retinal sections using the same secondary antibodies used to label $G_{q\alpha}$ and retinochrome (supplementary material Fig. S8).

$G_{q\alpha}$ antibody colocalizes with retinochrome antibody in chromatophore membranes, radial muscle fibers and sheath cells in mantle and fin tissues (Fig. 5). $G_{q\alpha}$ antibody also labels many regions of mantle and fin tissue that are not labeled by retinochrome antibody. Ventral mantle tissue was examined in an oblique orientation, showing labeling of retinochrome and $G_{q\alpha}$ in the pigment cell membrane and of a single radial muscle fiber pulling the pigment cell away from a sheath cell (Fig. 5A). Dorsal mantle tissue was visualized in an orthogonal orientation, showing two chromatophores with retinochrome and $G_{q\alpha}$ labeling of the pigment cell membrane and sheath cells surrounding each pigment cell

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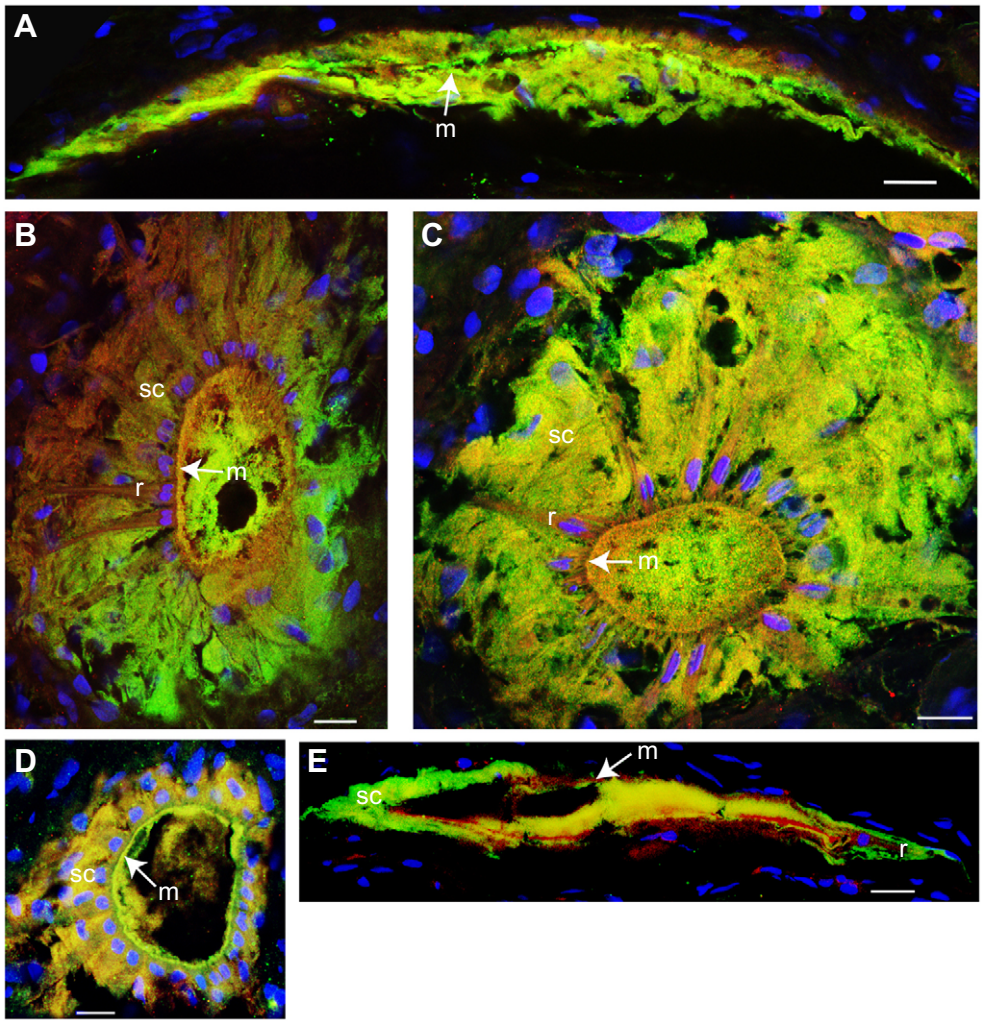

Fig. 3. Immunohistochemical labeling of rhodopsin and retinochrome in various tissues of *D. pealeii*. (A) Ventral mantle, (B) dorsal mantle, (C) fin, (D) arm 1, (E) tentacle. Rhodopsin (green) and retinochrome (red) are present in chromatophore (pigment cell) membranes, radial muscle fibers and sheath cells. Yellow indicates overlap of rhodopsin and retinochrome label, suggesting that some of these cells express both proteins. Blue represents DAPI labeling of nuclei. m, pigment cell membrane; r, radial muscle fiber; sc, sheath cell. Scale bars: 25 μm.

(Fig. 5B). Fin tissue was visualized in cross-section, showing the presence of a chromatophore with labeling of retinochrome and $G_{q\alpha}$ of the pigment cell membrane, a single radial muscle fiber, and a labeled sheath cell (Fig. 5C). $G_{q\alpha}$ antibody also labels connective tissue (Fig. 5A,B), the iridophore layer and muscle tissue underlying the chromatophore layer (Fig. 5C; for a review of dermal composition, see Cloney and Florey, 1968). Similar to the staining seen for rhodopsin and retinochrome double labels, there are sections where the outer membrane of the pigment sac is labeled with retinochrome (Fig. 5A,C). This is the case when the outer pigment membrane is present in the section, and other sections where outer pigment membrane is not present do not show this staining (Fig. 5B).

DISCUSSION

Cephalopods have extraocular photoreceptors in their light organs and parolfactory vesicles (Hara and Hara, 1980; Tong et al., 2009); both types of photoreceptors express rhodopsin protein and the parolfactory vesicles of the oceanic squid *T. pacificus* express retinochrome protein (expression of retinochrome protein in light organs was not reported). Ours is the first study to identify and localize several phototransduction components in cephalopod skin, where they may serve a distributed light-sensing system.

Cephalopod skin is unique because it produces dramatic color and pattern changes by modulating a number of specialized structures within the dermis (Hanlon, 2007). One set of these is the dermal chromatophore organs, which are complex structures composed of a pigment sac surrounded by the highly reticulated membrane of its pigment cell (Cloney and Florey, 1968) to which radially arranged muscle fibers are directly attached. Contraction of radial muscle fibers expands the pigment cell, while their relaxation allows the pigment cell to contract. Surrounding the membrane of the pigment cell are sheath cells whose function is unidentified; they are hypothesized to support the chromatophore organ throughout the dynamic movements that occur when the pigment sac is rapidly expanded, contracted or maintained in a partially expanded state (Cloney and Florey, 1968). Cephalopods have particularly complicated dermal architecture that, while often studied, is still poorly understood.

Rhodopsin, retinochrome, $G_{q\alpha}$ and sTRP transcripts were found in the retina and throughout dermal tissues. With the exception of $G_{q\alpha}$, dermal transcripts match the retinal transcript identified in each respective species, with no additional rhodopsin transcripts identified in any species. Variations in the $G_{q\alpha}$ transcripts reflect the presence of more than one $G_{q\alpha}$ class in cephalopods. Despite these small differences in $G_{q\alpha}$ transcripts, G_q proteins are known to target the PLC pathway. Whether these changes result in changes to the signaling cascade is unknown. The stellate ganglion and fin nerve of *D. pealeii* serve, in a sense, as negative controls since neither rhodopsin nor retinochrome transcripts were detected within these tissues. While Mathger et al. (2010) reported the presence of two

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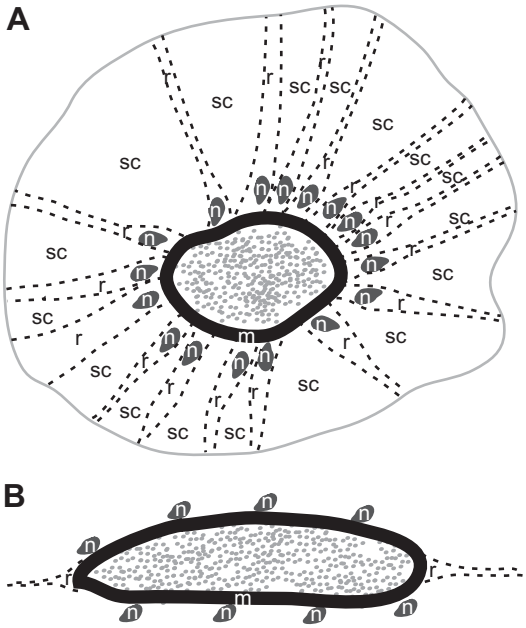

Fig. 4. Schematic representation of chromatophore structure. *En face* (A) and cross-section (B) orientation of immunohistochemically stained samples. Small stippled dots represent pigment granules within the pigment sac. m, outer membrane of pigment cell; r, radial muscle fiber; sc, sheath cell; n, nucleus.

distinct rhodopsin transcripts in ventral mantle skin of *S. officinalis* that differ by one predicted amino acid change from the retinal sequence, it is likely that DNA polymerase or sequencing errors produced such differences.

The presence of rhodopsin, $G_{q\alpha}$ and sTRP channels in cephalopod skin is particularly significant because all are components that could serve extraocular photoreception, duplicating their function in retinal photoreceptors. Retinochrome in cephalopods is also thought to be necessary for retinal photoreceptor function, even though its role is not well understood. Most significantly, antibody labeling of rhodopsin, retinochrome and $G_{q\alpha}$ in the highly folded membranes, radial muscle fibers and sheath cells of chromatophore organs suggests a photoreceptive function. Phototransduction in the retina is thought to rely on these same components, although the precise sequence of events is not clear. Nevertheless, our finding of identical or very similar molecular components in chromatophores strongly suggests that they function in phototransduction.

While physiological and behavioral assays are necessary to determine if, how and why these putative photoreceptors function, we propose three hypotheses based upon our current understanding of this system. Such chromatophore photoreceptors might act as a local system affecting individual cells, within a broader system of cells immediately adjacent to the photoreceptive cell or in coordination with the central nervous system. Thus one hypothesis posits that sensing by chromatophores could alter a single chromatophore component (pigment cell membrane, radial muscles, sheath cell), or the entire organ, to make it more or less likely to change its state of expansion or retraction. In this case, individual chromatophore organs would respond to light locally. Alternatively, local receptors could communicate with one another among the chromatophores via the gap junctions that exist between adjacent muscle cells and allow electrical interactions (Cloney and Florey, 1968), so that small regional areas of chromatophores would respond to light stimuli as a unit. Finally, phototransduction-

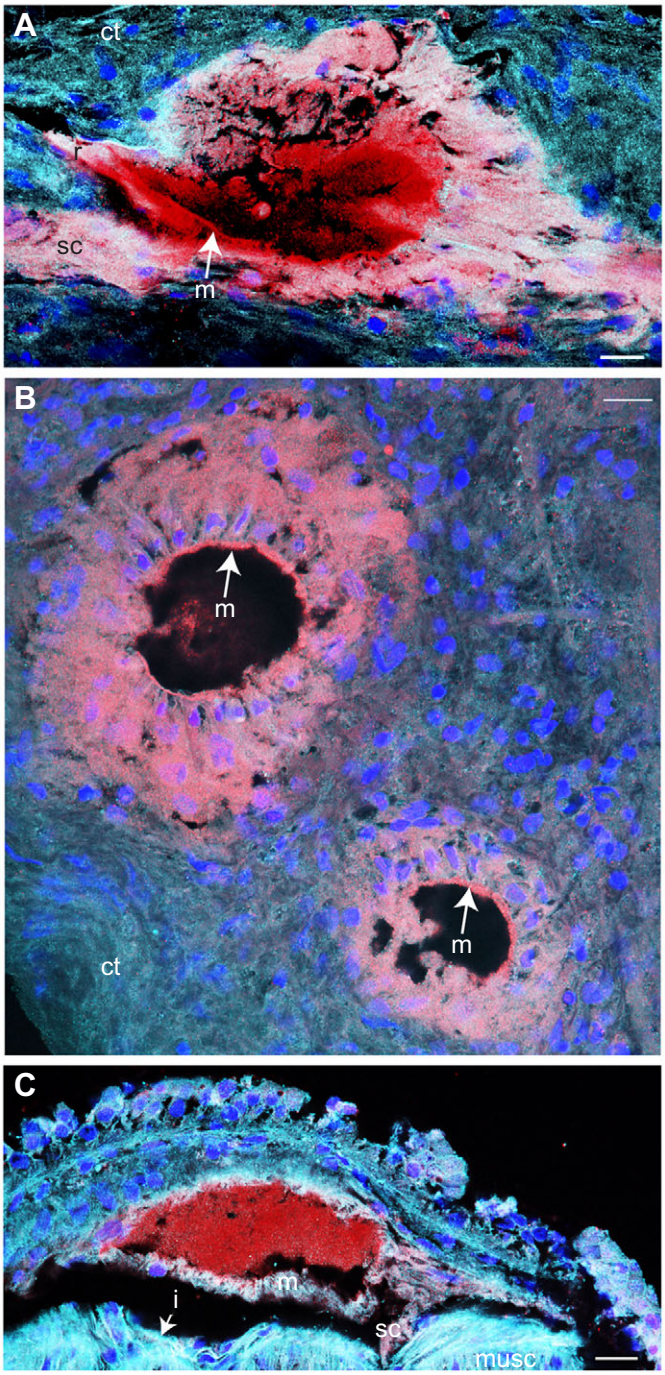

Fig. 5. Immunohistochemical labeling of $G_{q\alpha}$ and retinochrome in various tissues of *D. pealeii*. (A) ventral mantle, (B) dorsal mantle and (C) fin. $G_{q\alpha}$ (cyan) and retinochrome (red) labels are seen in pigment cell membranes, radial muscle fibers and sheath cells. Pink color apparent in some areas is due to overlap of $G_{q\alpha}$ and retinochrome labels. Blue represents DAPI labeling of nuclei. m, pigment cell membrane; r, radial muscle fiber; sc, sheath cell; ct, connective tissue; i, iridophore layer; musc, muscle tissue underlying the iridophore layer. Scale bars: 25 μm.

induced signals produced by chromatophores may travel by afferent nerve fibers to the central nervous system to provide additional information about the environment in which the animal exists. This information itself could serve ultimately to affect chromatophore behavior. While future research will clarify their function, the molecular evidence presented here suggests that cephalopod

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chromatophores contain the basic components required for a system of distributed light detectors.

MATERIALS AND METHODS

Tissue collection and fixation

The retina, ventral mantle, dorsal mantle, fin, arms and tentacles from each species were used for RT-PCR. Also included in the analyses of *D. pealeii* were fin muscle tissue, stellate ganglion and fin nerve. Retina and dermal tissues from *D. pealeii* were also analyzed immunohistochemically. Adult *Doryteuthis pealeii* Lesueur 1821 were collected in Vineyard Sound, by the Aquatic Resources Division at Marine Biological Laboratory (MBL) in Woods Hole, MA, USA. Adult *Sepia officinalis* Linnaeus 1758 were hatched from fertilized eggs obtained from England and reared to adulthood at MBL. A single adult *Sepia latimanus* Quoy and Gaimard 1832 was collected from Lizard Island Research Station in Queensland, Australia. *D. pealeii* and *S. officinalis* were killed by decapitation immediately prior to use. *S. latimanus* was killed by anesthetic overdose in 5% ethanol in natural seawater. Dissected tissues were stored in RNALater (Qiagen, Valencia, CA, USA) or fixed immediately for immunohistochemistry in 4% paraformaldehyde in 0.1 mol l^{-1} phosphate buffered saline (PBS) for 4–8 h at room temperature, followed by cryoprotection using a 10, 20, 30% sucrose gradient in PBS overnight at 4°C.

RNA isolation, PCR, cloning, sequencing

Total RNA was isolated using TRIzol Reagent (Life Technologies, Carlsbad, CA, USA) following the manufacturer's protocol. RNA was reverse-transcribed using Superscript III Reverse Transcriptase (Life Technologies, Carlsbad, CA, USA) and an Oligo$(dT)_{50}$ primer (Life Technologies, Carlsbad, CA, USA). RT-PCR was performed using PrimeSTAR HS Premix (Takara, Otsu, Japan) and gene-specific primers for rhodopsin, retinochrome, $G_{q\alpha}$ and sTRP (supplementary material Table S3). PCR products were sequenced using gene-specific primers or TA-cloned using pGEM T-easy vector system (Promega, Madison, WI, USA) and sequenced using M13 vector primers. Isolated dissociated chromatophores were obtained following a protocol from Lima et al. (2003). RNA isolation, PCR, cloning and sequencing of dissociated chromatophore tissue followed the methods cited above.

Antibodies

Custom anti-rhodopsin antibody (Covance, Princeton, NJ, USA) was designed against the first 15 amino acids of retinal opsin sequences from *D. pealeii*, *S. officinalis* and *S. latimanus* (predicted amino acid sequence: MGRDIPDNETWWYNP). The predicted amino acid sequences were identical in this region, in all three species (black bar in supplementary material Fig. S1). The 15 amino acid peptide was conjugated to thyroglobulin via a cysteine residue added at the C-terminus to maximize immune response of the host. The host for this antibody was rabbit and upon completion of the standard rabbit protocol (Covance, Princeton, NJ, USA), the antibody was affinity purified from 25 ml serum. Custom anti-retinochrome antibody (Covance, Princeton, NJ, USA) was designed against the terminal 11 amino acids of retinochrome sequences from *D. pealeii*, *S. officinalis* and *S. latimanus* (predicted amino acid sequence: RTIPKSDTKKP), whose predicted amino acid sequences in this region were identical (black bar in supplementary material Fig. S2). The 11 amino acid peptide was conjugated to bovine serum albumin (BSA) via a cysteine residue added to the N-terminus to maximize immune response of the host. The antibody was produced in chicken to avoid potential cross-reactivity when double labeled with anti-rhodopsin antibody, and affinity purified from egg yolks upon completion of the standard chicken protocol (Covance, Princeton, NJ, USA). Commercial anti-$G_{q/11\alpha}$ antibody (Millipore, Billerica, MA, USA, produced in rabbit) targets the terminal region of mouse and human $G_{q/11\alpha}$ (sequence: QLNLKEYNLV) that is also identical to the terminal ten amino acids of *D. pealeii* $G_{q/11\alpha}$ (supplementary material Fig. S3; denoted by black bar). Secondary antibodies used included Alexa Fluor 488 Goat Anti-Rabbit IgG (H+L), Alexa Fluor 555 Goat Anti-Chicken IgY (H+L), and Alexa Fluor 633 Goat Anti-Chicken IgY (H+L) (retina only) (Life Technologies, Carlsbad, CA, USA).

Western blot

Western blots were used to ensure that the custom and commercial antibodies were specific to proteins of the predicted molecular weights of target proteins for immunohistochemistry: rhodopsin, retinochrome and $G_{q\alpha}$. Proteins were solubilized using protein extraction buffer containing 2 mmol l^{-1} phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich, St. Louis, MO, USA) and 2 mmol l^{-1} dithiothreitol (DTT; Sigma-Aldrich, St. Louis, MO, USA) plus tissue protein extraction reagent (T-PER; Life Technologies, Carlsbad, CA, USA). Whole eyes lacking lenses from *D. pealeii* were homogenized in protein extraction buffer by vigorous shaking at 4°C for 3 h. Supernatant containing solubilized protein was added to an equal volume of Laemmli sample buffer (Bio-Rad, Hercules, CA, USA) plus 5% β-mercaptoethanol (Sigma-Aldrich, St Louis, MO, USA), and vigorously shaken at 4°C for 30 min. Protein mixtures were loaded on a 4–15% Mini-Protean TGX precast gel (Bio-Rad, Hercules, CA, USA) and run at 100 V for 2 h. Spectra Multicolor Broad Range Protein Ladder (Life Technologies, Carlsbad, CA, USA) was run in gels and used to estimate molecular mass of proteins. Proteins were transferred from gel to PVDF membrane at 100 V for 1 h. Membranes were blocked overnight at 4°C in Membrane Blocking Solution (Life Technologies, Carlsbad, CA, USA). Membranes were incubated in primary antibody for 1 h at room temperature, washed three times, incubated in secondary antibody for 1 h at room temperature and washed three times. Anti-rhodopsin was used at 1:5000, anti-retinochrome was used at 1:2000 and anti-$G_{q\alpha}$ was used at 1:5000.

Secondary-only control blots were incubated with blocking solution for 1 h at room temperature, washed three times, incubated in secondary antibody for 1 h at room temperature and washed three times (supplementary material Fig. S5).

Absorption controls were performed on retinal protein extracts, to ensure that affinity purified antibodies were specific to only the proteins against which they were designed. Primary antibody was incubated with the peptide used to make the antibody, overnight at 4°C. Primary antibody/peptide mixture was used as primary antibody, and the same protocol was used for antibody incubations and washes as when using primary antibodies. Primary antibody dilutions for absorption controls were the same used when probing for retinal proteins (supplementary material Fig. S6).

All membranes were visualized by incubating blots in HyGLO Chemiluminscent HRP Antibody Detection Reagent (Denville Scientific, Metuchen, NJ, USA) for 1 min, then placing HyBLOT Autoradiography Film (Denville Scientific, Metuchen, NJ, USA) on blots and developing. Secondary antibodies were conjugated to horseradish peroxidase and included anti-chicken IgY, HRP Conjugate (Promega, Madison, WI, USA) used at 1:1000 and anti-rabbit IgG, HRP Conjugate (Thermo Scientific, Rockford, IL, USA) used at 1:5000.

Immunolabeling

Following fixation and cryoprotection, tissues were cryosectioned at 12 µm, mounted on SuperfrostPlus slides (Fisher Scientific, Pittsburgh, PA, USA), and stored at −20°C until used. Sections were rehydrated at room temperature in three changes of PBS+0.3% Triton X-100 (PBS-TX) and blocked in PBS-TX +10% normal goat serum (NGS; Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature. Primary antibodies were diluted at a concentration of 1:100 in 300 µl PBS-TX+10% NGS and applied to sections. Slides were covered with Parafilm and stored horizontally at 4°C for 1–4 days. Subsequently, slides were washed three times in 0.1 mol l^{-1} PBS at room temperature. Secondary antibodies were diluted at a concentration of 1:400 in 300 µl PBS-TX+10% NGS and applied to sections. Slides were covered with Parafilm and stored horizontally at 4°C overnight. Slides were then washed in PBS three times for 30 min at room temperature, in the dark. Slides were mounted using Dapi-FluormountG (Southern Biotech, Birmingham, AL, USA), sealed with clear nail polish, and imaged using a Leica SP5 scanning confocal microscope. In all immunohistochemical images in this paper, blue represents DAPI (4′,6-diamidino-2-phenylindole) labeling of nuclei, green represents rhodopsin antibody labeling, red represents retinochrome antibody labeling and cyan represents $G_{q\alpha}$ antibody labeling. Overlap of rhodopsin and retinochrome labeling appears yellow, and overlap of $G_{q\alpha}$ and retinochrome labeling appears pink. For best visualization of tissues, retinal and dermal tissues were sectioned in orthogonal orientation or in cross-section (Fig. 4). Orthogonal sections revealed a single dermal layer with entire chromatophores. In these *en*

face images, chromatophores are viewed from 'above' where the chromatophores appear round, radial muscle fibers project circumferentially and sheath cells are present in between these muscles (e.g. Fig. 4A). In cross-section, all dermal layers are present from the surface epithelial layer to the underlying basal muscle tissue. Chromatophores are seen from the side with few or no radial muscle fibers or sheath cells apparent (e.g. Fig. 4B). More oblique sections allowed chromatophores to be viewed in an ovoid form.

Secondary-only tissue controls lacking primary antibodies were performed using the same protocol and conditions as tissues labeled with primary antibodies. Retina, ventral mantle, dorsal mantle, fin, tentacle and arm 1 tissue sections were labeled with anti-rabbit 488 and anti-chicken 555 to show lack of non-specific secondary antibody binding and minimal fluorescence due to secondary antibodies (supplementary material Figs S7 and S8).

Retina and mantle tissues were used for absorption controls to show that primary antibodies can be blocked with the antigenic peptides used to develop the antibody (supplementary material Figs S9 and S10). Absorption controls were performed by incubating rhodopsin or retinochrome antibody with the respective antigenic peptide at 4°C overnight. The mixture was then diluted to the working concentration of antibody used in primary antibody labeling experiments (1:100) and applied to tissue following the protocol used previously.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
A.C.N.K. designed and performed experiments. A.M.K. provided dissociated chromatophores and contributed to overall project strategy. R.T.H. and T.W.C. provided guidance, supervised the project and provided all laboratory resources.

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Supplementary material
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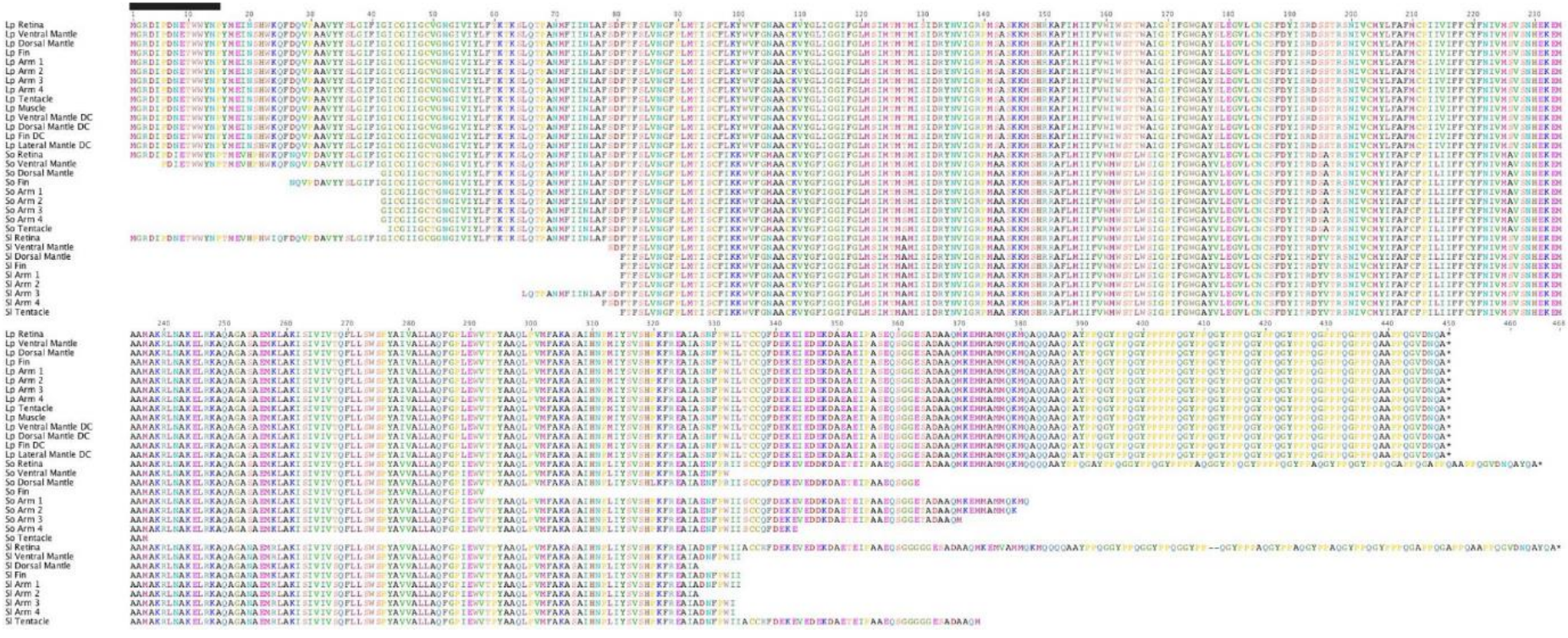

Fig. S1. Predicted amino acid alignment of rhodopsin identified by RT-PCR in *Doryteuthis pealeii, Sepia officinalis*, and *Sepia latimanus*. The black bar represents the region against which anti-rhodopsin antibody was designed.

Fig. S2. Predicted amino acid alignment of retinochrome identified by RT-PCR in *Doryteuthis pealeii, Sepia officinalis*, and *Sepia latimanus*. The black bar represents the region against which anti-retinochrome antibody was designed.

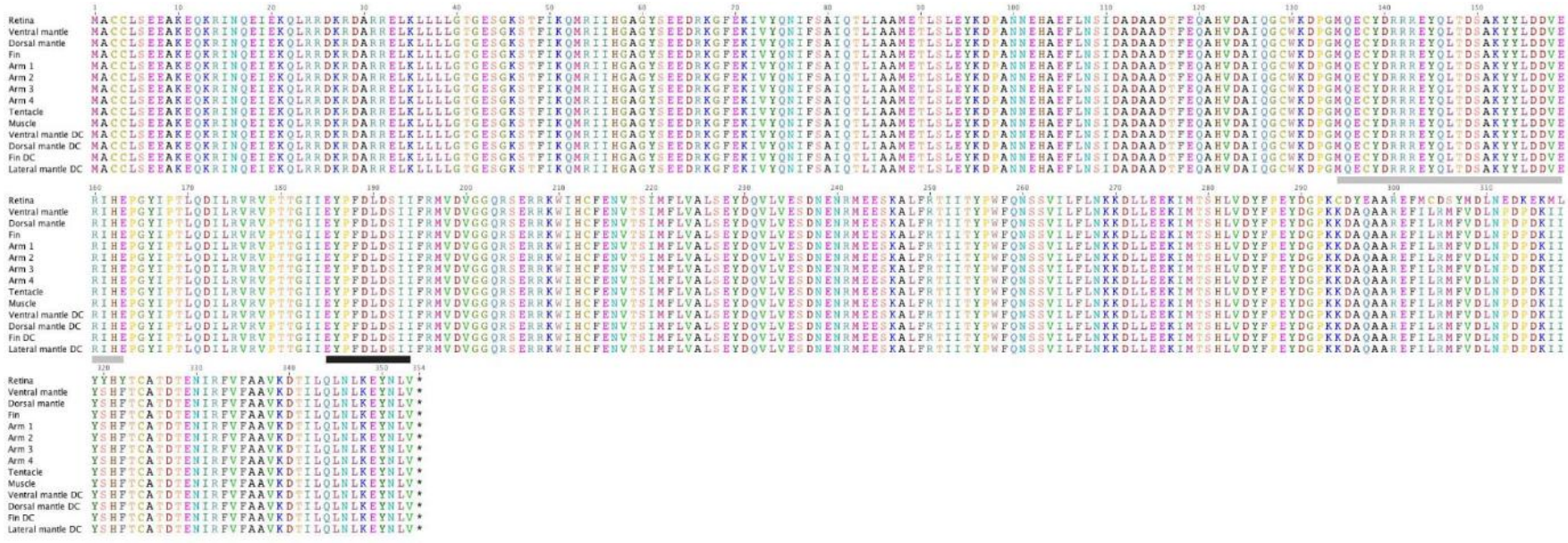

Fig. S3. Predicted amino acid alignment of full length Gqα identified by RT-PCR in *Doryteuthis pealeii*. The black bar represents the region of the protein against which anti-Gqα targets. The gray bar represents the region of the Gqα transcript identified in the retina that is different from the Gqα transcripts identified in dermal tissues.

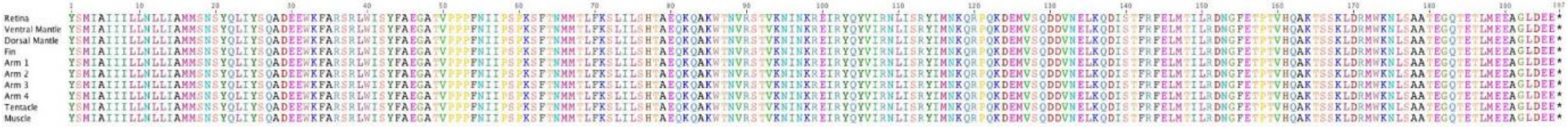

Fig. S4. Predicted amino acid alignment of partial transient receptor potential channel (TRP) identified by RT-PCR in *Doryteuthis pealeii*.

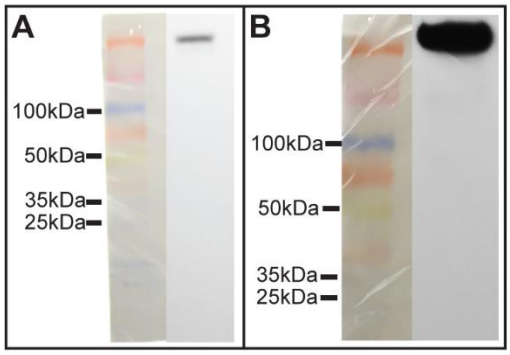

Fig. S5. Secondary only control for anti-rabbit horseradish peroxidase-conjugate (A) and anti-chicken horseradish peroxidase-conjugate (B) Western blots. Secondary-only controls lack primary antibody and show no labeling. Bands at the top of each blot represent excess protein.

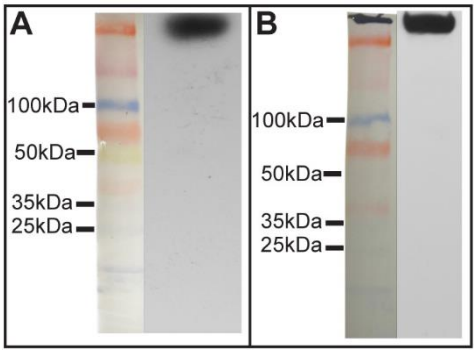

Fig. S6. Absorption controls for anti-rhodopsin (A) and anti-retinochrome (B) Western blots. Rhodopsin absorption control is labeled with rhodopsin primary antibody incubated with antigenic peptide to inhibit functionality of primary antibody (A). Retinochrome absorption control is labeled with retinochrome primary antibody incubated with peptide to inhibit functionality of primary antibody (B). Bands at the top of each blot represent excess protein.

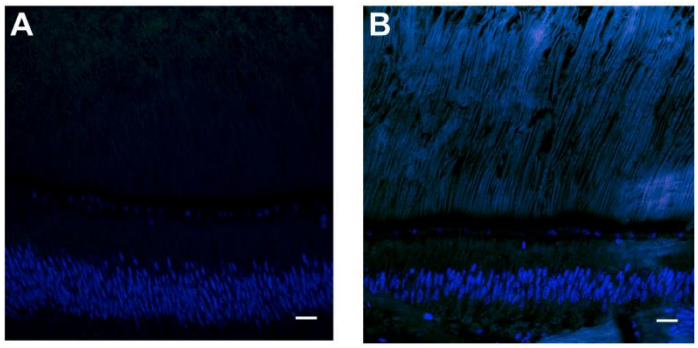

Fig. S7. Immunohistochemical secondary antibody-only control lacks primary antibody. *Doryteuthis pealeii* retina is labeled with (A) anti-rabbit 488 and anti-chicken 633 and (B) anti-rabbit 488 and anti-chicken 555 to ensure no non-specific labeling of secondary antibodies.

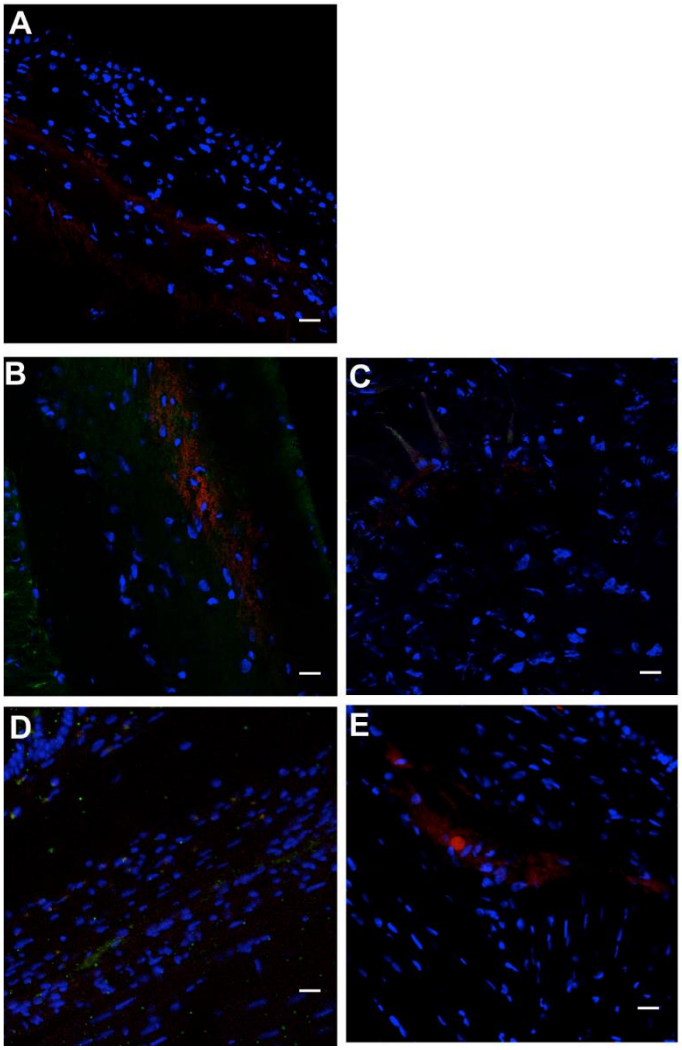

Fig. S8. Immunohistochemical secondary antibody-only control lacks primary antibody. *Doryteuthis pealeii* (A) ventral mantle, (B) dorsal mantle, (C) fin, (D) arm 1, and (E) tentacle labeled with anti-rabbit 488 and anti-chicken 555 to ensure no non-specific labeling of secondary antibodies.

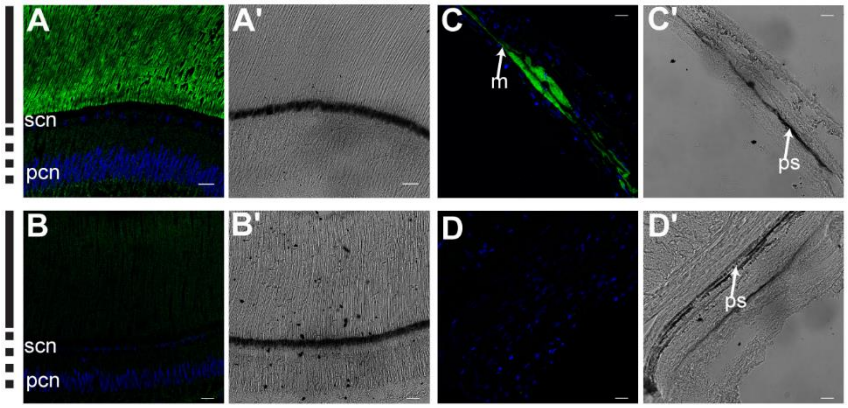

Fig. S9. Absorption control immunolabeling of rhodopsin protein. Rhodopsin protein is expressed in retina outer segments (A) and chromatophores (C). When antibody is absorbed with antigenic peptide, protein labeling is blocked in retinal sections (B) and mantle sections (D). Transmitted light images are included to show tissue structure (A″, B″, C″, D″). Blue represents DAPI labeling of nuclei. The location of the outer segments is represented by the vertical solid black line; that occupied by the inner segments is represented by the vertical dotted lines. Letter labels: supporting cell nuclei; pcn, photoreceptor cell nuclei; m, membrane; ps, pigment sac. Scale bar, 25µm.

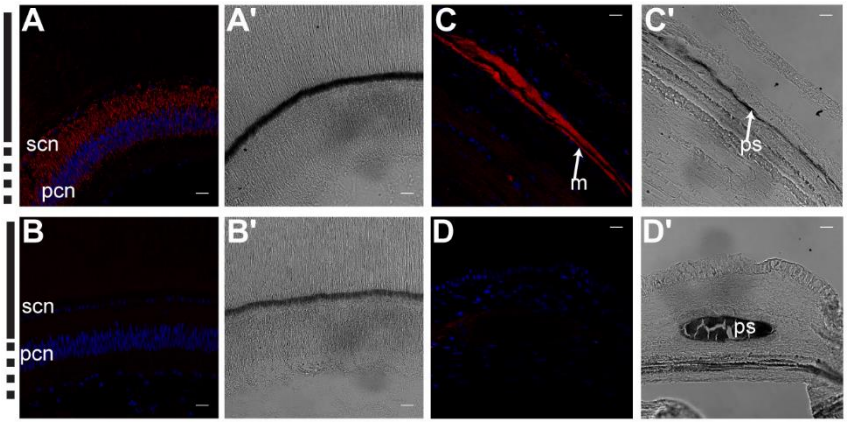

Fig. S10. Absorption control immunolabeling of retinochrome protein. Retinochrome protein is expressed in retina inner segments (A) and chromatophores (C). When antibody is absorbed with antigenic peptide, protein labeling is blocked in retinal sections (B) and mantle sections (D). Transmitted light images are included to show tissue structure (A″, B″, C″, D″). Blue represents DAPI labeling of nuclei. The location of the outer segments is represented by the vertical solid black line; that occupied by the inner segments is represented by the vertical dotted lines. Letter labels: supporting cell nuclei; pcn, photoreceptor cell nuclei; m, membrane; ps, pigment sac. Scale bar, 25µm.

Table S1. Presence of phototransduction component transcripts in dermal tissues from *D. pealeii* (black), *S. officinalis* (red), and *S. latimanus* (blue). + indicates transcript found; X indicates transcript not found.

	Rhodopsin	Retinochrome	Gqα	sTRP
Retina	+++	+++	+	+
Ventral mantle	+++	+++	+	+
Dorsal mantle	+++	+++	+	+
Fin	+++	+++	+	+
Arm 1	+++	+++	+	+
Arm 2	+++	+++	+	+
Arm 3	+++	+++	+	+
Arm 4	+++	+++	+	+
Tentacle	+++	+++	+	+
Muscle	+++	+++	+	+
Fin nerve	X	X		
Stellate ganglion	X	X		

Table S2. Presence of phototransduction transcripts in chromatophores dissociated from *D. pealeii* dermal tissue.

	Rhodopsin	Retinochrome	Gqα
Ventral mantle	+	+	+
Dorsal mantle	+	+	+
Lateral mantle	+	+	+
Fin	+	+	+

Table S3. Gene specific primer sequences used to characterize cephalopod phototransduction transcripts.

Gene	Primer	Sequence 5'>3'
Rhodopsin	**L.pealeiiRhoF1**	**ATGGGTCGCGATATCCCAGACAATG**
	L.pealeiiRhoR1325	**TTAGGCCTGGTTGTCAACCCCCTGAG**
	SepiaRhoF1	**ATGGGTAGAGACATCCCAGATA**
	SepiaRhoR1395end	**TCAAGCCTGGTAGGCCTGGTTGTCAA**
	S.latimanusF91	**GACGCTGTTTACTACTCCCTCGGTAT**
	S.latimanusF189	**TCCCTCCAGACTCCAGCCAACATG**
	L.pealeiiR860	**CATAAGGTGTTACCCATTCGAGTGGACC**
Retinochrome	**CephRetF1**	**ATGTTCGGAAATCCAGCAATGACTGG**
	CephRetR906	**TTAGGGCTTCTTGGTGTCACTTTTGG**
	S.latimanusRetR196	**GGGTCAATGGAGGAGTTGCTGCTC**
	L.pealeiiRetR775	**GTGACCTCCCAAGTGAGTAAGGCTGGC**
Gqα	**LpGq_F1short**	**ATGGCGTGCTGCCTCAGCG**
	LpGq_Rend	**TCAGACCAAGTTATACTCCTTCAAGTTAAG**
sTRP	**L.pealeiiTRP F1852**	**CAACTTGCTTATCGCTATGATGAGC**
	L.pealeiiTRP R2365	**CCATAAGTGTTTCGGTCTGGCCC**